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Capillary Electrophoresis of Carbohydrates

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Structural and Functional Diversity of Glycoconjugates

A Formidable Challenge to the Glycoanalyst

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1. Overview of Glycosylation

Glycoconjugates represent the most structurally and functionally diverse molecules in nature. They range in complexity from relatively simple glycosphingolipids and nuclear or cytosolic glycoproteins with dynamic monosaccharide modifications to extraordinarily complex mucins and proteoglycans (for review, *see* **refs.** ***1,2***). Some of the proteoglycans are perhaps the most complex molecules in biology, with more than 100 different saccharide side chains on a single polypeptide. We now realize that most proteins, even those within intracellular compartments, are co- and/or post-translationally modified by covalent attachment of saccharides ***(3)***.

1.1. The Glycocalyx and Extracellular Matrix

Many early electron microscopic studies using cationic stains, such as ruthenium red or alcian blue, documented that virtually all cells are surrounded by thick carbohydrate coats ***(4,5)***, termed the "glycocalyx." The glycocalyx is comprised of protein- and lipid-bound oligosaccharides and polysaccharides attached to membrane-associated proteins and lipids. Although electron micrographs visualize the glycocalyx as a distinct boundary many times the thickness of the lipid bilayer of the plasma membrane, in reality, the glycocalyx is probably even larger and is contiguous with the extrinsically associated extracellular matrix glycoconjugates, which are washed away during sample preparation for microscopy. Even the simplest eukaryotic cell, the erythrocyte, has a large and complex glycocalyx (**Fig. 1A**) about which we have consider-

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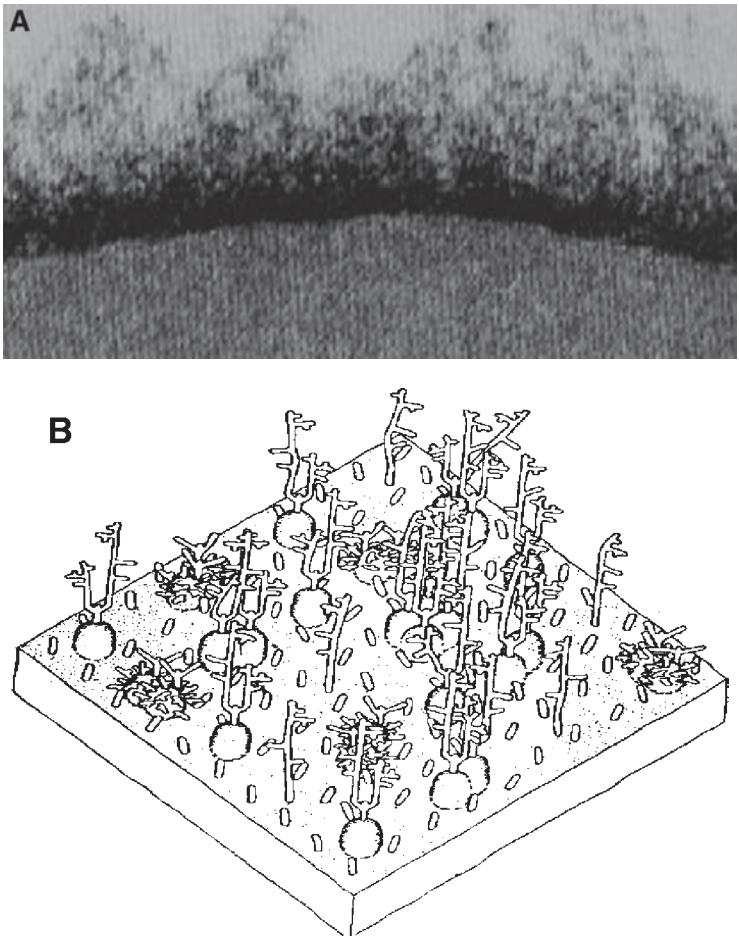

Fig. 1. **(A)** Electron micrograph of a human erythrocyte stained to illustrate the large size of the Glycocalyx with respect to the lipid bilayer of the plasma membrane. (From Voet and Voet, *Biochemistry, 2nd ed.*, with permission). **(B)** An axonometric projection of area 350×350 Å of the erythrocyte surface, representing approx 10^{-5} of the erythrocyte surface. (Both figures reproduced from **ref. *6*** with permission from Elsevier Science).

able structural information (**Fig. 1B**) ***(6)***. The glycocalyx of all cells is comprised of an astonishingly complex array of glycoconjugates. This saccharide "barrier" is critical to the biology of the cell by specifically mediating/modulating its interactions with small molecules, macromolecules, other cells, and with the extracellular matrix. In many respects, the glycocalyx has the physical properties of both gel filtration and ion-exhange resins, but is much more com-

plex and selective in its molecular interactions. The protein- and lipid-bound saccharides of the glycocalyx serve not only as recognition molecules in multicellular interactions, but also as binding sites for viral and bacterial pathogens. The saccharides play a crucial role in the concentration and activation of ligands for cell-surface receptors and in the lateral organization of membrane-associated proteins and lipids (for review, *see* **ref. *7***).

The spaces between cells of eukaryotic multicellular organisms are filled with secreted glycoproteins, such as collagens, laminins, fibronectin, and many others. In addition, the proteoglycans and glycosaminoglycans play an important role in fibrillogenesis and organization of the extracellular matrix. All of these secreted macromolecules self assemble to form highly organized structures such as basement membranes and lattices that define the elasticity and resiliency of various tissues. For example, the collagens and proteoglycans secreted by the three cell types of the cornea of the eye are highly organized to develop and maintain the transparency of this tissue ***(8–10)***. Similarly, the elasticity of cartilage is largely defined by the structural organization of water by the collagens and highly negatively charged proteoglycans that are synthesized in large quantities by chondrocytes ***(11–13)***. The glycoconjugates of the extracellular matrices are not only important for their physical properties, but they are also informational molecules regulating development and cellular trafficking. For example, we have only recently appreciated the enormous, almost DNA-like, information content encoded by the specific saccharide modifications along the sequence of the glycosaminoglycans, such as heparin ***(14–17)***. All of these glycoconjugates display cell-type specific glycoforms, termed "glycotypes," whose structures are also developmentally dependent. Not only do these glycotypes differ in saccharide linkages and chain lengths, but also in minor saccharide substituents, and nonsaccharide components such as sulfation. Clearly, elucidation of the structure/function of these macromolecules will require separation technologies of extraordinary resolution and sensitivities.

1.2. Extracellular Glycoconjugates Have Incredible Structural Diversity

Glycosylation of proteins can be thought of as a spectrum (**Fig. 2**). At one end of the spectrum are the collagens, which contain only a few mono- or disaccharide side chains, and nuclear or cytosolic glycoproteins that contain clusters of the monosaccharide, *N*-acetylglucosamine. In the middle of the spectrum are the mucins, which typically contain many shorter side chains often terminating in sialic acids ***(18,19)***, but may contain so many sugar chains that they can be mostly carbohydrate by weight. Next are the *N*-linked glycoproteins, which typically have only a few but longer, highly branched complex saccharide side chains, all having a common inner core structure added *en bloc*

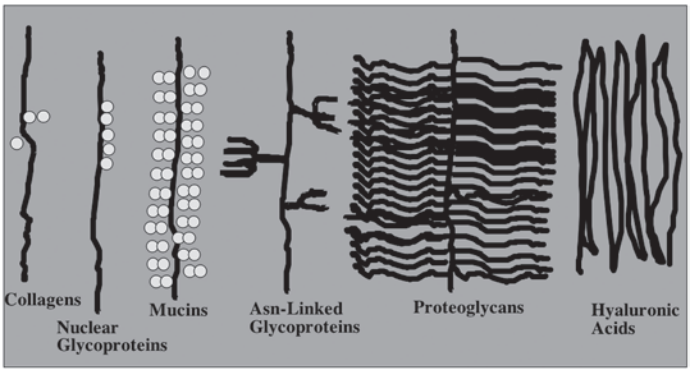

Fig. 2. A model depicting the "spectrum" of glycosylated proteins.

during polypeptide synthesis ***(20)***. At the far end of the spectrum are the proteoglycans, which can contain more than 100 large polysaccharide side chains, many *N*-linked and "mucin-type" *O*-linked saccharide chains attached to very large protein cores ***(21,22)***. For example, the cartilage proteoglycans are among the most complicated molecules known.

Even though in higher eukaryotes, saccharide side chains are comprised of only a few common monosaccharide components, including *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose, galactose, fucose, glucose, and sialic acids, the structural diversity possible is much larger than that for proteins or nucleic acids. This diversity results from the chirality about the glycosidic bond (anomericity) and the ability of monosaccharides to branch. For example, as illustrated in **Table 1** even a small oligosaccharide with relatively small chain length (*N*) has an enormous relative number of structural isomers possible. As discussed below, extracellular glycoproteins and glycolipids typically have complex glycans attached. The site-specific glycosylation of polypeptides is cell type and developmental stage specific, as well as being controlled by the environment surrounding the cell synthesizing the glycoprotein. Indeed, site-specific oligosaccharide heterogeneity is one of the most important biological features of cell surface and extracellular glycoproteins ***(23–26)***. In general, the outer glycans of glycosphingolipids, which typically are comprised of saccharides covalently attached to the lipid ceramide, resemble those of glycoproteins, and sometimes share similar recognition functions ***(27–29)***.

1.3. Intracellular Glyconjugates Have Simpler Glycans

Until recently, dogma in textbooks dictated that nuclear and cytosolic proteins were not glycosylated. However, we now realize that many (perhaps most?) of these intracellular proteins are dynamically modified by single

Table 1
Branching and Anomericity of Saccharides Generates Enormous Structural Diversity

	Number of linear oligomers of length *N*			
			Oligosaccharides	
N	DNA	Proteins	*N* = 4	*N* = 8
1	4	20	4	8
2	16	400	128	800
3	64	8000	4096	6.4×10^4
6	4096	6.4×10^7	1.34×10^8	3.27×10^{10}
10	1.04×10^6	1.28×10^{13}	1.4×10^{14}	1.34×10^{18}

N-acetylglucosamine moieties at specific serine or threonine hydroxyls (termed *O*-GlcNAc, *see* **Fig. 3**) ***(30)***. *O*-GlcNAc is not elongated to more complex structures, but is simply rapidly added and removed to proteins in a manner similar to protein phosphorylation. Stoichiometry of protein modification by *O*-GlcNAc ranges from less than one sugar per mole of polypeptide to proteins with more than 15 mol of sugar per mole of protein. Many *O*-GlcNAc proteins are modified at numerous sites, each of which is substoichiometrically occupied at any point in time, making separation of glycoforms and subsequent structural analyses very difficult. Recent data suggest that *O*-GlcNAc is as abundant as protein phosphorylation, and may be important to numerous cellular processes. Genetic knockouts have shown that *O*-GlcNAc is essential to the life of single cells and to mammalian ontogeny. Despite its potential biological importance, *O*-GlcNAc presents a formidable challenge to the analyst, as addition of the sugar generally does not affect polypeptide behavior in most of the commonly used separation methods such as, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), reverse-phase high-performance liquid chromatography or other chromatographic techniques, and the current methods of detection of the saccharide are insensitive ***(31)***. In contrast, capillary electrophoresis is readily capable of resolving unmodified and *O*-GlcNAcylated peptides, and with laser-induced fluorescent detection methods, may provide the sensitivity needed to study the glycosylation of low-abundance regulatory molecules ***(32)***.

Evidence is emerging for the presence of more complex glycoconjugates within the nucleoplasm and cytoplasm. For example, glycogenin is a glycoprotein glucosyltransferase that serves to prime glycogen synthesis by self-glucosylation of a tyrosine hydroxyl ***(33,34)***. Marchase and colleagues have shown that a key enzyme in energy metabolism, phosphoglucomutase, is *O*-mannosylated by a saccharide that is further modified by the attachment of

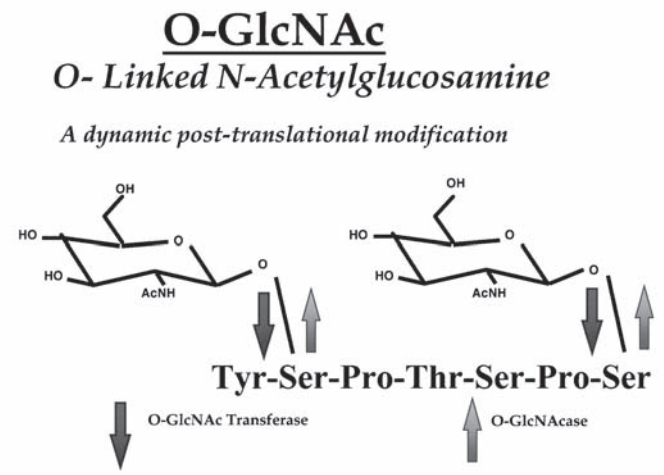

Fig. 3. *O*-Linked *N*-acetylglucosamine is a dynamic modification found exclusively in the nucleoplasmic and cytoplasmic compartments of cells.

α-glucose-1-phosphate ***(35–37)***. West and co-workers have shown that a cytosolic *Dictyostelium* protein that is involved in cell-cycle regulation is modified at hydroxy proline residues by complex oligosaccharides of the type Galα1-6-Galα1-Fucα1-2Galβ1-3GlcNAc-(HyPro) ***(38)***. Raikhel and co-workers have detected *O*-GlcNAc oligosaccharides attached to plant nuclear pore proteins ***(39 40)***, and recently sialic acid containing oligosaccharides were suggested to be on some mammalian nuclear pore proteins. Many studies, even as early as 1964, presented data supporting the presence of glycosaminoglycans within the nucleus and cytoplasm ***(41–43)***. However, these findings remain controversial in the mainstream proteoglycan community. Clearly, researchers studying intracellular processes, such as the cell cycle, transcription, nuclear transport, or cytoskeletal assembly, can no longer afford to be blissfully ignorant of protein glycosylation.

1.4. Classification of Glycolipids and Glycoproteins

The major glycoconjugates in higher eukaryotes are classified as shown in **Table 2** (*see* **ref.** ***1*** for review). This classification is somewhat arbitrary because many glycoconjugates may contain more than one type of saccharide component covalently attached. For example, many glycoproteins contain *N*-linked saccharides, *O*-linked saccharides, and a glycosylphosphatidylinositol (GPI) anchor. Glycoproteins are classified further based on the major type of linkage between the saccharide and the polypeptide backbone.

1.5. Factors Regulating the Attachment of Glycans to Lipids and Proteins

Even though the glycan moieties of complex glycoconjugates are not themselves directly encoded within the genomes of organisms, we now realize that the covalent glycan modifications of lipids and proteins at specific sites are carried out with high degrees of regulation and fidelity by specific glycosyltransferases. There is generally one type of glycosyltransferase activity for every specific carbohydrate–protein linkage known ***(44–46)***. However, molecular biological analyses have shown that there are also a very large number of different glycosyltransferase genes encoding enzymes that catalyze very similar reactions, but that display unique developmental expression and regulation. The sequential combined action of several glycosyltransferases to produce complex saccharides is controlled not only by the expression of the enzymes, but also by sugar nucleotide levels, protein synthetic and transport rates, protein folding rates, and by the regulated compartmentalization of both substrates and enzymes ***(47,48)***. Thus, unlike the structures of polypeptides or nucleic acids, which are "hard-wired" by the genetic makeup of the cell, the structures of complex glycans on proteins and lipids dynamically reflect the metabolic and developmental state, as well as the environment of the cell in which the glycoconjugate is made.

The responsiveness of the cell's "glycosylation machinery" to metabolism and environment provides a powerful mechanism of "fine-tuning" macromolecular structures for cell-specific biological functions. However, the inherent structural diversity of glycan structures and their highly varied physical properties also represent a formidable challenge to traditional separation technologies developed primarily for polypeptides and nucleic acids. Thus, elucidation of the structure/functions of complex glycoconjugates will require the development of new high-resolution, high-sensitivity analytical methods. Recent developments in capillary electrophoretic methods, as described in this book, represent a potential breakthrough in our ability to characterize small amounts of biologically important glycoconjugates ***(49–59)***.

2. Glycolipids

Glycosphinoglipids (GSLs), which are made up of glycans covalently attached to ceramide, are the most common type glycolipid in eukaryotes ***(29,60)***. Other types of glycolipids include rare glycosylated glycerolipids and free glycosyl inositol phospholipids (GIPLs; *see* **Subheading 3.5.**). GIPLs have mainly been studied in protozoan parasites, but are present in mammals. They appear to either be biosynthetic intermediates for GPI anchors or they may serve as signaling molecules ***(61–64)***.

Glycosphingolipids function in many biological processes in a manner similar to glycoproteins. They are blood group and tumor-specific antigens, they serve as receptors for microorganisms and toxins, and they mediate numerous cellular interactions. Recently, GSLs have been found to play an important role in growth regulation by modulating the activities of transmembrane receptor kinases. The abundance of GSLs varies considerably with the type of membrane. GSLs represent 5–10% of the total lipid in the erythrocyte membrane, as much as 30% of the total lipid of neuronal membranes, and are virtually absent in mitochondrial membranes.

GSLs are amphipathic molecules, and unlike glycoproteins or glycopeptides are readily analyzed by simple high-resolution chromatographic techiques, the most common of which is thin-layer chromatography. Glycosphingolipids are also comparatively very well behaved in mass spectrometric analyses.

2.1. Glycosphingolipid Structural Variability

As indicated in **Subheading 2.**,GSLs are composed of glycans glycosidically linked to ceramide. Ceramide is comprised of a long-chain amino alcohol, sphingosine, to which fatty acids are attached by an amide linkage. In mammalian GSLs, the glycan structures on GSLs typically range in size from one to ten monosaccharides, with some being much larger. There is also considerable variability in the structures and lengths of the fatty acid substituents, depending on the tissue, cell-type, and species of origin (**Fig. 4**). Acidic GSLs include the ganglio series, which contain sialic acids and the sulfatides, which often contain sulfate esters attached to galactosylceramides. Neutral GSLs range from those containing only one monosaccharide, such as globosides, to those containing variable length repeating structures such as the lactoside and globoside series. The structural variability of the glycan portions of GSLs is very large and rivals that seen for the glycosylation of proteins. In fact, glycoproteins and GSLs have many of the same terminal saccharide structures *(27)*. Unlike glycoproteins which display enormous numbers of glycan structures at a single glycosylation site, even when made by clonal cell populations *(23,65,66)*, each glycan structure on a GSL is classified as a different species. Given that the amphipathic character of GSLs greatly facilitates their separation and study, recent methods for the study of the glycans on glycoproteins have resorted to first releasing the glycans from the protein and chemically converting them to so-called “neoglycolipids” prior to study. Formation of neoglycolipids from released glycans not only improves the chromatographic or electrophoretic behavior of the glycans, but also allows for the introduction of fluorescent or charged residues which greatly facilitate physical separations and detection.

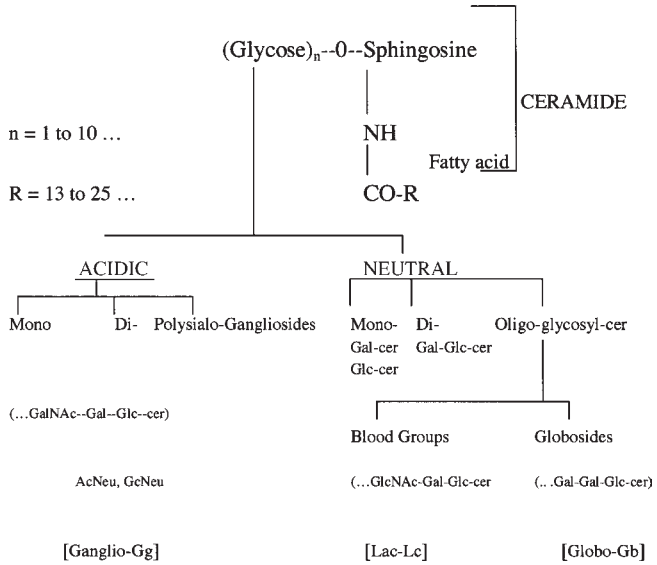

Fig. 4. Classification of glycosphingolipids according to their glycan structures.

Table 2
Major Types of Glyconjugates

Glycoproteins: Asn-linked; GalNAc-Ser(Thr); GlcNAc-Ser(Thr); collagens; glycogen
Proteoglycans: Many diverse types; contain one or more glycosaminoglycans
Glycosphingolipids: Glycosylated ceramides: gangliosides; neutral GSLs, sulfatides
Phosphatidylinositol Glycans: GPI anchors; free GPIs

3. Glycoproteins

As mentioned previously, glycoproteins are classified by how the major saccharide side chain is attached to the polypeptide core (**Table 2**). While most is known about the biosynthesis, structures and functions of the asparagine-linked (*N*-linked) glycoproteins ***(67)***, it is clear that the "mucin-type" *O*-linked glycoproteins, which contain saccharides linked via *N*-acetylgalactosamine to serine or threonine (GalNAc-Ser[Thr]) residues, are likely as abundant, and just as important to many biological processes, including the trafficking of blood cells, and defenses against microorganisms. Collagens are among the most abundant glycoproteins and represent the only common example of glycosylated hydroxylysine residues in higher organisms. Of the collagen types, those species found enriched in basement membranes are the most heavily glycosylated ***(68)***.

3.1. Mucin-Type O-Glycans

The complex glycans derived from mucin-type glycoproteins can readily be released from the protein by alkali-induced β-elimination ***(69–72)***. However, due to "peeling" reactions that destroy the saccharides from the newly exposed reducing end, these eliminations must generally be performed in the presence of a reducing agent such as borohydride, complicating the easy modification of the released saccharides with chromophores. Fortunately, as described in later chapters, methods such as hydrazinolysis ***(73–75)*** have circumvented these problems. Analysis of mucin-type saccharides has also been slowed by the lack of a nondelective enzyme that will release intact *O*-glycans from proteins, as exists for *N*-linked glycans (e.g., peptide:*N*-glycosidase F) ***(76,77)*** and GSLs (glycoceramidase) ***(78)***. *O*-glycanase, which is commercially available, is unfortunately specific only for Galβ1–3-GalNAc-Ser(Thr) structures and will not release glycans from more complex *O*-linked glycoproteins ***(79)***.

GalNAc-Ser(Thr)-linked saccharides have been most well studied on mucins, which contain a very heterogeneous population of clustered regions of short saccharides that often terminate in sialic acids. The protein core and saccharide modifications on mucins are different for each cell type in which they are made, and molecular biological studies have now described several distinct types of core proteins ***(18,80–82)***. Most of these core proteins have regions rich in proline, serine, threonine, glycine, and other amino acids that give rise to distinctive mucin-like motifs. These motifs are often very extensively glycosylated. Such mucin regions form rigid rod structures in solution owing to the close spacing of bulky hydrophilic groups and negative charges along their backbone. Mucins not only serve to lubricate epithelial surfaces and protect them from desiccation, but also, owing to their almost infinite structural diversity, they serve as "decoy" binding sites for pathogenic microorganisms, protecting host cells from invasion. Heavily glycosylated mucin domains also serve a structural role in many receptors by creating a rigid rod domain that allows the business end of the receptor to be displayed above the glycocalyx of the cell ***(83)***. Given their comparatively small size, enormous diversity, and the ability to be derivatized at their reducing termini, capillary electrophoresis should prove to be a valuable tool in the study of these important but largely neglected class of glycoproteins.

3.2. O-Linked N-Acetylglucosamine

The dynamic modification of nuclear and cytosolic proteins by *N*-acetylglucosamine at specific serine and threonine residues (termed *O*-GlcNAc, **Fig. 3**) is now known to be ubiquitous and abundant in virtually all eukaryotic cells ***(30,84)***, with the possible exception of baker's yeast. *O*-GlcNAc has not yet been described in prokaryotes and does not appear to

Table 3
Identified *O*-GlcNAc Proteins

Nucleus
Nucleoporins
RNA polymerase II
Transcription factors: TBP, SP1, SRF, IPF-1
Kinases and splicing proteins: CK2 and SRs
Nuclear oncoproteins: *c*-Myc, v-Erb, SV40
Estrogen receptors: α and β
Tumor suppressors: Rb, p53
Many chromatin proteins: polytene
Fungal DNA binding, tyrosine phosphatase
Cytoplasm
Intermediate filaments: cytokeratins, neurofilaments
Bridging proteins: talin, vinculin, ankyrin, synapsins, 4.1
Microtubule-associated proteins: (MAPS): tau
Clathrin assembly protein
Many synapse and neuron proteins: APP
Small heat shock proteins
Signaling proteins: Raf
Many viral and parasite proteins

occur in lumenal or extracellular compartments, locations where other forms of glycosylation predominate. *O*-GlcNAc is found on myriad proteins in the nucleus. As summarized in **Table 3**, many important regulatory proteins are dynamically modified by *O*-GlcNAc. *O*-GlcNAc is added to proteins by the *O*-GlcNAc transferase ***(85,86)***, which has been recently shown to be essential for single cell viability. *O*-GlcNAc is removed by *O*-GlcNAcase, one of which has been characterized ***(87)***. These two enzymes are analogous to kinases and phosphatases, respectively, for phosphorylation. In several cases, *O*-GlcNAcylation and phosphorylation are reciprocal events, suggesting a "yin–yang" relationship in terms of biological functions ***(88)***. Current evidence suggests that *O*-GlcNAcylation may play an important role in the regulation of transcription, translation, nuclear transport, cytoskeletal assembly, the cell cycle, diabetes, and in the regulation of protein turnover.

Biochemical analyses of *O*-GlcNAcylation is complicated by the low abundance and rapid turnover of most regulatory proteins, the low stoichiometry of *O*-GlcNAc at individual sites, and the lack of sensitive detection methods. As mentioned earlier, most currently used separation methods do not detect the addition and removal of *O*-GlcNAc on most proteins. In addition, *O*-GlcNAc is very labile, both due to the abundance of *N*-acetylglucosaminidases in cells,

and the chemical/physical stability of the linkage itself. For example, it is difficult to detect *O*-GlcNAcylation even by mass spectrometry (MS). In electrospray techniques, the saccharide is readily cleaved at commonly used orifice voltages and is almost always lost prior to peptide fragmentation, making MS/MS site mapping problematic. However, prior β-elimination of the saccharide followed by electrospray mass spectrometry has allowed for direct site mapping ***(89,90)***. In matrix-assisted laser desorption (MALDI) mass spectrometric methods the presence of the GlcNAc moiety typically lowers the sensitivity of detection by at least five fold compared to that for the unmodified peptide. In mixtures, suppression of the glycopeptide signals by unmodified peptides makes analyses even more difficult. Generally, reverse-phase HPLC does not readily resolve *O*-GlcNAc modified and unmodified peptides, but this depends a great deal on the relative hydrophobicity of the peptide to which the sugar is attached. In contrast, under the right conditions, capillary electrophoresis has the resolving power to readily separate *O*-GlcNAc, *O*-phosphate, and unmodified peptides from each other ***(32)***. We anticipate that the combined use of capillary electrophoresis and nanospray MS will play an important role in elucidating the functions of *O*-GlcNAc on many key regulatory proteins.

3.3. N-*Glycans*

Asparagine-linked (*N*-linked) glycans are the most extensively studied form of protein glycosylation ***(67)***. *N*-glycans are attached to nascent polypeptides as they enter the lumen of the rough endoplasmic reticulum (RER) at specific asparagine residues in the sequon Asn-X-Ser(Thr), where X can be almost any amino acid, but generally is not proline or aspartate. In the RER, a large oligosaccharide, $Glc_3Man_9GlcNAc_2$-, is transferred directly to the protein *en bloc* from a C_{95} isoprenoid lipid donor, dolichol phosphate. The oligosaccharyl dolichol phosphate donor substrate is preassembled in the RER. The enzyme complex that accomplishes the transfer of the oligosaccharide to the protein is the oligosaccharyl transferase ***(91)***. Immediately after transfer to the nascent chain, an unusual processing of the *N*-glycan begins in which the outer glucose residues and mannose residues are enzymatically removed as the protein is transported through the secretory pathway ***(20)***. We now realize that the glucose residues are part of an exquisite quality control mechanism involving the glucose binding lectins calreticulin and calnexin ***(92,93)*** and reglucosylation by an "unfolded protein" specific glucosyltransferase ***(94,95)*** that together prevent misfolded proteins from leaving the RER. On entering the Golgi, typically, trimming of the *N*-glycans reaches a branch point at the oligosaccharide $Man_5GlcNAc_2$, where if the saccharide is acted on by *N*-acetylglucosaminyltransferase I, it will be processed further to become a complex *N*-glycan, containing outer sugars such as galactose and sialic acids. If the *N*-glycan

is not acted upon by the *N*-acetylglucosaminyltransferase, it will remain a "high-mannose" type saccharide.

A characteristic feature of *N*-glycans is their extensive branching which is controlled by a number of specific *N*-acetylglucosaminyltransferases ***(96,97)***. Perhaps the most important aspect of *N*-linked glycosylation in terms of biology is site-specific oligosaccharide microheterogeneity. On most populations of a glycoprotein, there can be many different glycan structures at a single site, even though the amino acid sequences are identical in the population. The amount and distribution of these glycoforms are highly reproducible depending on the growth conditions of the cell and the glycoforms are usually cell-type specific (glycotypes). It appears that the purpose of the elaborate biosynthetic/processing pathway for *N*-linked glycoproteins is not only to regulate trafficking and folding, but also to allow the cell to structurally remodel the proteins it is synthesizing in response to its environment and developmental state.

3.4. Proteoglycans

By definition, a proteoglycan is any polypeptide that contains one or more glycosaminoglycan (GAG) side chains ***(98–100)***. Clearly, many proteoglycans also contain other types of sugar modifications. GAGs are long linear polymers composed of repeating disaccharide sequences typically containing an amino sugar and a uronic acid (except for keratan sulfates, which contain an amino sugar and galactose residues). Except for hyaluronic acids, GAGs are also extensively modified by sulfate esters. **Table 4** summarizes the major types of GAGs and their linkage to protein. Virtually every imaginable type of proteoglycan has been found in various cell types. Some proteoglycans are membrane proteins with only one or a few GAG chains, whereas others are secreted molecules with more than 100 different GAG and other saccharide modifications. Many of the proteoglycan core proteins have been cloned and characterized, yet we still know little about the detailed structures of the intact molecules of even the simplest proteoglycans. Proteoglycans are important structural components, they serve to regulate development and fibrillogenesis of collagen, and they regulate growth hormone functions. Capillary electrophoresis is playing an important role in the structural elucidation of GAG chains, particularly with respect to the separation of GAG fragments produced by controlled chemical or enzymatic degradations ***(101,102)***.

3.5. GPI Anchors

Until the mid-1980s it was widely believed that most integral membrane proteins were anchored to the lipid bilayer by stretches of hydrophobic amino acids. Initially studies with phospholipases ***(103)*** suggested that some proteins were anchored by covalently attached lipid components. Structural studies in

Table 4
Classification of Glycosaminoglycans

GAG	Mol Wt.	Repeating disaccharide $(A–B)_n$ Monosaccharide A	Monosaccharide B	Sulfate per disaccharide
Hyaluronic acids	4000–8×10^6	D-Glucuronic acid	*N*-acetylglucosamine	0
Chondroitin sulfates	5000–50,000	D-Glucuronic acid	*N*-Acetylgalactosamine	0.2–2.3
Dermatan sulfates	15,000–40,000	D-Glucuronic acid or L-Iduronic acid	*N*-Acetylgalactosamine	1.0–2.0
Heparan sulfates	5000–12,000	D-Glucuronic acid or L-Iduronic acid	*N*-Acetylglucosamine	0.2–2.0
Heparin	6000–25,000	D-Glucuronic acid or L-Iduronic acid (mostly)	*N*-Acetylglucosamine	2.0–3.0
Keratan sulfates	4000–19,000	D-Galactose	*N*-Acetylglucosamine	0.9–1.8

parasites documented that certain proteins are anchored to the membrane by GPI anchor structures ***(104)*** at their C-termini. **Figure 5** summarizes the structure of a GPI anchor and illustrates the growing structural heterogeneity that has been found in various organisms and cell types. The GPI anchor is assembled in the RER by first attaching GlcNAc to phosphatidylinositol ***(105)***. The GlcNAc is deacetylated and the mannosyl core is added. Ethanolamine phosphate is attached using phosphatidylethanolamine as the donor. Proteins to receive a GPI anchor have a hydrophobic signal sequence at their C-terminus, which serves to temporarily anchor them to the RER membrane. A transpeptidase cleaves the signal sequence and concomitantly transfers the peptide to the lipid anchor. Outer sugars, such as galactose, are added to the anchor in the Golgi (for review, *see* **ref. *106***). GPI anchors are another remarkable example of how important posttranslational modifications can be completely overlooked. In fact, it is now clear that most membrane proteins in protozoans are anchored by GPI anchors ***(107)***, whereas the majority of membrane proteins in eukaryotes are anchored by hydrophobic peptides. There are also several examples of proteins that are bound to the membrane by both GPI anchors and by peptide sequences, depending on RNA splicing. While there has been much speculation about the purpose of GPI anchors in terms of membrane mobility, role or lack thereof in signaling, and in the controlled release of proteins, the functions of this mode of attachment remain unclear ***(108)***.

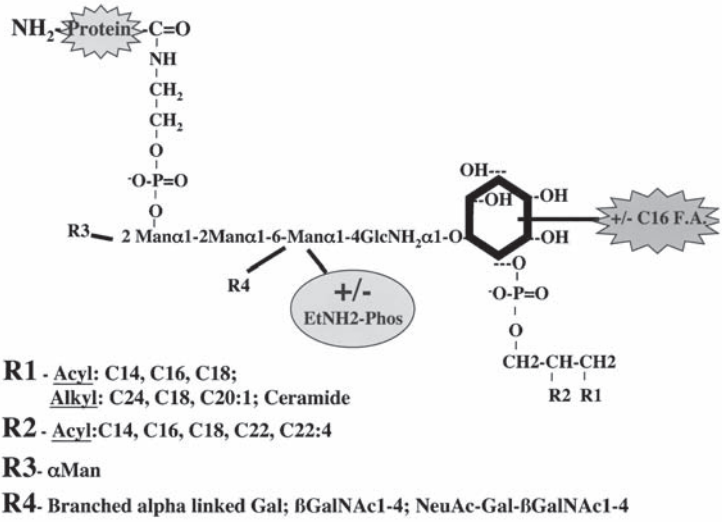

Fig. 5. Illustration of the structural diversity of GPI anchors.

4. Conclusions and Generalizations

In recent years, we have come to appreciate that most eukaryotic proteins are covalently modified by the attachment of sugars. Glycobiology, which is now the name of the field attempting to elucidate the structural/functional importance of protein glycosylation, has become one of the most rapidly growing areas of biochemistry and cell biology. The enormous structural diversity of complex glycans potentially allows the cell to express vast amounts of biological information. Indeed, glycoconjugates are critical molecules in virtually every biological process in eukaryotic organisms, including almost every infectious and noninfectious disease afflicting mankind. Protein-bound saccharides are thought to modify or fine-tune a protein's functions at the structural level. However, unlike proteins or nucleic acids, which are genetically encoded, the structures of glycans are highly responsive to, and dependent on, both the metabolic and developmental state of a cell. The study of glycoproteins has, until recently, been hindered by the inherent complexities and structural diversity of the molecules themselves, by the lack of tools for their study at the structural level, and by a lack of knowledge about multicellular systems in which many of the functions of protein-bound glycans reveal themselves. For convenience, the reader is referred to the Appendix of this book for a description of the structures of typical mono-, oligo-, and polysaccharides found in prokaryotic and eukaryotic cells. Many of the methods described herein provide much needed approaches toward our better understanding of these enigmatic molecules.

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2

Chemical and Enzymatic Release of Glycans from Glycoproteins

Tony Merry and Sviatlana Astrautsova

1. Introduction

The majority of proteins are posttranslationally modified, and the most significant modification to many secreted and membrane-associated proteins of eukaryotic cells is glycosylation, that is, the attachment of one or more oligosaccharide (glycan) chains. Glycans may be attached to the peptide backbone through different types of linkage but they usually are subdivided into those attached to glycoproteins primarily through an amide linkage to asparagine residues (*N*-linked glycans), and those attached through an *O*-glycosidic linkage to serine or threonine residues (*O*-linked glycans) or where the carbohydrates form part of a glycosylphosphatidyl inositol moiety (GPI) attached to the C-terminus of the peptide. Other types of linkage occur in certain other glycoconjugates such as the linkage to hydroxylysine residues in collagen and β-xylose of glycosaminoglycan chains in proteoglycans to serine residues in the peptide core.

The structural diversity of glycans attached to proteins ***(1)***, as well as the fact that each glycosylated polypeptide is generally associated with a population of different glycan structures ***(2)*** leads to the considerable glycosylation heterogeneity observed in many glycoproteins. With current techniques the analysis is generally not possible on the intact glycoprotein. For this reason oligosaccharide analysis is performed mainly following release of the oligosaccharides from the polypeptide. A number of important considerations need to be taken into account regarding the release procedure, and the following criteria may be set:

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1. Release should be nonselective with regard to the types of glycan; otherwise a representative profile will not be obtained.
2. The release should cause no modification of the glycan.
3. It should be suitably efficient to allow recovery of sufficient material for study of the chosen sample.
4. The peptide material should be separated from the released glycans.

An additional consideration is that a free reducing terminal will make subsequent derivatization for analysis of the glycans much simpler and is very desirable.

Techniques for glycan release have been devised based on either an enzymatic or a chemical procedure. Each type of technique has its own merits, and the choice of technique will depend on such factors as the type of glycosylation present and the nature and amount of the sample. In this chapter we concentrate on the release of the *O*- and *N*-linked and GPI-linked glycans attached to glycoproteins.

Historically, chemical methods have been used to release *O*- and *N*-linked oligosaccharides. A number of chemical techniques for release have been described and used for several years but principally those most commonly used are hydrazinolysis and alkali/reducing conditions (β-elimination) ***(3,4)***. The use of anhydrous hydrazine for release of *N*-linked glycans was developed mainly by the group of Kobata ***(4)*** and has now been applied to the analysis of a large number of glycoproteins by many groups. It is thus a well established and validated technique. More recently it has been shown ***(5,6)*** that the technique may be modified for the release of *O*-glycan structures.

In the last two decades, a growing repertoire of enzymes, including endoglycosidases and glycosamidases, able to release glycoprotein oligosaccharides under mild conditions have been available. The use of these enzymes enables convenient and nonselective release of *N*-linked oligosaccharides from glycoproteins. Some of these have a high degree of specificity with respect to the type of *N*-linked oligosaccharides released. These have been well characterized and some of them have been cloned ***(3)***. The specificity may cause problems; for example, endoglycosidases able to release *O*-linked sugars exhibit very restricted substrate specificity that limits their use.

In the cases when the protein is difficult to purify or when there are limited amounts of sample, the *N*-glycan may be released directly from a band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel or a spot on two-dimensional electrophoresis using peptide *N*-glycosidase F (PNGase F) ***(7)***. Following release, sequential exoglycosidase digestion using highly specific enzymes can be used for simultaneously sequencing the glycan in a standard panel of enzyme arrays, with analysis of the product using high-performance liquid chromatography (HPLC). The new approaches involve the digestion of aliquots of a total pool of oligosaccharides (flourescently labeled)

with a series of multiple enzyme arrays ***(8,9)***. New techniques for the oligosaccharide chain-cleaving enzymes are constantly developed using bacterial cells as a source.

The first described endo-β-*N*-acetylglucosaminidase able to release *N*-linked glycans from glycoproteins was endo D, isolated from the genus *Diplococcus*. The specificity of this enzyme is, however, relatively narrow compared to several other endoglycosidases applied for *N*-linked glycan release and it is not commercially available.

Four oligosaccharide chain-cleaving enzymes have been identified and purified to homogeneity from cultural filtrates of *Flavobacterium meningosepticum*: endoglycosidase F_1, F_2, and F_3 and the amidase peptide-N^4, *N*-acetyl-β-D-glycosaminyl-asparagine amidase (PNGase F) free from endo F and protease activity was isolated ***(10)*** and purified using direct fast protein liquid chromatography (FPLC)-controlled, hydrophobic interaction chromatography of the cultural filtrate on TSK-butyl and TSK-phenyl resins, followed by FPLC-developed, high-resolution sulfopropyl chromatography in >50% yield ***(11)***. Endo F_2 and endo F_3 were shown to represent new distinct endoglycosidases that prefer complex as compared to high-mannose asparagine as do glycans. Preliminary evaluation of the substrate specificity of these enzymes indicates that F2 cleaved biantennary oligosaccharides, whereas endo F_3 cleaved both bi- and triantennary oligosaccharides. Preparation of endoglycosidase from *Flavobacterium meningosepticum* are currently commercially available, generally being sold as "endoglycosidase F". These preparations are primarily endo F_1, with relatively small and variable amounts of endo F_2 and endo F_3 being present ***(12)***. Commercial suppliers include Europa Bioproducts, Ely, Cambridge, UK; Boehringer Mannheim (Indianapolis, IN); and Genzyme (Boston, MA).

Endoglycosidase H is in widespread use in glycoprotein research. This enzyme is active on *N*-linked glycoprotein oligosaccharides and is highly stable. It is able to release all high-mannose type oligosaccharides from glycoproteins, as well as most hybrid types of oligosaccharides ***(13–15)***. The utility of endo H is perhaps best realized in working with glycoprotein substrates that are known not to contain complex oligosaccharides ***(15)***. Endo H is commercially available from a number of sources, including Boehringer Mannheim (Indianapolis, IN) and; Genzyme (Boston, MA).

A variety of eukaryotic endoglycosidases specific for *N*-linked glycoprotein oligosaccharides have also been reported ***(3)***. The cellular slime mold *Dictyostellium disoideum* produces an endoglycosidase, termed endo S ***(16)***. Several fungal endoglycosidases were isolated from *Mucor heimalis*, termed endo M ***(17)***, and *Sporotrichum dimorphosporum*, termed endo B ***(18)***. Endo M and endo B are similar to endo F_2: they cleave high-mannose and some complex type oligosaccharides. Endoglycosidases have been also reported in higher plants and mammals ***(19–21)***.

The endoglycosidases capable of cleaving glycoprotein glycans at the *N*-acetylglucosamine–asparagine linkage are less susceptible to the steric hindrance sometimes found for glycoamidase ***(3)***. The endoglycosidases described in the foregoing cannot cleave tetraantennary glycans. To hydrolyze this class of *N*-linked oligosaccharide a different type of glycan releasing enzyme, the glycosamidases, may be applied ***(22)***. These enzymes cleave *N*-linked oligosaccharides directly between the asparaginyl residue and the reducing end *N*-acetylglucosamine residue of the glycan by cleavage of the amide linkage. In this reaction, the asparaginyl residue is converted to an aspartyl residue with consequent oligosaccharide releasing in the form of glycosylamine. These enzymes are known as glycopeptidases, peptide: *N*-glycosidases, PNGases, *N*-glycohydrolases, and *N*-glycanases. They are very similar in their substrate specificity, as they cleave all types of *N*-linked glycoprotein oligosaccharides—high-mannose, hybrid, and complex. Enzymatic release of oligosaccharides also provides the possibility to recover the protein part and to use this for analysis of the material, such as biological activity of deglycosylated protein, its functioning, and other properties ***(26)***.

The release of *O*-linked carbohydrates using enzymes is problematic. The enzymes will cleave only the disaccharide Gal-β-1,3-GalNAc *O*-linked structures ***(23,24)***. This structure can be cleaved by endo-GalNAc-ase D and endo-GalNAc-ase A, but these enzymes are active only toward the unmodified disaccharide and will not cleave the sialylated derivatives that are commonly found. *O*-linked oligosaccharides are quite variable in structure and are often extended with additional sugars ***(23–25)*** and therefore the use of the current so-called "*O*-glycanase" is of very limited value.

1.1. Points to Consider—Chemical Release

1. Chemical release will generally degrade the protein.
2. Release is frequently affected by salts and detergents.
3. Chemicals may be hazardous to handle.
4. The removal of byproducts may be difficult and samples generally require cleanup before analysis.
5. Chemical methods require specialized apparatus and knowledge.
6. The use of highly reactive material is required and may cause modification of the released glycan.

1.2. Points to Consider—Enzymatic Release

1. There may be problems of steric hindrance in the reaction of the enzyme with the glycoprotein.
2. Denaturation may be required and detergents can often interfere with subsequent labeling and analysis.
3. Selective release of more accessible glycans can occur.

4. Release may be more efficient with glycopeptides.
5. Efficient techniques for separation of the deglycosylated protein from the glycans must be used.
6. Frequently the deglycosylated protein is much less soluble and may precipitate.

2. Materials

2.1. Enzymatic Release by PNGase F and In Gel Release

1. Plastic 0.5- and 1.5-mL Eppendorf tubes washed in distilled water and dried.
2. PNGase F (either recombinant or natural form) 100 U/mL in 20 m*M* $NaHCO_3$, pH 7.0 (*see* **Note 1**). (This enzyme is frequently referred to as *N*-Glycanase, a trademark of Glyko Inc., Novato.)
3. 50 m*M* Ammonium formate, pH 8.6 (prepare by adding formic acid to ammonium hydroxide—titrate to pH 8.6), 0.4% SDS.
4. 1.2% (CHAPS), 0.1 *M* EDTA (add 2% dithiothreitol [DTT] before use).
5. Distilled water (*see* **Note 2**).
6. 20 m*M* $NaHCO_3$, pH 7.0.
7. 45 m*M* DTT.
8. 100 m*M* Iodoacetamide.
9. Dowex AG50X12 (H^+ form) (*see* **Note 3**).
10. Biogel P2 gel-filtration resin (Bio-Rad, Hercules, CA).
11. Microcon 10 concentrator (Amicon, Beverly, MA).
12. Toluene.

2.2. Endoglycosidase Release

1. 1 U/mL Endoglycosidase H (*see* **Note 4**) in 50 m*M* sodium phosphate buffer, pH 7.0.
2. 20,000 mU/mL Endoglycosidase F_1 (*see* **Note 5**) in 50 m*M* sodium acetate buffer, pH 6.0.
3. 50 m*M* Sodium citrate phosphate buffer, pH 7.0: 25 m*M* EDTA, 0.1% sodium azide.
4. 1.0 *M* Sodium acetate buffer, pH 6.0 (stock diluted 1:5 v/v to 200 m*M*).
5. Protein binding membrane.

2.3. Hydrazinolysis

1. Dialysis membranes, 10,000 mol wt cutoff (Gibco-BRL, Bethesda, MD).
2. Trifluoroacetic acid (TFA).
3. Anhydrous hydrazine (Ludger Ltd., Oxford, UK) (*see* **Note 6**).
4. Toluene.
5. Sodium hydrogen carbonate.
6. Acetic anhydride.
7. Chromatography paper.
8. Butanol–ethanol–water mixture (4:1:1, by vol).
9. Butanol–ethanol–water mixture (8:2:1, by vol).
10. Polytetrafluoroethylene (PTFE) filters.

2.4. Automated Hydrazinolysis

1. GlycoPrep 1000 automated hydrazinolysis system (Oxford GlycoSciences Ltd., Abingdon, UK).
2. Reagents and column sets (Oxford GlycoSciences Ltd., Abingdon, UK).
3. Rotavap or equivalent drying system for drying down aqueous samples ≤ 5.0 mL.

2.5. Release of GPI Anchor from Blotted Glycoproteins

1. HPLC-grade water.
2. Screw-top Eppendorf tubes.
3. 0.5-mL Microtubes (BDH-Merck, Poole, UK).
4. 48% Aqueous hydrofluoric acid (HF), Aristar-grade (BDH-Merck, Poole, UK). Store in 0.5-mL aliquots in Eppendorf tubes at –20°C. **Caution:** Highly corrosive.
5. Dewar container.
6. Access to a freeze-drying apparatus.
7. Access to a sonicating waterbath.
8. 0.3 *M* Sodium acetate buffer, pH 4.0. Prepare by titrating 0.3 *M* sodium acetate solution to pH 4.0 with glacial acetic acid. Stable at room temperature for several months.
9. 1.0 *M* Sodium nitrite. Always prepare freshly just before use.
10. C8 and NH_2 Isolute™ cartridges (IST, Mid-Glamorgan, UK).
11. Methanol, HPLC grade (BDH-Merck, Poole, UK).
12. Dowex AG5OX12, 200–400 mesh (Bio-Rad, Hemel Hempstead, UK), converted to the H^+ form by washing with >10 vol 1 *M* HCl and >20 vol water Store with an equal volume of water at 4°C .
13. Dowex AG3X4, 200–400 mesh (Bio-Rad, Hemel Hempstead, UK), converted to the OH– form by washing with >10 vol 1 of *M* NaOH and >20 vol of water. Store with an equal volume of water at 4°C .
14. Access to a SpeedVac or rotary evaporator.
15. Signal™ 2-AB labeling kit (Ludger Ltd., Oxford, UK).
16. Access to a heating block.
17. 3MM Whatman paper.
18. Small chromatography tank with rack.
19. Butan-1-ol–ethanol–water mixture (4:1:1, by vol). The paper chromatography tank should be lined with 3MM Whatman paper with some of the solvent in the bottom.
20. Access to a fume cupboard.
21. Long-wave ultraviolet (UV) lamp.
22. Access to a microcentrifuge.
23. Microcentrifuge filters (Sigma, Poole, UK).
24. 30% Acetic acid in water.
25. Acetonitrile, Aristar grade (BDH, Poole, UK).
26. Access to an SDS-PAGE system.
27. Polyvinylidene fluoride (PVDF) membrane (Amersham, Buckinghamshire, UK).

28. Access to a blotting apparatus (e.g., a semidry blotting apparatus from Hoefer Scientific Instruments, CA).
29. Amido black (Sigma, Poole, UK).
30. Razor blade.
31. Fluorescence detector (e.g., Gilson Model 121).
32. Access to a Microbore HPLC system, e.g., an Ultrafast Microprotein Analyzer (Michrom Bio Resources, CA).
33. Liquid nitrogen.
34. Powder-free gloves.
35. Dextran, grade C (BDH-Merck, Poole, UK).

3. Methods

3.1. PNGase F Release and Recovery

Suitable for analysis of *N*-linked glycans where sufficient material is available for optimization.

3.1.1. Preparation of Glycoprotein for Release by PNGase F (Method 1)

Method using denaturation—use this if in doubt about complete release:

1. Isolate the glycoprotein according to your usual procedures.
2. The sample should be relatively salt-free and contain no extraneous carbohydrates (e.g., Sephadex-purified material contains large amounts of glucose) (*see* **Notes 7–9**).
3. If the volume of the glycoprotein solution required is >100 µL, dry the glycoprotein in a 1.5-mL microcentrifuge tube. Generally 50–200 µg of glycoprotein is required.
4. The incubation of a control glycoprotein with known glycosylation alongside experimental samples is recommended. Suitable proteins for this purpose are bovine serum fetuin, ribonuclease B, or haptoglobin (*see* **Note 10**).
5. Proceed with the enzymatic digestion as described in **step 7**.
6. Store remaining glycoprotein at 4°C for future use.
7. Dissolve sample in 50 µL 50 m*M* ammonium formate, pH 8.6; 0.4% SDS.
8. Incubate for 3 min at 100°C.
9. Cool and add 50 µL of CHAPS detergent buffer.
10. Add 0.2 U (2 µL) of PNGase F.
11. Incubate for 24 h at 37°C (add 5 µL of toluene to prevent bacterial growth).
12. Remove 5 µL and analyze the reaction mixture by SDS-PAGE (*see* **Note 11**).
13. If sample is completely deglycosylated proceed with **step 14** otherwise continue with incubation.
14. Filter samples through protein binding membrane or perform gel filtration (*see* **Note 12**).
15. Dry sample in a rotary evaporator.

3.1.2. N*-Linked Oligosaccharide Release by PNGase F (Method 2)*

Method without denaturation—only use if complete release has been confirmed.

1. Dry pure, desalted glycoprotein into a 2-mL screw-top Eppendorf tube.
2. Suspend glycoprotein in 200 µL of PNGase F digestion buffer.
3. Add 1 U/0.5 mg of glycoprotein of PNGase F (*see* **Note 13**) and 5 µL of toluene (to prevent bacterial growth).
4. Incubate at 37°C for up to 72 h.
5. Centrifuge the sample briefly, and transfer to a Microcon 10 concentrator.
6. Centrifuge the concentrator at 14,000*g* for 20 min to separate protein and oligosaccharide components.
7. Transfer the filtrate to a 2-mL Bio-Gel P2 column equilibrated with water in a glass Pasteur pipet (*see* **Note 14**).
8. Elute oligosaccharides with 800 µL of HPLC grade water (*see* **Note 8**).
9. Dry desalted oligosaccharides for further analysis.

3.1.3. Release from Polyacrylamide Gels with PNGase F

Suitable for analysis of low microgram amounts of protein or for unpurified proteins separated by SDS-PAGE or two-dimensional electrophoresis.

1. Run gel and remove top glass plate.
2. Cut out gel pieces with band of interest using a washed scalpel blade, keeping the piece as small as possible.
3. Put into 1.5-mL tubes and wash with 1 mL of 20 m*M* $NaHCO_3$, pH 7.0, twice using a rotating mixer, leaving for 30 min. Discard the washings.
4. Add 300 µL $NaHCO_3$, pH 7.0.
5. Add 20 µL of 45 m*M* DTT.
6. Incubate at 60°C for 30 min.
7. Cool to room temperature and add 20 µL of 100 m*M* iodoacetamide.
8. Incubate for 30 min at room temperature in the dark. Discard solution.
9. Add 5 mL of 1:1 acetonitrile–20 m*M* $NaHCO_3$ pH 7.0.
10. Incubate for 60 min to wash out reducing agents and SDS.
11. Cut gel into pieces of 1 mM^2.
12. Place in SpeedVac to dry.
13. Add 30 µL (3 U) of PNGase F in 20 m*M* $NaHCO_3$, pH 7.0
14. Allow gel to swell and then add a further 100 µL aliquot of buffer.
15. Incubate at 37°C for 12–16 h.

3.2. Endoglycosidase Release

Suitable for selective release of different classes of *N*-linked glycans.

1. Prepare solution of glycoprotein.
2. Make up 20,000 mU/mL of endoglycosidase F_1 or 1 U/mL of endo glycosidase H in appropriate buffer.
3. For incubation with endoglycosidase F_1 add 200 mU of enzyme solution (10 µL).
4. For incubation with endoglycosidase H add 40 mU (25 µL) of enyme solution.
5. Incubate at 37°C for 18 h.
6. Pass through a protein binding membrane.
7. Evaporate to dryness.

3.3. Hydrazinolysis

Suitable for analysis of *N*- or *O*-linked glycans in which the amount of protein is limited, steric hindrance to enzymatic release is known, or selective release of glycans by enzymatic means is suspected.

3.3.1. Preparation of Samples for Hydrazinolysis

1. Desalt the samples completely.
2. Dissolve the sample in 0.1% TFA in as small a volume as possible.
3. Set up dialysis at 4°C (*see* **Note 15**).
4. Dialyze for a minimum of 48 h.
5. Recover sample from dialysis membrane. Wash membrane with 0.1% TFA to ensure recovery.
6. Transfer to a suitable tube for hydrazinolysis.
7. Lyophilize the sample.
8. For *O*-glycan analysis further drying is recommended.
9. Remove sample from the lyophilizer immediately prior to addition of hydrazine.

3.3.2. Manual Hydrazinolysis Procedure

Suitable for analysis of *N*- and *O*-linked glycans when expertise and equipment for procedure are available.

1. Place sample (dialyzed against 0.1% aqueous TFA) in acid-washed glass tube.
2. Completely lyophilize the sample for 2 d.
3. Remove tubes from drying immediately prior to hydrazine addition.
4. Flush tube with argon, taking care not to dislodge lyophilized protein.
5. Rinse a dried syringe fitted with a stainless steel needle with anhydrous hydrazine and discard the liquid.
6. Take up fresh hydrazine and dispense onto the sample; 0.1 mL hydrazine is sufficient to dissolve up to 2 mg of glycoprotein. For larger amounts add more hydrazine.
7. Seal the tube.
8. Gently shake tube—the protein should dissolve.
9. Place in an incubator (use water bath).
10. For release of *N*-linked glycans incubate at 95°C for 5 h, for *O*-glycan release incubate for 60°C for 6 h.
11. Allow to cool and remove hydrazine by evaporation.
12. Add 250 µL toluene and evaporate; repeat 5 times.
13. Place tube on ice and add 100 µL saturated sodium bicarbonate solution.
14. Add 20 µL acetic anhydride.
15. Mix gently and leave at 4°C for 10 min.
16. Add a further 20 µL acetic anhydride.
17. Incubate at room temperature for 50 min.
18. Pass solution through a column of Dowex AG50X12 (H^+ form)—0.5 mL bed volume.
19. Wash tube with 4 × 0.5 mL water and pass through a Dowex column.
20. Evaporate to dryness. This should be done in stages by redissolving in decreasing volumes of water.

21. Prepare a 80 × 2 cm strip of chromatography paper (prewashed in water by descending chromatography for 2 d).
22. Spot sample on strip, and perform descending chromatography in 4:1:1 Butanol-Ethanol-water for 3 d (*N*-glycans) or 8:2:1 Butanol-Ethanol-water for 2 d (*O*-glycans).
23. Elute region from –1 cm to + 3 cm of origin with 4 × 0.5 mL water. Filter through PTFE filter and dry.

3.3.3. Automated Hydrazinolysis **(27)**

Suitable for the routine release of *N*- or O-linked glycans, e.g., for quality control and where a high degree of reproducibility between samples is required.

1. Prepare samples by dialysis against 0.1 *M* TFA at 4°C.
2. Transfer samples to GlycoPrep reaction vials (*see* **Note 17**).
3. Lyophilize the sample in reaction vials.
4. Set up GlycoPrep 1000 with new column sets and collection tubes.
5. Select the desired program.
6. Load samples from the lyophilizer.
7. Start the instrument run.
8. Samples in collection vials may be removed when the system wash protocol commences (*see* **Note 18**).
9. Dry the sample down on Rotavap or similar apparatus. It is necessary to divide the sample into smaller aliquots for drying in centrifugal evaporators.
10. The sample is then ready for direct analysis or for derivatization.

3.3.4. Small Scale GPI Release and 2-AB Labeling "On the Blot"—Procedure of Zitzmann and Ferguson **(28)**

Suitable for the analysis of GPI anchors attached to proteins separated by SDS-PAGE.

1. Apply 5 µg of protein (or an equivalent of at least 100 pmol) on a 10% polyacrylamide gel and subject to SDS-PAGE.
2. Transfer proteins from the gel to a PVDF membrane by electroblotting.
3. Stain the PVDF membrane with amido black, cut out the protein bands of interest using a razor blade, and transfer into screw-top Eppendorf tubes.
4. Deaminate the samples by completely submerging the blot strips in 50 µL of 0.3 *M* NaAc, pH 4.0, and 50 µL of freshly prepared 1 *M* sodium nitrite.
5. Wash the strips three times with water to remove salt, transfer into 0.5-mL Eppendorf tubes and dry.
6. Prepare 2-AB labeling reagent as described by manufacturer.
7. Take care to completely wet each blot strip with the labeling reagent (usually 15 µL are sufficient), cap the tubes, and label the strips for 2–3 h at 65°C in a heating block.
8. Wash the blot strips three times with about 10 mL of 50% acetonitrile, transfer to screw-top Eppendorf tubes, and dry.
9. Add 40 µL (or as much as needed to submerge the strip) of ice-cold 48% aqueous HF acid and dephosphorylate the samples by leaving them for 60–72 h on ice-water.

10. Remove the HF by freeze-drying. Add 100 µL of water to each tube and freeze-dry again. Repeat this step.

4. Notes

1. Peptide *N*-glycosidase F is available from Sigma, Poole, UK. It is advisable to use a glycerol-free preparation of the enzyme, as this can interfere with subsequent fluorescence labeling reaction efficiencies.
2. For preparations in which analysis is to be performed with mass spectrometry, particularly matrix-assisted laser desorption–time-of-flight (MALDI-TOF), glass-distilled water should be used as deionized water may contain polymeric material which will interfere with the analysis.
3. Dowex AG50 × 12 (200–400 mesh) should be used for desalting of glycans as there will be minimal loss on this grade. It may be prepared in the H^+ form by washing with 10 vol of 1 *M* HCl followed by water until the pH is the same as that of the wash water (usually slightly acidic).
4. Endoglycosidase H from *Streptomyces plicatus* is available from Sigma, Poole, UK. The specificity of this enzyme is that it will cleave the chitobiose core structure of oligomannose or hybrid type glycans attached to the asparagine residue of a glycopeptide. The presence of 1, 6-linked fucose on the *N*-acetylglucosamine linked to the peptide will not affect activity. The free glycan or the dolichol pyrophosphate derivative will also be cleaved.
5. Endoglycosidase F is available from Europa Bioproducts, Ely, Cambridge, UK. Three different types of endoglycosidase F with distinct specificities have been cloned. The general specificity is such that it will cleave the chitobiose core of oligomannose or hybrid type glycans but will not cleave complex type glycans. The presence of fucose 1, 6-linked to *N*-acetylglucosamine attached to the peptide will reduce the rate of cleavage >50 times.
6. Hydrazine is toxic and flammable; discard ampoule and residual contents after using once. Dispose of safely according to your institution's regulations.
7. The glycoprotein sample should ideally first be dialyzed against distilled water and stored lyophilized in a 1.5-mL microfuge tube. If the sample needs to be in a buffered solution, one can place it in 50 m*M* sodium phosphate buffer, pH 7.5, at a final concentration of at least 100 µg/µL or 2 mg/mL. Best results are obtained if the total salt concentration of the solution is >100 m*M*. The use of a Tris-based buffer is not recommended. If desired, the sample may also contain 0.05% sodium azide.
8. It is recommended that at least 250 µg of glycoprotein is used for analysis.
9. The actual amount of glycoprotein required for profiling will depend on the size of the protein, the amount of glycosylation, and the degree of oligosaccharide heterogeneity. In general, the amount of glycoprotein required increases with the size of the protein or the degree of heterogeneity and decreases with the percent of glycosylation. As a general guideline, one would start with approx 50–100 µg to profile the *N*-linked oligosaccharides of a 60-kDa glycoprotein that contains 10–20% carbohydrate by weight. For *O*-linked oligosaccharide analysis we suggest 100–500 µg of starting glycoprotein. This amount would normally provide sufficient material for several electrophoretic runs. For isolation of individual oli-

gosaccharides, and carrying out sequencing, additional material is required.

10. A suitable control in which *N*-glycans have been well characterised should be used as a control for enzyme digestion Examples of such glycoproteins include ribonuclease B, bovine serum fetuin, and haptoglobin (all available from Sigma–Aldrich but there may be some variation in glycosylation profile between different batches). Use of the control ensures that the reagents are working and that release and labeling procedures were performed properly.
11. If is advisable to assay the degree of glycosylation both before and after PNGase F digestion by SDS-PAGE.
12. Extra care should be taken to thoroughly desalt denatured samples to ensure that subsequent fluorescence labeling reactions are not affected. Protein components can also be removed by precipitation with ice-cold ethanol. 75% v/v
13. Deglycosylatation can also be performed using PNGase A. But in this case the protein should be cleaved to glycopeptides by appropriate protease enzymes. This is especially relevant if plant glycoproteins are being studied, as PNGase F will not cleave oligosaccharides with core fucose residues in α1–3 linkage.
14. Bio-Gel P2 has an exclusion limit of approx 1.8 kDa. If a 2-mL column has an exclusion volume of about 600 µL, then elution of the column with 800 µL of water should elute all *N*-linked oligosaccharides. If in doubt, fractions can be assayed for hexose using the phenol–sulfuric acid method.
15. Dialysis against 0.1% TFA will remove most salts and detergents but should always be performed in the cold (about 4°C) to minimize desialyation under the acidic conditions. In certain cases this may not be feasible, however (e.g., if protein precipitates) and in these situations purification by reverse-phase chromatography in volatile solvents that may be removed under vacuum is recommended.
16. Protein samples for hydrazinolysis should be essentially salt free. Salts, heavy metal ions, dyes, and detergents may interfere with the hydrazinolysis reaction in an unpredictable way and need to be removed. Unless the sample has been desalted by other techniques such as reverse-phase chromatography (*see* **Note 15**) this is most conveniently performed by dialysis.
17. The maximum amount of sample is 2.0 mg. The minimum amount of sample depends on the glycoprotein and analytical technique to be used but is generally in the range of 10–100 µg.
18. All chemistry has been completed by this step and the system is being washed before the next run. The sample is delivered in dilute acetic acid (approx 5.0 mL). If it is left in this solution at elevated temperatures there is a possibility of desialylation of the released glycans and the sample should therefore be dried down as soon as possible after collection.

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3

Derivatization of Carbohydrates

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1. Introduction

The monosaccharides comprise a number of isomers and homologues having very similar structures, basically composed of hydroxymethyl or hydroxymethine groups linked to each other. In reducing monosaccharides the hemiacetal group is at the reducing ends in addition to these polyhydroxylated basal structures. There are other series of monosaccharides with the ketal or carboxyl group instead of or in addition to the hemiacetal group. Substitution of the hydroxyl group by the hydrogen atom or the amino group produces the deoxy sugar or the amino sugar, respectively. All these functional groups give monosaccharides hydrophilic properties. With the exception of monosaccharide residues containing a carboxyl group, detection of these compounds following chromatographic and electrophoretic separation is rendered difficult owing to their inherent lack of chromophores in the ultraviolet (UV) and visible spectrum regions. Obviously, oligosaccharides and polysaccharides composed of these monosaccharide units have a similar shortcoming with respect to their detection.

Chemical derivatization is one of the strategies used to enhance detection of carbohydrates by spectroscopic methods. Introduction of a chromophore and/or a fluorophore group(s) into a carbohydrate molecule not only facilitates their detection using absorption or fluorescence detectors, but also alters their physical properties such as hydrophobicity and electric charge. Thus, the change of hydrophobicity extends the separation mode, and the introduction of an electric charge makes the derivatives amenable to electrophoretic separation. Similarly, carbohydrate analysis based on electrospray mass spectrometry (ESMS) detection relies on chromatographically separable oligosaccharides compris-

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ing ionizable functionalites. From these reasons chemical derivatization of carbohydrates for capillary electrophoresis should involve the quantitative introduction of tags enhancing spectrometric detection while simultaneously giving rise to separation based on changes of overall electric charge.

A number of methods fulfilling these requirements have been developed for capillary electrophoresis (CE) of carbohydrates. Most of them are based on reductive amination with various aromatic or heterocyclic amines, utilizing the reactivity of the amino group in the reagent and the hemiacetal group in reducing carbohydrates. The method using 2-aminopyridine* (2-AP, ***1***), *p*-aminobenzoic acid ***(2)*** as well as its ethyl ester (ABEE, ***3***) and nitrile ***(4)***, *N*-(4-aminobenzoyl)-L-glutamate ***(5)***, 6-aminoquinoline ***(6)***, 2-aminoacridone** (AMAC, ***7***), mono- ***(8)***, di- ***(9)****, or tri- (ANTS, ***10***)** sulfonate of aminonaphthalene, 3,6,8-trisulfonate of 1-aminopyrene** ***(11)***, and so forth belong to this category. The derivatives can be detected by UV absorption and the amines identified by asterisks can also be detected by fluorescence. The amines marked with double asterisks can be excited by the conventional lasers (Ar, He–Cd, etc.) and give rise to intense fluorescence yield, thus allowing ultrahigh sensitive detection.

The selection of carbohydrate reagents for MS characterization can also be influenced by fragmentation characteristics that facilitate the interpretation of fragment ions from which branching and/or sequencing information can be derived. Sequence information can also be obtained from the combined CE–MS–MS experiment using fragment ions arising from sequential cleavages of glycosidic bonds. Such a task is facilitated in positive ion mode where the fragmentation proceeds via the formation of stable oxonium fragment ions spread through the entire mass range. However, the sequencing of negatively charged derivatives such as ANTS derivatives has been proven more difficult as the fragmentation is localized near the charge site, thus producing a limited number of fragment ions.

When considering separation of derivatized carbohydrates by CE, most of these analytes have an overall net charge under specific conditions, owing to the presence of the amino, carboxyl, or sulfonate group(s). Therefore, they can be separated simply by the zone electrophoresis mode. Addition of an alkaline metal salt or an oxoacid (e.g., boric acid) salt to the running buffer provides enhanced separation based on the configurational difference among carbohydrate species. The micellar electrokinetic chromatography mode can also separate the derivatives, especially when hydrophobicity is moderate and electric charge is weak. However, this type of derivatization method requires an acidic catalyst, and reducing carbohydrate samples are reacted with an amine reagent in a nonaqueous solution in the presence of a reductant and an acid as catalyst. The use of a strong acid for catalysis may cause partial release of acid-labile structures such as the sialic acid residue and the *O/N*-sulfate groups.

There are also a few variations of this type of derivatization, performed in two steps of reductive amination with the ammonium salt followed by introduction of fluorescent tags to the resultant glycamines. Thus, in the isoindole method** ***(12)*** the glycamines formed in the first step are reacted with 3-(4-carbobenzoyl)-2-quinolinecarboxaldehyde (CBQCA), and in the 7-nitro-2,1,3-benzoxadiazole-tagged *N*-methylglycamine (NBD-MG) method** ***(13)*** the *N*-methylglycamines formed by reductive *N*-methylamination are reacted with NBD-F. Although the derivatization is performed in two steps, the total reaction time is relatively short, especially in the latter method, because the presence of the *N*-methyl group facilitates both reductive amination and the subsequent tagging. In these two-step methods the derivatives are not so strongly charged, and the zone electrophoresis mode as complexes is recommended for separation of the derivatives.

Another type of derivatization was developed, which is based on the condensation of the hemiacetal group with the active carbon atom in 1-phenyl-3-methyl-5-pyrazolone (PMP) ***(14)***. Two PMP groups are introduced to each reducing carbohydrate to give the *bis*-PMP derivative which shows strong absorption in the UV region and is readily oxidized on a glassy carbon electrode. This method has an advantage that it proceeds under mild conditions using almost neutral aqueous media, and therefore causes no release of the sialic acid residues and the sulfate groups.

A great number of methods are available for precapillary derivatization as mentioned previously, and each of them has its characteristic features. **Table 1** summarizes the absorption/fluorescence wavelengths, limits of detection, and characteristic features of these methods. **Table 2** provides detection limits for several carbohydrate derivatives used in CE–ESMS experiments.

In this chapter, we selected typical methods from those described in the preceding paragraphs and showed the corresponding protocols. The selection is rather arbitrary, but the selected methods cover almost all types of mono- and oligosaccharides encountered in glycobiology.

1.1. Derivatization for CE with UV Detection

1.1.1. The PMP Method as a General Method for Routine Analysis of Diverse Combinations of Mono- and Oligosaccharides **(14–23)**

This method was first developed by Honda and co-workers for precolumn derivatization in high-performance liquid chromatography (HPLC) of reducing carbohydrates ***(15)*** and later applied to CE by the same group ***(14)***. It is simple, rapid, and robust, and accordingly a suitable method for routine analysis of reducing mono- and oligosaccharides. The derivatization reaction proceeds in aqueous methanol at almost neutral pH values to give quantitative

Table 1
Various Methods for the Derivatization of Reducing Carbohydrates for CE with Photometric and/or Fluorometric Detection

Derivatization reagent	λ_{max} (nm)	Limit of detection (*M*)	Reference[a]
2-Aminopyridine	237 320 (ex)/380 (em)	8 μ*M* 10 m*M*	***1***
4-Aminobenzoic acid	285 nm	4 μ*M* (aldoses), 0.8 m*M* (ketoses)	***2***
Ethyl 4-aminobenzoate	305 nm	2 μ*M*	***3***
4-Aminobenzonitrile	285 nm	0.3 μ*M*	***4***
N-(4-Amionobenzoyl)-L-glutamate	291 nm	170 fmol	***5***
6-Aminoquinoline	245 nm	10 μ*M*	***6***
2-Aminoacridone	442 (ex)/525 (em), He–Cd laser	~ 5 n*M*	***7***
5-Aminonaphthalene-2-sulfonate	325 (ex)/520 (em), He#-3Cd laser	50 n*M*	***8***
7-Aminonaphthalene-1,3-disulfonate	325 (ex)/ 425(em), He–Cd laser	5 n*M*	***9***
8-Aminonaphthalene-1,3,6-trisulfonate	325 (ex)/520 (em), He–Cd laser	50 n*M*	***10***
1-Aminopyrene-3,6,8-trisulfonate	488 (ex)/520 (em), Ar laser	pmol	***11***
$NH_4OAc/NaBH_3CN$-3-(4-carboxybenzoyl)-quinoline-2-carboxaldehyde	457 (ex)/522 (em), Ar laser	250 p*M*	***12***
$CH_3NH_2/(CH_3)_2NH:BH_3$-4-fluoro-7-nitrobenzoxadiazole	488 (ex)/520 (em), Ar laser	100 n*M*	***13***
1-Phenyl-3-methyl-5-pyrazolone	245 nm	1 μ*M*	***14***

[a]Represent typical references.

Table 2
Sensitivity and Detection Limits Using CE–ESMS

Derivatives[a] with	CE–ESMS (SIR)[b]		CE–ESMS (Scan)[c]	
	Mass (pg)	Conc. (ng/mL)	Mass (ng)	Conc. (μg/mL)
2-AP	35 (28 fmol)	700	1.8 (1.4 pmol)	35
PTMAA	15 (11 fmol)	300	0.75 (550 fmol)	15
ANTS	50 (33 fmol)	1000	2.5 (1.7 pmol)	50
Pyrimidine hydrazine	75 (59 fmol)	1500	3.8 (3.0 pmol)	75
Girard P	50 (39 fmol)	1000	2.5 (2.0 pmol)	50

[a]All except ANTS derivatives were separated on Polybrene-coated capillary (50 μm i.d. × 1 m), 0.1 *M* formic acid, –25 kV, 50 nL/ inj. Separation for ANTS: uncoated capillary, 50 m*M* morpholine–formate, pH 3.0, –30 kV.

[b]Conducted on heptaose derivatives using selected ion monitoring (SIM) acquisition mode.

[c]Full mass scan acquisition *m/z* 350–1200.

yields of *bis*-PMP derivatives (**Fig. 1**), which can be detected by UV absorption with detection limits in the low 10^{-6} *M* level.

Continuous-wave detectors using a deuterium lamp is preferable. Use of a mercury lamp with an interference filter can also detect the derivatives, although the emission wavelength (254 nm) somewhat deviates from the wavelength of the maximum absorption (245 nm). Owing to the mildness of reaction condition the sialic acid residue and the sulfate group are not released during derivatization, as evidenced by model experiments using neuraminyllactose ***(16)*** and 4,5-unsaturated disaccharide sulfates derived from chondroitin sulfates by chondroitinase ABC ***(17)***. This is especially useful for the analysis of sialylated *N*- as well as *O*-glycans in glycoconjugates ***(16)***.

This method has an additional advantage in that it allows analysis by multiple separation modes, including plain zone electrophoresis ***(18)***, zone electrophoresis as oxoacid complexes ***(14)***, zone electrophoresis as alkaline earth metal complexes ***(19)***, electrokinetic chromatography in micelles ***(20)***, ion-interaction electrokinetic chromatography in a solution of an ionic polymer such as Polybrene (hexadimethrine bromide) ***(21)***, affinity electrophoresis in the presence of lectins ***(22)***, hydrogen bonding electrophoresis with chiral selectors such as optically active dodecoxycarbonylvalines ***(23)***, and so forth, because each derivative can form an enol group and the magnitude of electric charge is controlled by the pH value of running buffer. The multiplicity in separation modes allows application to a wide range of carbohydrate combinations.

The PMP method has a further advantage in that the derivatives can be most sensitively detected in ESMS ***(24)***. Comparison of the lower limits of detection

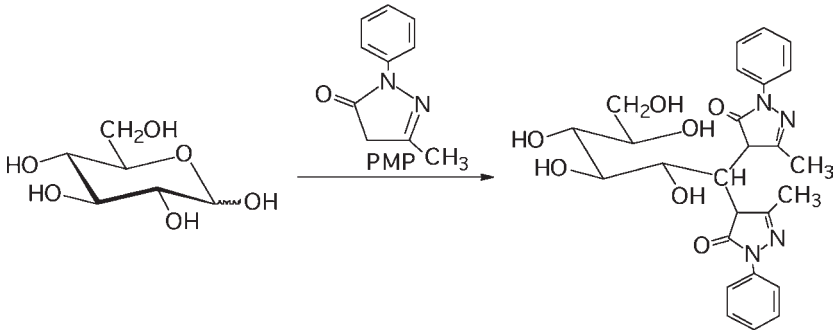

Fig. 1. Derivatization of reducing carbohydrates with PMP.

of the $[M+H]^+$ peak among the PMP, 2-AP, ABEE, aminoethanethiol, and *o*-aminobenzene–thiol derivatives of maltopentaose by the infusion mode indicated that the PMP derivative had by a far lower detection limit than any other derivatives.

Applications to simple mono- and oligosaccharides are described in Chapters 5 and 6, respectively. In Chapter 6 an example of an application to the analysis of *N*-glycans in a glycoprotein sample is also presented ***(16)***. In these chapters it is indicated that quantification is reliable, because the yields of *bis*-PMP derivatives are quantitative. Since typical examples of analysis of mono- and oligosaccharides by the plain zone electrophoresis mode and the zone electrophoresis mode as borate complexes are presented in the following chapters, examples of the separation of disaccharides by affinity electrophoresis mode (**Fig. 2**) and separation of D/L enantiomers by hydrogen bonding electrophoresis mode (**Fig. 3**) are given here. In these examples the procedure for derivatization is the same as that described in Chapter 10 for monosaccharide analysis, but the sample loading was reduced by about 10-fold.

In the example of affinity capillary electrophoresis (**Fig. 2**), the PMP derivatives of glucobioses and galatosylglucoses were subjected to CE in the presence of RCA_{60}, a galactose-recognizing lectin from *Ricinus communis* seeds. Addition of higher concentrations of RCA_{60} caused more marked retardation of the PMP derivatives of galactosylglucoses (melibiose and lactose), and the derivatives of galactosylglucoses together with those of glucobioses were clearly separated from each other at a RCA_{60} concentration of 5 mg/mL ***(22)***. It is noteworthy that without RCA_{60} such retardation and separation could not be attained.

The PMP derivatives of the D- and L- enantiomers of arabinose, xylose, galactose, and glucose were baseline separated with resolution (the ratio of the migration time difference to the average peak width) values of 1.84, 1.81, 2.04, and 2.11, respectively, in a neutral phosphate buffer containing 50 m*M*

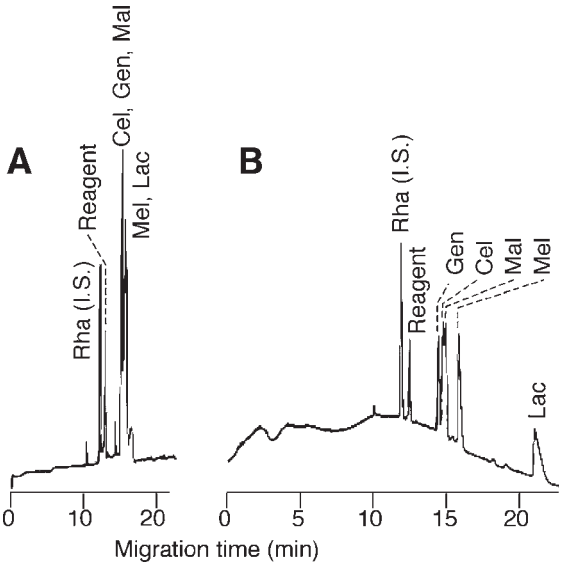

Fig. 2. Capillary electrophoretic analysis of glucobioses and galactosylglucoses as PMP derivatives by affinity capillary electrophoresis in the presence of RCA_{60} with UV detection. Capillary, uncoated fused silica (50 µm i.d., 50 cm) **(A)** or linear polyacrylamide-coated fused silica (50 µm i.d., 50 cm) **(B)**; running buffer, 50 m*M* phosphate buffer, pH 6.8 **(A)** or 50 m*M* phosphate buffer, pH 6.8, containing to a concentration of 5 mg/µL **(B)**; applied voltage, 15 kV **(A)** or 18 kV **(B)**; detection, UV absorbance at 245 nm. (Reproduced with permission from **ref. *29***).

(*R*)-dodecoxycarbonylvaline as a chiral selector (**Fig. 3**) ***(23)***. Such excellent separation is due to the difference in the extent of the intermolecular hydrogen bonding between PMP-aldoses and the chiral selector which together form a ring structure.

1.1.2. The ABEE Method for Routine Analysis of Neutral Mono- and Oligosaccharides **(3)**

This method, reported by Wang and co-workers for HPLC of reducing carbohydrates ***(25)***, was applied for capillary electrophoresis by Oefner and coworkers ***(3)*** with modification. It gives the glycamines in which the amino group is substituted by the ethyl benzoate group (**Fig. 4**). The reaction conditions employed by these groups are basically similar to those in the earlier paper by Hase and coworkers for reductive amination with 2-AP ***(26)***, which involves reaction in a methanolic solution of an amine reagent containing sodium cyanoborohydride and acetic acid. The procedure by Oefner and coworkers required reaction for 2 h at 50°C. We used the procedure of Wang

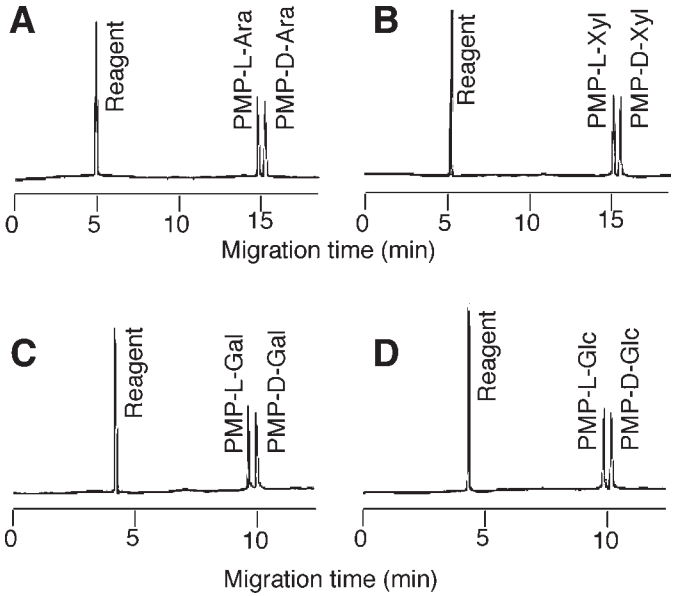

Fig. 3. Capillary electrophoretic analysis of the D-/L-enantiomers of various aldoses in the presence of (*R*)-dodecoxycarbonylvaline with UV detection. Capillary, uncoated fused silica (50 µm i.d., 54 cm); running buffer, 50 m*M* phosphate buffer, pH 7.0, containing (*R*)-dodecoxycarbonylvaline to a concentration of 50 m*M*; applied voltage, 20 kV; detection., absorbance at 245 nm. (Reproduced with permission from **ref. *23***).

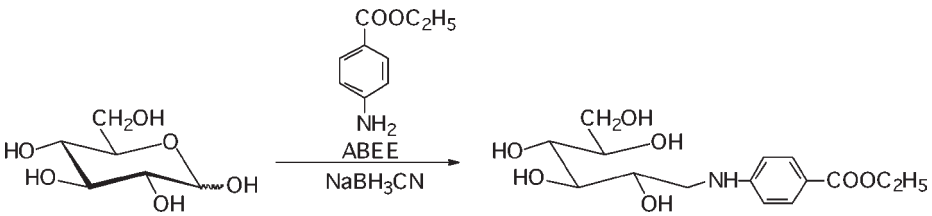

Fig. 4. Derivatization of a reducing carbohydrate with ABEE.

and coworkers ***(25)*** with minor modifications using the reagent solution prepared by mixing ABEE (1 µmol), acetic acid (41 µL), sodium cyanoborohydride (35 mg), and anhydrous methanol (350 µL). The derivatization was performed at a higher temperature of 80°C.

The derivatives from neutral reducing carbohydrates have a positive charge in acidic media owing to the protonated imino group, but electroosmotic flow (EOF) in an uncoated fused silica capillary is slow under these conditions. Introduction of ABEE derivatives from the anodic end of the capillary will

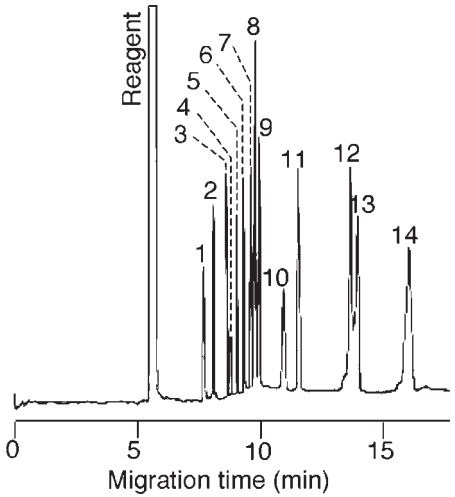

Fig. 5. Capillary electrophoretic analysis of reducing carbohydrates by zone electrophoresis as the borate complexes of the ABEE derivatives with UV detection. Capillary, uncoated fused silica (50 µm i.d., 72 cm); running buffer, 175 m*M* borate buffer, pH 10.5; applied voltage, 25 kV; detection, UV absorbance at 305 nm. (Reproduced with permission from **ref. *3***).

result in very rapid analysis without sufficient separation. In neutral or alkaline media ABEE derivatives have no net electric charge and migrate quickly without adequate resolution. The use of borate buffer, as reported previously ***(1)***, gives improved resolution as the derivatives react *in situ* with the borate ion to form complexes having a net negative charge. The ABEE derivatives introduced to the anodic end of an uncoated fused silica capillary move to the cathode owing to the strong EOF which has a direction opposite to that of the electrophoretic mobility of the analyte. The electropherogram observed at 305 nm (**Fig. 5**) gave well resolved peaks of monosaccharides as in the electropherogram of 2-AP derivatives obtained under similar conditions ***(1)***.

Excess reagent can be removed by precipitating the derivatives following the addition of a twofold volume of ether ***(3)***. However, purification is not complete by this procedure alone, and our studies indicated that extraction with ethyl acetate is more effective to remove excess reagent.

The reaction yield is not necessarily quantitative, probably owing to the formation of alditol groups at the reducing end of the carbohydrates. Therefore, determination is possible only when an internal standard is added, although this may not be ideal in all conditions. The alditols as byproducts and the unreacted carbohydrate samples are not detected at 305 nm, and hence do not interfere with the determination of the derivatives.

Thus, the use of acetic acid is often found necessary for derivatization, although this results in more drastic conditions than those of the PMP method. Owing to the high concentration of this acid the integrity of the sialic acid residue and sulfate groups cannot be preserved. Therefore, this method is suitable for routine analysis of neutral reducing carbohydrates. The results of fast atom bombardment mass spectrometric analysis of ABEE derivatives have been well documented *(25)*.

The following method describes our modification of the earlier procedure of Wang and co-workers *(25)*.

1.2. Derivatization for CE with Fluorescence Detection

1.2.1. The 8-Amino-1,3,6-Naphthalenetrisulfonate (ANTS) Method for Microanalysis of Oligosaccharides Based on Degree of Polymerization **(10)**

Among various methods for precapillary derivatization the ANTS method gives the strongest negative charge to the derivatives by introducing a reagent having three sulfonate groups (**Fig. 6**).

The sulfonate groups in the derivatives are dissociated in a wide pH range to give ions having a strongly negative charge. Because the charge-to-mass ratio is different among oligosaccharides having varying degrees of polymerization, they are well separated from each other by plain zone electrophoresis even at neutral pH values. This method for derivatization was first reported by Jackson *(27)* and applied to gel electrophoresis of oligosaccharides by the Glyco group *(28)*. Chiesa and Horváth *(10)* first reported the separation of ANTS derivatives of homologous series of oligosaccharides by CE and observed the relationship between the mobility and the degree of polymerization. It was reported that the yield of the ANTS derivative from ^{14}C-labeled glucose was almost quantitative *(27)*, although the data were not shown. One of the shortcomings of this method is the possible release of the sialic acid residue during derivatization due to the use of acid catalyst. Another drawback of this method is the possible influence of such a strongly negative group on the studies of carbohydrate–protein interaction *(29)*.

Figure 7 shows an example of separation of ANTS derivatives of maltooligosaccharides having various degrees of polymerization.

1.2.2. The 7-Nitro-2,1,3-Benzoxadiazole-Tagged Methylglycamine (NBD-MG) Method for Ultramicro Analysis of Glycans in Glycoconjugates and the Component Monosaccharides **(13)**

One of the drawbacks of the derivatization by reductive amination is the use of an acid catalyst, which may cause partial degradation of the acid-labile groups. The reaction must be performed in nonaqueous media in the presence of a reduc-

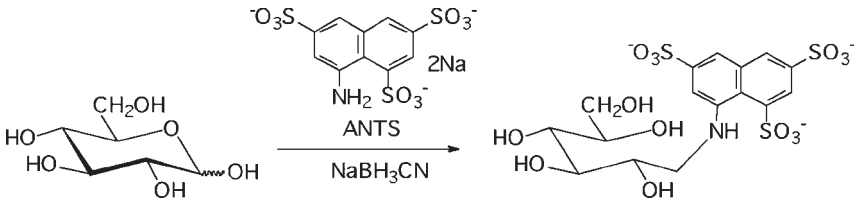

Fig. 6. Derivatization of a reducing carbohydrate with ANTS.

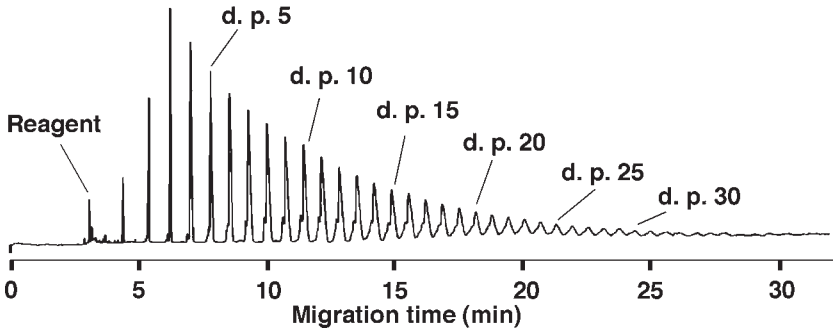

Fig. 7. Capillary electrophoretic analysis of isomaltooligosaccahrides having various degrees of polymerization as the ANTS derivatives. Capillary, polyacrylamide-coated fused silica (50 µm i.d., 50 cm); running buffer, 50 m*M* phosphate buffer, pH 6.8; sample introduction, from the cathode; applied voltage, 15 kV; detection, UV absorption at 280 nm. (Reproduced with permission from **ref. *29***).

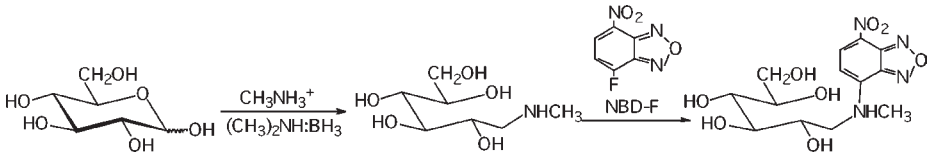

Fig. 8. Derivatization of reducing carbohydrates to NBD-tagged *N*-methylglycamines.

tant, to avoid such partial decomposition. Enrichment of electron density on the nitrogen atom of the amine reagent by introducing an electron-donating group such as a methyl group enhances the reactivity of the reductive amination and gives higher yields of *N*-methylglycamines (MGs) under much milder conditions (**Fig. 8**).

The reaction can be carried out in an aqueous medium, and the resulting glycamines can be labeled by 7-nitro-2,1,3-benzoxadiazole (NBD) following

the condensation with NBD 4-fluoride (NBD-F). The presence of the methyl group on the nitrogen atom is again beneficial for accelerating this condensation, giving higher yields of NBD-tagged MGs in a short time. The final derivatives can be excited by the argon laser at 488 nm and detected by monitoring the fluorescence at approx 520 nm. The limit of detection of these derivatives is typically in the order of 10^{-9} – 10^{-8} *M*. Therefore, this method is suitable for the analysis of minute amounts of carbohydrates obtained from biological samples. The use of mild reaction conditions does not cause release of the sialic acid, as evidenced by an experiment using neuraminyllactose as a model sialooligosaccharide ***(13)***.

The chemical properties of the NBD-MG derivatives are somewhat different from those of other derivatives. For example, the hydrophobicity is lower than that of PMP derivatives. Therefore, the electrokinetic chromatography mode in sodium dodecyl sulfate (SDS) micelles does not give good separation, but zone electrophoresis as borate complexes gives separation as good as that of other derivatives. Addition of SDS to alkaline borate buffer also improves separation. Examples of ultrahigh sensitive analyses of sialylated *N*-glycans and the component monosaccharides were reported previously in the literature ***(13)***. **Figure 9** shows the analysis of sialo-*N*-glycans released from 10 μg of bovine fetuin using the NBD-MG reagents ***(13)***.

1.3. Derivatization for Analysis by CE-ESM

The analysis of neutral oligosaccharides by CE–ESMS also requires the formation of suitable derivatives providing convenient ionizable groups for separations based on electrophoretic mobilities. The molecular weight determined by the CE–ESMS analysis can be used to deduce possible carbohydrate compositions and potential structures based on typical *N*- and *O*-linked glycans. Sequence information can also be obtained from the combined CE–MS–MS experiment using fragment ions arising from sequential cleavages of glycosic bonds ***(30)***. Such a task is facilitated in positive ion mode where the fragmentation proceeds via. the formation of stable oxonium fragment ions spread through the entire mass range. However, the sequencing of negatively charged derivatives has proven more difficult as the fragmentation is localized near the charge site, thus producing a limited number of fragment ions.

Reductive amination has been used extensively to incorporate chromophores and/or fluorophore for UV and fluorescence detections into carbohydrates, some of them also compatible with mass spectral analysis using electrospray ionization. A number of previous investigations have reported the use of suitable derivatives for ESMS detection including PMP ***(24)***, AMAC ***(31)***, and 3-(acetylamino)-6-aminoacridine ***(32)*** derivatives.

In this section we report simple methods for the preparation of oligosaccharide derivatives using commercially available reagents (2-AP and

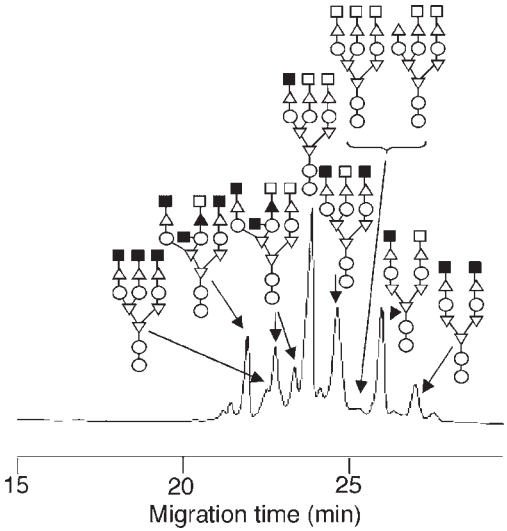

Fig. 9. Capillary electrophoretic analysis of the sialo-*N*-glycans from bovine fetuin as the NBD-tagged *N*-methylglycamine derivatives with argon laser induced fluorescence detection. Capillary, uncoated fused silica (50 µm i.d., 50 cm); running buffer, 200 m*M* borate buffer, pH 10.8, containing SDS (100 m*M*). Applied voltage, 13 kV; detection, fluorescence at 520 nm with irradiation by an argon laser (488 nm). ○, *N*-Acetylglucosamine; ▽, mannose; △, galactose; ■, 2,3-linked *N*-acetylneuraminic acid; □, 2,6-linked *N*-acetylneuraminic acid. (Reproduced with permission from **ref. *13***).

p-trimethyaminoaniline [PTMAA]). Approaches for enhancing sample loading and sensitivity using on-line isotachophoresis preconcentration are also presented.

1.3.1. CE-ESMS Analyses of Oligosaccharides Using 2-AP and PTMAA Derivatives

Electrophoretic conditions for the separation of carbohydrate derivatives can be tailored depending on the type of reagents used. Oligosaccharide derivatives with a basic functionality (e.g., amino groups) can be separated and detected in positive ion mode, while acidic oligosaccharide derivatives with a carboxylic or sulfonic acid group are analyzed in negative ion mode. Volatile buffers with low proton affinity are most often selected in ESMS experiments to prevent deposit of salts on the sampling interface plate of the mass spectrometer. Enhancement of resolution for the separation of oligomeric carbohydrates can be achieved by selecting separation conditions and using coated capillaries to prevent analyte adsorption on silica walls. For example, separation of glycopeptides in positive ion CE–ESMS was previously reported that

used formic acid and dynamic coating reagents such as Polybrene ***(33)***, which imparts an overall positive charge on the capillary surface and anodic EOF. Dynamically coated capillaries can also be reconditioned periodically to maintain separation performance. This is more easily performed with an ionization source accessible to the user to prevent and/or remove alkali salts used during this procedure. Alternatively, capillaries derivatized with covalent reagents (*see* Chapter 13, **Subheading 2.5.**) can be prepared to minimize down time and to avoid the reconditioning steps required with dynamic coating ***(34)***.

Preliminary experiments on different reagents for carbohydrate derivatization indicated that both 2-AP and PTMAA provided lower limits of detection compared to other derivatives (**Table 2**). Low femtomole detection limits were also reported recently for LC-ESMS experiments on *N*-linked oligosaccharides ***(32)***, although sample loading in HPLC is two to three orders of magnitude higher than that typically available with zone electrophoresis.

A comparison of separation performance obtained for 2-AP and PTMAA derivatives is presented in **Fig. 10** for a mixture of maltooligosaccharides using both UV and mass spectrometric detections. Components migrate according to their respective electrophoretic mobilities dependent on decreasing degrees of polymerization. PTMAA-derivatized glycans detected by UV absorption (**Fig. 10B**), generally yielded higher electrophoretic mobilities compared to their corresponding 2-AP congeners (**Fig. 10A**) thereby providing enhanced resolution between related glycans. This was also clearly visible in CE–ESMS experiments on these derivatives from the same sample (**Figs. 10C** and **10D**). The electrophoretic mobility of the corresponding oligosaccharides is dictated not only by the number of ionizable groups but also by the molecular weight of each component. Thus, the derivatives of glycans comprising a fixed number of ionizable group assigned by the reagent tag can be separated from one another based on their hydrodynamic volume approximated by their molecular masses. This is better visualized by the diagonal lines of negative slope in the contour profile of the corresponding analysis (**Fig. 11B**).

This representation corresponds to a two-dimensional depiction of *m/z* values vs time, whereby the order of migration is inversely proportional to the molecular mass of the derivatized glycans (the direction of electrophoretic mobility is opposite to that of the EOF). Contour profiles are also convenient to identify unusual migration patterns and to identify isomeric components that can be present in mixtures of glycans released from complex glycoproteins.

1.3.2. Enhancement of Sample Loading for the Analysis of Oligosaccharide Derivatives Using CITP-CZE-ESMS

One of the obvious difficulties in the analysis of trace level oligosaccharides by CE–ESMS is the relatively small sample loading typically used in zone

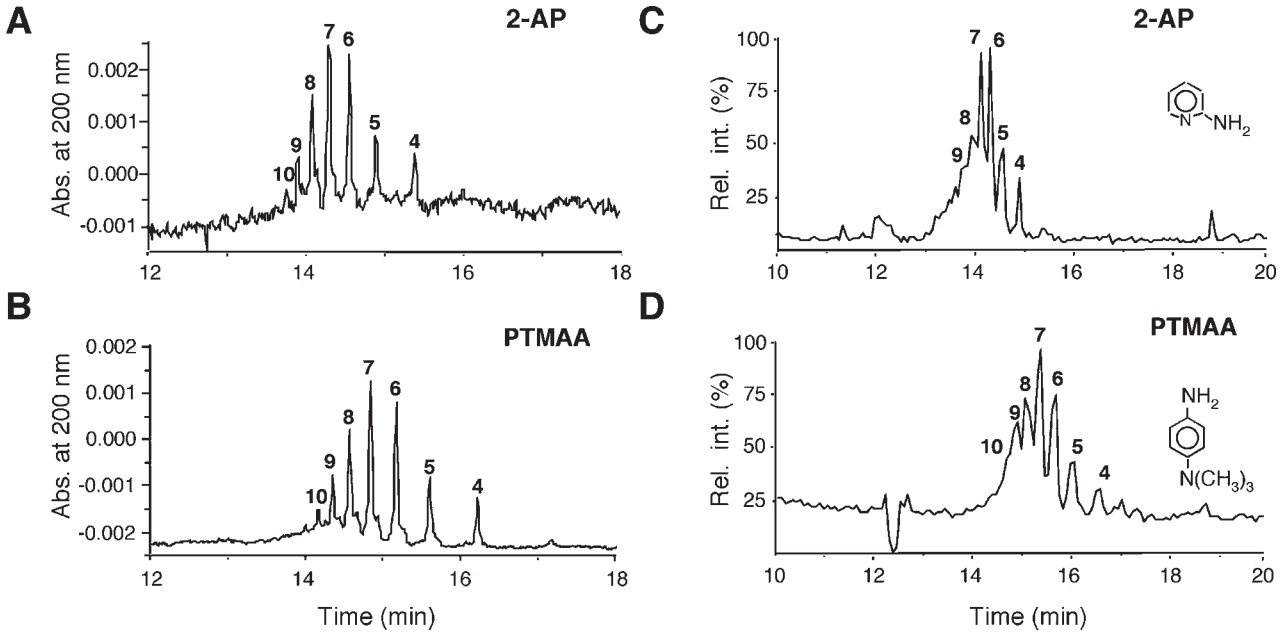

Fig. 10. Separation of 2-AP and PTMAA-derivatized maltooligosaccharides by CE-UV (**A**,**B**) and CE-ESMS (**C**,**D**). Conditions: 40 nL inj. of 0.1 mg/mL of glycan mixture, 2 *M* HCOOH, 10% acetonitrile. The numbers above each peak indicate the degree of polymerization.

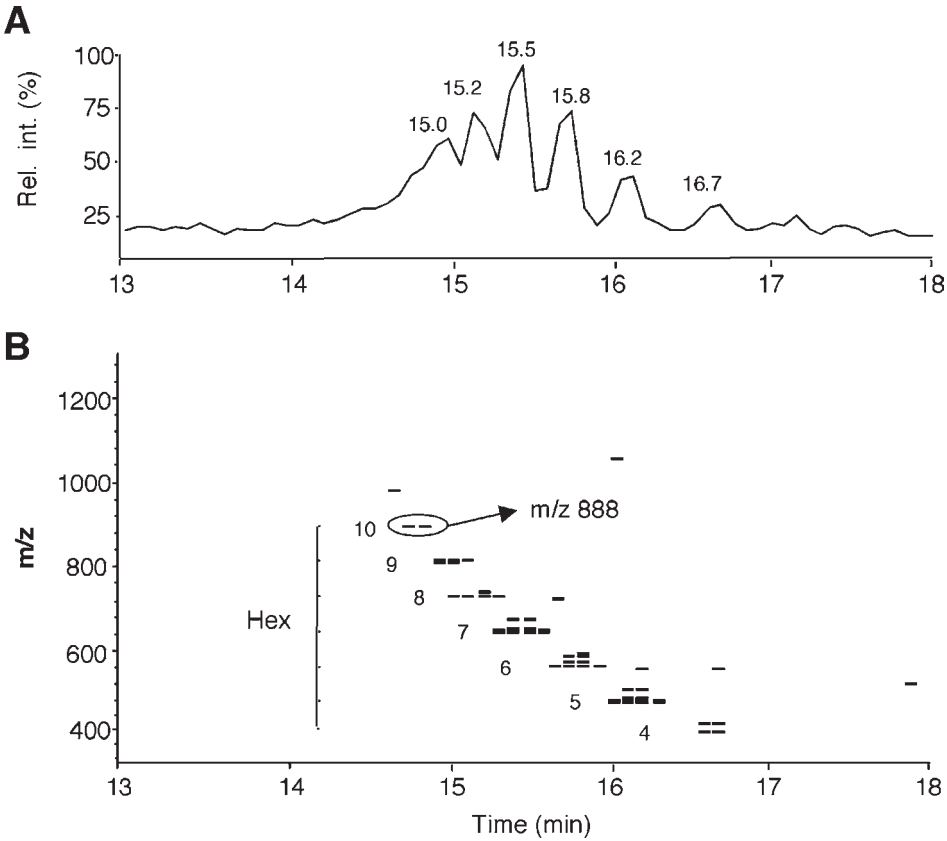

Fig. 11. Separation of PTMAA-derivatized maltooligosaccharides by CE-ESMS. **(A)** Total ion electropherogram, *m/z* 400–1300 and **(B)** Contour profile of *m/z* vs time (intensity of each peak is orthogonal to the plane). Conditions: 40 nL inj. of 0.1 mg/mL of glycan mixture, 2 *M* HCOOH, 10% acetonitrile. The numbers beside each peak indicate the degree of polymerization. Peaks shown are doubly protonated ions each spaced by *m/z* 81.

electrophoresis. To maintain separation efficiency in this separation format, sample loading is typically limited to 2% of the capillary volume (e.g., 40 nL for a 1 m × 50 μm i.d. capillary). Although 50–200 fmol detection are typically available in ESMS, this translates into a low micromolar concentration detection limit. The application of on-column sample preconcentration with capillary isotachophoresis (CITP) and discontinuous buffer systems prior to capillary zone electrophoresis (CZE) separation can be used to enhance sample loading without compromising separation performance. The judicious choice of leading and terminating electrolytes for the preconcentration step can pro-

vide an improvement of the concentration detection limit of at least one to two orders of magnitude over that obtainable using the conventional CZE format. Separations are performed in capillaries where EOF is significantly reduced to facilitate proper band focusing. Examples of application of CITP preconcentration were previously demonstrated for peptides ***(38)*** and marine toxins ***(39)***. Such improvements in sample loadings now enable characterization of oligosaccharide derivatives present at nanomolar concentration levels in extracts obtained from glycoprotein chemical and enzymatic digests. A simple example of this approach is presented below for peptide-N^4, *N*-acetyl-β-D-glucosaminyl-asparagine amidase from *Flovobacterium meningosepticum* (PNGase F) digest of ribonuclease B.

2. Materials

2.1. The ABEE Method

1. Carbohydrate sample: Pipet a 10-µL aliquot of an aqueous solution of a reducing mono- or oligosaccharide (~1×10^{-3} *M*) or a mixture of reducing mono- and/or oligosaccharides (~1×10^{-3} *M* each) into a screw-cap vial and evaporate the solvent (water) by leaving the vial in a desiccator containing pellets of sodium hydroxide and phosphorus pentoxide (*see* **Note 1**).
2. Reagent solution (*see* **Note 2**): Dissolve 0.1 nmol of ABEE (*see* **Note 3**), 41 µL of acetic acid and 35 mg of sodium cyanoborohydride (*see* **Note 4)** in 350 µL of anhydrous methanol.
3. LH-20 column.

2.2. The ANTS Method

1. Carbohydrate sample: Pipet a 5-µL aliquot of an aqueous solution of a reducing mono- or oligosaccharide (~10^{-6} *M*) or a mixture of reducing mono- and/or oligosaccharides (~10^{-6} *M* each), and evaporate the solvent (water) *in vacuo* (*see* **Note 5**).
2. Amine reagent solution for tagging: A 150 m*M* solution of ANTS (*see* **Note 6**) in an acetic acid–water (3:17, v/v) mixture.
3. Reducing agent solution: A 1 *M* solution of sodium cyanoborohydride (*see* **Note 7**) in dimethyl sulfoxide (DMSO).

2.3. The NBD-MG Method

1. Carbohydrate sample: Pipet a 10-µL aliquot of an aqueous solution of a reducing mono- or oligosaccharide (~10^{-6} *M*) or a mixture of reducing mono- and/or oligosaccharides (~10^{-6} *M* each) in an Eppendorf tube, and evaporate the solvent (water) *in vacuo* (*see* **Note 8**). For the preparation of *N*-glycan samples from glycoproteins refer to the procedure in **Subheading 3.3.** Derivatize the released *N*-glycans directly without purification (*see* **Note 9**).
2. Methylamine solution: Make a 200 m*M* aqueous solution of methylamine by diluting commercial methylamine sample with water (*see* **Note 10**), and adjust the pH of the solution to 4.5 by adding acetic acid.

3. Dimethylamine–borane complex: Use a commercial sample of the highest grade.
4. Reagent solution for tagging: 300 m*M* solution of NBD-F (*see* **Note 11**) in ethanol.
5. Reaction medium for tagging: 10 m*M* borate buffer, pH 8.5.
6. Enzyme used for *N*-glycan release: PNGase F.
7. Reagents used for *N*-glycan release: SDS, 2-mercaptoethanol, potassium dihydrogen phosphate, Nonidet P-40 (NP-40; use the highest grade commercially available).

2.4. CE–ESMS

1. Carbohydrate libraries such as maltooligosaccharides and heptaose are available from Sigma Biochemicals. Glycans can also be released from glycoproteins using chemical and enzymatic methods as described in **Subheadings 3.3.** and **3.5.** and in Chapter 6.
2. AnalaR grade toluene, ammonium hydroxide, sodium hydroxide, and glacial acetic acid (BDH, Toronto, ON, Canada).
3. Reagent solutions: 2-AP, PTMAA, and sodium cyanoborohydride are available from Aldrich Chemicals. For derivatization with PTMAA, dissolve the aminating reagent (10 mg, 45 µmol) in 350 µL of water, to which 40 µL of glacial acetic acid is added. Prepare the reducing solution by dissolving the reducing reagent (sodium cyanoborohydride, 4 mg, 18 µmol) in 350 µL of water, to which 40 µL of glacial acetic acid is added. For derivatization with 2-AP, dissolve 526 mg of the aminating reagent and 100 mg of sodium cyanoborohydride in 229 µL of glacial acetic acid and methanol (1000 µL).
4. Reacti-vials or sealable glass vials (Pierce Chemicals).
5. CE buffers: Aqueous formic acid in 10% acetonitrile (for positive ion detection). Prepare all solutions with deionized water from a Millipore water filtration system and filter through a Millipore 0.45-µm filter.
6. Capillaries: 90 cm length × 50 µm i.d., available from Polymicro Technologies (Phoenix, AZ) polybrene-coated.
7. Polybrene coating solution: 5% w/v Polybrene (Aldrich), 2% v/v ethylene glycol in deionized water.
8. CE–ESMS apparatus: In the present case a Crystal model 310 CE instrument (ATI Unicam Boston, MA) coupled to the mass spectrometer via a coaxial sheath-flow interface (*see* Chapter 13) delivered by a syringe pump. Mass spectral analyses were conducted using an API III$^+$ triple quadrupole mass spectrometer (Perkin Elmer/Sciex, Concord, ON, Canada).

2.5. Enhancement of Sample Loading

1. Samples: Ribonuclease B and other glycoproteins were obtained from Sigma Biochemicals.
2. PNGase F: Available from Boehringer Manheim. A 1 mg/mL solution was prepared in 20 m*M* Tris-HCl, pH 7.1, containing 100 m*M* NaCl.
3. Digestion buffer solution: 200 m*M* Na_2HPO_4, pH 4.0 containing 10 m*M* EDTA (disodium salt).

4. Reductive amination reagents (*see* **Subheading 2.4.** above) (*see* **Note 12**).
5. CE buffers: Prepare all solutions with deionized water from a Millipore water filtration system and filter through a Millipore 0.45-µm filter. Leading electrolyte buffer: 50 m*M* morpholine formate pH 4.0. Terminating electrolyte buffer: 10 m*M* formic acid. Prepare sample solution in 0.1 *M* acetic acid containing 5 m*M* ammonium hydroxide.
6. Preparation of linear polyacrylamide-coated capillay: CE column: 3 m length × 50 µm i.d. capillary (Polymicro Technologies, Phoenix, AZ). Rinsing: GC capillary column rinsing kit (Supelco), high-pressure ultrapure nitrogen. Silanization reagent solution: prepared by dissolving 20 µL of 7-oct-1-enyltrimethoxysilane (Huls America Inc., Bristol, PA) and 20 µL of glacial acetic acid in 4 mL of mthanol. Polymerization solution: prepared from 4% w/v acrylamide (Crescent Chemical Co., Inc., Hauppage, NY) in deionized water, 10% w/v ammonium persulfate (Sigma/Aldrich) in deionized water and *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED, International Biotechnologies Inc., New Haven, CT).
7. CE apparatus: In the present case a Crystal model 310 CE instrument (ATI Unicam, Boston, MA) was coupled to the mass spectrometer via a coaxial sheath flow (5 m*M* morpholine formate, 25% methanol, pH 4.0) delivered by a syringe pump (*see* Chapter 13). Mass spectral analyses were conducted using an API III$^+$ triple quadrupole mass spectrometer (Perkin Elmer/Sciex, Concord, ON, Canada) (*see* **Note 13**).
8. Ultrafree-MC 10,000 molecular weight centrifuge filters (Millipore Corp., Bedford, MA).
9. Centrifuge.
10. Centrifugal evaporator (SpeedVac).

3. Methods

3.1. The ABEE Method

1. Derivatization: Add 40 µL ofthe reagent solution to a carbohydrate sample and heat the mixture for 30 min at 80°C. After cooling the reaction solution to room temperature evaporate the solvent using a SpeedVac (a centrifugal concentrator), add 200 µL of water and 500 µL of ethyl acetate (*see* **Note 14**) to the residue, and shake vigorously to extract the excess reagent. Discard the upper layer and add 500 µL of ethyl acetate again, and repeat extraction of the excess reagent. After repeating this extraction process once more (three times in total), evaporate the final aqueous layer to dryness by a SpeedVac. Dissolve the residue in a minimum volume of water and apply the solution to a Sephadex LH-20 column (*see* **Note 15**) and elute the carbohydrate derivative with a 1:1 (v/v) mixture of methanol and water. Monitor the eluates with a fluorimeter and collect the first fraction giving a fluorescence signal at 370 nm ($\lambda_{ex.}$ = 305 nm) (*see* **Note 16**). Evaporate the collected fraction and prepare the analytical sample solution by dissolving the residue in a relevant volume of water (*see* **Note 17**).
2. Analysis of the ABEE derivatives by CE: Any apparatus for CE can be used, provided that it is equipped with a sampling system, a high voltage supply

(~30 kV), a UV detector, and a data processor. Normally use a capillary having an inner diameter of 50 μm (*see* **Note 18**) for the analysis of the ABEE derivatives. Make a detection window (*see* **Note 19**) on the capillary and mount the capillary on the detector block. Before starting the analysis, rinse the capillary with sufficient volumes of 100 m*M* sodium hydroxide followed by water, and equilibrate with the running buffer to be used for analysis. Introduce the analytical sample solution from one end of the capillary (*see* **Note 20**), start analysis by applying the separation voltage (normally 15–25 kV), and monitor the ABEE derivatives at 305 nm.

3.2. The ANTS Method

1. Derivatization: Add a 2-μL portion of the amine reagent solution to the carbohydrate sample and dissolve it by gentle swirling. Add 2 μL of the reducing agent to the solution and vortex the mixture. Heat the mixture on a heating block at 40°C overnight (16 h) (*see* **Note 21**). Dilute the reaction solution by 200-fold with the running buffer, and analyze this solution by CE.
2. CE: Use any apparatus equipped with a high-voltage power supply, a sample injection device, and a laser-induced fluorescence detector. Make a detection window on a capillary by burning a short segment (3–4 mm) of the polyimide coating and mount the capillary on the capillary holder. Prior to analysis, flush the capillary with 0.1 *M* sodium hydroxide and rinse it with the running buffer (*see* **Note 22**) for 2 min each. Detect the separated derivatives at approx 520 nm with irradiation by He–Cd laser.

3.3. The NBD-MG Method

1. Release of *N*-glycans from glycoproteins (*see* **Note 23**): Add 10 μL of a 5% w/v SDS solution in 10 w/v% aqueous solution of 2-mercaptoethanol to a glycoprotein sample (~100 μg) and heat the mixture for 5 min on a boiling water bath for denaturation of the glycoprotein sample. Subsequently add 5 μL of 20 m*M* potassium phosphate buffer, pH 7.5; 5 μL of 10% w/v NP-40; and 500 U of PNGase F and incubate the mixture for 24 h at 37°C. Terminate the enzyme reaction by heating the mixture for 3 min on a boiling water bath.
2. Derivatization: Add 100 μL of the methylamine solution for reductive amination (*see* **Note 24**) to the carbohydrate sample and dissolve it by gentle swirling. Add dimethylamine–borane complex to the solution to make a concentration of 40 m*M*. Heat the mixture for 30–45 min at 40°C, and evaporate the solution to dryness by a SpeedVac. To the residue add 5 μL of the reaction medium (borate buffer, pH 8.0) and 5 μL of the reagent solution for tagging, heat the mixture for 5 min at 70°C, and evaporate the solvent by a SpeedVac. For the derivatization of samples containing simple mono- and/or oligosaccharides free of proteinaceous substances, make an analytical solution by dissolving the residue in an appropriate volume of the running buffer, and analyze the derivatives using this buffer. In cases of the derivatization of the *N*-glycans from glycoproteins, however, purify the derivatives by the following procedure. Add the same volume of ethyl acetate (*see* **Note 25**) as that of the analytical sample solution, and vigorously shake the

mixture. Discard the upper layer and add again the same volume of ethyl acetate. Repeat this procedure three times in total, and analyze the final aqueous solution by CE.

3. CE: Use an apparatus equipped with a sample introduction device, a high-voltage supply, an argon laser capable of supplying approx 3 mW power (*see* **Note 26**), a fluorescence detector, and a data processor. Uncoated fused silica capillaries are commercially available, for example, from Polymicro Technologies, Phoenix, AZ (*see* **Note 27**). Make a detection window on a capillary and install the capillary by adjusting the position of the detection window to the laser beam (*see* **Note 28**). Because the three-dimensional positioning of the detection window to the laser beam requires a delicate technique, the use of a commercial system is recommended to obtain the highest sensitivity.

3.4. CE-ESMS

1. Derivatization procedure (*see* **Note 29**): The PTMAA derivatization is based on a method described earlier ***(35)*** with minor modifications. Add 5 µL of the aminating reagent solution to the lyophilized carbohydrate sample and heat the mixture at 90°C for 5 min. After cooling to room temperature, add a 20-µL aliquot of the reducing reagent solution to the reaction mixture. Mix the solution and incubate for a further 60 min at 90°C. Then evaporate the reaction mixture to dryness and reconstitute in water prior to analysis. For the derivatization with 2-AP, use the following method ***(36)***. Add approx 40 µL of the derivatizing solution to the oligosaccharide solution in a sealed 200-µL glass vial. Incubate the solution overnight at 80°C. After cooling to room temperature, adjust the pH of the solution to approx 10 by adding 5 *M* ammonium hydroxide dropwise. Extract the solution twice with an equivalent volume of toluene to remove the excess 2-AP. Isolate the aqueous layer, evaporate to dryness, and reconstitute in deionized water at a concentration of 1 mg/mL relative to the initial weight of the glycoprotein (if applicable).
2. Capillary conditioning (*see* **Note 30**): Rinse the bare fused silica capillary in turn with 1 *M* sodium hydroxide (20 min), deionized water (20 min), 5% (w/v) Polybrene, and 2% (v/v) ethylene glycol in deionized water (20 min), and finally with 2 *M* formic acid (20 min). Then flush the CE buffer through the capillary for approx 10 min at 20 Psi. Between analyses it is recommended to recondition the capillary with sodium hydroxide (3 min, 20 Psi), dionized water (1.5 min, 20 Psi), Polybrene solution (1.5 min, 20 Psi), and buffer (2 min, 20 Psi).
3. CE–ESMS interface and mass spectral acquisition (*see* **Note 31**):
   Remove a 3-mm portion of the polyimide coating from the electrospray emitter end of the capillary to ensure proper wetting of the tip with the sheath liquid. This is achieved by gently burning the coating with an open flame and removing the char with a tissue impregnated with methanol. Insert column in the coaxial CE–ESMS interface from the emitter end and cut a 1-cm portion of the capillary from the injector end to avoid blockage. Introduce a sheath buffer (0.1 *M* formic acid in 2% methanol) to the back tee of the CE–ESMS interface (*see* Chapter 13, **Fig. 2**). Optimization of the mass spectrometer (emitter position, nebulizer, and

sheath solution flow rates) is achieved by electrokinetically infusing a solution of angiotensin I (10 µg/mL in the separation buffer) at a voltage of –20 kV (current 30 µA) and maximizing the signal of the triply protonated ion at *m/z* 433. Redissolve derivatized glycans in water to give a concentration of approx 100 µg/mL. Inject approx 40 nL (head pressure 300 mbar for 0.1 min) of the sample on the capillary using the CE instrument. Record mass spectra by acquiring mass spectrum over the range *m/z* 350–1500 with a duty cycle of 3 s/scan. Set the orifice voltage to a minimal value (typically 50 V) to avoid in-source fragmentation.

3.5. Enhancement of Sample Loading

1. Release of oligosaccharides from ribonuclease B (*see also* **Subheading 3.3.** and Chapter 6): Dissolve approximately 2 mg of a glycoprotein sample in 460 µL of the digestion buffer to which then add 25 µL of a PNGase F solution. Digest the sample at 37°C overnight with occasional monitoring by CE-UV using 10-µL aliquots of the solution. Details of the CE analysis procedure are provided below. Collect the free oligosaccharide fraction by filtering the solution through an Ultrafree-MC 10,000 molecular weight centrifuge filter. Evaporate the filtrate to dryness on the centrifugal evaporator and redissolve in 10 µL deionized water.
2. Preparation of 2-AP or PTMAA derivatives (*see* **Subheading 3.4.**).
3. Preparation of linear polyacrylamide (LPA)-coated capillaries: The procedure used is based on a published method ***(40)***. Rinse a fused silica capillary (50 µm i.d., 180 µm o.d., 3 m) for derivatization in turn with 1 *M* NaOH, deionized water, and methanol, each for 1 h. This can be done using the capillary column rinsing kit. Fill the capillary with 20 µL of a solution of 7-oct-1-enyltrimethoxysilane and 20 µL of acetic acid in 4 mL of methanol and allow to stand overnight. Apply low pressure of nitrogen (5 Psi) to ensure constant flow through the capillary at all times. Subsequently, rinse the capillary with methanol and deionized water, each for 1 h. Then fill the capillary with a filtered and degassed solution of 1 mL of 4% w/v acrylamide, to which add 14 µL of 10% w/v ammonium persulfate and 2 µL of TEMED. Maintain a constant flow through the column overnight. Finally, rinse the capillary for 1 h with water and store until required.
4. Analysis of 2-AP-derivatized oligosaccharides by CZE–ESMS with isotachophoretic preconcentration (*see* **Fig. 10** and **Note 32**): The coaxial CE–ESMS interface described in **Subheading 3.4.** was used in the present experiment. However, an LPA-coated capillary (50 µm i.d., 180 µm o.d., 0.90 m) was used for separation. Fill the capillary first with the leading electrolyte buffer. Fill approx 20% of the column with sample dissolved in the sample dissolution solution (approx 360 nL). Maintain the sheath liquid flow rate at 2–3 µL/min. Perform isotachophoretic preconcentration for 1 min at 25 kV with the inlet of the capillary submerged in terminating electrolyte solution. Then move the capillary to a vial containing the leading electrolyte and carry out capillary zone electrophoresis at 25 kV. The ESMS acquisition conditions are as described in **Subheading 3.4.**

4. Notes

4.1. Materials, the ABEE Method

1. First evaporate the solutions under moderate vacuum to avoid bumping. Gradual evaporation under these conditions causes temperature decrease, which helps further evaporation in higher vacuum without bumping.
2. The reagent solution should be prepared fresh before derivatization.
3. We usually use a sample from Wako Pure Chemicals (Dosho-machi, Chuo-ku, Osaka, Japan), but samples from other sources are also suitable provided they are of reagent grade.
4. Because this reagent is hygroscopic and tends to decompose in moisture, the original sample should be subdivided in several vials under a nitrogen stream and a relevant amount of reagent can be used when required.

4.2. Materials, the ANTS Method

5. A 0.5-mL volume polyethylene tube with a screw cap is recommended.
6. A highly purified specimen of disodium salt of ANTS is commercially available from Molecular Probes (4849 Pitchford Avenue, Eugene, OR).
7. *See* **Note 4**.

4.3. Materials, the NBD-MG Method

8. We use this evaporation technique widely for carbohydrate analysis. For further details *see* **Subheading 3.1.** in this chapter for the ABEE method. *See also* Chapters 5 and 10.
9. Purification might be performed in an earlier stage such as the completion of *N*-glycan release, but our strategy is to purify all at once at the final stage, because it is considered to be simple. The major impurities in the final derivatization products can be conveniently removed by solvent extraction, and the use of ethyl acetate as an extractant proved to be the most effective. Removal by ultrafiltration and solid-phase extraction did not give good results.
10. Use a commercial sample of an aqueous solution of methylamine of the highest quality.
11. We use a sample from Tokyo Kasei Kogyo (Nihonbashi-honcho, Chuo-ku, Tokyo, Japan) but it is also available from Sigma and Acros. Solvents other than ethanol gave a number of peaks of unidentified byproducts on derivatization.

4.4. Materials, Enhancement of Sample Loading

12. All reagents including 2-AP should be prepared fresh before use.
13. CE–ESMS analyses were conducted using a coaxial sheath flow interface. In contrast to that described previously for CE–ESMS experiments, tapering of the capillary end was not required. Both ends of the capillary should be leveled to minimize losses of separation efficiency. The injection end of the capillary could be kept elevated (2 cm above the electrospray end) to shorten analysis time. The nebulizer flow rate should be adjusted to prevent siphoning effects at the end of

the capillary. Under selected ion recording (SIR), scanning mode, detection limits of 3 fmol of 2-AP-maltoheptaose (or 10 ng/mL for approx 300 nL inj.) were typically achieved with the present electrophoretic conditions.

4.5. Methods, the ABEE Method

14. Solvents with higher dielectric constants do not extract the excess reagent effectively, and solvents with lower dielectric constants give low recovery yields.
15. This is effective to remove byproducts. A column dimension of 1 cm i.d., 50 cm length is recommended.
16. ABEE derivatives give weak fluorescence at 305 nm (excitation)/370 nm (emission) under the conditions used. Monitoring the UV absorption is not recommended, because considerable amounts of byproducts are eluted together with ABEE derivatives.
17. The use of a running buffer as sample matrix is desirable, but the use of distilled water is more practical even though separation may degrade slightly because the derivatives can easily be recovered simply by evaporation.
18. Commercially available capillaries with an inner diameter of 50 μm generally have an outer diameter of 375 μm. A length of 40–50 cm is preferable to reduce analysis time.
19. It can be created easily by burning a part of the polyimide coating of a capillary. A small flame can be used for this purpose, but the use of a stick of incense or a commercial window maker is recommended, because it can make a small window with high reproducibility.
20. When an uncoated fused silica capillary is used, EOF is toward the cathode. Use of an alkaline borate buffer facilitates complexation of ABEE derivatives with the borate ion with varying magnitudes. Therefore, the ABEE derivatives are held back to the anode at varying velocities, whereas EOF is rapid and constant toward the cathode independent of this electrophoretic effect. As a result the ABEE derivatives move to the cathode at different total velocities.

4.6. Methods, the ANTS Method

21. Closing the vial tightly with a screw cap is essential to prevent the vaporization of the liquid.
22. Acidic buffers such as 100 m*M* phosphate buffer, pH 2.5, are recommended. Under such conditions the sulfonate group is dissociated to give a strong negative charge to the derivatives. ANTS-derivatized mono- and oligosaccharides introduced from the cathodic end of a fused silica capillary migrate quickly to the anode and are typically separated from each other as the strong electrophoretic mobility of the negatively charged derivatives overtakes the opposite electroosmotic flow of correspondingly smaller mobility.

4.7. Methods, the NBD-MG Method

23. *See also* Chapter 10.
24. Larger excess amounts of methylamine and dimethylamine–borane complex are added, but they can be completely removed simply by evaporation after reaction.

25. Investigation of many other solvents indicated that this solvent was best. Almost all substances giving their peaks in the NBD-MG-oligosaccharide region could be removed by this simple procedure.
26. Use of an argon laser of 10–25 mW is recommended from the aspect of durability, although the output power in analytical runs is actually approx 3 mW.
27. Use of a narrow (e.g., 10 µm i.d.)-bore capillary is recommended to obtain better separation. In addition the amount of sample can be reduced
28. Well established apparatus are commercially available, for example, from Beckman (Palo Alto, CA) and Bio-Rad (Richmond, CA) instrument companies.

4.8. Methods, CE–ESMS

29. The 2-AP and PTMAA reagent solutions should be prepared fresh before use.
30. Loss of separation performance can be observed when using CE buffer of high concentration (e.g., >1 *M* formic acid) and column reconditioning between analysis is required. The user can perform the reconditioning by retracting the ESMS probe to prevent accumulation of salt on the interface plate or by using a retractable arm to collect conditioning reagent ***(33)***. Alternatively, a capillary modified with a cationic reagent can be used (*see* Chapter 13, **Subheading 3.4.**).
31. CE–ESMS analyses are conducted using a co-axial sheath flow interface (*see* Chapter 13, **Fig. 2**). Enhanced sensitivity and spray stability can be obtained by tapering the end of the CE capillary to 15 µm i.d. using a microtorch or a laser capillary puller ***(37)***. Acetonitrile (10% v/v) is added to the separation buffer to reduce the magnitude of EOF and to provide better spray stability conditions. Both ends of the capillary should be leveled to minimize losses of separation efficiency. The nebulizer flow rate should be adjusted to prevent siphoning effects at the end of the capillary. Under the present operating conditions, a detection limit of 10 µg/mL is typically achievable in full mass scan acquisition using a triple quadrupole mass spectrometer. Separation requiring faster and more sensitive mass spectral acquisition (>1 s/scan) can be achieved using a time-of-flight instrument that also enables isotopic resolution of closely related oligosaccharides. **Figure 10** shows the CE–ESMS analysis of PTMAA-derivatized maltooligosaccharides for on-column injection of 5 ng of the total mixture. The contour profile (**Fig. 10B**) shows a series of doubly protonated molecules from which the molecular mass profile can be reconstructed. For example, the peak at 14.8 min gave $[M+2H]^{2+}$ at *m/z* 888 molecular mass (M_{obs}) of 1774.0 Da, consistent with the expected decaose (M_{calc}.: 1773.1 Da).

4.9. Methods, Enhancement of Sample Loading

32. **Figure 12** shows the CE–ESMS analysis of AP-derivatized oligosaccharides obtained from ribonuclease B for an on-column injection of 400 ng of the total mixture. Reconstructed ion electropherograms are shown for different glycoforms ranging from Man_5 to Man_9, Man_5 being the most abundant. Each peak monitored corresponds to the singly protonated ion, $[M+H]^+$ of the corresponding oligosaccharide derivative.

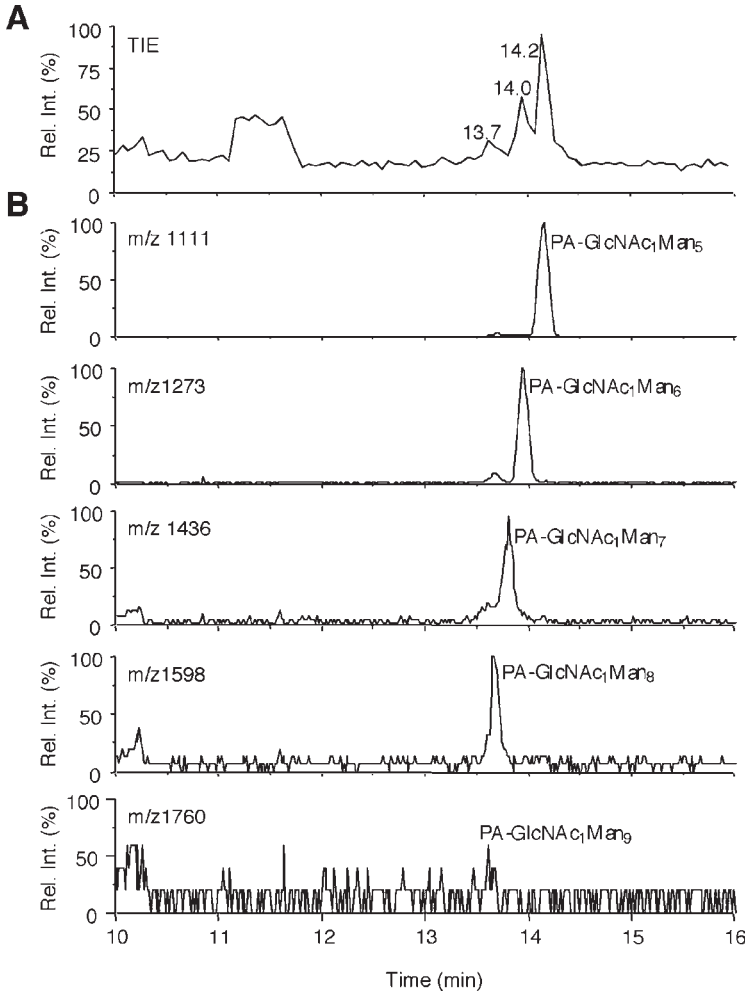

Fig. 12. CITP–CZE–ESMS of the 2-AP derivatives of glycans released from ribonuclease B.

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4

Preparation of Oligosaccharides from Sulfated Glycosaminoglycans Using Bacterial Enzymes

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1. Introduction

Although the polysaccharide backbone Glycosaminoglycans (GAGs) have been demonstrated to interact with a variety of proteins and such interactions are thought to be involved in the regulation of the physiological functions of these proteins ***(1)***. Although the polysaccharide backbone of GAGs is a linear polymer composed of alternating amino sugar and hexuronic acid residues, this simple repeat structure acquires a considerable degree of variability by extensive modifications involving sulfations and uronate epimerization ***(2)***. The structural variability is the basis for the wide variety of domain structures with biological activities ***(1)***. Investigation of the structure–function relationship of GAGs has been hindered by the difficulty in microanalyzing their complicated structure. GAG molecules are so heterogeneous that sequence analysis on unfractionated GAG chains can give only statistical structural information. Actual sequencing is possible, however, on oligosaccharide fragments, which can be obtained by chemical or enzymatic degradation of GAG chains followed by separation by means of various chromatographies. In this chapter, we describe the methods for enzymatic degradation of GAG chains and fractionation of the oligosaccharide products. Because many kinds of highly purified GAG-degrading enzymes, which are not contaminated by sulfatases, are commercially available and cleave polysaccharides with high specificities under mild conditions, enzymatic cleavage is a useful method for the preparation of GAG oligosaccharides. Although treatment with bacterial eliminases converts the original uronic acid structure in the polysaccharides into an artificial structure, the 4,5-unsaturated uronic acid, 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid, can be detected with high sensitivity and quantitated by its

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absorbance at 232 nm ***(3,4)***. Thus, bacterial eliminases have proved valuable for the structural study of GAGs.

2. Materials

2.1. Enzymes

1. Chondroitinase ABC (a conventional and a highly purified preparation), AC-I, AC-II or B are utilized for depolymerization of chondroitin sulfate/dermatan sulfate. Highly purified chondroitinase ABC is commercially available as a protease-free preparation from Seikagaku Corp. These chondroitinases have distinct substrate specificities respectively and produce different sets of oligosaccharides from chondroitin and/or dermatan sulfate ***(5)***.
2. Hyaluronidases can also be used for depolymerization of chondroitin sulfate ***(5)***. Hyaluronidase from sheep testes and hyaluronate lyase from *Streptococcus dysgalactiae* (hyaluronidase SD) are commercially available. For depolymerization of heparin/heparan sulfate, heparinase and heparitinases I and II isolated from *Flavobacterium heparinum* are usually utilized ***(5,6)***. Recombinant forms of these three enzymes are also used ***(7–9)***.
3. Other heparin/heparan sulfate-degrading enzymes, heparitinases I_0, IV, and V, have also been purified from *Flavobacterium* sp. Hp206 and their substrate specificities have been examined ***(10)***, although they are not commercially available yet. Because substrate specificities are different among individual heparin/heparan sulfate-degrading enzymes, these enzymes produce different sets of oligosaccharides ***(6)***. The substrate specificities of the above GAG-degrading enzymes have been extensively studied ***(5)***.

2.2. Buffers for Enzymatic Digestion

1. Chondroitinase ABC (conventional and highly purified preparations): 50 m*M* Tris-HCl, pH 8.0 containing 60 m*M* CH_3COONa.
2. Chondroitinase AC-I: 50 m*M* Tris-HCl, pH 7.3.
3. Chondroitinase AC-II: 50 m*M* Acetate-Na buffer, pH 6.0.
4. Chondroitinase B: 100 m*M* Tris-HCl, pH 8.0.
5. Hyaluronidase (from sheep testes): 50 m*M* Phosphate-Na buffer, pH 6.0, containing 150 m*M* NaCl.
6. Hyaluronidase SD: 40 m*M* Phosphate-Na buffer, pH 6.2.
7. Heparinase: 100 m*M* Acetate-Na, pH 7.0, containing 3 m*M* $Ca(CH_3COO)_2$.
8. Heparitinases: 20 m*M* Acetate-Na, pH 7.0, containing 2 m*M* $Ca(CH_3COO)_2$.

3. Methods

A given GAG preparation is incubated with a suitable degradation enzyme in the buffer described in the preceding at 37°C (*see* **Note 1**). Aliquots are withdrawn to monitor the reaction by measurement of UV absorption (*see* **Note 2**). The reaction is terminated by treatment in boiling water for 2 min when the reaction reaches the degradation level aimed at. To complete the reaction, an

additional amount of the enzyme is usually added. The digest is subjected to gel filtration chromatography and is fractionated into a series of even-numbered oligosaccharides. The separated fractions are pooled, concentrated, desalted by gel filtration, and lyophilized. Each fraction is subfractionated by HPLC on an amine-bound silica column ***(11–13)***.

3.1. Extensive Heparinase Digestion of a Large Amount of Commercial Heparin

Using this procedure, 5–30 µmol of heparinase-resistant oligosaccharides (a mixture of tetra-, hexa-, octa-, and decasaccharides) is obtained in addition to disaccharide products ***(14)***.

1. Dissolve 300 mg of commercial porcine intestinal heparin (Na salt) (American Diagnostica Inc., New York) in 5.6 mL of distilled water.
2. Add 1.6 mL of 500 m*M* acetate-Na, pH 7.0 containing 15 m*M* $Ca(CH_3COO)_2$ and 656 µL of 0.1% bovine serum albumin (*see* **Note 3**).
3. Add 2.4 IU (144 µL) of heparinase solution (5 IU/300 µL) (*see* **Note 4**).
4. Incubate at 37°C for 20 h.
5. Withdraw aliquots at 0.5- to 1-h intervals to monitor the reaction by measurement of absorption at 232 nm (*see* **Note 5**).
6. Boil for 2 min to terminate the reaction.
7. Store at –20°C until fractionation.

3.2. Extensive Digestion of Whale Cartilage Chondroitin Sulfate with a Highly Purified Preparation of Chondroitinase ABC

Using this procedure, approx 48 and 12 µmol of unsaturated di- and tetrasaccharides is obtained, respectively ***(15)***, as a highly purified preparation of chondroitinase ABC does not degrade tetrasaccharides into disaccharides ***(15,16)***. This procedure is applicable to digestion of other types of chondroitin sulfate. To prepare larger oligosaccharides, partial digestions can be used by reducing the amount of the enzyme or by shortening the incubation period.

1. Dissolve 50 mg of whale cartilage chondroitin sulfate A (Seikagaku Corp., Tokyo, Japan) in 1.2 mL of distilled water.
2. Add 0.4 mL of 250 m*M* Tris-HCl, pH 8.0, containing 300 m*M* CH_3COO Na and 200 µL of 0.1% albumin (*see* **Note 3**).
3. Add 1.0 IU (200 µL) of highly purified chondroitinase ABC (1 IU/200 µL) (*see* **Note 4**).
4. Incubate at 37°C for 30 h.
5. Withdraw aliquots at 0.5- to 1-h intervals to monitor the reaction by measurement of absorption at 232 nm (*see* **Note 5**).
6. Boil for 2 min to terminate the reaction.
7. Store at –20°C until fractionation.

3.3. Complete Digestion of Shark Cartilage Chondroitin Sulfate with Sheep Testicular Hyaluronidase

Using this procedure, even-numbered oligosaccharides, mainly tetra-, hexa-, octa-, and decasaccharides with glucuronic acid residues at the nonreducing ends, are obtained. The amounts of these oligosaccharide fractions are 1.2, 0.84, 0.61, and 0.51 μmol, respectively ***(17)***.

1. Dissolve 100 mg of shark cartilage chondroitin sulfate D (Seikagaku Corp., Tokyo, Japan) in 1.6 mL of distilled water.
2. Add 0.4 mL of 250 m*M* phosphate-Na buffer, pH 6.0 containing 750 m*M* NaCl.
3. Add 14 mg (approx 21,000 National Formulary Units [NFUs]) of sheep testicular hyaluronidase (*see* **Note 6**).
4. Incubate at 37°C for 25 h.
5. Withdraw aliquots at 0.5- to 1-h intervals to monitor the reaction by the turbidity assay.
6. Boil for 2 min to terminate the reaction.
7. Store at –20°C until fractionation.

3.4. Fractionation of Sulfated Oligosaccharides

Enzyme digests prepared as described in the preceding are fractionated by gel filtration. Separated fractions are desalted by gel filtration and subfractionated by HPLC as described in the following.

1. Gel filtration conditions for fractionation of enzyme digests into disaccharide and oligosaccharide fractions (**Fig. 1**, *inset*):
   Column: Sephadex G-25 fine (46 × 1.5 cm inner diameter [i.d.]) (Amersham Pharmacia Biotech, Tokyo, Japan).
   Eluent: 0.25 *M* NH_4HCO_3/7% 1-propanol.
   Detection: Absorbance at 232 nm (*see* **Note 7**).
2. Gel filtration conditions for separation of enzyme digests into a series of even-numbered oligosaccharide fractions (**Fig. 1**) ***(14)***.
   Column: Bio-Gel P-10 (95 × 1.6 cm [i.d.]) (Bio-Rad, Hercules, CA).
   Eluent: 1.0 *M* NaCl/10% ethanol.
   Detection: Absorbance at 232 nm (*see* **Note 7**).
3. Gel filtration conditions for desalting:
   Column: Sephadex G-25 fine (46 × 1.5 cm [i.d.]).
   Eluent: Distilled water.
   Detection: Absorbance at 232 nm (*see* **Note 7**).
4. Experimental conditions for HPLC subfractionation (*see* **Note 8** and **Fig. 2**) ***(18)***:
   Column: YMC-Pack PA-03 (250 × 4.6 mm [i.d.]) (YMC Co., Kyoto, Japan).
   Flow rate: 1 mL/min.
   Elution conditions: A linear gradient of NaH_2PO_4. The concentration is increased from 200 to 800 m*M* over 70 min.
   Detection: Absorbance at 232 nm (*see* **Note 7**).

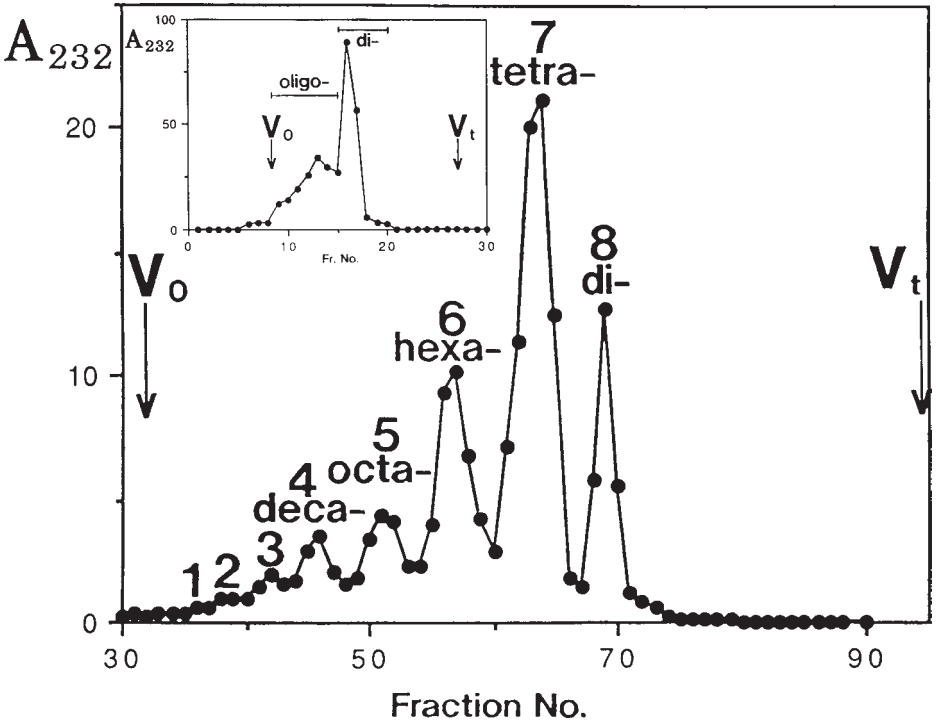

Fig. 1. Size fractionation of the heparinase digest of porcine intestinal heparin by gel filtration chromatography ***(14)***. For digestion conditions, *see* **Subheading 3.1.** Since a large amount of disaccharides is produced by this digestion experiment, disaccharides are first separated from larger oligosaccharides as shown in the *inset*. Then, the oligosaccharide fraction is fractionated further on a column of Bio-Gel P-10. It may be possible to fractionate the heparinase digest directly through a column of Bio-Gel P-10 without removing disaccharides by gel filtration on Sephadex G-25. (Reproduced with premission from **ref. *14***, Shujunsha Co., Ltd.).

4. Notes

1. The optimum temperature for maximum activity of heparitinase I is 43°C ***(5,19)***.
2. Bacterial eliminases produce oligosaccharides with 4,5-unsaturated uronic acid at the nonreducing ends. Unsaturated uronic acid-containing oligosaccharides can be detected with high sensitivity owing to absorbance at 232 nm and can be spectrophotometrically quantified based on an average millimolar absorption coefficient of 5.5 at 232 nm ***(4)***. On the other hand, oligosaccharides produced by mammalian testicular hyaluronidases, which are hydrolases, contain glucuronic acid residues, but not unsaturated uronic acid, at the nonreducing ends. Hence, to monitor the hyaluronidase digestion, the turbidity of the reaction mixture caused by formation of the complex between GAGs and cetrimide is measured ***(20)***. As the reaction proceeds and GAGs are degraded into oligosaccharides, the turbidity decreases.

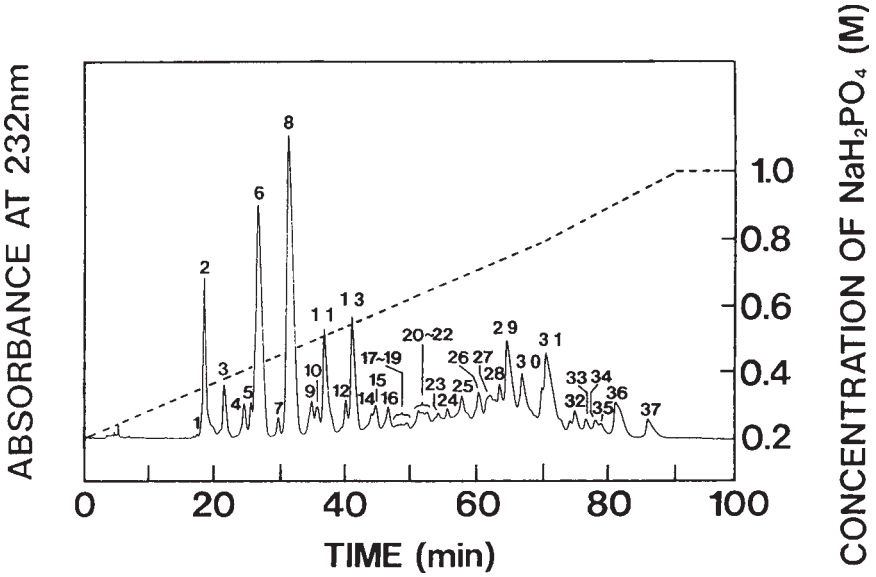

Fig. 2. HPLC fractionation of the octasaccharide fraction on a PA-03 column ***(18)***. The octasaccharide fraction obtained from gel filtration on Bio-Gel P-10 (fraction 5 in **Fig. 1**) was separated into subfractions 5-1 to 5-37 using an NaH_2PO_4 gradient as indicated by the *dashed line*. (Reproduced with permission from **ref. *14***. Shujunsha Co., Ltd.).

3. Bovine serum albumin is usually added as a stabilizer of the enzymes.
4. Enzymes are dissolved in each appropriate incubation buffer containing 50% glycerin that has been precooled, and stored at –20°C. Chondroitinases may be dissolved in distilled water containing 50% glycerin. Most of the enzymes in the respective buffers containing 50% glycerin are stable for several months when stored at –20 ˚C.
5. Aliquots of the reaction mixture are diluted 600–700 × with 0.01 *N* HCl and used for monitoring the digestion. When the reaction reaches the degradation level aimed at, the digestion is terminated by heat treatment in boiling water. Unless the reaction is completed, an additional amount (usually one tenth the initial amount used) of the enzyme is added.
6. One NFU (National Formulary Unit) corresponds to the amount of the enzyme that hydrolyses 74 μg of hyaluronate/min ***(21)***.
7. Bacterial eliminases produce unsaturated oligosaccharides, which are detected by absorbance at 232 nm. Because testicular hyaluronidases are hydrolases and the digestion products do not contain unsaturated uronic acid residues, these oligosaccharides are detected by UV absorbance at 210 nm caused by *N*-acetyl groups of GalNAc residues ***(17)***.
8. Oligosaccharides obtained by enzymatic digestion followed by gel filtration are treated with a 0.45-μm C3HV membrane filter (Millipore), and an aliquot is sub-

jected to HPLC analysis ***(11–13)***. For preparative HPLC, up to 1 µmol of oligosaccharides is applied to an analytical PA-03 column by a single injection. To prepare a larger amount of oligosaccharides, up to 3 µmol of oligosaccharides is applied to a preparative YMC-Pack PA-13 column (250 × 6 mm [i.d.]) by a single injection at a flow rate of 1.7 mL/min.

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5

Monosaccharide Analysis by Capillary Electrophoresis

Susumu Honda

1. Introduction

In carbohydrate analysis efficient separation is indispensable, because a sample is usually accompanied by analogous compounds having very similar structures and properties. All carbohydrates contain hydroxyl group and most of them in addition have a characteristic functional group, such as the hemiacetal (aldoses), keto (ketoses), and carboxyl (aldonic and saccharic acids) groups. There are also compounds having two or more of such functional groups (e.g., uronic acids having the hemiacetal and carboxyl groups; sialic acids having the keto and carboxyl groups). Alditols are exceptional in having no such functional groups. In deoxy sugars a part of the hydroxyl group is eliminated, and in amino sugars, aminocyclitols, muramic acid, and sialic acids, it is substituted by the amino or acylamino group. Each of the aforementioned groups comprises homologues having different numbers of carbon atoms. The homologues composed of carbon atoms more than five have ring structures and have various epimers based on configurational differences. Thus, the number of possible monosaccharide species will reach almost an astronomical figure.

Although the possible number of monosaccharide species is extremely large, we need not necessarily analyze all species in one run. Each monosaccharide group has a characteristic chemical property depending on the functional group(s), hence can be separated by a particular combination of solid and stationary phases and detected by utilizing the selective reactivity to a certain kind of reagent in high-performance liquid chromatography (HPLC) ***(1)***. For example, aldose epimers can be simultaneously analyzed as borate complexes on an anion-exchange column with fluorimetric detection after postcolumn

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labeling with 2-cyanoacetamide ***(2)***. On the other hand, amino sugars are analyzed on a cation-exchange column with ultraviolet (UV) detection after postcolumn labeling by the Hantzsch reaction ***(3)***. In addition to this concept of group analysis based on chemical properties, there is another kind of group analysis that is inherent to sample source. For example, samples from seaweeds contain a few types of aldoses and uronic acids together with an anhydroaldose, whereas samples from bacterial cell walls contain several kinds of aldoses and deoxy sugars, together with muramic acid. These component monosaccharides are analyzed using systems specially designed for such combinations.

Analysis of monosaccharides by high-performance capillary electrophoresis (HPCE) has a rather different feature from that by HPLC, in that it is primarily a method for ions. Direct separation of monosaccharides as anions can be achieved by ionization in strong alkali based on the same principle as in anion-exchange chromatography using alkali-stable peripheral resins ***(4)***. The monosaccharides thus separated by HPLC can be detected by the electrochemical method, which is easily achieved using a commercially available detector. In HPCE, however, such electrochemical detection requires an ingenious technique, because an analytical column is extremely narrow. Colón et al. ***(5)*** succeeded in sensitive on-column detection of mono- and oligosaccharides separated by HPCE in strong alkali using a specially made narrow copper wire as electrode. However, this technique is too delicate to be used in average laboratories. Pulse amperometric detection on a gold electrode was also attempted for HPCE of carbohydrates ***(6)***, but it has been still left as a challenging technique.

Under these circumstances chemical derivatization to ions is promising. The derivatization in HPCE must involve conversion of the components of a sample to not only ionic but also chromophore/fluorophore-bearing derivatives. Among various methods for precapillary derivatization the 1-phenyl-3-methyl-5-pyrazolone (PMP) method ***(7)*** is preferable, because it proceeds rapidly under mild conditions to give quantitatively *bis*-PMP derivatives from reducing carbohydrates (**Fig. 1**).

The keto groups in the PMP derivatives are interchangeable to the enol groups by tautomerism, and the molar proportion of the keto form to the enol form depends on pH. Either form absorbs strongly in the UV region, though in different magnitudes. Unlike most of the reported methods based on reductive amination in nonaqueous acidic solvents, the PMP method can be applied to aqueous neutral solutions of reducing carbohydrates.

The PMP method is applicable to all reducing monosaccharides including aldoses, acylamino sugars, and uronic acids (*see* **Note 1**). In this chapter details are described for the derivatization of aldoses that constitute the basic group of monosaccharides. As special interest of this volume is in the analysis of the carbohydrates of animal origin, especially glycans in glycoconjugates, it is pri-

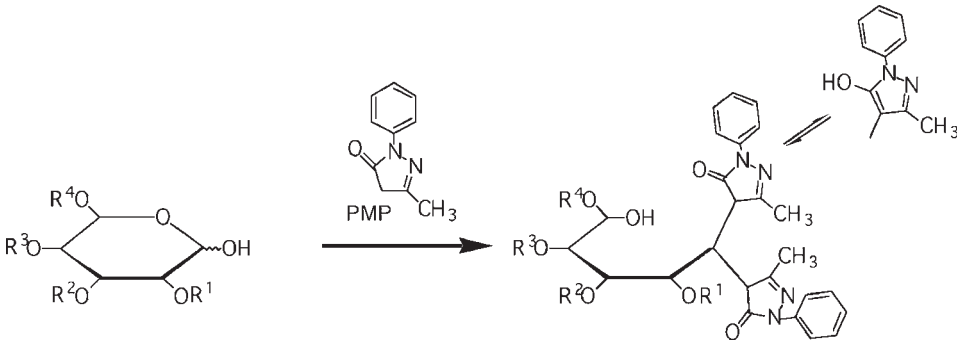

Fig. 1. Derivatization of a reducing carbohydrate with PMP. The derivatization involves consecutive introduction of the PMP group to a carbohydrate up to two. With low molar ratios of PMP to the carbohydrate the principal product is the *bis*-PMP derivative, but a small amount of mono-PMP derivative is concurrently formed. With sufficient excess of PMP, the *bis*-PMP derivative is formed quantitatively.

marily important to determine their monosaccharide composition. Therefore, analysis of the monosaccharides in glycoproteins, including several aldoses and *N*-acetylhexosamines, is also emphasized.

2. Materials and Apparatus

2.1. Derivatization

1. The PMP reagent (*see* **Note 2**).
2. The reagent solution: 0.5 mol/L of PMP in methanol (*see* **Note 3**).
3. Monosaccharide samples (*see* **Note 4**).
4. 0.3 *M* of sodium hydroxide (*see* **Note 5**).
5. 0.3 *M* of hydrochloric acid (*see* **Note 5**).
6. Ethyl acetate: Reagent grade (*see* **Note 6**).
7. Microtubes: Screw-capped microtubes having inner capacity of approx 1 mL.
8. A centrifugal vacuum concentrator (a SpeedVac).
9. A vacuum desiccator containing pellets of sodium hydroxide as desiccant.

2.2. HPCE

1. Instruments: An HPCE apparatus, homemade or marketed, equipped with a high voltage supply, a sample introducing system, and a UV detector (*see* **Note 7**).
2. Capillary: A fused silica tube having an inner diameter of 50 μm (*see* **Note 8**).
3. 1 mol/L of sodium hydroxide for capillary cleaning.
4. Electrophoretic solution (running buffer): 200 m*M* borate buffer, pH 9.5, degassed before use (*see* **Note 9**).

2.3. Hydrolysis of Glycoproteins

1. Glycoprotein samples (*see* **Note 10**).
2. Acid catalyst for hydrolysis: 2 mol/L trifluoroacetic acid (*see* **Note 11**).
3. Microtubes: Screw-capped glass tubes having an inner capacity of approx 1 mL.
5. Acetic anhydride: Reagent grade.
6. A saturated aqueous solution of sodium bicarbonate.
7. An Amberlite CG-120 column (hydrogen form) (*see* **Note 12**).
8. A rotary evaporator.

3. Methods

3.1. Derivatization of Monosaccharides with PMP

1. Add 50 µL of the reagent solution of PMP (*see* **Note 3**) to a mixture of monosaccharides or an acid hydrolysate of a carbohydrate material such as a poly- or oligosaccharides, a glycoconjugate (glycoprotein, glycolipid, etc.), and a plant glycoside, in a screw-capped tube (*see* **Note 4**) and dissolve the monosaccharide(s) completely by gentle swirling.
2. Add 0.3 *M* of sodium hydroxide (50 µL) to the resultant solution to convert PMP to its sodium salt (*see* **Note 5**).
3. Heat the solution at 70°C for 30 min to allow for the condensation to complete.
4. Cool the reaction mixture to room temperature.
5. Add 0.3 *M* hydrochloric acid (50 µL) for neutralization of the reaction mixture (*see* **Note 5**).
6. Evaporate the solution to dryness under reduced pressure (*see* **Note 13**).
7. Dissolve the residue in 200 µL of water.
8. Add 200 µL of ethyl acetate, shake the mixture vigorously, and discard the upper layer (ethyl acetate layer). Repeat this process two more times (*see* **Note 6**).
9. Evaporate the final aqueous layer to dryness under reduced pressure by a SpeedVac (*see* **Note 13**).
10. Reconstitute the residue with an indicated volume of water for HPCE analysis (*see* **Note 14**).

3.2. HPCE of the PMP Derivatives of Monosaccharides

1. Remove a 1-mm portion of the polyimide coating at an approx 5-cm position from the outlet (the cathodic end) of the capillary to make a window for UV detection (*see* **Note 15**).
2. Mount the capillary on a cassette, fixing the window at the center of the UV beam.
3. Install the cassette on a HPCE apparatus, and keep it at a specified temperature by circulating temperature-controlled air or liquid depending on the apparatus used.
4. Flash the capillary with 1 mol/L sodium hydroxide at least for 10 min to clean its inner wall, rinse it with water for 5 min, and equilibrate it with the running buffer with application of a high voltage (20 kV) for 10 min.
5. Refill the capillary with the running buffer to make the stand-by state.
6. Introduce a sample solution from the inlet (the anodic end) (*see* **Note 16**).

7. Apply a specified voltage of 15–20 kV between both ends of the capillary, and start recording.
8. Maintain this voltage until all the peaks of the monosaccharides are detected.

The keto group in the PMP derivatives of aldoses in an aqueous solution is changeable to the enol group by tautomerism, and the PMP derivatives exist as an equilibrium mixture of the keto and the enol forms. In addition, the enol group is dissociated to give the enolate group, which is anionic. Therefore, the PMP derivatives are negatively charged in neutral and alkaline media, and move to the cathode in the electric field. However, no separation is expected for the PMP derivatives of aldose epimers, as all derivatives of aldose epimers, in either the pentose or hexose family, have the same molecular weight and the same number of the protecting group (two PMP groups). Nevertheless all the derivatives of aldose epimers are separated from each other by simple zone electrophoresis at pH 7–8. This unusual separation is based on the difference in the ease of intramolecular ring formation by hydrogen bonding ***(8)***. The separation by this mode is fairly good but liable to be affected by a slight pH change.

Separation by zone electrophoresis as borate complexes, as described here, is much more insensitive to pH change. As seen from **Fig. 2** all aldopentose epimers including arabinose, lyxose, ribose and xylose, of the D-series, gave single peaks of the individual *bis*-PMP derivatives well separated from each other, when analyzed in the optimized running buffer (200 m*M* borate buffer, pH 9.5) (*see* **Note 17**) ***(9)***.

Under the same conditions the PMP derivatives of all epimers of aldohexoses (allose, altrose, idose, galactose, glucose, gulose, mannose, talose) of the D-series could also be completely separated from each other (**Fig. 3**) ***(9)***. This is much simpler and better than any other reported separations of aldohexoses. Separation by zone electrophoresis as borate complexes can be further improved by addition of Polybrene to the electrophoretic solution, because of the concerted exertion of ion-interaction electrokinetic chromatography with zone electrophoresis as borate complexes ***(10)***. However, the latter mode alone suffices for the separation of the aldose sets.

3.3. Analysis of the Monosaccharide Composition of Glycoproteins

The monosaccharide analysis mentioned in the preceding subheading can be applied to the analysis of the component monosaccharides in poly- as well as oligosaccharides, glycoconjugates including glycoproteins and glycolipids, and plant glycosides. Examples of its application to a few glycoproteins are given below.

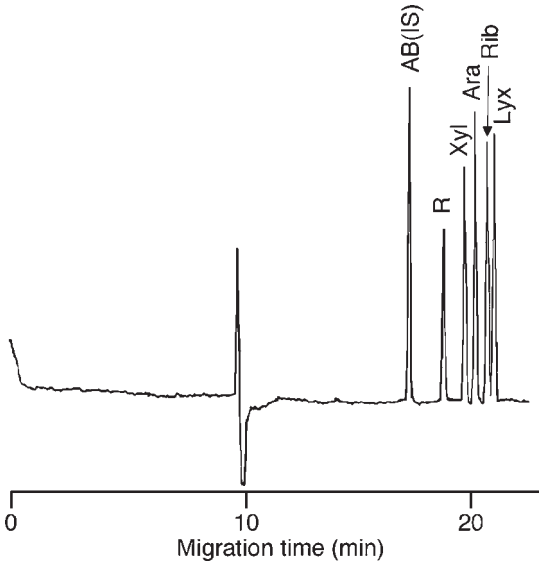

Fig. 2. Analysis of aldopentoses of the D-series by zone electrophoresis as borate complexes of their PMP derivatives. Apparatus, a JASCO capillary electrophoresis system 875-CE (semi-automatic) equipped with a JASCO 890-CE UV detector; capillary, fused silica (50 µm i.d., 78 cm); running buffer, 200 m*M* borate buffer, pH 9.5; applied voltage, 15 kV; detection, UV absorption at 245 nm. Xyl, xylose; Ara, arabinose; Rib, ribose; Lyx, lyxose; AB, Amobarbital (internal standard), R, reagent (PMP). (Reproduced with permission from **ref. *9***).

3.3.1. Separation and Preparation of the Standard Monosaccharides

1. Prepare samples containing 1, 2.5, 5, 7.5, and 10 nmol each of L-fucose, D-galactose, D-mannose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine, together with L-rhamnose and amobarbital (internal standards; *see* **Note 18**), as described in **Subheading 2.1.** (*see* **Note 4**).
2. Derivatize all monosaccharides in each sample with PMP by the procedure described in **Subheading 3.1.**
3. Reconstitute each of the final residues of the derivatization products with 100 µL of water.
4. Inject each of the analytical sample solutions thus prepared to the inlet of the capillary, and apply a voltage of 15 kV according to the procedure described in **Subheading 3.2.**

The aforementioned monosaccharides, which are commonly found in glycoproteins, could also be separated under the same conditions as used for the separation of aldopentose and aldohexose epimers. **Figure 4** shows an example of separation at 5 nmol each of these monosaccharides and rhamnose (internal

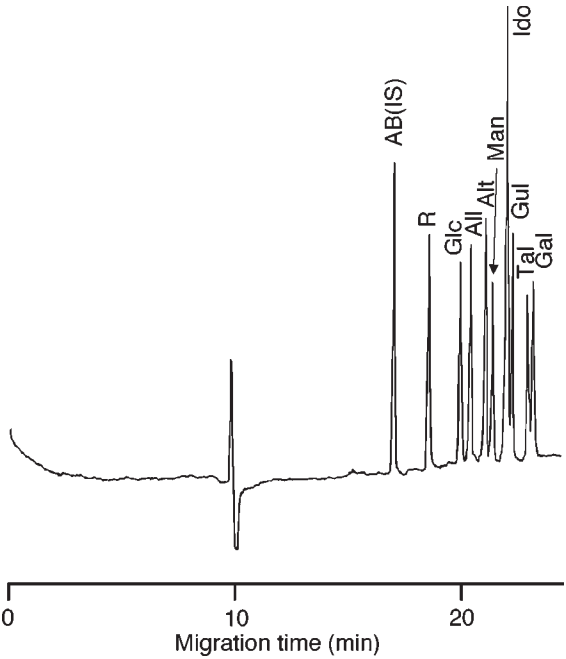

Fig. 3. Analysis of aldohexoses of the D-series by zone electrophoresis as borate complexes of their PMP derivatives. Glc, glucose; All, allose; Alt, altrose; Man, mannose; Ido, idose; Gul, gulose; Tal, talose; Gal, galactose. The analytical conditions and other abbreviations are as in **Fig. 1**. (Reproduced with permission from **ref. *9***).

standard) (Honda, S. and Sasaoka, I., *unpublished results*). In this case unprotected hexosamines could not be completely converted to their *bis*-PMP derivative even under stronger conditions, because the presence of the amino group at the C-2 position of the pyranose ring reduces the reactivity of C-1 to PMP. *N*-Acetylation, however, eliminated this problem, and thus *N*-acetylgalactosamine as well as *N*-acetylglucosamine gave their *bis*-PMP derivatives as easily as hexoses.

The calibration lines can be prepared by plotting peak response of each monosaccharide to rhamnose against its concentration. All lines are straight with high coefficients of correlation, >0.999), although the slope is varied among lines, presumably due to varied molar proportions of the keto form to the enol form. Low relative standard deviations less than <2% can be obtained for each monosaccharide with careful operation, at least in a concentration range of 1–100 nmol/100 µL of analytical samples. The detection limit is approx 50 pmol/100 µL, which corresponds to approx 5 fmol as the injected amount, if the injected volume is assumed to be 10 nL. If the volume of the

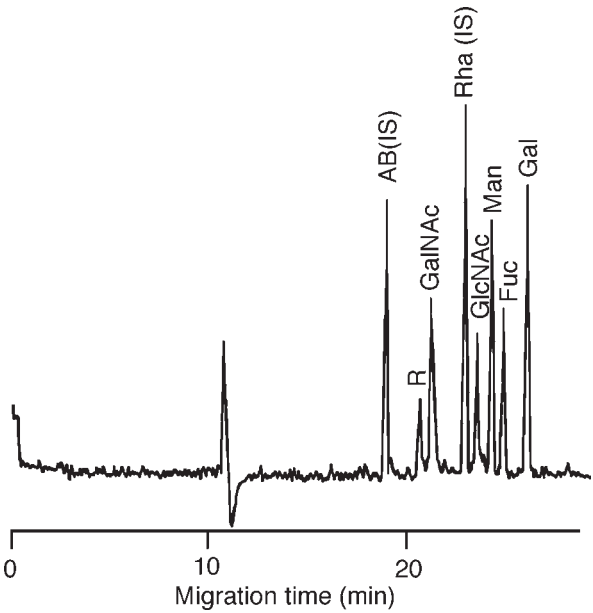

Fig. 4. Analysis of the monosaccharides commonly found in glycoproteins, as borate complexes of their PMP derivatives. The analytical conditions as in **Fig. 1**. GalNAc, *N*-acetyl-D-galactosamine; Rha, L-rhamnose (internal standard); GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Fuc, L-fucose. The analytical conditions and other abbreviations are as in **Fig. 1**.

analytical solution is reduced to 5 µL by skillful operation, monosaccharides as small as 2.5 pmol (approx 250 amol as injected amount) can be analyzed.

This combination of monosaccharides cannot be completely separated from each other by the simple zone electrophoresis mode but can also be well separated by micellar electrokinetic chromatography (MEKC) in 50 m*M* phosphate buffer, pH 6.5, containing sodium dodecyl sulfate (SDS) to a concentration of 20 m*M* (Honda, S. and Sasaoka, I., unpublished results). In SDS-MEKC the migration order was mannose, rhamnose, *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, fucose.

3.3.2. Determination of the Monosaccharide Composition of Glycoprotein Samples

1. Weigh a glycoprotein sample containing approx 1–10 µg of carbohydrates in total into a screw-capped tube and add 2 mol/L trifluoroacetic acid (100 µL).
2. Seal the tube and heat it for 2 h in a boiling water bath.
3. Open the tube, and evaporate the solution to dryness under reduced pressure in a similar manner as described for the preparation of monosaccharide samples (*see* **Note 4**).

4. Add 100 µL of a saturated aqueous solution of sodium bicarbonate to the residue and dissolve it completely.
5. Add 20 µL acetic anhydride, vortex the mixture, and stand the mixture overnight.
6. Apply the mixture onto a small column of Amberlite CG-120 (hydrogen form, 2 mL), and wash the column with 10 mL of water (*see* **Note 12**).
7. Concentrate the combined eluate and the washing fluids under reduced pressure to <100 µL using a rotary evaporator.
8. Transfer the concentrate to the screw-capped tube with a small volume of water, and evaporate the combined fluids to dryness under reduced pressure as in sample preparation (*see* **Note 4**).
9. Derivatize the monosaccharides in the residue with PMP according to the procedure in **Subheading 3.1.**
10. Reconstitute the final residue of the products with 5–50 µL of water.
11. Introduce the analytical sample solution thus prepared to the inlet of the capillary and apply a voltage of 15 kV.
12. Maintain this voltage until all the peaks of the monosaccharides are detected.
13. Read the concentration of each monosaccharide from the corresponding calibration line in **Subheading 3.3.1.**, and calculate its content in the glycoprotein sample taking the volume of the analytical solution (100 µL) and the sample amount used into account.

N-Acetylation can be easily achieved by reacting the hydrolysate with an excess amount of acetic anhydride in a saturated aqueous solution of sodium hydrogen carbonate. The *N*-acetates can be recovered, together with aldoses, by passing the reaction solution through a column of a cation-exchanger (Amberlite CG-120, hydrogen form), followed by evaporation of the eluates.

The acid-released and re-*N*-acetylated monosaccharides thus obtained can be derivatized with PMP to give *bis*-PMP derivatives, which can be analyzed by HPCE without any interference from accompanying substances. For example, our data of monosaccharide composition (w/w%) for calf fetal fetuin and human serum transferrin were as follows. Fetuin: fucose, 0.043% (0.033); galactose, 5.31 (3.49); mannose, 2.07 (2.45); *N*-acetylgalactosamine, 0.45 (0.54); *N*-acetylglucosamine, 3.14 (2.62). Transferrin: fucose, 0.055 (0.040); galactose, 1.02 (1.00); mannose, 0.73 (1.08); *N*-acetylgalactosamine, 0.078 (0.053); *N*-acetylglucosamine, 2.11 (2.52). The numbers in parentheses are the reported values obtained by HPLC ***(11)***. The samples obtained from the manufacturers were used as obtained, but there may be differences of sample lot between the present and reported analyses.

4. Notes

1. Application of the PMP method to oligosaccharide analysis is described in Chapters 5 and 9.
2. The PMP reagent is commercially available, for example, from Kishida (Doshomachi, Chuo-ku, Osaka, Japan) and Sigma (St. Louis, MO; under a different

name: 3-methyl-1-phenyl-2-pyrazolin-5-one). Recrystallization from methanol is encouraged.

3. The reagent solution is prepared by dissolving the purified sample of PMP in methanol to make a 0.5 mol/L solution.
4. For the preparation of a mixture of the authentic specimens of monosaccharides pipet 10 μL each of aqueous solutions of monosaccharides of known concentrations (~1×10^{-4} mol/L) into a screw-capped tube, and evaporate the mixed solution by placing the tube in a vacuum desiccator containing pellets of sodium hydroxide. Care must be taken to avoid bumping. Gentle suction at the initial stage of evaporation is advised. After gentle suction for a short period the temperature of the solution drops down due to evaporation heat and further suction at higher vacuum can safely be carried out. For the preparation of samples for component monosaccharide analysis see **Subheading 3.3.2.**
5. The reaction solution is neutralized to facilitate removal of the excess PMP reagent by extraction with ethyl acetate. A slightly insufficient or excess volume of the sodium hydroxide solution can result in contamination with minor peaks due to the reagent blank and/or the mono-PMP derivative as byproduct. Therefore, the neutralization should be carried out by using exactly measured volumes of normalized sodium hydroxide and hydrochloric acid solutions.
6. Complete removal of the excess reagent is important, because the reagent peak may interfere with monosaccharide analysis. Extraction three times with the same volume of the solvent is desirable. Chloroform is used for the cleanup of oligosaccharide derivatives (Chapter 5), but it is not suitable for the cleanup in monosaccharide analysis, because it causes loss of the derivatives.
7. HPCE apparatus. Any high voltage supplier capable of supplying ~30 kV can be used. Sample solutions can be introduced by pressure drop utilizing either compression, suction, or siphoning. Introduction by electromigration may cause introduction of the component ions in a sample at different velocities. There are two types of UV detectors, one designed for a particular wavelength using a special lamp and an interference filter and another for multiple wavelengths using a deuterium or tungusten lamp and a grating spectroscope. The latter type is preferable in the present analysis, because the PMP derivatives absorb strongly at 245 nm. The detectors of the former type equipped with a mercury lamp that emits most abundantly at 254 nm can also be used, but sensitivity is not high at this wavelength.
8. The velocity of electroosmotic flow varies depending on the material of the capillary tube. We use fused silica capillaries with i.d. 50 μL from Polymicro Technologies (Phoenix, AZ) as the standards.
9. Degassing the electrophoretic solution can be done by aspiration of the stock solution of the electrophoretic solution in a suction bottle under sonication. Filtration through a membrane filter before use is recommended.
10. The purity of the glycoprotein samples may vary among sample lots. Commercial (from Sigma) samples of calf fetal fetuin and human serum transferrin were used as obtained in the adopted examples. Their purity may differ from that of the samples used in the literature ***(12)***.

11. Trifluoroacetic acid is the most suitable acid catalyst for hydrolysis. Heating in 2 mol/L trifluoroacetic acid at 100°C can liberate all monosaccharides quantitatively in 2 h without non-hydrolytic degradation ***(12)***. Galactosamine and glucosamine exist as their *N*-acetates in glycoconjugates, but the acetyl group is removed during acid hydrolysis. Because the free bases of hexosamines cannot be derivatized efficiently with PMP, they should be re-*N*-acetylated.
12. Preparation of the ion-exchange column: Suspend the Amberlite resin (approx 2 g) in water and pour the slurry into a small glass column (5–8 mm i.d.). Regenerate the resin to the hydrogen form by elution with 1 mol/L of hydrochloric acid, followed by rinsing with water.
13. Evaporation of the neutralized solution and the aqueous layer in the ethyl acetate extraction process can be carried out in a similar manner as in sample preparation (*see* **Note 4**).
14. The analytical sample solution contains a high concentration of sodium chloride, which may interfere with the monosaccharide analysis at low levels of sample amount. Electric dialysis by a microdialyzer using a neutral membrane can eliminate this inorganic salt. The isolated products (*bis*-PMP derivatives) are hydrolyzed to the mono-PMP derivatives in neutral or alkaline solutions at high temperatures, but the *bis*-PMP derivatives in analytical sample solutions prepared according to the procedure described in **Subheading 3.1.** are stable for at least a month if stored in a refrigerator.
15. A detection window on a capillary can be made by burning out a part of the polyimide coating on a capillary by a thin flame or application of electric current to a Nichrome wire wound around the capillary at the position where the coating is to be eliminated. More convenient is the use of an incense stick. One end of the stick is lighted and applied to a portion of polyimide coating until it is completely burned out.
16. The sample introduction period should be controlled to create a 1–2-mm sample plug at the inlet of the capillary. The appropriate period of time for introduction depends on the apparatus and conditions. For example, 10 s is appropriate for the siphoning method using a 10-cm difference of levels between the sample solution and the cationic electrode solution.
17. pH and concentration dependence of borate complexation: The concentration of the borate ion is increased with increasing amounts of sodium tetraborate used. Increase of pH also causes an increase of borate ion concentration. With all aldose epimers the molar fraction of the complex increases, as the borate ion concentration increases, but the increasing rate is varied among epimers. This is the reason why the optimum conditions exist for good separation.
18. The use of amobarbital is controlled by law because it is a hypnotic. This internal standard is not indispensable and may be omitted.

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6

Separation of Oligosaccharides by Capillary Electrophoresis Using Buffer Modifiers

Susumu Honda and Shigeo Suzuki

1. Introduction

Zone electrophoresis is the basic mode of capillary electrophoresis, and it allows separation of ions having different charge-to-size ratios. Carbohydrates having ionic groups can also be the objects of separation by this mode. A typical example is the separation of carboxylated/sulfated oligosaccharides from glycosaminoglycans, as described in Chapter 15. Most carbohydrates, however, have no electric charge under normal conditions. Therefore, introduction of ionic tags plays an important role in zone electrophoresis of such carbohydrates.

A number of methods have been developed for the introduction of ionic groups to reducing carbohydrates for capillary electrophoresis. Most of them are based on reductive amination using an acidic catalyst. For example, the methods using 2-aminopyridine ***(1)***, 6-aminoquinoline ***(2)***, 2/9-aminoacridone ***(3)***, 4-aminobenzoic acid ***(4)*** and its derivatives ***(5,6)***, 8-aminonaphthalene-1,3,6-trisulfonate ***(7)***, 2-aminopyrene-3,6,8-trisulfonate ***(8)***, and so forth, as amino group sources are found in the literature. Two-step or three-step introduction of isoindole ***(9)*** and 5-carboxytetramethyl rhodamine succinimidyl ester ***(10)*** groups have also been reported. In all these methods there exists a problem of acid instability of the sialic acid residue, resulting in its partial release during derivatization, as reductive amination requires an acidic catalyst. In addition, the procedures must be performed in nonaqueous solvents. A few of these methods have been reported to give quantitative yields of derivatives, but high yields are obtainable under strict control of reaction condition. Furthermore, many of them require cleanup of derivatives, which is not easy. A number of applications of these methods have been reported, but such limitations should be taken into account if they are to be used. Some of these methods

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give fluorescent derivatives, and a few of them can be excited by laser beams. This is profitable for ultramicroanalysis of carbohydrates, but such ultramicroanalysis is very sensitive to sample matrix and is liable to be affected by slight changes of various factors in cleanup and analysis of the derivatives. In this chapter we focus on a robust method using 1-phenyl-3- methyl-5-pyrazolone (PMP), which is suitable for routine analysis of oligosaccharides.

The PMP method is based on the condensation of the hemiacetal group in the reducing end of a carbohydrate with the active methylene group in the pyrazolone ring of PMP. This is a novel reaction inherent to reducing carbohydrates and is quite different from those described in the preceding. Two PMP groups are introduced quantitatively to each carbohydrate molecule under very mild conditions using aqueous methanol as solvent (*see* **Note 1**). The yield is quantitative in 20 min at 70°C for small oligosaccharides. For higher oligosaccharides heating for a longer time is recommended. The sialic acid residue is neither damaged nor released from oligosaccharide chains during derivatization, as demonstrated by a model experiment using 6'-*N*-acetylsialyllactose, as shown in **Fig. 1**. A commercial sample of 6'-*N*-acetylneuraminyllactose gave an intense peak of its PMP derivative, but did not give the peak of PMP-lactose that is expected to be formed when the sialic acid is released. This is strong evidence for the safety of the sialic acid residue.

The keto group of the PMP moiety in these derivatives is changeable to the enol form by tautomerism, and the enol form provides the enolate ion, which functions as a weak anion. For this reason the PMP derivative of each carbohydrate is weakly dissociated to give an anion in alkaline media. **Figure 2** shows an example of the separation of the PMP derivatives of isomaltooligosaccharides having different degrees of polymerization (DPs). Although each derivative has the same electric charge, the size is different among the homologues. Therefore, the charge-to-size ratio is varied among the homologs and they were separated from each other by the zone electrophoresis mode.

Thus, zone electrophoresis is effective for the separation of PMP derivatives of oligosaccharides, but the separation is not satisfactory for more complex mixtures, especially those containing isomeric oligosaccharides, as it is based simply on the difference of charge-to-size ratio.

In this chapter we present much more improved separation of the PMP derivatives of oligosaccharides attainable by changing separation mode with addition of various modifiers to the electrophoretic solution.

2. Materials

2.1. Derivatization

1. The PMP reagent (*see* **Note 2**).
2. The reagent solution: 0.5 *M* PMP in methanol (*see* **Note 3**).

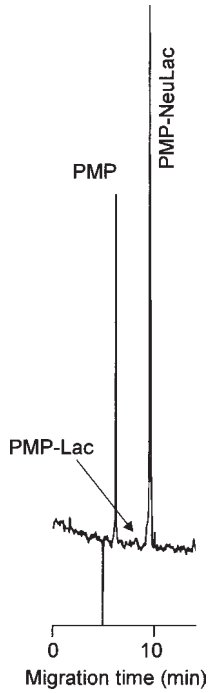

Fig. 1. Analysis of the products of derivatization of commercial 6'-*N*-acetylneuraminyllactose with PMP by capillary electrophoresis. Capillary, fused silica (50 µm i.d., 70 cm); running buffer, 50 m*M* phosphate buffer, pH 6.5, containing 20 m*M* SDS; applied voltage, 25 kV; detection, UV absorption at 245 nm. PMP-NeuLac, the PMP derivative of 6'-*N*-acetylneuraminyllalctose; PMP-Lac, the peak position of the PMP derivative of lactose.

3. Oligosaccharide samples (*see* **Note 4**).
4. 0.3 *M* of sodium hydroxide (*see* **Note 5**).
5. 0.3 *M* of hydrochloric acid (*see* **Note 5**).
6. Chloroform: Reagent grade (*see* **Note 6**).
7. Micro tubes: Screw-capped microtubes of 500-µL capacity.
8. A centrifugal concentrator (a SpeedVac).

2.2. High-Performance Capillary Electrophoresis (HPCE)

1. Instruments: An HPCE apparatus, homemade or marketed, equipped with a high-voltage supply, a sample introducing system, and an ultraviolet (UV) detector (*see* **Note 7**).
2. Capillary: A fused silica tube having an inner diameter of 50 µm. We use the capillaries prepared by Polymicro Technologies (Phoenix, AZ) as the standards.
3. 1 mol/L of sodium hydroxide for capillary cleaning.

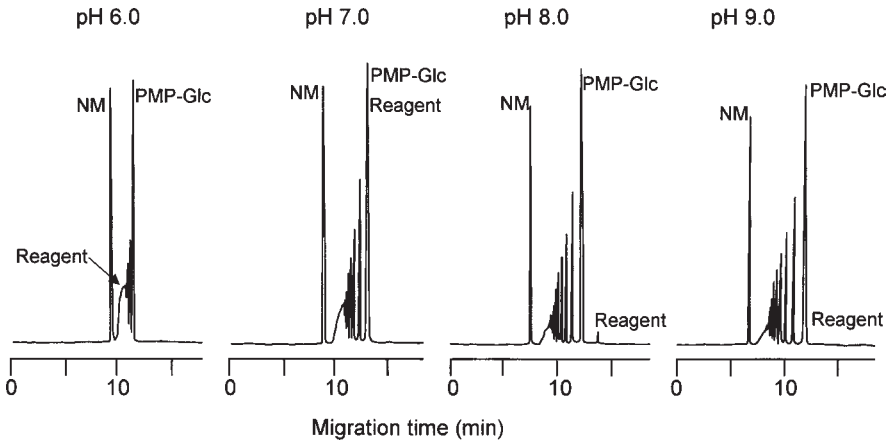

Fig. 2. Separation of the PMP derivatives of isomaltooligosaccharides having various DPs by zone electrophoresis. Capillary, fused silica (50 µm i.d., 70 cm); running buffer, 50 m*M* phosphate buffer; applied voltage, 15 kV; detection, UV absorption at 245 nm. PMP-Glc, the PMP derivative of glucose (DP = 1). Other peaks in each electropherogram are in the order of descending DPs. Reagent is the remaining PMP; NM, neutral marker (cinnamyl alcohol). The peaks were better separated as pH increased.

4. Electrophoretic solutions (running buffers): 200 m*M* borate buffer, pH 9.5 (A, for zone electrophoresis as borate complexes, *see* **Note 8**) or 50 m*M* phosphate buffer, pH 6.0, containing sodium dodecyl sulfate (SDS) to a concentration of 30 m*M* (B, for SDS micellar electrokinetic chromatography). Degas before use (*see* **Note 9**).

2.3. Liberation of Oligosaccharides from Glycoproteins

1. SDS reagent grade.
2. 2-Mercaptoethanol, reagent grade.
3. Nonidet P-40 (NP-40 nonylphenyloxy polyethoxy ethanol), reagent grade.
4. EDTA, reagent grade.
5. PNGase F: Glycopeptidase F from *Flavobacetrium meningosepticum* (*see* **Note 10**).
6. The denaturing reagent: Prepared by adding SDS (5 w/v%) to an aqueous 10% v/v solution of 2-mercaptoethanol.
7. The deglycosylation medium: 20 m*M* phosphate buffer, pH 7.2, containing EDTA to a concentration of 50 m*M*.
8. The NP-40 solution: A 10% aqueous solution of NP-40.
9. The enzyme solution: A solution containing 50 U of PNGase F in 3 µL (*see* **Note 11**).
10. Micropipets.
11. A water bath.

3. Methods

3.1. Separation of a Standard Mixture of Oligosaccharides by Zone Electrophoresis as Borate Complexes

3.1.1. Derivatization

1. Add the reagent solution (50 µL) of PMP (*see* **Note 3**) to a mixture of oligosaccharides (*see* **Note 4**) in a screw-capped microtube and dissolve the mixture completely by gentle swirling.
2. Add 0.3 *M* of sodium hydroxide (50 µL) to the resultant solution to convert PMP to its sodium salt (*see* **Note 5**).
3. Heat the mixed solution at 70°C for 30 min to allow for the condensation to complete.
4. Cool the reaction mixture to room temperature.
5. Add 0.3 *M* of hydrochloric acid (50 µL) for neutralization of the reaction mixture (*see* **Note 5**).
6. Evaporate the solution to dryness under reduced pressure with a SpeedVac.
7. Dissolve the residue in 200 µL water.
8. Add 200 µL of chloroform, shake the mixture vigorously, and discard the lower layer (chloroform layer) with a small syringe (*see* **Note 6**). Repeat this extraction process twice more.
9. Evaporate the final upper layer (aqueous layer) to dryness under reduced pressure with a SpeedVac.
10. Reconstitute the residue with an indicated volume of water for HPCE analysis (*see* **Note 12**).

3.1.2. HPCE

1. Remove a 1-mm portion of the polyimide coating at an approx 5-cm position from the cathodic end of the capillary, to make a window for UV detection (*see* **Note 13**).
2. Mount the capillary on a cassette, and fix the window at the center of the UV beam.
3. Install the cassette in an HPCE apparatus, and keep it at a specified temperature by circulating temperature-controlled air or liquid, depending on the apparatus used.
4. Flash the capillary with 1 mol/L of sodium hydroxide at least for 10 min to clean its inner wall, rinse it with water for 5 min, and equilibrate it with the running buffer with application of a high voltage (20 kV) for 10 min.
5. Refill the capillary with running buffer A (200 m*M* borate buffer, pH 9.5) (*see* **Subheading 2.2.**), to make the stand-by state.
6. Introduce an analytical sample solution from the anodic end (*see* **Note 14**).
7. Apply a specified voltage between both ends of the capillary, and start recording.

Zone electrophoresis as borate complexes is quite efficient for the separation of oligosaccharides containing positional and/or epimeric isomers. **Figure 3** demonstrates the high efficiency of this mode in the separation of isomeric

disaccharides. As described in the chapter on separation of the derivatives of monosaccharides (Chapter 10), the hydroxyl groups in a carbohydrate react with the borate ion to form an anionic borate complex. The molar fraction of the complex increases with the borate ion. Increase of pH is also favorable for increasing molar fraction of the complex, because higher pH values cause increased borate concentrations. However, the increasing rate of molar fraction is varied among the positional isomers. Optimization of borate concentration and pH indicated that the best separation was achieved at 200 m*M* borate concentration, pH 9.5, in this case. It is notable that all positional isomers of the glucobiose set were completely separated from each other under these simple conditions. These are the same conditions as those used for the separation of aldopentoses and aldohexoses in Chapter 10.

For other samples containing oligosaccharide isomers it is recommended to examine first the effect of pH at a borate concentration of 200 m*M*, which is almost the upper limit. If complete separation is not obtained, subsequently examine the effect of borate concentration down from 200 m*M*.

3.2. Separation of a Mixture of Oligosaccharides Derived from Glycoproteins by SDS Micellar Electrokinetic Chromatography

3.2.1. Liberation of Oligosaccharides from Glycoproteins

As described in Chapter 2, the asparagine-linked oligosaccharides in glycoproteins can be released by either chemical or enzymatic means. The method based on heating in anhydrous hydrazine or hydrazine atmosphere is a representative chemical method, but it has a drawback in that a part of the sialic acid residue is liable to be removed during heating and/or the cleanup process. In addition, this method is not selective to asparagine-linked oligosaccharides but causes the release of some parts of serine- or threonine-linked oligosaccharides at the same time, unless the prior sample treatment is not adequate. On the other hand, there are two types of enzyme that cleave asparagine-linked oligosaccharides; one at the *N*-acetylglucosamine–asparagine linkage in the innermost position of the oligosaccharide chains and the other between the two *N*-acetylglucosamine residues in the innermost *N,N*-diacetylchitobiose structure. To the best of our knowledge the glycanase of the former type, represented by PNGase F from *Flavobacterium meningosepticum*, has the widest substrate specificity. Although prior degradation of the polypeptide core by a certain kind of peptidase such as trypsin is reported to facilitate the release of asparagine-linked oligosaccharides, the liberation is almost complete without this depolymerization, provided a small amount of glycoproteins denatured by surfactant is incubated with a sufficient amount of PNGase F. The following represents typical procedure.

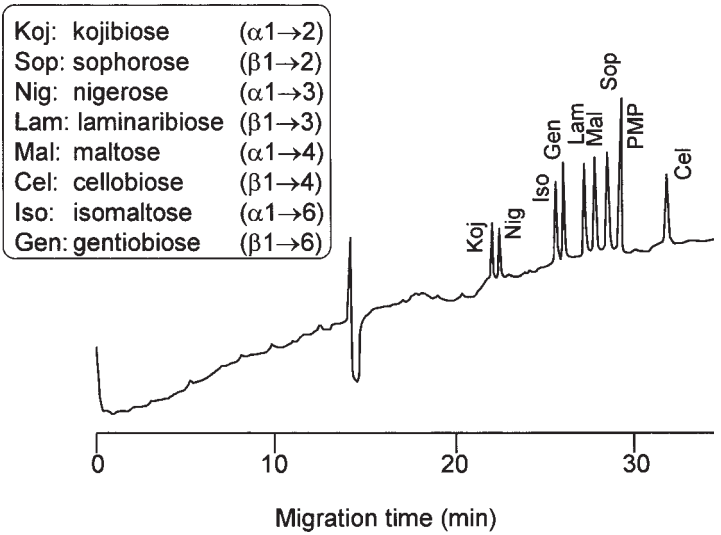

Fig. 3. Separation of all members of glucobiose by zone electrophoresis as borate complexes of their PMP derivatives. Capillary, fused silica (50 µm i.d., 78 cm); running buffer, 200 m*M* borate buffer, pH 9.5; applied voltage, l5 kV; detection, UV absorption at 245 nm.

1. Take a 1–200-µg glycoprotein sample in a screw-capped microtube (*see* **Note 15**).
2. Add 10 µL of a denaturing reagent and swirl the mixture for dissolution (*see* **Note 16**).
3. Heat the tube for 5 min in a boiling water bath (*see* **Note 16**).
4. Add 5 µL of the deglycosylation medium, followed by 5 µL of a NP-40 solution.
5. Add 3 µL of the enzyme solution and incubate the mixture for 24 h at 37°C.

3.2.2. Derivatization

1. Add 25 µL of the PMP reagent solution (*see* **Note 3**) to the incubation mixture (total volume 23 µL) described in **Subheading 3.2.1.**, followed by 25 µL of 0.3 mol/L sodium hydroxide (*see* **Note 5**).
2. Heat the mixed solution for 30 min at 70°C.
3. After cool add 25 µL of 0.3 mol/L hydrochloric acid for neutralization (*see* **Note 5**).
4. Evaporate the neutralized solution to dryness under reduced pressure with a SpeedVac (*see* **Note 7**).
5. Dissolve the residue in 50 µL of water and add 50 µL of chloroform.
6. Shake the mixture vigorously and discard the lower (chloroform) layer with a small syringe. Repeat this extraction process twice more (*see* **Note 6**).
7. Evaporate the final upper (aqueous) layer to dryness under reduced pressure with a SpeedVac (*see* **Note 7**).
8. Reconstitute the residue with an appropriate volume of water for HPCE analysis (*see* **Note 12**).

3.2.3. HPCE

1. Remove a 1-mm portion of the polyimide coating at an approx 5-cm position from the cathodic end of the capillary, to make a window for UV detection (*see* **Note 13**).
2. Mount the capillary on a cassette, and fix the window at the center of the UV beam.
3. Install the cassette in a HPCE apparatus, and keep it at a specified temperature by circulating temperature-controlled air or liquid, depending on the apparatus used.
4. Flash the capillary with 1 mol/L of sodium hydroxide at least for 10 min to clean its inner wall, rinse it with water for 5 min, and equilibrate it with the running buffer with application of a high voltage (20 kV) for 10 min.
5. Refill the capillary with the running buffer B, 50 m*M* phosphate buffer, pH 6.0, containing 30 m*M* SDS (*see* **Subheading 2.2.**), to make the stand-by state.
6. Introduce a sample solution from the anodic end (*see* **Note 14**).
7. Apply a specified voltage between both ends of the capillary, and start recording.

Oligosaccharides in glycoproteins are diverse not only in molecular size but also monosaccharide composition. The PMP derivatives of such oligosaccharides have commonly moderate hydrophobicity owing to the presence of the PMP group. In addition, their hydrophobicity differs from species to species, owing to the difference of the molecular size and the number of the *N*-acetyl group. Therefore, they are good objects of micellar electrokinetic chromatographic separation.

Figure 4 shows a typical example of separation of the asparagine-linked oligosaccharides ***(11)***. Calf fetal fetuin is known to contain sialylated di- and triantennary complex-type oligosaccharides as the major oligosaccharides. The peaks around 20 min were assigned to the PMP derivatives of these oligosaccharides by comigration with the authentic specimens obtained by fractionation by high-performance liquid chromatography. A pair of the two faster moving peaks of moderate intensity at 16–17 min were of the triantennary tetrasialooligosaccharides differing in the attaching position of the sialic acid residue. A pair of two major slower moving peaks with strong intensity at 18–19 min were similarly those of the sialic acid positional isomers of diantennary trisialooligosaccharides. A pair of peaks at approx 23 and 25 min were assigned to diantennary disialooligosaccharides as indicated. The other peaks were of the contaminants. This electropherogram was obtained from a 20-μg sample of fetuin under the described procedure and conditions. The use of a narrower capillary and the removal of the salt prior to analysis by electricmicrodialysis (*see* **Note 12**) will reduce sample amount by sensitization of detection.

4. Notes

1. Application of the PMP method to monosaccharide analysis is discussed in Chapter 10. Protocols for the application to affinity capillary electrophoresis of oligosaccharides are also described in Chapter 9.

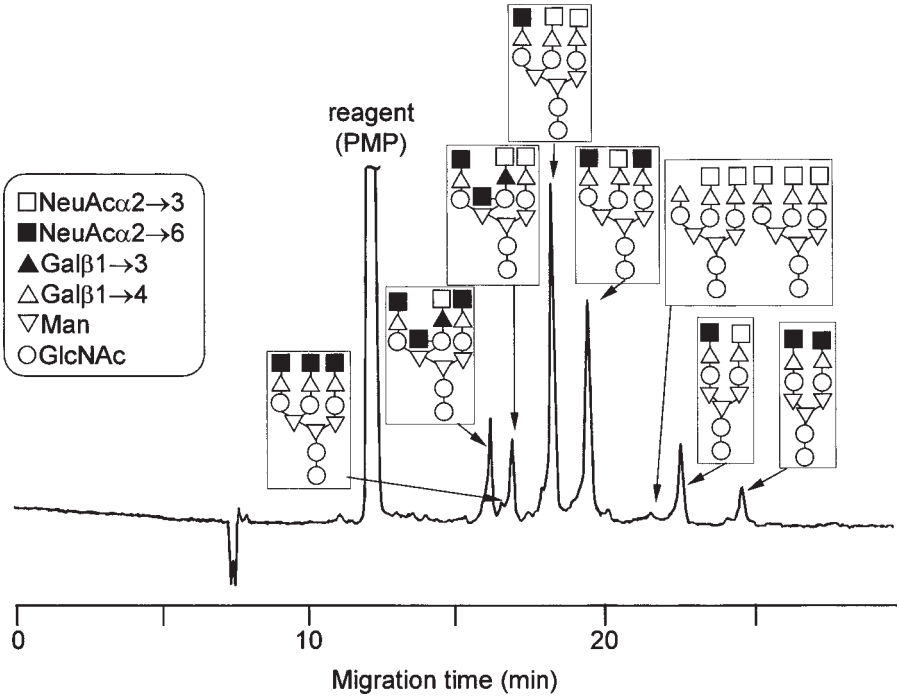

Fig. 4. Separation of the oligosaccharides derived from calf fetuin by SDS micellar electrokinetic chromatography of their PMP derivatives. Capillary, fused silica (50 μm i.d., 47 cm); running buffer, 50 m*M* phosphate buffer, pH 6.0, containing 30 m*M* SDS; applied voltage, 15 kV; detection, UV absorption at 245 nm. (Reproduced with permission from **ref. *11***).

2. The PMP reagent is commercially available, for example, from Kishida (Doshomachi, Chuo-ku, Osaka, Japan) and Sigma (St. Louis, MO; under a different name: 3-methyl-1-phenyl-2-pyrazolin-5-one). Recrystallization from methanol is encouraged.
3. The reagent solution is prepared by dissolving the purified sample of PMP in reagent grade methanol to a concentration of 0.5 mol/L.
4. For the preparation of a standard mixture of oligosaccharides pipet a 10-μL portion each of a $1 \times 10^{-5} - 1 \times 10^{-3}$ mol/L aqueous solution of an oligosaccharide into a screwed microtube, and evaporate the mixed solution to dryness under reduced pressure. For the preparation of an oligosaccharide sample from a glycoprotein *see* **Subheading 3.2.1.**
5. The neutralization of the reaction solution is to facilitate removal of the excess PMP reagent by extraction. Slightly insufficient or excess volume of sodium hydroxide can result in contamination with minor peaks due to the reagent blank and/or the mono-PMP derivative as byproduct. Therefore, the neutralization

should be performed by using exactly the indicated volumes of normalized sodium hydroxide and hydrochloric acid solutions.

6. Complete removal of the excess reagent is important, because the reagent peak may interfere with oligosaccharide analysis. Three-times extraction with the same volume ethyl acetate is recommended. Although ethyl acetate is used for the cleanup of monosaccharide derivatives (Chapter 10), chloroform is preferable for the cleanup of oligosaccharide derivatives, because it has stronger and weaker dissolving power to PMP and PMP derivatives, respectively, than ethyl acetate.
7. HPCE apparatus: Any high-voltage supplier capable of supplying. 30 kV can be used. Sample solutions can be introduced by pressure drop utilizing either compression, suction, or siphoning. Introduction by electromigration may cause introduction of the component ions in a sample at different velocities. There are two types of UV detectors, one designed for a particular wavelength using a special lamp and an interference filter and another for multiple wavelengths using a deuterium or tungsten lamp and grating spectroscope. The latter type is preferable in the present analysis, because the PMP derivatives absorb most strongly at 245 nm. The detectors of the former type equipped with a mercury lamp, which emits most abundantly at 254 nm, can also be used, but sensitivity is not high owing to the deviation of the wavelength.
8. Borate buffer A as running buffer is conveniently prepared by cautiously adding hydrochloric acid to 50 mmo/L sodium tetraborate to pH 9.5 with constant stirring. It should be noted that 1 mol of sodium tetraborate forms 4 mols of the borate ion.
9. Degassing the electrophoretic solution can be achieved by aspiration of a freshly prepared or stock solution of a running buffer in a suction bottle under sonication. It is recommended to filter the degassed solution through a membrane filter before use.
10. One unit of PNGase F hydrolyzes 1 nmol of *N*-dansylfetuin per min at pH 7.2 at 37°C.
11. Solutions of PNGase F in 50% glycerol containing sodium 100 m*M* phosphate buffer, 25 m*M* EDTA, and 5 m*M* sodium azide, pH 7.2, are commercially available (e.g., from Sigma, Biolabs, etc.).
12. Preparation of analytical sample solutions: In the derivatization with PMP by the described procedure a high concentration of sodium chloride is produced by the neutralization of the sodium salt of the large excess of PMP with hydrochloric acid. Analysis of a small amount of a sample near the lower limit is liable to be disturbed by this inorganic salt. The cleanup of the derivatized sample by electric microdialysis using an amphoteric membrane is effective to eliminate this interference. The isolated product (*bis*-PMP derivatives) may be hydrolyzed to the mono-PMP derivatives at high temperatures, but the products in analytical sample solutions or the dialysates are stable for at least a month if stored in a refrigerator.
13. A detection window on a capillary can be made by burning out a part of the polyimide coating on a capillary either by thin flame or application of electric current to a Nichrome wire wound around the capillary at the position where the coating is to be eliminated. More convenient is the use of an incense stick. One end of the stick is lighted and applied on a portion of polyimide coating until it is completely burned out.

14. The sample introduction should be controlled to create an approx 2-mm sample plug at the inlet of the capillary. The appropriate period of time for introduction depends on the apparatus and conditions. For example, 10 s is appropriate for the siphoning method using a 10-cm difference of the levels between the sample solution and the cathodic electrode solution.
15. Sampling of a glycoprotein can be done in a similar manner as the sampling of mono- and oligosaccharides (*see* **Note 4**). A 10-µL aliquot of a 0.01–2% aqueous solution of a glycoprotein is taken in a small sample tube or a screw-capped microtube, and evaporated to dryness under reduced pressure.
16. Denaturation of glycoprotein samples: Heating in a solution containing SDS and 2-mercaptoethanol causes reduction of the disulfide bond and change of the three-dimensional structure. This series of changes facilitates the attack of the PNGase F on the cleaving site (the *N*-acetylglucosamine–asparagine linkage).

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7

Characterization of Oligosaccharides from Starch, Dextran, Cellulose, and Glycoproteins by Capillary Electrophoresis

Fu-Tai A. Chen

1. Introduction

Neutral oligosaccharides are complex in terms of their linkage, either in the linear or branched form. An enormous number of variants are therefore possible. A homopolymer of glucose with a glycosidic bond may assume its stereochemical configuration at either the α or β position. Glucose units could be joined through 1,2; 1,3; 1,4; or 1,6 linkages in linear sequence or extended through different linkages, which eventually could lead to a two- or three-dimensional network of homopolymers. Starch and dextran are homopolymer of glucose with α-1,4 and 1, 6 linkages, respectively, and are substantially different in physical and chemical characteristics from the cellulose with the β-1,4 linkage. Because all neutral sugars do not have a charge and seldom contain chromophores, methods for sugar analysis by capillary electrophoresis (CE) are thus limited in separation mechanism as well as detection methods. Sugars do complex well with borate at high pH that provides the charge and yield significant incremental absorbance at 195 nm but still with relatively low molar absorptivity ***(1,2)***. Alternatively, a chemical tag may be introduced to the reducing end of the neutral oligosaccharides to provide enhanced sensitivity and introduce charges to the sugars for electrophoretic separation. The earliest CE-based analysis of derivatized sugars was the reductive amination adducts of sugar with 2-aminopyridine (2-AP). The method was first explored by Honda et al. ***(3,4)*** for high-resolution CE analysis of mono- and oligosaccharides. The pyridylamino sugar derivatives do not carry a charge; complexation of vicinal hydroxyl groups with borate at high pH or protonation with acidic buffer with the amino moiety in the AP sugars was utilized for electro-

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phoretic separation ***(4)***. Since then, CE analysis of oligosaccharides derivatized with chromogenic and fluorogenic tags has flourished and two extensively reviewed articles have been published recently ***(5,6)***.

This chapter describes protocols for the characterization of oligosaccharides derived from fractionated starch, dextrans, and cellulose and *N*-linked oligosaccharides from glycoproteins. All oligosaccharides are derivatized with 8-aminopyrene-1,3,6-trisulfonate (APTS), a fluorogenic reagent that contains three negatively charged sulfonate groups that provide electrophoretic mobility of the sugar adducts. CE analysis was monitored by fluorescence emission induced by the excitation with a 488-nm argon ion laser. A few reports on the application of APTS for profiling glycoprotein *N*-linked oligosaccharides were reported recently ***(7–10)***. The derivatization chemistry was also used for characterization of starch structures by Morell et al. ***(11)***, carrageenan by Roberts et al. ***(12)***, and other polysaccharides ***(13–15)***.

2. Materials

2.1. Reductive Amination with APTS

1. 8-Aminopyrene-1,3,6-trisulfonate (APTS); high purity APTS from Beckman Coulter, Inc., Fullerton, CA.
2. Sodium cyanoborohydride ($NaCNBH_3$): 1.0 *M* in tetrahydrofuran (THF) (Aldrich Chemicals, Milwaukee, WI).
3. 1.0 *M* Citric acid.
4. Maltooligosaccharide DE-5: Grain Processing Co.
5. Dextran 1000, 5000, and 12,0000 (Fluka Co., Flushing, NY).
6. Glucose, maltoheptaose, and cellooligosaccharides (Sigma, St. Louis, MO).

2.2. Electrophoresis Buffer and Capillary

1. 25 m*M* lithium acetate with 0.4% polyethylene oxide (mol wt 300,000; Aldrich Chemicals), pH 4.75.
2. 50 m*M* Triethylamine phosphate, pH 2.5.

2.3. Capillary Electrophoresis System

1. Instrument: P/ACE 5500 equipped with a laser induced fluorescence (LIF) detector.
2. Capillary: Polyvinyl alcohol-coated capillary (N-CHO capillary by Beckman Coulter, Inc., Fullerton, CA), 50 μm inner diameter (i.d.) × 47 cm length (40 cm to detector window) assembled in a cartridge format.
3. Detection window containing an ellipsoidal mirror to collect fluorescence.
4. Laser: 488-nm argon ion laser with output of 3 mW.
5. Fluorescence emission filter: 520 nm ± 9 nm (Oriel Optics, Stratford, CT) and notch filter, 460–500 nm.
6. Data processing: P/ACE window software in an IBM PC.
7. Mobility calculation: Caesar 4.1 software.

3. Methods

3.1. Reductive Amination of Reducing Sugars with APTS (see Note 1 and Fig. 1)

1. Dissolve reducing sugars (oligosaccharide ladders of DE-5 and dextrans) in water, divide into 500-µL aliquots in clean microfuge tubes, and dry under reduced pressure (SpeedVac) to yield 0.1–10 nmol of sugar samples. *N*-linked oligosaccharides from bovine ribonuclease B (20–100 µg of glycoproteins) and commercial sources (5–20 µg) are dried similarly to the sample for reductive amination reaction with APTS.
2. Start derivatization by adding 5 µL of 0.1 *M* APTS solution–1.0 *M* citric acid to dissolve sample, followed by the addition of 2.5 µL of 1.0 *M* $NaCNBH_3$ –THF.
3. Vortex-mix well and centrifuge briefly to bring down the liquid, cap the vial tightly, and heat in a sand bath at 80°C for 1 h.
4. Dilute the resulting mixture to 200 µL with water and dilute further by 50-fold prior to capillary electrophoresis–laser-induced fluorescence (CE-LIF) analysis.
5. Measure the ultraviolet (UV)/visible spectra of APTS and its adducts using a Beckman DU-7500 diode-array spectrometer.

3.2. Analysis of Oligosaccharide Ladder DE-5 and Dextran and Cello-Oligosaccharide Adducts with APTS by CE-LIF (see Notes 2 and 3; see Figs. 2 and 3)

1. Place the capillary in the cartridge format is placed on the P/ACE-LIF system, and samples and buffers on the carousel of the instrument. The system software in an IBM PC controls buffer rinsing, sample injection, separation, and detection. One buffer vial is used exclusively for rinsing the capillary at 20 psi for 2 min for each cycle of analysis.
2. Place 10–50 µL of diluted samples in a sample holder on the inlet side of the system. Inject sample at 0.5 psi for 5–10 s. Afterward, dip the inlet capillary tip in water vial to avoid carryover.
3. Start CE separation with buffers on both inlet and outlet, applying a the separation voltage of 23.5 kV (500 V/cm) for 15–20 min depending on the samples.
4. At end of each run, rinse the capillary with running buffer for 20 psi for 2 min.

3.3. Analysis of N-Linked Oligosaccharide Adducts by CE-LIF (see Notes 4–6 and Figs. 4–6)

The protocol for the analysis of *N*-linked oligosaccharides is identical to that of the oligosaccharide ladders (*see* **Subheading 3.2.**). CE-LIF analysis of the *N*-linked oligosaccharide sample provides the profile of the glycoproteins. Analysis of sample containing oligosaccharide ladders DE-5 or dextran 1000 provides mobility data using maltopentaose (G5) or isomaltopentaose (D5) as the reference mobility value of 14.947 or 14.810×10^{-5} $cm^2/V{\cdot}s$, respectively (*see* **Table 1**).

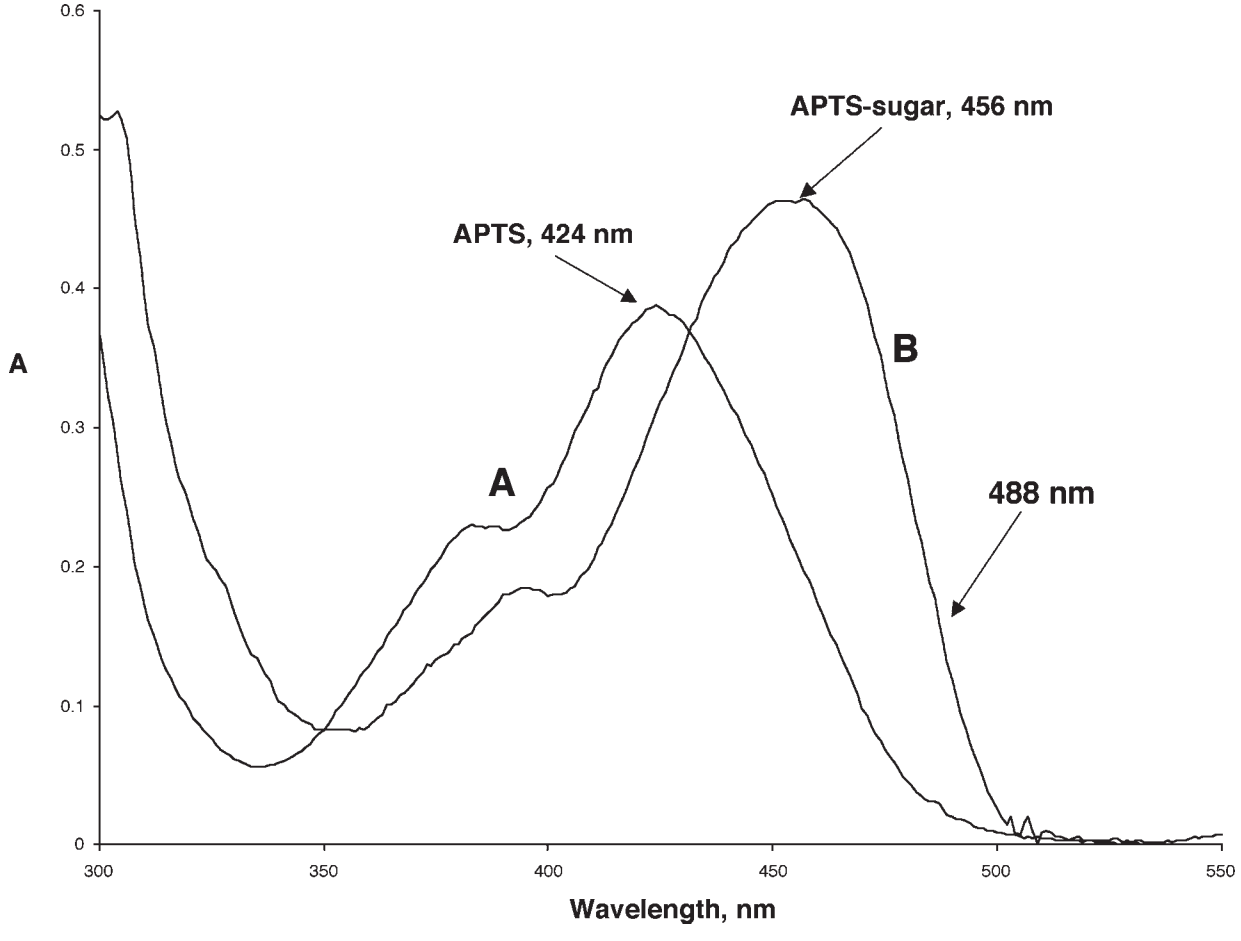


Fig. 1. UV/visible spectra of APTS and APTS–dextran 1000 adduct at 27 μ*M* each.

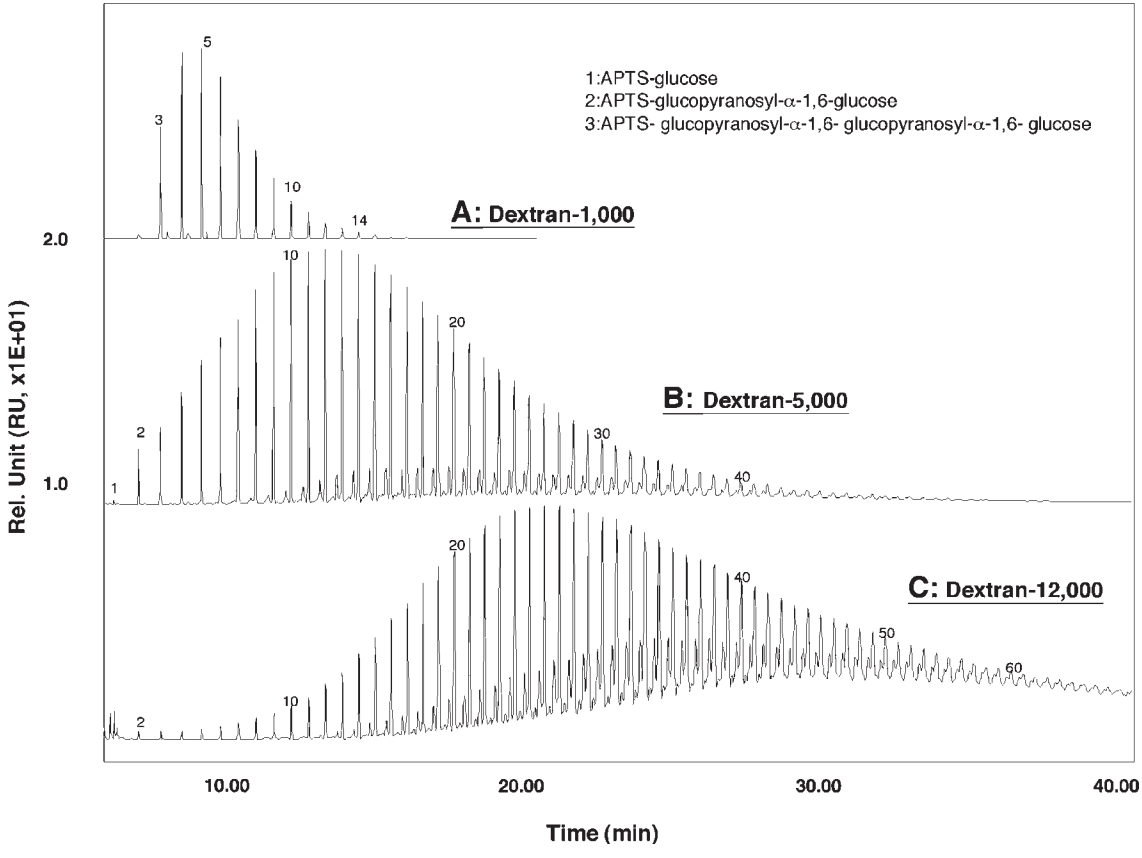


Fig. 2. Electropherograms of APTS-derivatized dextran 1000 **(A)**, dextran 5000 **(B)**, and dextran 12,000 **(C)**. Conditions: 50 μm × 47 cm PVA-coated capillary; buffer: 25 m*M* acetate, pH 4.75, containing 0.4% PEO; injection: 0.5 psi for 5 s; 23.5 kV/12.6 μA; ex: 488 nm/em: 520 nm.

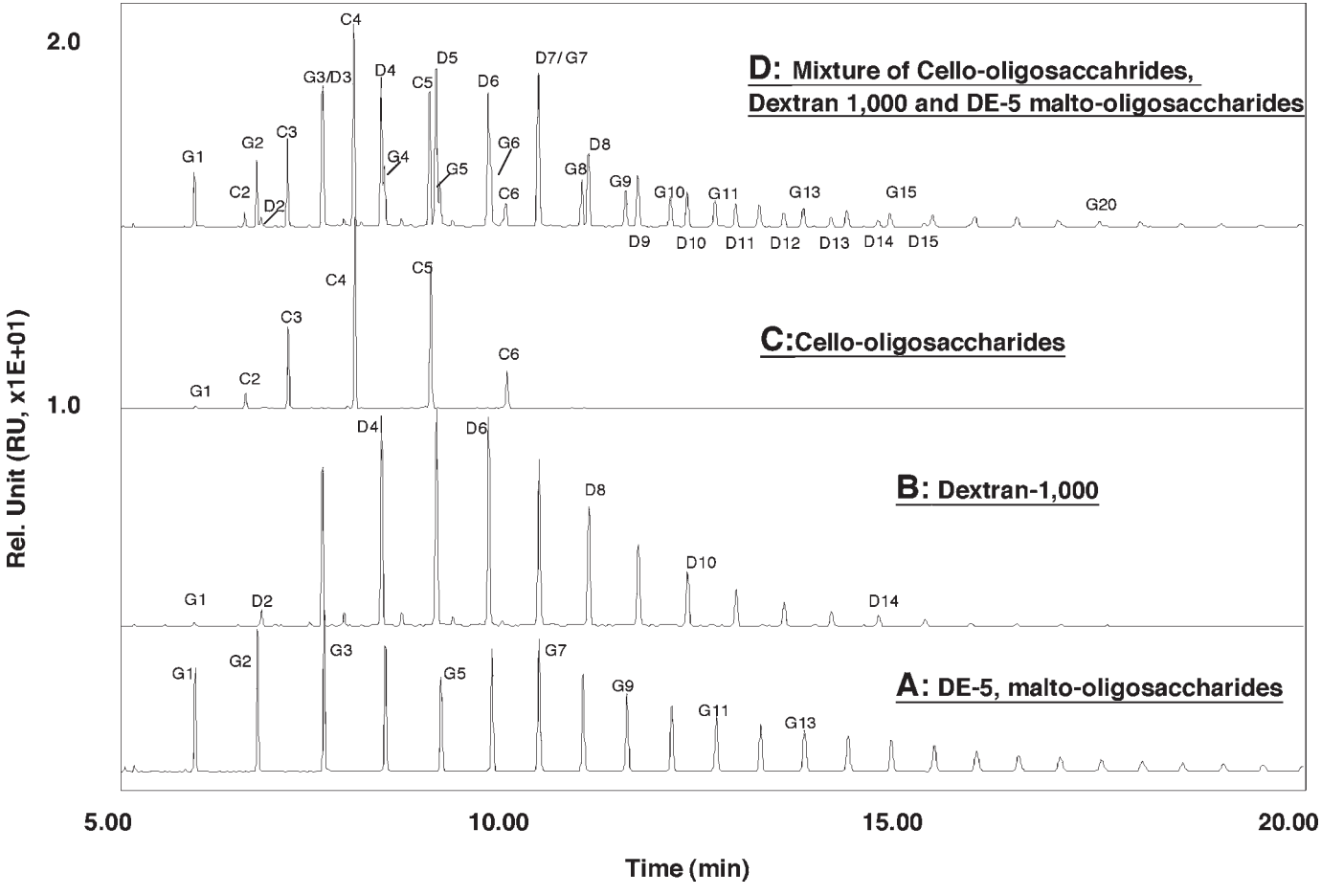


Fig. 3. Electropherograms of APTS-derivatized DE-5 **(A)**, dextran 1000 **(B)**, cellooligocellulose **(C)**, and a mixture of **A**, **B**, and **C** **(D)**. Conditions as for **Fig. 2.**

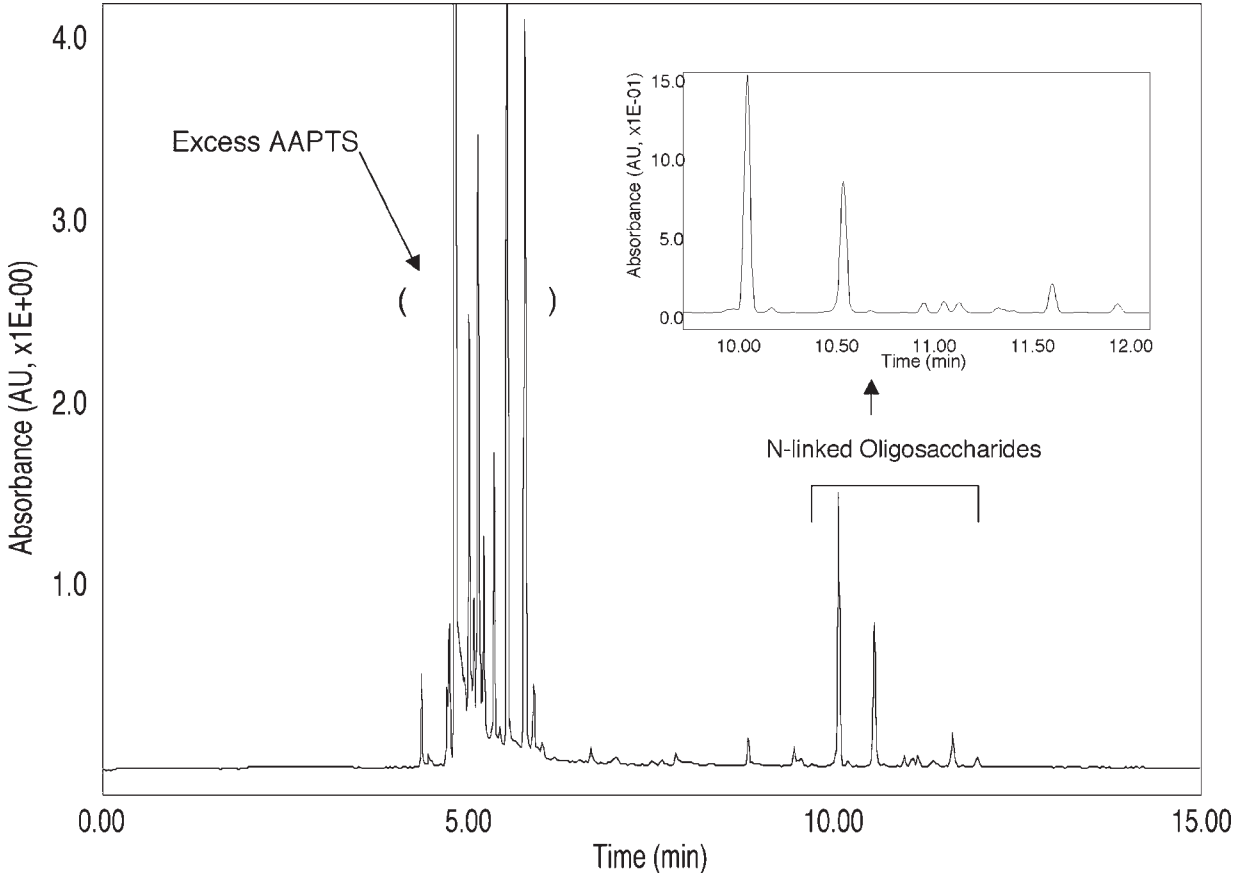


Fig. 4. Electropherograms of APTS–derivatized *N*-linked oligosaccharides obtained from PNGase-catalyzed cleavage of ribonuclease B. *Inset* shows the *N*-linked oligosaccharide portion of the electropherogram. Conditions as for **Fig. 2**.

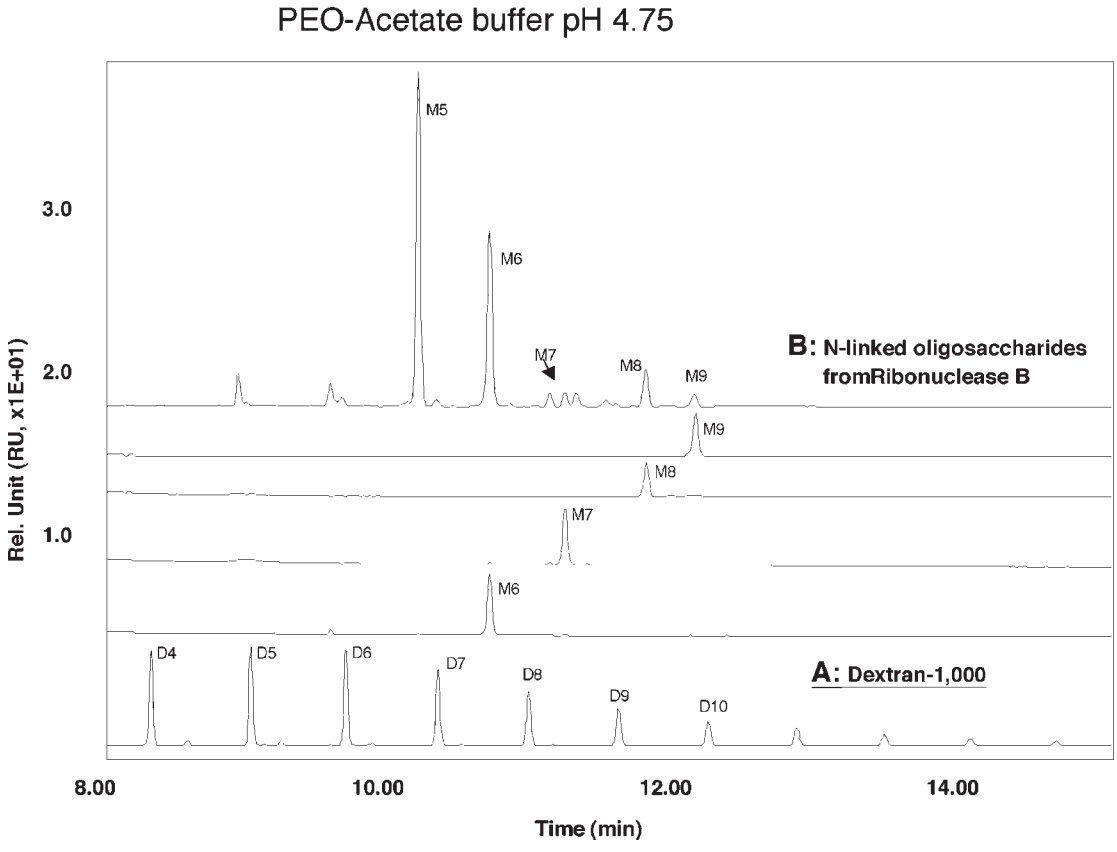


Fig. 5. Electropherograms of APTS-derivatized dextran 1000 **(A)**, APTS-derivatized *N*-linked oligosaccharides obtained from PNGase-catalyzed cleavage of ribonuclease B **(B)**, and electropherograms of M6, M7, M8, and M9 in between **(A)** and **(B)**. Conditions as for **Fig. 2**.

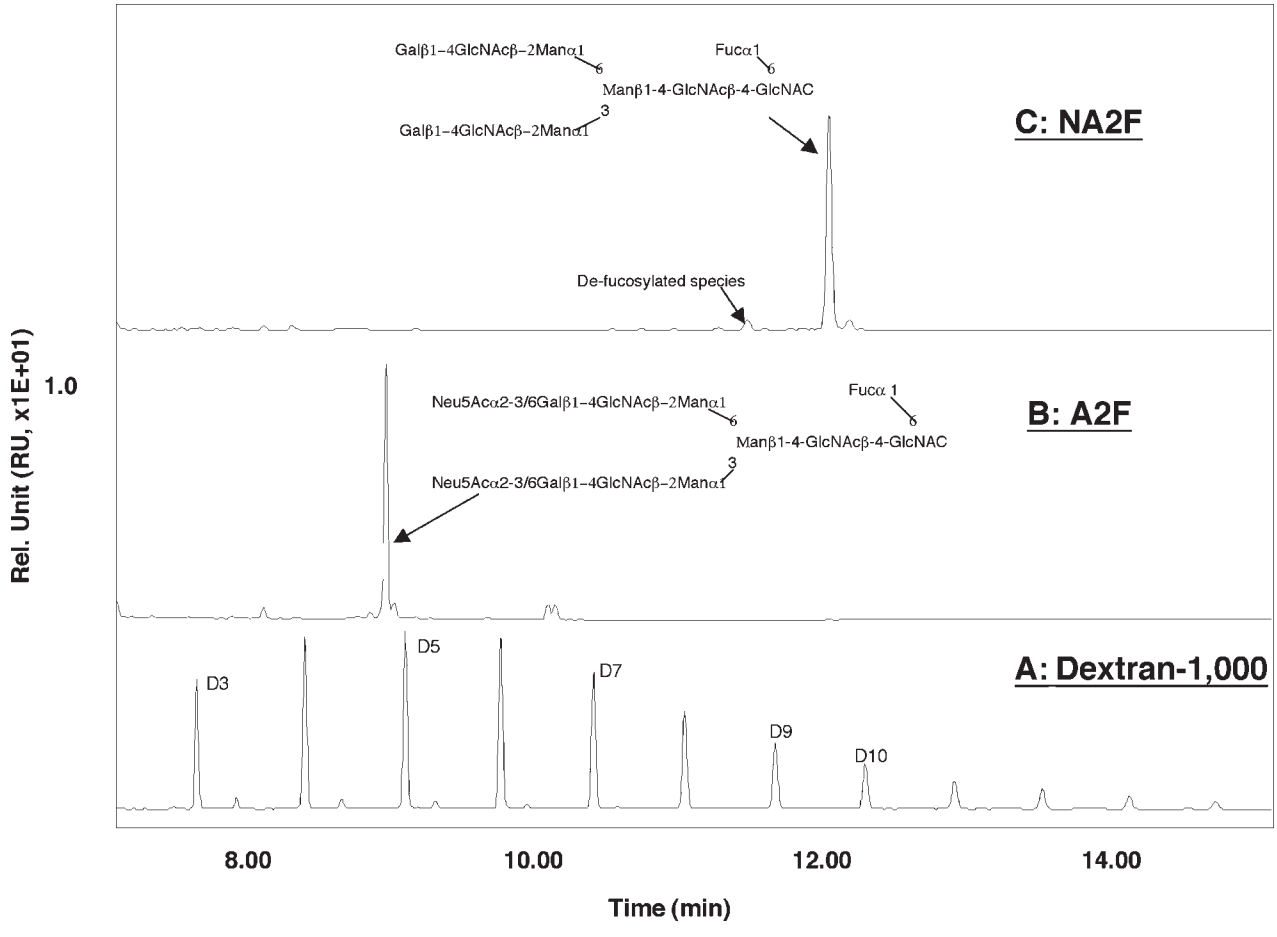


Fig. 6. Electropherograms of APTS-derivatized dextran 1000 (**A**), A2F (**B**), and NA2F (**C**). Conditions as for **Fig. 2**.

Table 1
Electrophoretic Mobility of Oligosaccharide Ladders

	Linkage	Monosaccharide units	Electrophoretic mobility, $\mu \times 10^{-5}$ cm^2/Vs
G1	α-1,4	1	22.390
C2	β-1,4	2	20.224
C3	β-1,4	3	18.698
C4	β-1,4	4	16.739
C5	β-1,4	5	14.947
D5	α-1,6	**5**	14.810
G5	α-1,4	**5**	14.775
G10	α-1,4	10	11.158
D10	α-1,6	10	10.969
G15	α-1,4	15	9.074
D15	α-1,6	15	8.815

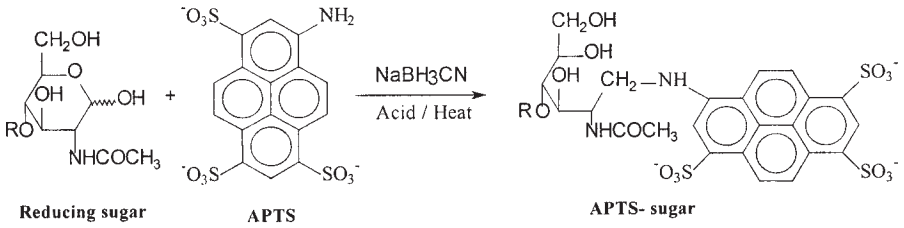
G, Maltooligosaccharide; C, cello oligosaccharide; D, dextran.

3.4. Conclusion

The application of APTS-based derivatization chemistry for the characterization of oligosaccharides by CE-LIF has demonstrated that high-resolution, rapid and reproducible analysis of reducing sugars can be readily achieved. In the analysis of oligosaccharides obtained from glycoproteins, profiling *N*-linked oligosaccharides, for example, provides a qualitative analysis of the distribution of oligosaccharides. Ultimately, the sequencing information of each oligosaccharide would be desirable. Such information may be obtained with the help of the consecutive enzymatic digestion of labeled oligosaccharides using exoglycosidase arrays, coupled with CE separation of the digests. The present results on mobility measurement using an internal standard as the mobility reference provide great accuracy in the measurement of mobility of each oligosaccharide species in the digests. Such results may provide an opportunity for direct reading of sequences from the oligosaccharide mobility database obtained by the present method.

4. Notes

1. The reductive amination of reducing sugars with APTS is a general acid catalysis reaction that has been studied extensively ***(16)***. Citric acid is one of the most effective catalysts for APTS-based reductive amination of oligosaccharides. Citric acid promotes adduct formation most efficiently at room temperature without causing degradation of some oligosaccharides containing labile groups such as sialic acid and fucose. Quantitative recovery of the common monosaccharides



Scheme 1. Labeling of reducing oligosaccharide with APTS.

with reducing ends as the APTS adducts was obtained ***(17)***. Under the standard condition, the recovery of adducts was essentially quantitative for all neutral reducing sugars up to maltoheptaose (G7) including the pentasaccharide core (M3N2) with GlcNAc at the reducing end ***(9)***. Alternatively, for oligosaccharides containing temperature-labile moieties such as the fucose and sialic acid residues, the reaction may be carried out at room temperature (23°C) for 15 h ***(9)***. The resulting reaction mixture was diluted with water to 200 µL and was diluted further 50-fold prior to CE-LIF analysis. The reductive amination reaction of reducing sugars with APTS is shown in **Scheme 1**. The stoichiometry of labeling is one molecule of fluorophore per reducing sugar and the fluorescent intensity is independent of the buffer pH. The three negatively charged sulfonate moieties of APTS-derivatized oligosaccharides provide mechanism for electrophoretic separation and the mobility of adduct is dependent on the degree of polymerization (DP) of the oligosaccharides.

The APTS-derivatized sugars have essentially the same UV/visible spectra between 300 and 500 nm, represented in **Fig. 1**. The λ_{max} of APTS (**Fig. 1A**) and the APTS-derivatized dextran 1000 (**Fig. 1B**) at 27 µ*M* each in aqueous solution were 424 and 456 nm, respectively. Molar absorptivity of the APTS–sugar adduct was reported as 17,100 $M^{-1}cm^{-1}$ ***(18)***. The red shift of λ_{max} of APTS–sugar versus that of the APTS is expected as the result of the formation of an alkylated aromatic amine group by reductive amination. The λ_{max} of the APTS adducts is between 455 and 457 nm with significant absorption at 488-nm, while APTS itself has λ_{max} at 424 nm with a relatively low absorption at 488-nm. When excited with a 488-nm argon ion laser, APTS–sugar derivatives fluoresce with substantially higher intensity (em λ_{max} = 512 nm) than the APTS emission (λ_{max} = 501 nm). Using a selective emission filter, preferential detection of the APTS–sugar derivatives can be achieved. The use of the 488-nm argon ion laser for excitation is more advantageous than the 442- or 325-nm He/Cd laser, which has a relatively short lifetime and considerable intensity fluctuation due to the sensitivity of its output to temperature variations ***(19)***.

2. CE separations can be performed on a P/ACE 5500 equipped with laser induced fluorescence system (Beckman Coulter Inc., Fullerton, CA). The separation of oligosaccharide ladders in CE system with buffer containing polymer networks and the influence of operating variables on thze separation were studied exten-

sively by Guttman et al. ***(20)***. The addition of a polymeric network did not seem to provide any sieving effect at concentrations above the entanglement threshold of sieving polymer (0.41%), polyethylene oxide (PEO). Thus, the presence of PEO in the buffer solution simply provides increased viscosity η that leads to a higher friction coefficient ($f = 6\pi\eta r$, where r is the root mean square of the solute ion radius) which results in reduced mobility of solute ion. Furthermore, the use of a polyvinyl alcohol coated capillary provides a neutral hydrophilic surface that eliminates residual electroendoosmotic flow. The charges on the APTS moiety of derivatized oligosaccharides provide the electrophoretic mobility and the mobility of adduct is inversely proportional to the DP. APTS–oligosaccharide adducts migrate according to their charge-to-mass ratio, or more appropriately, the charge-to-hydrodynamic volume. **Figures 2A–C** show the electropherograms of the APTS-derivatized dextran with average molecular weight of 1000, 5000, and 12,000, respectively. Baseline separation of oligosaccharides is visible up to 70 DP. Minor peaks migrating between the linear dextran α-1,6 linkage are branched oligosaccharides and are well resolved also. For dextran 1000 shown in **Fig. 2A**, the weight average was calculated to be 6.2 DP, very close to the molecular weight of 1000 provided from the vendor. The adducts of APTS with dextran 5000 and 12,000 shown in **Figs. 2B**,**C**, respectively, contain considerable amount of the small oligosaccharides of 20-mer or less. On the other hand, the dextran-1000 adduct shown in **Fig. 2A** exhibits major peaks from 3-mer to 10-mer with a negligible amount of maltose–APTS and DP above 14. The possibility that APTS-based reductive amination reaction efficiency is attenuated for oligosaccharides with larger DP could be consistent with the above observation. However, Morell et al. ***(11)*** found that the APTS derivatization efficiency is essentially identical for linear maltooligosaccharides derived from starch. Because all three dextrans were derivatized with APTS under identical conditions, it was unlikely that the hydrolysis would occur during the reductive amination process. Thus the hydrolysis presumably took place prior to derivatization and the derivatization efficiency for each oligomer appeared to be similar if not identical.

3. **Figures 3A–C** correspond to the electropherograms of APTS-derivatized DE-5, dextran 1000, and cellooligosaccharides, respectively. The common species in all electropherograms in **Fig. 3** is APTS–glucose (G1) at 5.94 min. The electropherogram of the mixture of all three types of the oligosaccharides shown in **Fig. 3D** provides an overview on the resolution of the CE separation system. Despite identical mass in APTS-derivatized C2, G2, and D2, cellobiose–APTS (C2–APTS) migrates ahead of the maltose–APTS (G2–APTS) followed by D2–APTS. Cellooligosaccharides with DP of <6 exhibit faster mobility than that of the maltooligosaccharides and dextrans with the corresponding DP, presumably because they are more compact in aqueous solution. Dextrans with DP of >8 migrate increasingly slower than their corresponding maltooligosaccharides, and are clearly resolved. As the DP increases above 8, the α-1,6 linkage in dextran favors a more extended structure than that of the conformation in the α-1,4 link-

Table 2
Mobilities of *N*-Linked Oligosaccharide in Two Different Buffer Systems

		Electrophoretic mobility, $\mu \times 10^{-5}$ cm^2/V.s	
	No. DP	PEO-acetate buffer, pH 4.75	TEA-phosphate buffer, pH 2.5
M5	7	12.770	12.773
M6	8	12.160	12.159
M7-D1	9	11.690	11.693
M7-D2	9	11.575	11.566
M7-D3	9	11.494	11.420
M8	10	11.007	10.936
M9	11	10.688	10.630
A2F	12	11.263	ND
NA2F	10	15.157	ND

age in starch. The PEO-based acetate buffer coupled with the polyvinyl alcohol (PVA)-coated capillary system provided good resolution and reproducibility between runs. The electrophoretic mobility of each species in **Fig. 3D** is shown in **Table 1**. Using G5 as a reference standard with an electrophoretic mobility of 14.755×10^{-5} cm^2/V·s, the relative mobilities of all the remaining maltooligosaccharides were measured with great accuracy. Coefficient of variation (CV) was consistently <0.15% on a day-to-day basis over a period of 9 mo using two different instruments with three different batches of capillaries. Within-run CV of >0.025% was observed for the mobility measurement of each maltooligosaccharide.

4. The APTS-derivatized *N*-linked oligosaccharides are most effectively characterized by CE-LIF. The *N*-linked oligosaccharides obtained from PNGase-catalyzed cleavage of ribonuclease B showed a well defined profile in **Fig. 4**, similar to that obtained previously ***(9,21)***. Excess APTS and its degradation products migrated between 4.5 and 6 min and they were also observed in an APTS sample blank. The *inset* in **Fig. 4** is an expanded scale showing the *N*-linked oligosaccharide portion of the electropherogram. The electropherogram of *N*-linked oligosaccharides from ribonuclease B shown in **Fig. 5** is compared with that of known oligosaccharide standards for proper assignment. The electropherograms of dextran ladders and the *N*-linked oligosaccharides from ribonuclease B are shown in **Fig. 5A**, **B**, respectively, while that of the APTS–M6, –M7, –M8, and –M9 are aligned in between. APTS–M5, the APTS derivative of pentamannosyl *N*,*N*'-diacetylchitobiose migrates slightly ahead of D7, while mobilities of M6, M7, M8, and M9 are increasingly faster than each of their corresponding dextran oligomers (D8, D9, D10, and D11) with M9 (DP of 11) migrating even faster than the D10, because M5, M6, M7, M8, and M9 were branched oligosaccharides, and assume a more compact structure than the linear oligosaccharides. A2F, a

biantennary oligosaccharide with a disialylated galactosylated core structure containing a fucosyl residue at the reducing end, was derivatized with APTS at room temperature overnight. The considerably high electrophoretic mobility of A2F (composed of 12 monosaccharides with two sialic acids) shown in **Fig. 6B**, migrating slightly ahead of D5, is indicative of complete dissociation of the carboxylic acid in both sialic acid residues. As the A2F is desialyated to become NA2F that contains 10 monosaccharides, it migrates slightly ahead of D10 (**Fig. 6C**).

5. CE separation of the APTS-derivatized *N*-linked oligosaccharides from ribonuclease B in phosphate buffer, pH 2.5 (data not shown), was compared with that in the standard buffer of PEO-acetate, pH 4.75. The pattern of the electropherogram is essentially the same as that in **Fig. 4**, and each species migrates slightly faster but without any reduction of resolution. Using D5 (mobility shown in **Table 1**: 14.810×10^{-5} cm/V·s) in dextran 1000 as the internal standard with the *N*-linked oligosaccharide sample (data not shown), the electrophoretic mobilities of the *N*-linked oligosaccharides were calculated in columns 3 and 4 of **Table 2** for PEO-acetate, pH 4.75, and phosphate, pH 2.5, respectively. For mobility values with two completely different buffer systems, the coefficient of variation is 0.5% or less, indicative of the reliability of mobility measurement.

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8

Affinity Capillary Electrophoresis of Oligosaccharides

Atsushi Taga and Susumu Honda

1. Introduction

The basic separation mode of capillary electrophoresis is zone electrophoresis (ZE). In this mode solutes having different charge-to-size ratios migrate through an electrophoretic solution with different velocities, forming separate zones. ZE is a simple mode performable in an electrophoretic solution composed of a common salt, such as sodium acetate, sodium phosphate, and so forth, but is not applicable to nonionic compounds. Because most carbohydrates are electrically neutral under normal conditions, such analytes must be changed to ions prior to or during analytical runs. A number of methods have been developed for the precapillary conversion to cationic (e.g., *see* **refs. *1–4***) or anionic (e.g., ***5–8***) derivatives based on reductive amination. The derivatives of the former type are positively charged in acidic media as a result of the introduction of the imino group. The derivatives of the latter type have either the carboxyl group or the sulfonate group in addition to the imino group. They give cations or anions dependent on the pH of the electrophoretic solution. The derivatives having the sulfonate group, which is strongly dissociated, behave as high-mobility anions in neutral or alkaline media.

In situ conversion to ions includes complexation with the borate and the divalent metal ions. These inorganic ions form the anionic and cationic charges, respectively, in the carbohydrate molecules. The separation by ZE involving such complexations is based on the *in situ* conversion of the analytes to ions by chemical reactions in running buffers containing the corresponding inorganic salts as ion sources ***(9)***. In cases in which the analytes are ionic derivatives, these complexations result in change of its mobility ***(10,11)***. For example, when a fused silica capillary is used, 2-pyridylaminated glucose migrates faster than

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a neutral marker in acidic phosphate buffers, whereas it moves slower than the neutral marker in alkaline borate buffers owing to the creation of the negative charge by the *in situ* complexation with the borate ions ***(1)***.

Physical interaction can also change mobility. One typical example is the specific binding to a protein. When such a protein is added to an electrophoretic solution, an ionic ligand as sample binds to the protein while moving and changes its velocity, because its mobility is affected by the protein. This type of modified ZE is called affinity capillary electrophoresis (ACE), because it is based on the affinity between a ligand as sample and a protein as additive. Affinity electrophoresis in slab or disc gels is a well established technique, but ACE has a different feature in that the interaction process can be continuously observed in free solution. This chapter describes the application of ACE to carbohydrate separation.

Although there are a number of methods for precapillary derivatization based on reductive amination, the 1-phenyl-3-methyl-5-pyrazolone (PMP) method ***(12)*** is adopted in this chapter because of the simplicity of the procedure and the appropriate mobilities of derivatives under interacting conditions. In this method two PMP groups are introduced to the reducing end of each carbohydrate molecule, and the derivatives have strong absorptivity in the ultraviolet (UV) region. They have weakly negative charge in neutral or alkaline media, because the keto group in the pyrazolone ring changes to the enol group, and the latter is dissociated to give an enolate ion in an equilibrium fashion (*see* **Note 1**). The presence of the PMP groups does not significantly affect the binding to proteins, as compared to the sulfonate group in 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) derivatives (*see* **Note 2**). The diversity of applicable separation modes is another reason why this derivatization method is selected. PMP derivatives of mono- and oligosaccharides can be separated by ZE directly ***(13)*** or with modification by complexation with the borate ***(10)*** or metal ions ***(11)***. They can also be separated by micellar electrokinetic chromatography (***14***; *see also* Chapter 5), because the derivatives have relevant hydrophobicity. They can also be the objects of ion-interaction electrokinetic chromatography with a cationic polymer such as Polybrene ***(15)***. The separation mode based on the intramolecular hydrogen bonding with an optically active *N*-dodecoxycarbonylvaline ***(16)*** is a unique method of enantioseparation inherent to PMP derivatives. All these separation modes are based on the selective interaction of the carbohydrate moiety with a certain kind of small molecule as an additive.

The ACE described in this chapter is somewhat different from the aforementioned modes in that the interaction occurs to a macromolecule of natural origin and is quite specific. We present here an example of ACE, taking PMP-derivatized disaccharides and a few lectins as models ***(17)***. The proposed pro-

tocol can be applied to any other carbohydrate–protein interactions. ACE causes a specific migration delay of the carbohydrate derivatives by a protein in the electrophoretic solution, and it is useful to identify them in complex mixtures from biological sources. Thus, the use of even a single lectin is beneficial for carbohydrate separation. The concurrent use of multiple lectins gives much better separation as demonstrated.

In ACE there is an inherent problem of protein adhesion on the capillary inner wall. This problem could be solved by careful coating with linear polyacrylamide, and special attention should be paid to this technique.

2. Materials

2.1. Derivatization (12)

1. Sample solution: A mixture of 10 µL each of –10^{-2} *M* aqueous solutions of oligosaccharides and L-rhamnose (internal standard).
2. PMP reagent: Purify a commercial sample of PMP by recrystallization from methanol.
3. Reagent solution: 0.50 *M* of PMP in methanol.
4. 0.30 *M* of sodium hydroxide (*see* **Notes 3** and **4**).
5. 0.30 *M* of hydrochloric acid (*see* **Note 4**).
6. Chloroform: Reagent grade (*see* **Note 5**).
7. Centrifugal concentrator (SpeedVac).

2.2. Chemical Coating of Capillary (18)

1. Capillary: A commercial sample having an inner diameter (i.d.) of 50 µm, made of fused silica (*see* **Note 6**).
2. 1 *M* Sodium hydroxide.
3. Acetonitrile: Reagent grade.
4. 3-Methacryloxypropyltrimethoxysilane (MOPTMS) solution: An acetonitrile solution containing MOPTMS (reagent grade) and acetic acid (reagent grade) to a common concentration of 0.4% v/v.
5. The *N,N,N',N'*-tetramethylethylenediamine (TEMED) solution: An aqueous 10% v/v solution of TEMED (reagent grade).
6. Ammonium peroxydisulfate solution: An aqueous 10% w/v solution of ammonium peroxydisulfate (reagent grade) (*see* **Note 7**).
7. Acrylamide solution: Prepare by dissolving acrylamide in a buffer solution containing tri(hydroxymethyl)aminomethane (Tris, reagent grade, 5×10^{-2} *M*), boric acid (reagent grade, 5×10^{-2} *M*), and the disodium salt of ethylenediamine-*N,N,N',N'*-tetracetic acid (EDTA-2Na, reagent grade, 2.5×10^{-3} *M*) to a concentration of 3% w/v. It should be prepared fresh before use.

2.3. Capillary Electrophoresis

1. Instrument: A HPCE apparatus, home-made or marketed, equipped with a high voltage supply, a sample introducing system, and a UV detector (*see* **Note 8**).

2. 1 *M* Sodium hydroxide for capillary cleaning.
3. Phosphate buffer (PB, 5×10^{-2} *M*, pH 6.8): Prepare it by adding 5×10^{-2} *M* sodium dihydrogen phosphate to 5×10^{-2} *M* disodium hydrogen phosphate until the pH reaches 6.8 (*see* **Note 9**).
4. Electrophoretic solutions (*see* **Note 10**).
   LCA solution, PB containing *Lens culinaris* agglutinin (LCA).
   RCA_{60} solution, PB containing *Ricinus communis* 60-kDa agglutinin (RCA_{60}).
   SBA solution, PB containing soybean agglutinin from *glycine max* (SBA).
   LCA/RCA_{60} solution, PB containing LCA and RCA_{60}.
   LCA/SBA solution, PB containing LCA and SBA.

3. Methods

3.1. Derivatization (12)

1. Evaporate the sample solution to dryness under reduced pressure with a SpeedVac.
2. Dissolve the residue in 50 µL of 0.3 *M* sodium hydroxide (*see* **Notes 3** and **4**).
3. Add 50 µL of the reagent solution of 0.5 *M* PMP in methanol.
4. Heat the mixture for 30 min at 70°C, then cool it to room temperature.
5. Neutralize the cooled solution with 50 µL of 0.3 *M* hydrochloric acid (*see* **Note 4**).
6. Evaporate the neutralized solution to dryness under reduced pressure with a SpeedVac.
7. Dissolve the residue in 200 µL of water.
8. Extract the aforementioned solution 3× with 200 µL of chloroform (*see* **Note 5**).
9. Evaporate the final aqueous layer to dryness under reduced pressure with a SpeedVac.
10. Reconstitute the residue in 50 µL of water for ACE.

3.2. Chemical Coating of a Capillary (18)

1. Cut out a 50-cm portion of capillary from a roll, and make a detection window at an approx 30-cm position from the inlet (*see* **Note 6**).
2. Rinse the cutout capillary with 1 mol/L sodium hydroxide followed by water, for 15 min each, by connecting the outlet to an aspirator.
3. Finally rinse the capillary with acetonitrile for 15 min.
4. Flow the MOPTMS solution through the capillary for 1 h to modify the silanol group on the inner wall.
5. Degas the acrylamide solution in an ultrasonic bath.
6. Prepare the polymerizing solution fresh before use by adding 16 µL of the TEMED solution and 4 µL of the ammonium peroxydisulfate solution to 1 mL of the polyacryamide solution.
7. Flow the final mixture in **step 6** through the capillary for 10 min and leave the solution in the capillary for a further 30 min to allow for polymerization.
8. Rinse this coated capillary with water for 15 min to remove polyacrylamide not bound to the silanol group.

3.3. ACE

1. Mount the coated capillary on a cassette, and fix the detector window at the center of the UV beam.
2. Install the cassette in a high-performance CE (HPCE) apparatus, and keep it at a specified temperature by circulating temperature-controlled air or liquid, depending on the apparatus used. Rinse the capillary with PB for ample time to clean the inner wall of the coated capillary.
3. Flow an electrophoretic solution through the capillary in the minimum period to completely replace PB by the electrophoretic solution (*see* **Note 11**).
4. Introduce the oligosaccharide mixture derivatized with PMP to the cathodic end of the capillary (*see* **Note 12**).
5. Soak both ends of the capillary in the electrode solution (PB) contained in reservoirs and apply a voltage.

4. Notes

1. PMP method: *See also* Chapters 5 and 6 for more details.
2. Effect of the protecting group on affinity: The authors noted the enhancement of the association constant by the introduction of the ANTS group in a binding study of an oligosaccharide to a lectin.
3. Sodium hydroxide is added to facilitate the condensation of a carbohydrate with PMP by changing PMP to its sodium salt.
4. Neutralization of the reaction solution effects removal of the excess PMP reagent by extraction. Slightly insufficient or excess volume of sodium hydroxide can result in contamination with minor peaks due to the formation of the mono-PMP derivative as byproduct and/or the reagent blank. Therefore, the neutralization should be performed by using exactly an indicated volume of normalized sodium hydroxide and hydrochloric acid solutions.
5. Complete removal of the excess reagent is important, because the reagent peak may interfere with oligosaccharide analysis. Extraction for three times with the same volume of the solvent is recommended. Although ethyl acetate is used for the cleanup of monosaccharide derivatives (Chapter 5), chloroform is preferable for the cleanup of oligosaccharide derivatives, because it has stronger and weaker dissolving power to PMP and PMP derivatives, respectively, than ethyl acetate.
6. The outer wall of commercial fused silica capillaries are mostly coated with polyimide to prevent breakage. A detection window must be made by removing an approx 1-mm portion of the polyimide coat by burning out with a thin flame, because polyimide is not transparent to UV light. (*See also* Chapter 5.)
7. Ammonium peroxydisulfate is used as a catalyst for the polymerization reaction.
8. The HPCE apparatus should be equipped with a high voltage supplier capable of supplying ~30 kV. Sample solutions can be introduced by pressure drop utilizing either compression, suction, or siphoning. Introduction by electromigration is not desirable, because it may cause introduction of the component ions in a sample at different velocities. There are two types of UV detectors, one designed

for a particular wavelength using a special lamp and an interference filter and another for multiple wavelengths using a deuterium or tungsten lamp and a grating spectroscope. The latter type is preferable in the present analysis, because the PMP derivatives absorb at 245 nm. The detectors of the former type equipped with a mercury lamp, which emits most abundantly at 254 nm, can also be used, but sensitivity is not high due to the deviation of the wavelength.

9. PB: PB is used as either a running buffer or the medium for the lectin solutions. It is also used as an electrode solution throughout analysis. It should be degassed under reduced pressure before it is used as a running buffer or an electrode solution by passing it through a membrane filter with suction.
10. Lectin solutions: Prepare the stock solutions by dissolving 1 mg each of a lectin in 100 µL of PB and store them at 4°C. When necessary mix any two stock solutions or dilutions with PB in appropriate proportions. Each solution should be degassed before analysis by use of a membrane filter. LCA is a lectin having specificity to the glucose and mannose residues in oligosaccharide chains. Both RCA_{60} and SBA recognize the galactose residue, but the affinity is dependent on the carbohydrate structure in the vicinity of the galactose residue.
11. A capillary is filled with a lectin solution because of the high cost of lectins.
12. The sample introduction period should be controlled to create an approx 4-mm sample plug at the inlet of a capillary. The appropriate period of time for introduction depends on the apparatus and conditions. For example, 10 s is appropriate for the siphoning method using a 10-cm difference of the levels between the sample solution and the cathodic electrode solution.
13. Examples of analysis:

    Figure 1 shows an example of the separation of five disaccharides as PMP derivatives. Because all these disaccharides have the same molecular weight and the same number (two) of PMP groups, they are isomers of each other. The separation of these disaccharides in PB not containing lectins was poor, as expected. The appearance of two close peaks of glucobioses and galactosylglucoses was rather surprising. This is presumably due to the participation of intramolecular hydrogen bonding, as observed in the separation of monosaccharides under similar conditions ***(13)***.

    Addition of a lectin caused a drastic change of electropherogram ***(17)***. The addition of LCA, which is known to bind to the glucose residue, to a concentration of 10 µg/µL, resulted in specific retardation of the peaks of glucobioses (maltose, cellobiose, and gentiobiose), and the magnitude of retardation was in the increasing order of cellobiose, gentiobiose, and maltose (**Fig. 2A**). Of the two galactosylglucoses (lactose and melibiose) lactose showed slight retardation but melibose remained at the original position. As a result these five disaccharides were separated into four peaks under the conditions employed. The gentiobiose and lactose peaks overlapped each other. The addition of RCA_{60} was much more effective than that of LCA even at a lower concentration (5 µg/µL) (**Fig. 2B**). In this case the galactosylglucoses were specifically retarded. The lactose peak appeared last, and the melibiose peak was next to last. Glucobioses were only

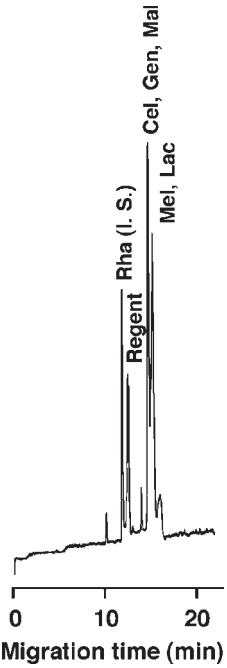

Fig. 1. Electropherogram of PMP derivatives of disaccharides by CZE. Capillary, linear polyacrylamide-coated fused silica (50 μm i.d., 50 cm); capillary temperature, 30°C; electrophoretic solution, 50 m*M* phosphate buffer, pH 6.8; applied voltage, 15 kV; detection, UV absorption at 245 nm; sample introduction, vacuum method (1.5 s) from the cathodic end. Mal, Cel, Gen, Lac, Mel, and Rha (IS) are the abbreviations of the PMP derivatives of maltose, cellobiose, gentiobiose, lactose, melibiose, and L-rhamnose (internal standard), respectively (*see* **Note 13**). (Reproduced with permission from **ref. *17***).

slightly retarded, giving poorly resolved peaks. These two examples demonstrate that the separation by ACE allows isomer separation with quite a different feature from other separation modes.

Addition of two lectins exerted concerted bindings to the disaccharides. **Figures 3A** and **B** show examples of separation of the PMP derivatives of the same five disaccharides by appropriate combinations of lectins. Since there is no mutual interaction between the lectins employed, each of the two lectins bound independently to the analytes. The concentrations of these lectins are important to obtain good separation. **Figure 3A** was obtained using LCA (7 μg/μL) and RCA_{60} (3 μg/μL). The concentration of RCA_{60} was less than half that of LCA, because the former gave much more pronounced retardation in **Figs. 2A** and **B**, especially to lactose. Although the result was not necessarily satisfactory, these disaccharides gave five separated peak tops under these conditions. **Figure 3B** is another

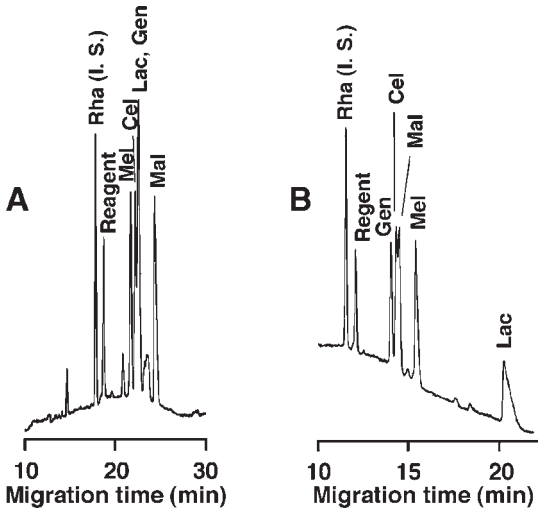

Fig. 2. Separation of the PMP derivatives of disaccharides by ACE in single lectin systems. Electrophoretic solutions: **(A)** LCA (10) solutions; **(B)** RCA_{60} (5) solution. The numbers in the parentheses are the lectin concentrations in μg/μL. Applied voltage: **A**, 15 kV; **B**, 18 kV. Other analytical conditions and abbreviations as in **Fig. 1**. (Reproduced with permission from **ref.** ***17***).

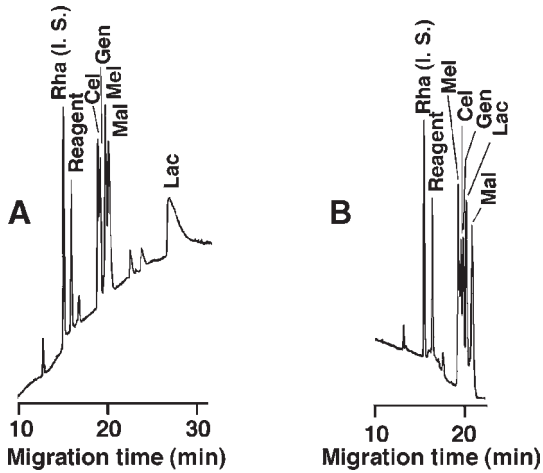

Fig. 3. Separation of PMP derivatives of disaccharides by ACE in dual lectin systems. Electrophoretic solutions: **(A)** LCA (7)/RCA_{60} (3) solution; **(B)** LCA (8)/SBA (2) solution. The numbers in the parentheses are the lectin concentrations in μg/μL. Other analytical conditions and abbreviations as in **Fig. 1**. Mal, maltose (Glcα1→4Glc); Cel, cellobiose (Glcβ1→4Glc); Gen, gentiobiose (Glcβ→6Glc); Lac, lactose (Galβ1→4Glc); Mel, melibiose (Galα1→6Glc); where Glc and Gal designate the D-glucose and D-galactose residues, respectively. (Reproduced with permission from **ref.** ***17***).

example obtained by the combination of LCA (8 μg/μL) and SBA (2 μg/μL). Because SBA is not so interactive to galactosylglucoses as compared to RCA_{60} and the affinities to lactose and melibiose were reversed, the electropherogram was rather compact, giving five peaks in a narrow range at around 20 min. Further optimization on the lectin concentration will improve separation.

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9

Characterization of Glycosaminoglycans by Capillary Electrophoresis

Robert J. Linhardt and Toshihiko Toida

1. Introduction

Glycosaminoglycans are linear, sulfated polysaccharides that are found in virtually all animal tissues. With the exception of hyaluronic acid, glycosaminoglycans are biosynthesized as proteoglycans in which one or more glycosaminoglycan chains are attached to a core protein ***(1)***. Chondroitin and dermatan sulfates are comprised of alternating 1→3, 1→4 linked *N*-acetyl-D-galactosamine and hexuronic acid (either D-glucuronic or L-iduronic acid) residues. These saccharide residues can be *O*-sulfonated at various positions on both sugar residues. Heparin and heparan sulfate are structurally related and are comprised of a hexuronic acid residue (either L-iduronic or D-glucuronic acid) 1→4 linked to a D-glucosamine (either *N*-sulfo or *N*-acetyl-D-glucosamine) residue. Both saccharide residues can be *O*-sulfonated at a variety of different positions, making these glycosaminoglycans structurally complex. Hyaluronic acid contains a simple repeating structure of 1→3, 1→4 linked *N*-acetyl-D-glucosamine and D-glucuronic acid residues. Hyaluronic acid is an unusual glycosaminoglycan as it is not sulfonated and can have a very high molecular weight, up to 2×10^6 Da compared to $1–3 \times 10^5$ for the other glycosaminoglycans. Keratan sulfate is a glycosaminoglycan that contains no uronic acid and is comprised of a repeating 1→3, 1→4 linked, *O*-sulfonated *N*-acetyl-D-glucosamine and D-galactose residues.

Glycosaminoglycans are most often found in the extracellular environment such as on cell surfaces or in the extracellular matrix. These extracellular glycosaminoglycans contribute important structural properties to cells and at the same time serve as receptors for a large number of extracellular signaling pro-

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teins, such as cytokines and growth factors, that are important in cell growth and cell–cell communication ***(1)***. It is because of their important functions and complex structure that extensive efforts have been made to develop methods for glycosaminoglycan analysis.

The direct analysis of intact glycosaminoglycans poses a number of challenges. First, they are highly charged (–25 to >–100), quite large (10^5 – 10^6 Da), polydisperse (M_W/M_N 1.1–3), and often contain a high degree of sequence variability owing primarily to an uneven distribution of sulfate groups. Second, in tissue samples they are often difficult to separate from one another because their physical–chemical properties are very similar. Third, glycosaminoglycans do not contain an ultraviolet (UV) chromophore, complicating their detection. Although direct analysis of glycosaminoglycans by capillary electrophoresis (CE) has recently been reported ***(2)***, in most analyses, glycosaminoglycans are first broken down into the disaccharide or oligosaccharide components ***(3–5)***.

Two methods are routinely used to depolymerize glycosaminoglycans. The first, chemical depolymerization, relies on nitrous acid, which is effective in breaking down heparin and heparan sulfate into disaccharides and oligosaccharides through a deaminative mechanism ***(6)***. Chondroitin and dermatan sulfates, hyaluronic acid, and keratan sulfate are insensitive to nitrous acid and must first be chemically de-*N*-acetylated to be depolymerized by nitrous acid. This selectivity provides a way to distinguish different families of glycosaminoglycans and to prepare smaller, easy to analyze disaccharide and oligosaccharide components. Despite the value of nitrous acid for the preparation of disaccharides and oligosaccharides, detection of these products typically requires the introduction of radiolabel. The second method of depolymerization utilizes enzymes called polysaccharide lyases ***(7–9)***. These enzymes are specific for different glycosaminoglycans and act to break each type of glycosaminoglycan down into disaccharide or oligosaccharide products. One unusual and useful aspect of these enzymes is that they act through an elimination mechanism affording an unsaturated uronate residue (ΔUA) at the nonreducing terminus of product. This unsaturated uronate is UV active with a molar absorptivity (ε_M) of from 3800 (at pH 7.0) to 5500 (at pH 2.0) at a wavelength λ of 230–235 nm. Enzymatic depolymerization offers advantages of both high specificity and high sensitivity but results in a loss of information about the chirality of the uronic acid end group.

The polysaccharide lyases that are widely used for the analysis of glycosaminoglycans and their specificity are presented in **Table 1**. These enzymes generally act at temperatures ranging from 25 to 37°C in a variety of buffers and do not require the addition of cofactors. The optimal conditions for each enzyme have been determined and are reviewed elsewhere ***(10)***. Detailed protocols are

Table 1
Polysaccharide Lyases Used in Glycosaminoglycan Analysis

Enzyme	EC number[a]	Substrate/ action pattern[b]	Specificity[c]
Chondroitin lyase ACI	EC4.2.2.5(endo)	CS (endo)	GalNAc → GlcA
Chondroitin lyase ACII	EC4.2.2.5(exo)	CS(exo)	GalNac → GlcA
Chondroitin lyase B	No EC number	DS (endo)	GalNAc → IdoA
Chondroitin lyase ABC	EC4.2.2.4 (endo)	CS/DS (endo)	GalNAc → HexUA
Chondroitin lyase ABC	EC4.2.2.4 (exo)	CS/DS (exo)	GalNAc → HexUA
Heparin lyase I	EC4.2.2.7	H/HS (endo)	GlcNS (6S/OH) → IdoA2S
Heparin lyase II	No EC number	H/HS (endo)	GlcN(S/Ac)(6S/OH) → IdoA/GlcA (2S/OH)
Heparin lyase III	4.2.2.8	HS (endo)	GlcN(Ac/S)(6OH/S) → (GlcA/IdoA)
Hyaluronate lyase	EC4.2.2.1	HA (endo)	GlcNAc → GlcA

[a]*See* **ref. *10***.
[b]*See* **refs. *14*,*15*,*17***.
[c]*See* **refs. *18–20***.

also described in Chapter 4 of this volume. Although the present chapter places emphasis on the CE analysis of the oligosaccharides, it also includes a brief description of a brief depolymerization procedure, because it is required for sample preparation in CE analysis. Typically, glycosaminoglycan is conveniently depolymerized by overnight treatment with enzyme in a buffer solution. The resulting oligosaccharide mixture is then subject to analysis. It is often possible to obtain only disaccharides for analysis by using a mixture of enzymes (i.e., heparin lyase [heparinase] I, II, and III) or an enzyme with a particularly broad specificity (i.e., chondroitin lyase [chondroitinase] ABC). (*see* **Table 1**.)

CE can analyze intact glycosaminoglycans at high voltage in normal polarity mode. A mixture of enzymatically prepared glycosaminoglycan-derived oligosaccharides or disaccharides can be analyzed using CE performed at high voltage in normal polarity or reverse polarity ***(3)***. In normal polarity a basic solution is used, maintaining a negative charge on the silanol groups of the fused silica capillary. This negatively charged capillary results in electroendoosmosis, causing a bulk flow of buffer from the anode to the cathode. This flow carries the disaccharide and oligosaccharide analytes from the anode where they are applied to the cathode where they are detected. In reverse polarity, an acidic solution is used, suppressing the dissociation of the silanol residues in the capillary. Thus, the disaccharide or oligosaccharide analyte applied at the cathode migrates to the anode under electrophoresis.

The following are typical examples for the analysis of intact glycosaminoglycans and the oligosaccharides.

2. Materials

2.1. Analysis of Intact Glycosaminoglycans

1. Glycosaminoglycans can be prepared from a variety of different species and tissues sources ***(11)*** and are commercially available from various companies. Hyaluronic acid, mol wt 100,000, from human umbilical cord (Seikagaku America, Ijamsville, MD); chondroitin-4-sulfate, mol wt 50,000, from shark cartilage (Seikagaku America); dermatan sulfate, mol wt 16,000, from porcine skin (Seikagaku America); heparin, mol wt 14,800, from porcine intestinal mucosa (Celsus Laboratories, Cincinnati, OH); low molecular weight heparin, mol wt 4800 (Celsus); heparan sulfate, mol wt 11,000, from porcine intestinal mucosa (Celsus).
2. 40 m*M* Sodium phosphate–40 m*M* sodium dodecyl sulfate (SDS)–10 m*M* sodium borate buffer, pH 9.2, was prepared by making 7.2 g of dibasic sodium phosphate (dihydrate), 11.6 gm of SDS, and 3.6 g of sodium borate (decahydrate) up to 1 L with distilled water. The pH of the solution was adjusted to 9.2 using 0.2 *M* sodium hydroxide.

3. Normal polarity capillary electrophoresis on a Beckman P/ACE 5010 System (Beckman Corp., Fullerton, CA) utilized a 75-µm inner diameter (i.d.) × 57 cm fused silica capillary (Supelco Co., Bellefonte, PA).
4. 5 m*M* Copper (II) sulfate solutions was prepared by making 0.8 g of copper (II) sulfate (anhydrous) up to 1 L volume with distilled water and adjusting the pH to 4.5 with 0.1 *M* sulfuric acid.
5. Reverse polarity capillary electrophoresis on a Dionex Capillary Electrophoresis System (Sunnyvale, CA) utilized a 75-µm i.d. × 68 cm fused silica capillary (Supelco).

2.2. Enzymatic Depolymerization of Glycosaminoglycans

1. The polysaccharide lyases (*see* **Table 1**) can be obtained from a number of commercial sources including Seikagaku America and Sigma Chemical Co. (St. Louis, MO). Lyophilized enzyme should be reconstituted with water or buffer according to the manufacturer's instructions and divided into small aliquots (typically 10 µL containing 1 mU/µL, where 1 U = 1 µmol of product formed/min) and stored frozen at -20 to -70°C (*see* **Note 1**).
2. To completely depolymerize chondroitin (4- or 6-) sulfate or dermatan sulfate to disaccharide products chondroitinase ABC is used in 50 m*M* Tris-Cl–sodium acetate buffer prepared by adding 6.05 g of Tris base and 8.17 g sodium acetate to 1 L of water adjusted to pH 8.0 with glacial acetic acid.
3. To completely depolymerize heparin or heparan sulfate to disaccharide products an equi-unit mixture of heparin lyase I, II, and III is used in 50 m*M* sodium 100 m*M* phosphate–sodium chloride buffer prepared by adding 7.1 g dibasic sodium phosphate and 5.8 g sodium chloride to 1 L of water adjusted to pH to 7.1 with concentrated phosphoric acid.
4. To completely depolymerize hyaluronic acid to disaccharide product hyaluronate lyase (*Streptomyces hyalurolyticus*) is used in 50 m*M* sodium acetate–125 m*M* sodium chloride buffer prepared by adding 4.1 g of sodium acetate and 7.3 g of sodium chloride to 1 L of water adjusted to pH 5.2 with glacial acetic acid.

2.3. Disaccharide Analysis by CE

1. Disaccharides prepared by complete enzymatic depolymerization were desalted using a 1 × 42 cm Bio-Gel P-2 column (Bio-Rad) eluted with water and the peak eluting at the column void volume having an absorbance at 232 nm was collected, lyophilized and prepared at 1 mg/mL in water for CE analysis.
2. Disaccharide standards are commercially available (Seikagaku America or Sigma Chemical Co.). The common abbreviated name, for these standards are (chondro/dermato disaccharide standards): ΔDi-0S, ΔUA (1→3) GalNAc; ΔDi-4S, ΔUA (1→3) GalNAc4S; ΔDi-6S, ΔUA (1→3) GalNAc 6S; ΔDi-diS_E, ΔUA (1→3) GalNAc4S6S; ΔDi-diS_B, ΔUA2S (1→3) GalNAc4S; ΔDi-diS_D, ΔUA2S(1→3) GalNAc6S; ΔDi-triS, ΔUA2S (1→3) GalNAc4S6S (heparin/heparan sulfate disaccharide standards): ΔDiHS-0S, ΔUA(1→4) GlcNAc; ΔDiHS-6S, ΔUA (1→4) GlcNAc6S; ΔDiHS-NS, ΔUA (1→4) GlcNS; ΔDiHS-diS_1, ΔUA(1→4) GlcNS6S;

ΔDiHS-diS_2, ΔUA2S(1→4) GlcNS; ΔUAHS-triS, ΔUA2S (1→4) GlcNS6S. Mixtures of chondro/dermato disaccharide standards (1 mg/mL in water) and heparin/heparan sulfate disaccharide standards (1 mg/mL in water) were used for CE analysis.

3. Normal polarity disaccharide analysis was performed on a Beckman P/ACE System by pressure injection of 8.7 nL of analyte at the anode side of a fused silica capillary (75 μm i.d. × 67 cm) containing 20 m*M* sodium phosphate buffer, pH 9.0, prepared by making 2.76 g of monobasic sodium phosphate up to 1 L and adjusting to pH 9 with 1 *M* sodium hydroxide. Migration controlled by electroosmotic flow at 18 kV resulted in the disaccharide having the smallest negative charge migrating fastest. Detection was by UV absorbance at 230 nm at the cathode.
4. Reverse polarity disaccharide analysis was performed on a Beckman P/ACE System by pressure injection of 8.7 nL of analyte at the cathode side of a fused silica capillary (75 μm i.d. × 47 cm) containing 20 m*M* sodium phosphate buffer, pH 3.6, prepared by adjusting 20 m*M* phosphoric acid to pH 3.6 with 1 *M* sodium hydroxide. Migration controlled by electrophoresis at 18 kV resulted in the disaccharide having the highest negative charge migrating fastest. Detection was by UV absorbance at 230 nm at the cathode.

2.4. CE Analysis of Higher Oligosaccharides

1. A 2 mg/mL solution of partially depolymerized bovine lung heparin was analyzed by CE (*see* **Note 4**).
2. Using a Dionex Electrophoresis System normal polarity analysis was performed, as it provided the best resolution of this complex mixture. A 9.2-nL sample (2 mg/mL) was gravity injected at the anode of a fused silica capillary (75 μm i.d. × 78 cm) filled with 10 m*M* sodium borate buffer, pH 8.8, containing 50 m*M* SDS prepared by making 3.6 g of sodium borate (decahydrate) and 14.5 g SDS up to 1 L and adjusting the pH to 8.8 with 0.2 *M* sodium hydroxide. Migration controlled by electroosmotic flow at 12 kV resulted in the largest oligosaccharides migrating fastest (*see* **Note 3**).

2.5. Fluorescent Labeling of Glycosaminoglycan-Derived Oligosaccharides

1. The monopotassium salt of 7-amino-1, 3-naphthalene disulfonic acid (AGA) of 85% purity (Aldrich Chemical Co., Milwaukee, WI) was crystallized repeatedly from water and 50 mg was dissolved in 1 mL of acetic acid–water (3:17, v/v) to prepare a 0.15 *M* solution.
2. A 1 *M* solution of sodium cyanoborohydride (Aldrich) was prepared by dissolving 63 mg in 1 mL of high-purity grade dimethyl sulfoxide (DMSO)(Aldrich).
3. A glass column (1 × 42 cm), packed with Bio-Gel P-2 resin (Bio-Rad), was equilibrated with water and used for desalting.

2.6. CE Analysis of Fluorescently Labeled Oligosaccharide Mixture

1. A Dionex Capillary Electrophoresis System with fluorescence detection was used.

2. The 20 m*M* sodium phosphate buffer, pH 2.6, was prepared by adjusting 20 m*M* phosphoric acid to pH 2.6 with 1 *M* sodium hydroxide.

3. Methods

3.1. Analysis of Intact Glycosaminoglycans Using Normal Polarity Capillary Electrophoresis

1. Wash the capillary by manually injecting 0.5 mL of 0.5 *M* sodium hydroxide followed by 0.5 mL of distilled water and 0.5 mL of running solution. Insert the clean capillary into the instrument.
2. Prepare 10 mg/mL stock solutions of glycosaminoglycans by dissolving 1 mg each of lycosaminoglycan or a mixture of glycosaminoglycans (1 mg each) into 100 µL of distilled water.
3. Using the sodium phosphate–SDS–sodium borate running solution, 8.7 nL of glycosaminoglycan sample was applied to the capillary at the anode side using pressure injection (5 s) using nitrogen gas. Applying a potential of 18 kV the glycosaminoglycan analyte migrated to the cathode side and was detected by UV absorbance at 200 nm (*see* **Fig. 1**).
4. Using the copper (II) sulfate running solution, 9.2 nL of glycosaminoglycan sample was applied to the capillary at the anode side using gravity injection (12 s) by hydrostatic pressure (45 m*M*). Applying a potential of 20 kV the glycosaminoglycan analyte migrated to the cathode side and was detected by its UV absorbance at 240 nm (*see* **Fig. 2** and **Note 4**).

3.2. Enzymatic Depolymerization of Glycosaminoglycans

1. To chondroitin (4- or 6-) sulfate or dermatan sulfate (100 µL at 10 mg/mL) in Tris-Cl–sodium acetate buffer, pH 8, 10 µL of 1 mU/µL chondroitin lyase ABC is added and the mixture incubated at 30°C overnight. Removal of a 10-µL aliquot and dilution in 1 mL of 30 m*M* HCl should afford an A_{232} of 0.8-1.0 if the reaction is complete.
2. To heparin or heparan sulfate (100 µL at 10 mg/mL) in sodium phosphate–sodium chloride buffer, pH 7.1, 3.3 µL each of heparin lyase I, II, and III (each at 1 mU/µL) is added and the mixture incubated at 30°C overnight. Removal of a 10-µL aliquot and dilution in 1 mL of 30 m*M* HCl should afford an A_{232} of 0.7–1.1 if the reaction is complete.
3. To hyaluronic acid (100 µL at 10 mg/µL) in sodium acetate–sodium chloride buffer (pH 5.2), 10 µL of hyaluronate lyase (1 mU/µL) is added and the mixture incubated at 30°C overnight. Removal of a 10-µL aliquot and dilation in 1 mL of 30 m*M* HCl should afford an A_{232} of 0.9–1.0 if the reaction is complete.
4. Bovine lung heparin (Sigma Chemical Co.) was partially depolymerized using heparin lyase I. Heparin (200 µL at 10 mg/mL) in sodium phosphate–sodium chloride buffer, pH 7.1, was treated with 2 mU of heparin lyase I at 30°C and small 10-µL aliquots were removed over the course of 24 h and the reaction terminated by heating at 100(C for 5 min. A 10-µL portion of each aliquot was

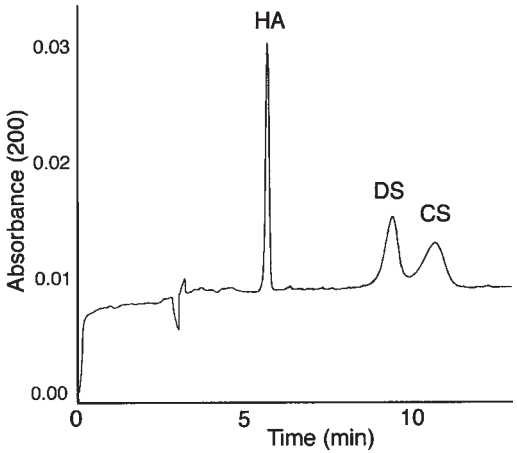

Fig. 1. Normal polarity CE analysis of intact glycosaminoglycans using UV detection. Hyaluronic acid (HA), dermatan sulfate (DS), and chondroitin sulfate (CS) were analyzed.

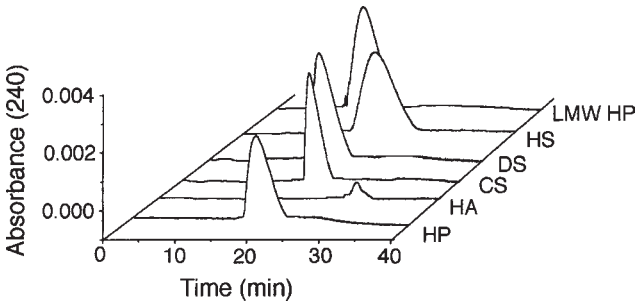

Fig. 2. Reverse polarity CE analysis of intact glycosaminoglycans as copper II complexes. Heparin (HP), hyaluronic acid (HA), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and low molecular weight heparin (LMW HP) were analyzed.

added to 1 mL of 30 m*M* HCl and absorbance at 232 nm was measured. The A_{232} value of the final time point represented reaction completion (100%). The reaction product to the depolymerization time point giving 30% of the final A_{232} value, corresponding to a partial (30%) depolymerization product, was diluted to 2 mg/mL with water and retained for analysis by CE (**Fig. 3**).

5. Hyaluronic acid was partially depolymerized using hyaluronate lyase. Hyaluronic acid (1 mL at 2.5 mg/mL) in sodium acetate–sodium chloride buffer, pH 5.2, was treated with ~5 mU of hyaluronate lyase (corresponding to ~10 turbidity reducing units [TRU]) at 30°C and small 10-µL aliquots were removed from the reaction mixture, heated to 100°C for 5 min, and used to calculate %

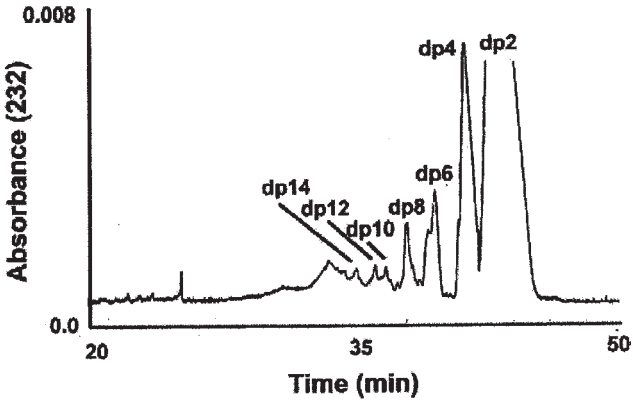

Fig. 3. Normal polarity CE analysis of intact glycosaminoglycans disaccharides prepared using polysaccharide lyases. **(A)** Chondro/dermato disaccharides; **(B)** heparin/heparan.

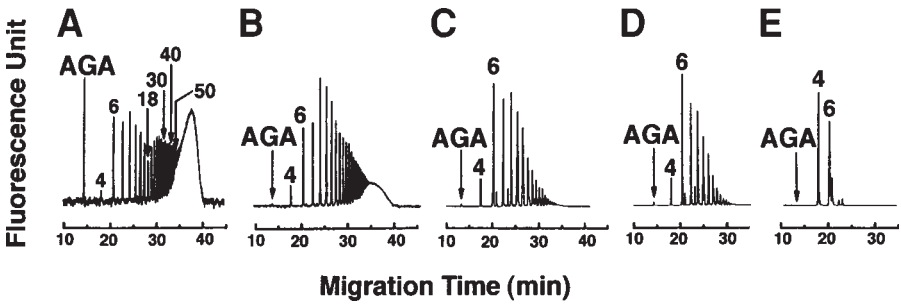

Fig. 4. Reverse polarity CE analysis of intact glycosaminoglycans disaccharides prepared using polysaccharide lyases. **(A)** Chondro/dermato disaccharides; **(B)** heparin/heparan sulfate disaccharides.

reaction completion as described in **Subheading 3.2.** The products at time points corresponding to 17, 23, 44, 61, and 100% reaction completion were desalted on a 1 × 42 Bio-Gel P-2 column (Bio-Rad) eluted with water, lyophilized, and saved for fluorescent labeling and analysis by CE (**Fig. 4**).

3.3. Disaccharide Analysis by CE

1. A mixture (1–10 mg/mL in water) of chondro/dermato disaccharides prepared through the complete depolymerization of chondroitin (4- or 6-) sulfate/dermatan sulfate with chondroitin lyase ABC, or a mixture of heparin/heparan sulfate disaccharides prepared through the complete depolymerization of heparin/heparan sulfate with heparin lyase I, II, and III, can be subjected to CE analysis to obtain the disaccharide composition of the glycosaminoglycan sample. Similarly, an

artificial equimolar mixture (250 μ*M* in water) of purified chondro/dermato disaccharides or heparin/heparan sulfate disaccharides (commercially available from Seikegaku America or Sigma Chemical Co.) can be prepared for CE analysis (**Figs. 5** and **6**).

2. In normal polarity CE, 8.7 nL of disaccharide mixture (250 μ*M* in water) is injected at the anode of a washed (*see* **Subheading 3.1.**) capillary containing sodium phosphate running solution, pH 9.0, and 18 kV is applied. Disaccharides migrate under electroosmotic flow, with those disaccharides having the lowest negative charge migrating the fastest (shortest migration time). Detection (A_{230}) is at the cathode side. The analysis of a mixture of chondro/dermato disaccharides and heparin/heparan sulfate disaccharides are shown in **Figs. 5A** and **B**, respectively.
3. In reverse polarity CE, 8.7 nL of disaccharide mixture (250 μ*M* in water) is injected at the cathode of a washed (*see* **Subheading 3.1.**) capillary containing sodium phosphate running solution, pH 3.6, and 18 kV is applied. Disaccharides migrate under electrophoresis, with those disaccharides having the highest negative charge migrating fastest (shortest migration time). Detection (A_{230}) is at the anode side. The analyses of a mixture of chondro/dermato disaccharides and heparin/heparan sulfate disaccharides are shown in **Figs. 6A** and **B**, respectively.

3.4. CE Analysis of Higher Oligosaccharides

1. A mixture of heparin oligosaccharides (2 mg/mL in water) prepared from the partial (30%) depolymerization of bovine lung heparin with heparin lyase I was used in this analysis.
2. Analysis by reverse polarity CE afforded the highest resolution of this mixture. Injection of 9.2 nL of sample at the cathode of a capillary containing 10 m*M* sodium borate buffer with 50 m*M* SDS, pH 8.8, was followed by the application of 12 kV and detection at the anode side by A_{232}. Peaks were assigned using oligosaccharides standards that have been previously characterized by nuclear magnetic resonance (NMR) spectroscopy ***(12)***. The oligosaccharides migrated in order of decreasing size with tetradecasaccharides (degree of polymerization [DP] 14) eluting first and disaccharides (DP 2) eluting last (*see* **Fig. 3** and **Note 3**).

3.5. Fluorescent Labeling of Glycosaminoglycan-Derived Oligosaccharides

1. Mixtures of hyaluronic acid oligosaccharides prepared by partial digestion with hyaluronate lyase were used for fluorescent labeling.
2. Reductive amination with AGA was accomplished by adding 125 μg of dry oligosaccharide mixture to 50 μL of 0.15 *M* AGA solution in acetic acid–water (3:17, v/v) and 50 μL of 1 *M* sodium cyanoborohydride solution in DMSO and incubating for 6 h at 45°C.
3. The labeled oligosaccharides mixtures were purified using a Bio-Gel P-2 column (1 × 42 cm) eluted with water to remove excess AGA. The earliest peak (A_{232}) eluting corresponded to the AGA-labeled oligosaccharides and the larger peak eluting late from the column corresponded to excess AGA reagent. The AGA-labeled oligosaccharide peaks were lyophilized and saved for CE analysis.

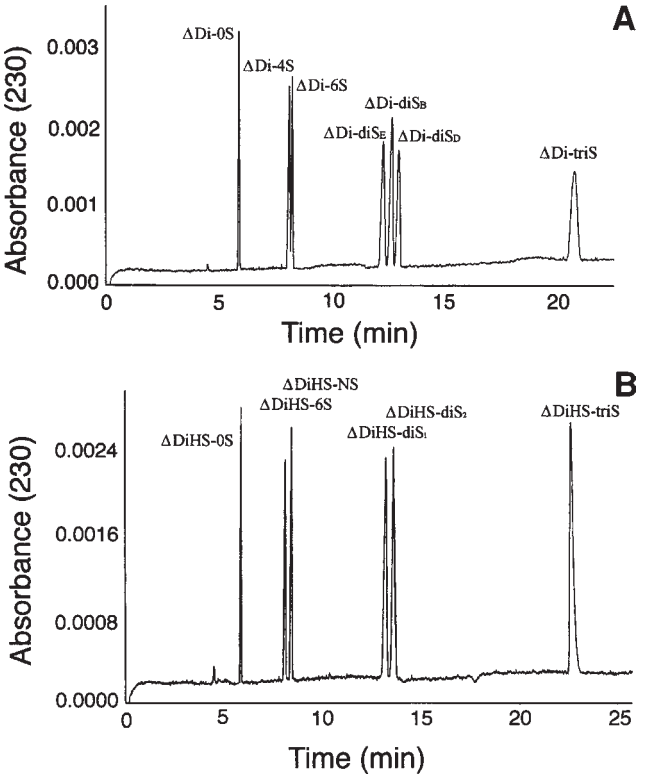

Fig. 5. Reverse polarity CE analysis of an oligosaccharide mixture prepared by partial depolymerization of heparin with heparin lyase I . Peaks (disaccharide [DP 2] through tetradecasaccharide [DP 14]) were assigned based on comigration with defined oligosaccharide standards ***(12)***.

3.6. CE Analysis of Fluorescently Labeled Oligosaccharide Mixtures

1. Mixtures of fluorescently labeled hyaluronic acid oligosaccharides were dissolved in water at a concentration of 1 mg/mL for CE analysis.
2. CE analysis was performed on a Dionex instrument in reverse polarity mode by gravity injection of 9.2 nL of sample at the cathode side of washed capillary (*see* **Subheading 3.1.**). The application of 18 kV resulted in sample migration under electrophoresis through a capillary containing 20 m*M* sodium phosphate buffer at pH 2.5. Detection relied on fluorescence (λ_{ex} 250 nm, λ_{em}420 nm) (**Fig. 4**).
3. Peaks were assigned by peak counting based on their relative position to the first peak corresponding to residual AGA and the second peak corresponding to an AGA-labeled hyaluronic acid tetrasaccharide standard that had been characterized by NMR spectroscopy ***(13)***. A linear plot of log mol wt as a function of migration time was obtained based on this peak assignment.

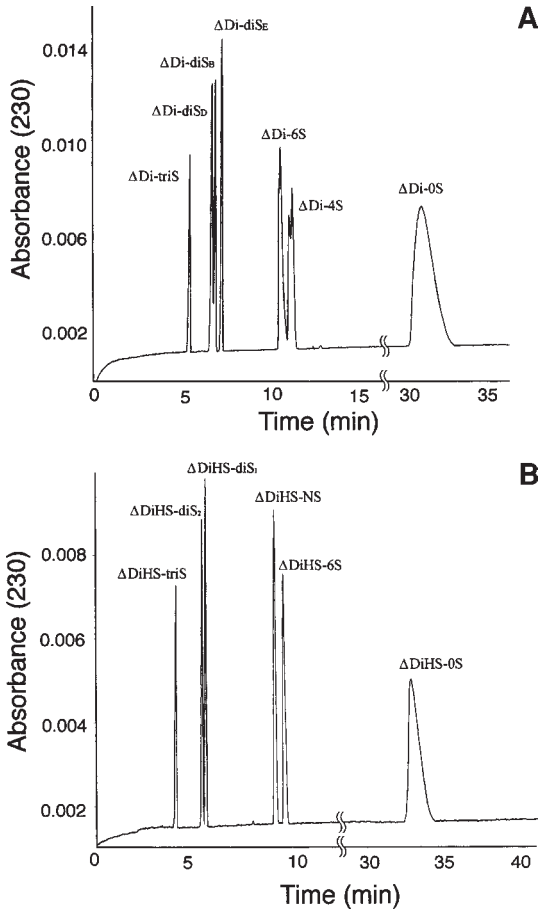

Fig. 6. Reverse polarity CE analysis of fluorescently labeled oligosaccharide mixture prepared by partial depolymerization of hyaluronic acid with hyaluronate lyase. Peaks (tetrasaccharide [DP 2] through [DP 50]) were assigned by peak counting from AGA and AGA-tetrasaccharide standards ***(13)***. Fluorescence detection was used.

4. Notes

1. The enzyme activities used by different manufacturers differ. The enzyme can be assayed by adding enzyme (~10 mU) to 1 mg of glycosaminoglycan substrate in 700 µL of the appropriate buffer in a 1-mL cuvette in a temperature-controlled (30°C) spectrophotometer ***(10)***. The change in absorbance at 232 nm is determined as a function of time (initial rate measurement at <5% reaction completion). Enzyme activity = $(\Delta A_{232}/\text{min})$ (700 µL)/(3800 M^{-1}).
2. Bovine lung heparin has a very homogeneous structure and is comprised primarily (> 90%) of repeating trisulfated disaccharide residues of the structure

IdoA2S→GlcNS6S ***(16)***. Thus, most of the oligosaccharides have identical charge density (equal charge-to-mass ratio). It is somewhat surprising that the oligosaccharides with the highest net negative charge eluted first under normal polarity conditions. This phenomenon, also observed in the normal polarity CE analysis of hyaluronic acid derived oligosaccharides, probably results from the effects of oligosaccharide conformation on migration velocity.

3. To prepare oligosaccharide products from a glycosaminoglycan two strategies can be used. First, an enzyme with broad specificity (one capable of cleaving all the linkages present in the glycosaminoglycan substrate) can be used in deficient amounts or for inadequate reaction times to afford incomplete reactions ***(13,14)***. For example, chondroitin lyase ABC can be used to partially depolymerize chondroitin (4- or 6-) sulfate or dermatan sulfate and the reaction can be terminated early by thermally inactivating the enzyme (heating at 100(C for 5 min). Hyaluronate lyase can be similarly used to partially depolymerize hyaluronic acid ***(13)*** (*see* **Fig. 4**). Second, an enzyme with narrow specificity can be used to completely depolymerize the susceptible linkages in a glycosaminoglycan substrate ***(15)***, for example, heparin lyase I treatment of heparan sulfate or heparin ***(12)***, chondroitin lyase AC treatment of a dermatan sulfate, or chondroitin lyase B treatment of chondroitin (4- or 6-) sulfate ***(14)***.
4. When using copper (II) sulfate as an electrolyte, the cathode platinum wire was plated by copper ***(2)***. While plating of the cathode had no effect on reproducibility or the baseline noise, in separations using copper (II) sulfates electrolyte it did result in severe baseline noise on the subsequent use of other buffer systems with UV detection. Thus, it was important to clean the cathode after the use of copper (II) sulfate and prior to the use of a second buffer system. The cathode was cleaned by washing it with concentrated nitric acid followed by water.

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10

Analysis of 2-Aminoacridone-Derivatized Complex Oligosaccharides Using Micellar Electrokinetic Capillary Chromatography and Laser-Induced Fluorescence Detection

Helen C. Birrell and Patrick Camilleri

1. Introduction

It is now widely accepted that oligosaccharides, covalently linked to proteins, can modulate both the physicochemical and biological properties of glycoproteins ***(1–3)***. Thus glycans can play a number of important roles: they can ensure that a protein is correctly folded; they can stabilize a particular conformation of a glycoprotein; they can prevent protein aggregation; and they can protect a protein from protease activity. Some of the biological roles of oligosaccharides include participation in cell–cell adhesion and immunological and reproduction processes ***(4,5)***. The clinical efficacy of a therapeutic glycoprotein may also be affected by the nature of its carbohydrate. Therefore it is important to minimize carbohydrate content from one batch to another, as this may lead to variations in solubility, metabolism, pharmacokinetics, and ultimately functional activity. Engineering of oligosaccharide structures in therapeutic glycoproteins can be useful in tailoring their desirable efficacy.

Linear biopolymers such as proteins and nucleic acids are biosynthesized by well established template mechanisms. In contrast, the synthesis of oligosaccharides involves a series of glycosidases and glycosyltransferases that act sequentially on the manufactured oligosaccharide as this moves along the lumen in the endoplasmic reticulum and the Golgi apparatus ***(6)***. Oligosaccharides in glycoproteins are highly branched and are usually characterized by multiple linkage types. This structural diversity of glycans gives them recognition indispensable in a number of cell–cell and cell–ligand interaction processes.

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The absence of intense chromophores in oligosaccharides has traditionally made them more difficult to analyze, compared to proteins and nucleic acids. Moreover, amplification using methodology such as polymerase chain reaction (PCR) is not possible to enhance trace levels of carbohydrate. Thus, over recent years, it has been necessary to develop derivatization methods that are suitable for the analysis of picomole quantities of oligosaccharides released from equivalent low levels of glycoprotein ***(7–10)***.

Most glycans of interest lack a suitable chromophore so that their ultraviolet absorbance at wavelengths above 200 nm is very low, preventing direct high sensitivity detection. Carbohydrate released from glycoproteins can be derivatized at the reducing end using aromatic amines via a Schiff base mechanism, followed by reduction to a secondary amine. Both reactions can conveniently be carried out "in one pot." In most cases aromatic amines that fluoresce intensely have been used, allowing the detection of trace amounts of carbohydrate with high sensitivity. Derivatizing agents that have been used include 2-aminopyridine ***(8)***, 6-aminoquinoline ***(9)***, and 8-aminonaphthalene-1,3,6-trisulfonic acid ***(10)***. These reagents are either positively or negatively charged.

We have developed methodology for the analysis of complex oligosaccharides, using 2-aminoacridone (2-AMAC) ***(11–14)***. Our studies have shown that the physicochemical properties of this probe (hydrophobic, stable over a wide pH range, and intensely fluorescent) make it suitable for the analysis of glycans by a wide variety of separation and detection techniques.

2-AMAC-derivatized glycans have a structure of the type shown for the derivative of an α-1,6-linked disaccharide (I) from dextran hydrolysis. These derivatives can be separated by micellar electrokinetic capillary chromatography (MEKC) and reverse- and normal-phase chromatography ***(15,16)***. These separation techniques are orthogonal to one another, and together with mass spectrometric analysis allow a considerable amount of complementary information to be obtained about the relative concentrations and structural identity of individual oligosaccharides in a glycan "pool."

The MEKC methodology we developed involves the addition of taurodeoxycholate to the separation buffer at a concentration higher than its critical micelle concentration (CMC). The order of migration of neutral 2-AMAC-derivatized oligosaccharides is to a great extent related to the size of the carbohydrate. The largest analyte usually migrates first and the smallest last. This may be explained in terms of the hydrophobicity and partitioning characteristics of these derivatives: as derivatives contain more carbohydrate they become less hydrophobic. However, the mechanism that determines the order of migration of neutral 2-AMAC-derivatized oligosaccharides is much more complex. As borate is included in the separation buffer, complexation of the carbohydrate derivatives with this inorganic anion is inevitably involved in the separation mechanism of these analytes.

2-AMAC-derivatized sialylated glycans are ionized at pH values above 5.0 so that the migration of these molecules under the influence of an electric field most probably takes place by "free-zone" capillary electrophoresis. Sialylated glycans are also expected to complex less readily with borate anions owing to electrostatic repulsion. As the sialic acid residue has a molecular volume relatively larger than that of neutral monosaccharides, the charge density of sialylated glycans is relatively lower. Thus sialylated species migrate faster toward the negative electrode, principally under the influence of the electroosmotic flow.

2-AMAC itself is both inherently unionized at the pH of analysis and does not complex with borate ions. These properties ensure that 2-AMAC is the most hydrophobic component in a derivatized mixture of oligosaccharides. Thus excess reagent is trapped by the taurodeoxycholate micelles, conveniently migrates at a much later time than the analytes of interest, and consequently does not interfere with analysis. This MEKC method has considerably reduced time of analysis compared to the high-performace liquid chromatographic (HPLC) methods, and combined with limited enzymatic treatment can provide valuable preliminary information about glycan mixtures.

In this chapter we first outline the experimental MEKC methodology used for the resolution of components of an enzymatically hydrolyzed dextran "ladder." This is followed by a protocol for the enzymatic release of glycans from a typical glycoprotein (we have chosen human immunoglobulin G [IgG] for this purpose) and the procedure of the MEKC analysis of the released glycans. We have also included digestion of 2-AMAC-derivatized glycans from IgG with sialidase and fucosidase. These enzymes were used either on their own or as an array.

For maximum sensitivity, detection of derivatives was carried out by laser-induced fluorescence (LIF). To ensure reproducibility from one electrophoretic run to another we used two internal standards in the analysis of the IgG glycans. These standards were 2-AMAC-derivatized polysaccharides containing ten and three (or two) glucose residues. The migration times of these two standards were just before and after, respectively, those of the derivatized glycans from IgG. Software on the Beckman instrument used for the present analysis allowed alignment of these two standards in all electropherograms. This procedure made the comparison of migration times from different runs possible.

Movement of peaks after enzymatic hydrolysis gave information of the degree of sialylation and core-fucosylation in the original glycan mixture released from IgG. This capillary electrophoresis technique is a powerful tool in the preliminary and rapid determination of the complexity of a glycan mixture. It is invaluable in the quality control of glycan mixtures from different fermentations. If necessary, differences in MEKC profiles are followed by complementary HPLC and mass spectrometric methods ***(14,17–19)***.

2. Materials

2.1. Dextran Ladder and Glycoproteins

1. Dextran calibration standard from Oxford GlycoSciences.
2. Human IgG, reagent grade from Sigma.
3. Recombinant IgGs.

2.2. Trypsin and Glycopeptidase F from Flavobacterium Meningosepticum (PNGase F) Digestion of Human IgG (See Note 1)

1. Tris buffer: 0.6 *M* Tris base, pH 8.5, with hydrochloric acid; bubble through with nitrogen for 30 min before use.
2. Reduction buffer: 1 *M* Dithiothreitol (DTT) in 0.6 *M* Tris-HCl, pH 8.5.
3. Carboxymethylation reagent: 1 *M* Iodoacetic acid (IAA) in 0.6 *M* Tris-HCl, pH 8.5; protect from light.
4. Slide-A-Lyzer Dialysis Cassette, 3500 MWCO: part no. 66330, fitted with a buoyancy collar from Pierce.
5. Dialysis buffer: Deionized water at 4°C.
6. Sodium phosphate buffer: 50 m*M* Sodium phosphate, pH 8.4.
7. Trypsin solution: 1 mg/mL of trypsin, type Xlll, tosyl-L-phenylalanine chloromethyl ketone (TCPK) treated, from Sigma.
8. *N*-Glycosidase F (in 50% glycerol) from Roche.
9. GlycoClean H cartridges: part no. I-4025, from Oxford GlycoSciences.
10. GlycoClean H cartridges reagents: 1 *M* sodium hydroxide, 30% glacial acetic acid, deionized water, eluent C: 5% acetonitrile in 0.1% aqueous trifluoroacetic acid, eluent D: 50% acetonitrile in 0.1% aqueous trifluoroacetic acid.

2.3. 2-Aminoacridone Derivatization of Released Glycans

1. Derivatization solvent: Dimethyl sulfoxide (DMSO), 17 parts, in glacial acetic acid, 3 parts (protect from moisture).
2. Derivatization reagent: 0.05 *M* 2-AMAC from Fluka in DMSO–acetic acid.
3. Reducing agent: 1 *M* Sodium cyanoborohydride from Sigma in DMSO–acetic acid.

2.4. Digestion with Exoglycosidases

1. Sialidase (*Arthrobacter ureafaciens*): 0.2 U (7 μg) in 100 μL of 100 m*M* sodium acetate, pH 5.0, from Oxford GlycoSciences.
2. α-Fucosidase (bovine kidney): 100 mU (50 μg) in 100 μL of 100 m*M* sodium citrate, pH 6.0, from Oxford GlycoSciences.

2.5. Preparation of Dextran Ladder Components as Internal Standards by HPLC (See Note 2)

1. An HPLC equipped with a fluorescence detector, λ_{ex} 428 nm, λ_{em} 525 nm (an ultraviolet detector will suffice, λ 276 nm) and fitted with a GlycoSep N column (250 mm × 3.9 mm), from Oxford GlycoSciences.
2. Mobile phase A: Acetonitrile. Mobile phase B: 250 m*M* ammonium formate, pH 4.4.

3. Gradient conditions: 65% A–35% B equilibration conditions then to 53% B over 72 min, linear gradient. Flow rate: 0.4 mL/min.
4. HPLC sample diluent: 75% acetonitrile–25% water.

2.6. Separation of Derivatized Carbohydrates by CE-LIF (See Note 3)

1. Beckman P/ACE 5510 series capillary electrophoresis (CE) instrument, fitted with a 50 cm × 50 μm inner diameter (i.d.) quartz capillary. Detection: He–Cd LIF at λ_{ex} 442 nm, λ_{em} 525 nm, from Omnichrome.
2. Capillary regeneration solution: 0.1 *M* sodium hydroxide.
3. Deionized water.
4. Separation buffer: 240 m*M* Boric acid in 80 m*M* taurodeoxycholic acid, from Sigma, adjusted to pH 9.2 with 1 *M* sodium hydroxide.
5. CE sample diluent: 20% DMSO in deionized water.

3. Methods

3.1. Trypsin and PNGase F Digestion of IgG Samples (See Note 1)

1. Dissolve up to 500 μg of IgG sample in 200 μL 0.6 *M* of Tris-HCl, pH 8.5, and add 1 μL of DTT. Vortex mix and incubate at 37°C for 30 min.
2. Add 5 μL of IAA solution, vortex mix, and incubate for 30 min at room temperature in the dark.
3. Hydrate a 3.5K dialysis cassette for 2–3 min before use by suspending in deionized water.
4. Dilute the sample to 1 mL with deionized water, draw into a syringe, and introduce into the dialysis cassette. Dialyze for 6 h at 4°C, changing the dialysis water every 2 h.
5. Remove the sample solution with a clean syringe and place in a 1.5-mL Eppendorf tube. Freeze in solid carbon dioxide and dry under reduced pressure. If necessary, store at –20°C.
6. Dissolve the sample in 200 μL phosphate buffer, and add 5 μL of trypsin solution. Incubate for 5 h at 37°C. Stop the digestion by heating at 100°C for 3 min and freeze-drying.
7. Dissolve the sample in 200 μL of phosphate buffer, and add 5 μL of PNGase F solution. Incubate for 18–24 h at 37°C, and freeze-dry.
8. Wash a GlycoClean H cartridge: Wash with 3 mL of 1 *M* sodium hydroxide, followed by 6 mL of deionized water, then 3 mL of 30% acetic acid and finally 6 mL of deionized water.
9. Prime the GlycoClean H cartridge: Prime with 3 mL of eluent D, followed by 6 mL of eluent C.
10. Dissolve the freeze-dried sample from **step 7** in 200 μL of eluent C and load onto cartridge. Wash with 3 mL of deionized water, followed by 3 mL of eluent C.
11. Elute with 2 × 1 mL with eluent D (filter, if necessary, through a 0.2-μm filter), and reduce the volume by freeze-drying. Combine the fractions into one Eppendorf tube. Freeze-dry.

3.2. 2-Aminoacridone-Derivatization of Released Glycans and Carbohydrates

1. Dissolve released IgG glycans or 0.2 mg of dextran calibration standard in 20 µL of 2-AMAC reagent.
2. Add 20 µL of sodium cyanoborohydride solution.
3. Heat in a closed Eppendorf tube at 70°C for 2 h.
4. Briefly centrifuge the reaction tube at 5000*g* to collect all the sample in the bottom of the tube.
5. Freeze in solid carbon dioxide to stop the reaction.

3.3. Digestion of Derivatized Glycans with Exoglycosidases

1. Sub-aliquot the derivatized released glycans from human IgG into 4 × 10 µL portions. Freeze-dry.
2. Store one portion at –20°C.
3. Dissolve one freeze-dried aliquot in 20 µL of sialidase solution, and incubate at 37°C for 18 h. Add a further 20 µL of sialidase solution and incubate for the another 6 h.
4. To a second freeze-dried aliquot add 20 µL α-fucosidase solution, and incubate at 37°C for 18 h. Add a further 20 of µL of α-fucosidase solution and incubate for another 6 h.
5. To the third freeze-dried aliquot add 20 µL of each enyzme solution and incubate for 18 h. Add further 20-µL aliquots of each enzyme solution and continue the incubation for a further 6 h.
6. Freeze-dry the enzyme digests.

3.4. Preparation of Dextran "Ladder" Components as Internal Standards by HPLC

1. Establish stable operating conditions.
2. Dissolve the derivatized dextran "ladder" in 30 µL of sample diluent.
3. Inject 25 µL of dextran "ladder" (reserve remaining sample for CE separation) and collect the individual ladder components up to the 12th oligomer.
4. Freeze-dry collected components.

3.5. Separation of Derivatized Carbohydrates by CE-LIF (See Notes 2–8 and Figs. 1–5)

1. Establish stable operating conditions of both laser and instrument.
2. Filter all CE solutions through a 0.2-µm filter immediately before use.
3. Flush capillary to waste with 0.1 *M* sodium hydroxide for 1 min.
4. Flush capillary to waste with running buffer for 2 min.
5. Refill the capillary with fresh running buffer.
6. Dissolve the samples with 10 µL of CE sample diluent.
7. Dilute 2 µL from (**step 5**) with 2 µL deionised water.
8. Pressure inject sample for 5 s, followed by deionized water for 5 s.
9. Apply 25 kV across the capillary for 20 min to separate sample components.

3.6. Suggested Sample Sequence for CE Separation (See Note 5 and Figs. 2–5)

1. 2-AMAC-derivatized dextran calibration standard.
2. Derivatized released glycans from human IgG and recombinant IgGs.
3. Select appropriate dextran internal standards to bracket sample components. Add to samples.
4. Separate by CE "spiked" samples from **step 3**.

4. Notes

1. It is essential to reduce disulfide bonds in a glycoprotein with DTT and to carboxymethylate the resulting thiol groups with IAA before trypsin digestion is attempted. The yield of glycans by PNGase F digestion of peptides formed in the tryptic digestion is also usually much better than that obtained via digestion of the intact glycoprotein.
2. The excitation and emission wavelengths of 2-AMAC and its carbohydrate derivatives are at 428 and 525 nm, respectively. These wavelengths are most suitable for the use of a He–Cd laser set at excitation and emission wavelengths of 442 and 520 nm. LIF detection at the latter wavelengths was used throughout this study. An argon laser detector (excitation wavelength, 488 nm and emission wavelength, 525 nm) is also available commercially. However, the sensitivity of such a system is more than an order of magnitude lower than that of the HE–Cd laser.
3. In MEKC, the taurodeoxycholate in the separation buffer can be replaced by SDS at a concentration of about 30 m*M*. However, we prefer the inclusion of taurodeoxycholate as this gives superior resolution of the derivatized glycans and better reproducibility between one run and another. This detergent is also more gentle on the capillary than SDS. In the case where a new capillary is used it is advisable to repeat the first analysis.
4. It is our practice to analyze a hydrolyzed dextran "ladder" before we attempt the analysis of complex glycan mixtures. The order of migration of this mixture of polysaccharides is related to their size (**Fig. 1**). The larger molecules (i.e., the ones of the lowest intensity) migrate first, whereas the smaller sugars have the longer migration times. The glucose derivative has the highest intensity and the longest migration time. If the conditions of analysis are correct then at least 12 derivatized glycans should be clearly visible. Analysis of a dextran "ladder" is especially useful in the conditioning of a new capillary.
   As mentioned in the Introduction, 2-AMAC is very hydrophobic compared to the 2-AMAC-derivatized glycans. Thus excess fluorophore is trapped by the micelle and migrates at a much longer retention time. In our analysis we usually stop the analysis at about 20 min and apply pressure to flush off excess 2-AMAC.
5. When it is necessary to compare a number of electropherograms then reproducibility in migration times from one electrophoresis run to another is essential for meaningful conclusions to be drawn from the results. We find that without the use of internal standards, comparison of electropherograms run either consecutively or on different days becomes difficult. Two standards with migration times

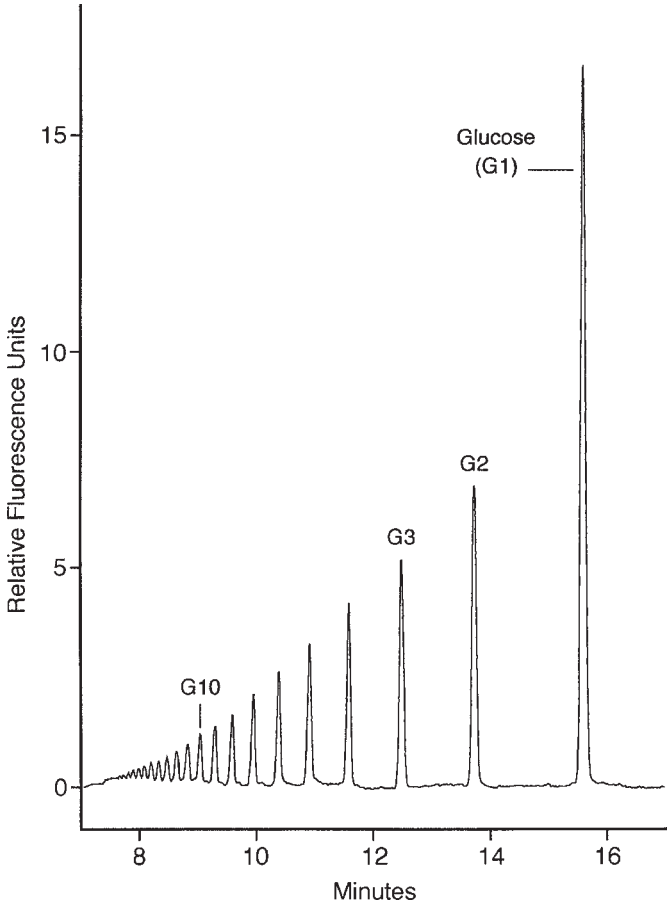

Fig. 1. Electropherogram of an enzmatically hydrolyzed dextran "ladder" derivatized with 2-AMAC. At least 25 components are resolved.

before and after the migration range of glycan mixtures under study are sufficient for this exercise.

A hydrolyzed dextran "ladder" provides a range of possible standards that can be coinjected with a glycan pool. The judicious choice of standards is carried out by comparing profiles from the dextran "ladder" to that of the glycan pool of interest. For effective internal standardization it is also desirable for the chemical class of the standards to be similar to that of the glycan mixtures analyzed so that the mechanism of separation of the two sets of carbohydrates is as close as possible. The individual components of a hydrolyzed dextran "ladder" can be prepared in good yield using an analytical normal phase column as detailed in **Subheading 3. Figures 2–4** demonstrate the use of two internal standards, the 2-AMAC-

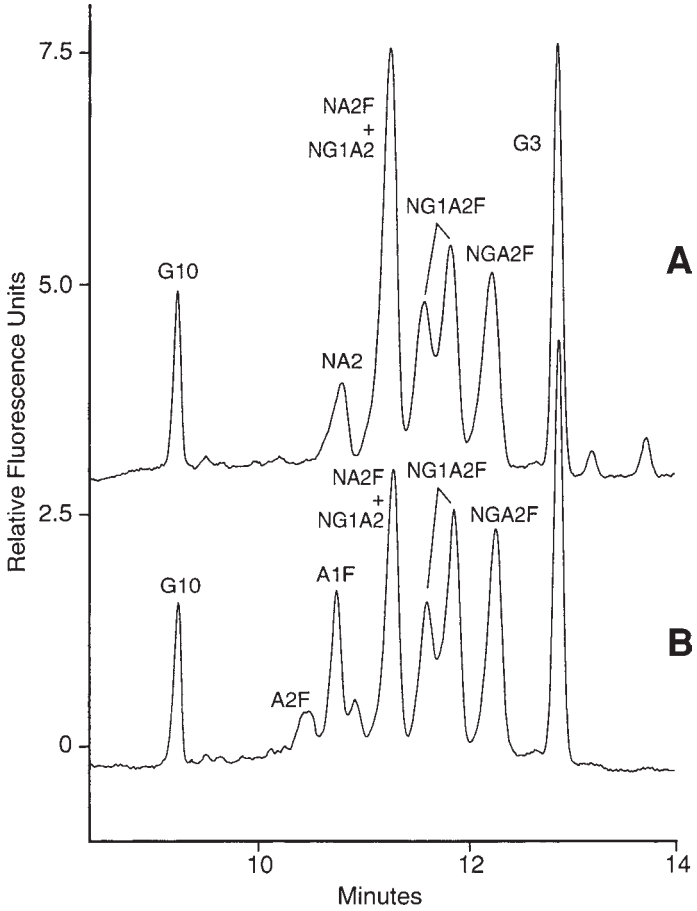

Fig. 2. A comparison of electropherograms of 2-AMAC-derivatized glycans released from human IgG **(A)** after and **(B)** before treatment with sialidase, using internal standards G3 and G10.

derivatives of tri- and deca-glucose linear polysaccharide, in the analysis of glycans released from a sample of human IgG. These two standards have been denoted as G3 and G10 in **Figs. 2–4**. The Beckman instrument used for this analysis has built-in software for the electronic alignment of electropherograms.

6. The structures of glycans that are *N*-linked to an asparagine residue usually fall within three main types: high-mannose, complex, and hybrid. The three types differ in branching patterns mainly to the nonreducing end, whereas they all share a common trimannose core, attached to two *N*-acetylglucosamine residues at the reducing end, which is the end that is involved in the Schiff reaction, followed by reduction. The number of mannose residues in high-mannose structures ranges

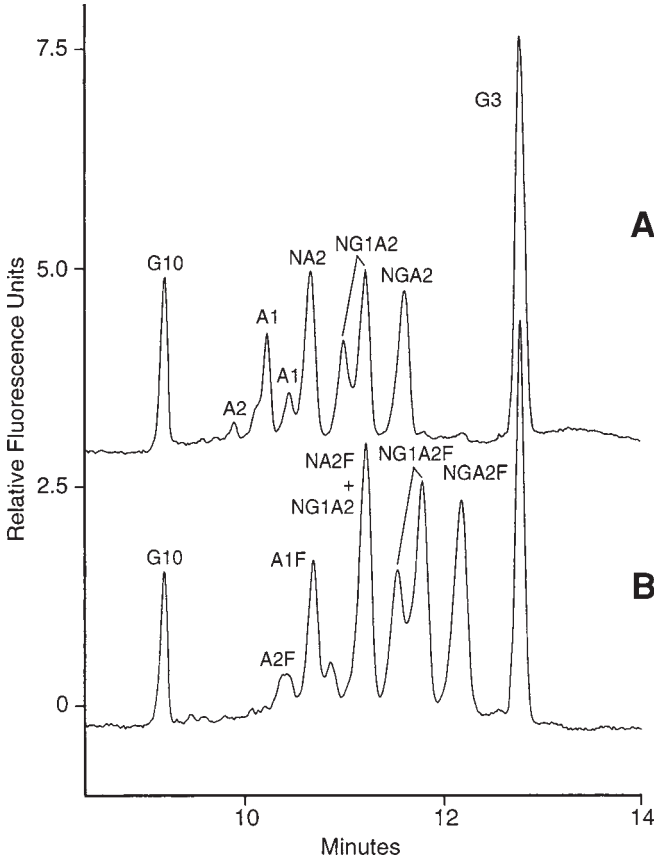

Fig. 3. A comparison of electropherograms of 2-AMAC derivatised glycans released from human IgG **(A)** after and **(B)** before treatment with fucosidase, using internal standards G3 and G10.

from five to nine. Elimination of these mannose residues by the action of α-mannosidases in the endoplasmic reticulum and the Golgi apparatus, followed by the action of glycosyltransferase enzymes (*N*-acetylglucosaminetransferase, galactosyltransferase, fucosyltransferase, and sialyltransferase) leads to the formation of complex and hybrid glycans.

Human IgG contains only complex-type biantennary glycans (**Fig. 6**). These are attached at Asn297 in the Fc region of the antibody. Removal of this oligosaccharide is reported to affect several effector functions of IgG ***(20)***. Moreover, the core glycosylation of IgG can affect recognition by complement and can modulate superoxide production ***(21)***. Engineering the glycosylation of monoclonal IgG molecules appears to be an important step forward as part of the design of therapeutic effects ***(22,23)***.

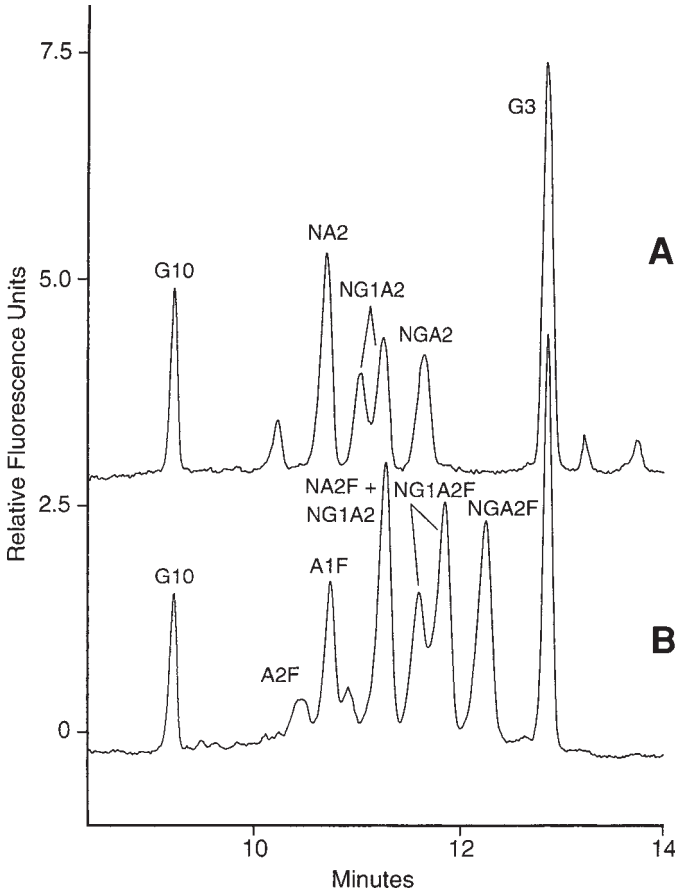

Fig. 4. A comparison of electropherograms of 2-AMAC-derivatized glycans released from human IgG **(A)** after and **(B)** before treatment with a two-enzyme array made up of sialidase and fucosidase, using internal standards G3 and G10.

7. Using the methodology described, it is possible to make meaningful conclusions about the movement of peaks after glycan mixtures have been digested with one or more glycosidases. To demonstrate this we have treated the glycan mixture from IgG either with sialidase (**Fig. 2**) or fucosidase (**Fig. 3**) or a mixture of these two enzymes (**Fig. 4**). The result from these enzyme treatments is compared to the untreated glycan mixture in all these figures after aligning of the two internal standards.

   Figure 2B shows that there are at least seven components in this sample of glycans from IgG. Treatment with sialidase leads to disappearance of the peak with the shortest migration time and considerable reduction in the intensity of the peak with the second longest migration time. From previous studies ***(12)*** we identified

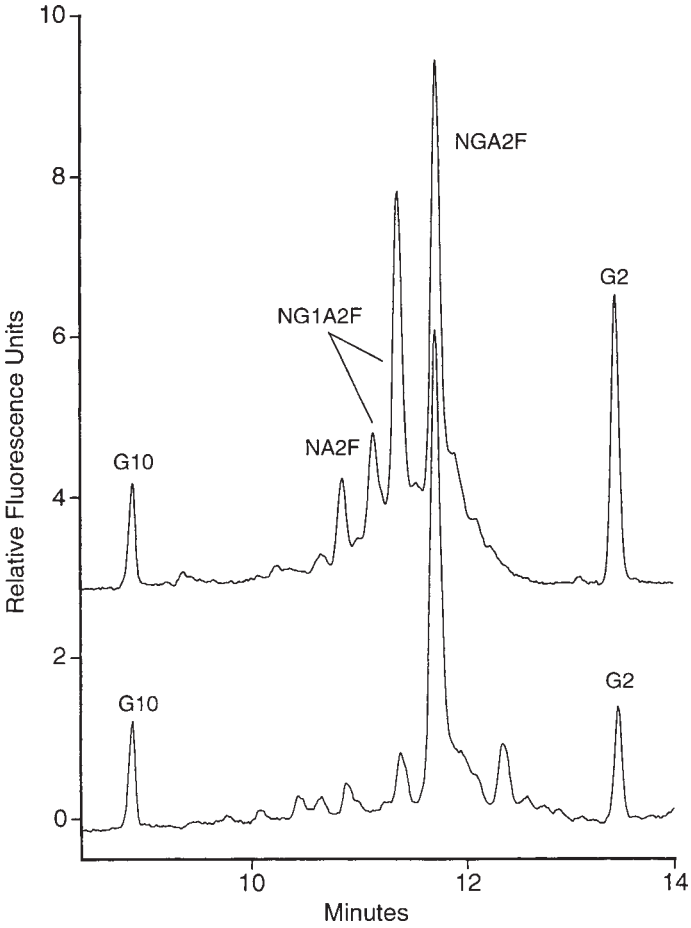

Fig. 5. Electropherograms of the 2-AMAC-derivatized glycans released from two commercial monoclonal antibodies. The internal standards used were G2 and G10.

these components as A2F and A1F, respectively (**Fig. 6**). The remaining peaks show no change in their migration characteristics on sialidase treatment, confirming that these components are neutral oligosaccharides, which differ by the number of galactose residues at the nonreducing end of these molecules (**Fig. 6**). The positional isomers NG1AF and NG'1AF can be separated by this method. It is interesting that on desialylation the ratio between the two isomers has changed. From this result it appears that one of the terminal galactose residues in A1F shows a preference for sialylation.

Treatment of the glycan mixture from IgG with fucosidase leads to the movement of all peaks to a shorter migration time (**Fig. 3**). The relative intensity of all the major signals has not changed, confirming that the majority of glycans in IgG

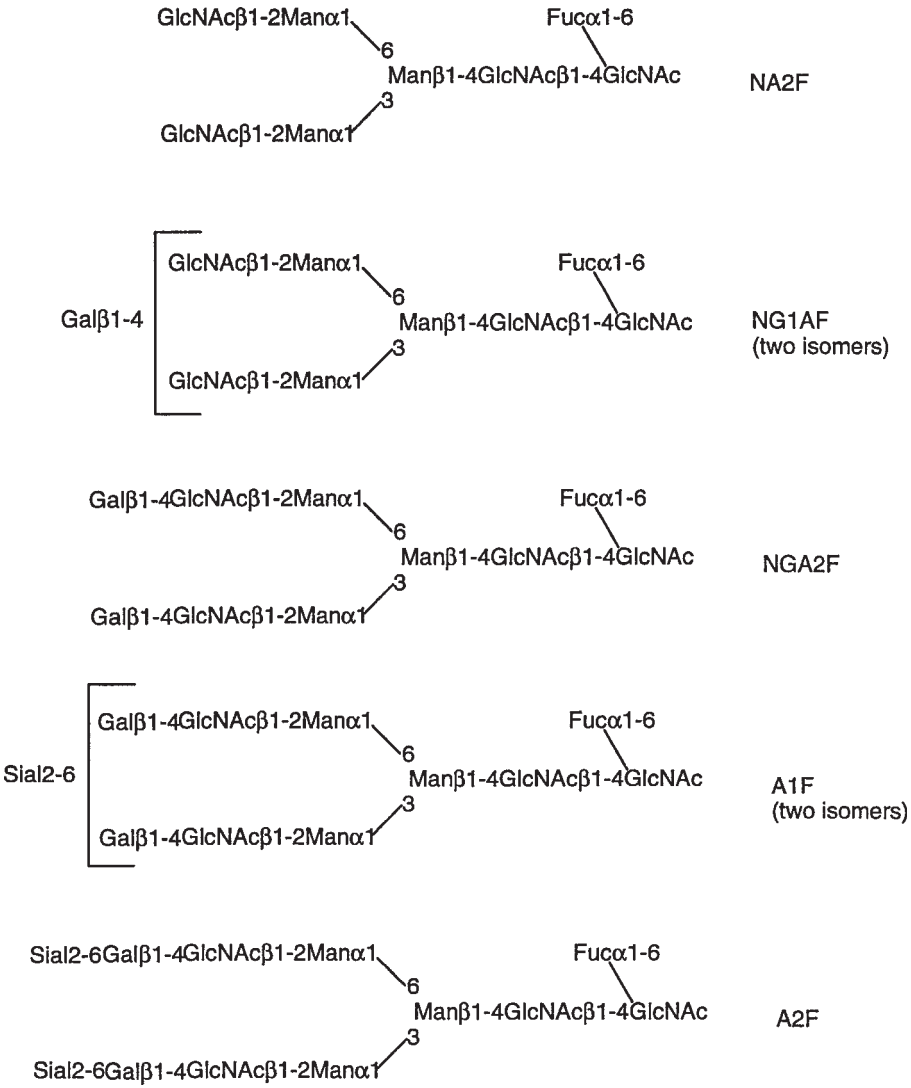

Fig. 6. Structures of the principal neutral and sialylated components commonly found in human IgG.

are core-fucosylated. The shorter migration time of fucosylated glycans may be due to a change in conformation of the resulting glycans affecting their overall charge density.

Figure 4 shows the electropherogram obtained after the sample of the derivatized glycans was treated simultaneously with sialidase and fucosidase. It is interesting that using this two-enzyme array led to the appearance of another component

at about 10 min. This 2-AMAC glycan comigrated with another glycan on defucosylation and did not appear on desialylation. It is likely that this component is a fucosylated oligosaccharide that differs from NGA2F by an *N*-acetylglucosamine residue attached to the center mannose in the core structure.

8. The methodology described was applied to the analysis of glycan pools from two commercial therapeutic antibodies. Results are compared in **Fig. 5**. In this case G2 and G10 were used as internal standards to align the two electropherograms. A number of points can be made from this rapid analysis: both glycan pools contain a large excess of neutral oligosaccharides; one of the antibodies contains almost exclusively NA2F; the second antibody contains predominantly NA2F and NG1AF; the relative concentrations of the two positional isomers differ by about a factor of 5.

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11

Separation of Protein Glycoforms by Capillary Electrophoresis

Myriam Taverna, Nguyet Thuy Tran, and Danielle Ferrier

1. Introduction

Glycoproteins are generally composed of a population of different variants having in common the same polypeptide chain but varying by their carbohydrate moiety. This carbohydrate-mediated heterogeneity is often called the microheterogeneity of glycoproteins and the different variants referred to glycoforms. This heterogeneity arises from the presence of different glycannic structures attached at one glycosylation site. The carbohydrate groups are covalently attached to the polypeptidic backbone through the amide nitrogen of an asparagin residue (*N*-glycosylation) or through an *O*-linkage to a serine, a threonine, or sometimes a hydroxyproline residue (*O*-glycosylation). *N*-linked oligosaccharides may be classified on the basis of the nature of monosaccharides that constitute the oligosaccharides. High-mannose type oligosaccharides consist of the only mannose and *N*-acetylglucosamine residues and are not charged. Complex type oligosaccharides may be negatively charged and contain galactose, fucose, and sialic acid residues in addition to the monosaccharides found in the high-mannose chains. Hybrid structures are intermediate structures between the high mannose and the complex type oligosaccharides (*see* Appendix, *this volume*). The three family of oligosaccharides share the same pentasaccharide structure (GlcNAc-GlcNAc-Man3) by which the oligosaccharide is attached to the protein. *O*-linked oligosaccharides are generally shorter than the *N*-oligosaccharides but exhibit larger differences in their monosaccharide composition. In addition to the monosaccharides found in *N*-oligosaccharides, these structures may contain also glucose, xylose, arabinose, and *N*-acetylgalactosamine residues.

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Glycosylation is often a critical determinant in many properties of glycoproteins and this is particularly important in the case of therapeutic glycoproteins, as the biological activity, the clearance rate, the immunogenicity, the solubility, and the stability may be significantly affected by the type of oligosaccharide attached to the protein ***(1–2)***.

Microheterogeneity of glycoproteins may be evaluated by the analysis of either oligosaccharides released enzymatically or chemically thereof (*see* Chapter 2, *this volume*), or glycopeptides obtained after the digestion of the glycoprotein by proteases . Alternatively, separation of the different glycoforms may represent an attractive and fast way to analyze the heterogeneity of an intact glycoprotein. The main advantage of this straightforward approach is that it does not require extensive sample preparation, and information on the identity, the heterogeneity, and the purity of the glycoprotein is readily obtained. Among the most efficient methods reported for this purpose isoelectricfocusing (IEF), sodium-dodecyl sulfate (SDS-PAGE), and two-dimensional (2D) gel electrophoretic methods have been widely employed.

The emergence of capillary electrophoresis with various modes in which separation can be performed has brought new possibilities in the field of glycoform separations not only for process and purification monitoring, but also for purity assessment and quality control evaluation. The potential of this technique for the analysis of glycoproteins produced by DNA technologies has gained interest in the quality control environments within biotechnology industries ***(3–9)***. Capillary zone electrophoresis (CZE) has been the most studied mode for glycoforms separation. CZE separations are based on differences in the charge-to-mass ratio of the proteins and glycoforms may be separated according to the number of sialic acids or to their extent of glycosylation. Glycoforms bearing only neutral oligosaccharides have also been successfully separated by a careful selection of the separation conditions of a special mode. In this mode a major problem encoutered is the adsorption of (glyco)proteins to the capillary wall. Micellar electrokinetic capillary chromatography (MECC) performed in the presence of surfactants added at concentrations above the critical micellar concentration (CMC) offers separation based on a different criterion ***(10)*** resulting from both electrostatic and hydrophobic interaction of glycoproteins with micelles. Capillary isoelectric focusing (CIEF) represents indubitably the method of choice for glycoform separations, while the lower resolving power of capillary gel electrophoresis (CGE) has limited its application in the field of glycoform analyses to specific glycoproteins exhibiting important structural variations.

The following are examples of glycoform analysis by various separation models.

2. Materials

2.1. Capillary Zone Electrophoresis (CZE)

2.1.1. CZE in Uncoated Capillaries

2.1.1.1. Separation of Ovalbumin Glycoforms

1. Instrument: Beckman P/ACE 2050 (Beckman, Fullerton, CA).
2. Capillary: Uncoated fused silica capillary (Beckman).
3. Buffer: Borate buffer made by titration of 25 m*M* sodium tetraborate with 100 m*M* boric acid to pH 8.5. Prepare a 1 *M* stock solution of 1,4-diaminobutane in water (*see* **Note 1**) and add an aliquot of this solution to the running buffer to obtain a final concentration of 1,4-diaminobutane of 1 m*M* (*see* **Note 2**).

2.1.1.2. Separation of Recombinant Human Erythropoietin (rhuEPO) Glycoforms

1. Instrument: Beckman P/ACE system 2050 (Beckman).
2. Capillary: Uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ).
3. Reagents: Tricine, sodium chloride, 1,4-diaminobutane.

2.1.2. CZE in Coated Capillaries

2.1.2.1. Separation of Recombinant Hauman Bone Morphogenic Protein-2 (rhBMP-2) Glycoforms

1. Instrument: Biofocus-3000 (Bio-Rad Laboratories, Hercules, CA).
2. Capillary: Polyacrylamide (PAA) coated capillary (Bio-Rad Laboratories)
3. Reagent: Phosphoric acid.

2.1.2.2. Separation of recombinant human Erythropoietin (rhuEPO) Glycoforms

1. Instrument: P/ACE 5500 (Beckman).
2. Capillary: eCap amine capillary (Beckman).
3. Reagent: Sodium phosphate.

2.1.2.3. Separation of Human α-acid glycoprotein (α-AG) Glycoforms

1. Instrument: Crystal 310 (ATI-UNICAM, Cambridge, UK), equipped with a variable wavelength UV-VIS detector,
2. Capillary: A Durawax FSOT capillary for gas chromatography (GC), internally coated with a 0.1-µm thick layer of DB-WAX stationnary phase (J & W Scientific, Folsom, CA).
3. Reagents: 2-*N*-morpholinoethanesulfonic acid (MES) (Sigma) sample: human α-AG, purified from Cohn factor VI in99%, urea.
4. Buffer: 50 m*M* MES buffer adjusted to the desired pH using Tris. Urea was added to a final concentration of 8 *M*.

2.2. Micellar Electrokinetic Capillary Chromatography (MECC)

2.2.1. Separation of Human Recombinant Interferon-γ (h-IFN Glycoforms)

1. Instrument: Beckman P/ACE 2100 (Beckman).
2. Capillary: Uncoated fused silica capillary (Beckman).
3. Reagents: Boric acid, 100 m*M* sodium hydroxide, sodium dodecyl sulfate (SDS).

2.2.2. Separation of Ribonuclease B Glycoforms (RNase B Glycoforms)

1. Instrument: P/ACE 2100 (Beckman).
2. Capillary: Uncoated fused silica capillary (Beckman).
3. Reagents: Sodium phosphate, sodium tetraborate, and SDS (*see* **Note 3**).

2.3. Capillary Isoelectric Focusing

2.3.1. Two-Step CIEF: Separation of a Mouse Monoclonal Antibody

1. Instrument: Beckman P/ACE 5510, equipped with an UV detector and a 280-nm filter.
2. Capillary: Coated capillaries, μSIL DB-1 (J&W Scientific, Folsom, CA).
3. Reagents: Anolyte, 10 m*M* phosphoric acid (*see* **Note 4**), catholyte, 20 m*M* sodium hydroxide. Pharmalyte, pH 3.0–10.0 (Pharmacia Biotech, Pitscataway, NJ, USA); *N*,*N*,*N*',*N*'' tetramethylethylenediamine (TEMED) (Bio-Rad); methyl cellulose (Sigma, Deisenhofen, Germany); Protein markers, β-lactoglobulin A and B (pI 5.3 and 5.15, respectively) and myoglobulin (pI 7.2 and 6.8) (Sigma).

2.3.2. One-Step CIEF: CIEF of Recombinant Tissue Plasminogen Activator (rtPA) Glycoforms

1. Instrument: Beckman P/ACE 5510.
2. Capillary: eCap neutral (PAA) coated capillary (Beckman).
3. Reagents: anolyte, 10 m*M* phosphoric acid; catholyte, 20 m*M* sodium hydroxide; pharmalyte, pH 3–10 and pH 5–8 (Pharmacia Biotech); *N*,*N*,*N*',*N*'' tetramethyl ethylenediamine (TEMED) (Bio-Rad Laboratories); hydroxypropylmethyl-cellulose (Sigma).

2.4. Capillary Gel Electrophoresis

2.4.1. Separation of Antithrombin IIIα and Antithrombin IIIβ

1. Instrument: BioFocus 3000 (Bio-Rad).
2. Capillary: Uncoated fused silica capillary (Bio-Rad).
3. Reagent: CE-SDS buffer (Bio-Rad).

2.4.2. Separation of Plasminogen-Treated rtPA Variants

1. Instrument: Beckman P/ACE 2100 or 5500.
2. Capillary: eCAP SDS-coated capillary (Beckman).

3. Reagents: SDS 14–200 kit from Beckman including the SDS14–200 gel buffer, the sample buffer (120 m*M* Tris-hydrochloric acid, 1% SDS, pH 6.6), mercaptoethanol and an internal standard, orange G.

3. Methods

3.1. Capillary Zone Electrophoresis

In CZE the proteins migrate in free solution in either fused capillaries (uncoated) or in modified capillaries (coated) and are separated on the basis of their charge to mass ratio. This is the simplest mode for glycoforms analysis generally carried out as the first stage in the development of a new method. As glycoforms differ mainly by the extent of glycosylation and the nature of their oligosaccharides, they may be separated on the basis of their sialic acid content for the complex ones or on the basis of the length of the oligosaccharide chain(s) for the less heavily glycosylated ones. The beneficial effect of borate complexation in the resolution of glycoforms has been reported by several authors ***(11–13)*** and this buffer is often preferred over phosphate buffer to achieve glycoform separations at alkaline pHs. In addition, as the electroosmotic flow (EOF) is fast at these pHs, diaminoalkanes may be added at low concentrations (1–10 m*M*) to the running buffer. The usefulness of diaminoalkanes relies on their ability to reduce the electroosmotic flow but also to reduce electrostatic interactions between the protein and the capillary surface. Diaminoalkanes have been shown to influence notably selectivity of glycoprotein separation ***(11,14–16)***.

Fused silica is regarded as a cation exchanger and the adsorption can be interpreted as an ion exchange mechanism between the positively charged regions of the protein and the ionized silanol groups of silica. Adsorption is critical for large proteins (Mw >5 kDa) and the tendency of proteins to stick to the capillary wall increases with increasing Mw (*see* **Note 5**). There is no universal solution to the adsorption, and depending on the protein analyzed several strategies may be employed to minimize this problem ***(17–19)***.

Electrostatic interactions may be weakened by reducing the charge of capillary wall by selecting low pHs for the buffer; typically buffers such as sodium phosphate, pH 2.5 may be employed. The alternative is to induce electrostatic repulsion by working at pHs above the pI of the protein. However, glycoform resolution is rarely achieved at extreme pHs, selectivity is generally better achieved at pHs close to the pI of the protein where the net charge of the protein is sufficiently lowered. Moreover, not all the proteins are stable at these extreme pHs and structural change of the native protein is probable at these pHs.

An increase in the buffer concentration generally decreases the electrostatic interactions. However, the increase in the conductivity will contribute to Joule

heating. In addition proteins can be denaturated by high temperatures or high salt concentrations. Owing to its low UV absorbance, potassium sulfate represents one of the most appropriate inorganic buffer additive to reduce wall adsorption ***(20,21).***

Phosphate has been demonstrated to reduce adsorption of proteins, one possible mechanism being the formation of more easily protonated complexes between phosphate and silanol ***(22,23)***. This buffer (phosphate buffer)-deactivated capillaries should be preferably used at acidic pHs when adsorption is suspected. Zwitterionic buffers have the property of ion pairing with proteins while not contributing to conductivity. Not only protein wall interaction but also protein-protein interaction can be reduced. In particular buffers such as tricine or betaine are useful for proteins having a tendency to self aggregate due to hydrophobic interactions.

Amines such as alkylamine, diaminoalkanes (diaminobutane, diaminopropane) or cationic polymers (chitosan, Polybrene) may assist protein separation by shielding the negative charges of the silanol groups (by hydrogen bonding and ionic interaction). Surfactant may be incorporated into the buffer to minimize protein/wall interactions. **Table 1** gives a list of the additives that have already been employed successfully to solve the problem of adsorption in glycoprotein analyses.

Permanent coating can also be employed for glycoform separation; capillaries coated with PAA and polyvinylalcohol (PVA) are the most widely employed. **Table 2** summarizes the different commercially available coated capillaries that have been reported for glycoform separations. However, adsorption at coatings may be suspected, therefore uncoated and dynamically coated capillaries are simpler to prepare and might be preferred.

3.1.1. CZE in Uncoated Capillaries

3.1.1.1. Separation of Ovalbumin Glycoforms ***(14)***

1. Prepare a new uncoated fused silica capillary (87 cm in total length, 75 µm in i.d.) (*see* **Note 6**). The capillary is then conditioned by 20-column-volume rinse with successively 100 m*M* sodium hydroxide, water, and the running buffer.
2. Dissolve ovalbumin (500 µg/mL) in the running buffer devoid of 1,4-diaminobutane.
3. For the protein analysis, the capillary is equilibrated by 3-column-volume rinse with the running buffer: 100 m*M* borate buffer, pH 8.5, containing 1,4-diaminobutane (1 m*M*) (*see* **Note 7**).
4. Separation conditions: injection, hydrodynamic (3 s); column temperature, 28°C; applies voltage, 25 kV; detection, 200 nm (*see* **Note 8**). **Figure 1** illustrates the usefulness of the addition of 1,4-diaminobutane on the separation of ovalbumin glycoforms. Five major peaks and a number of minor peaks are observed on the

Table 1
Additives Employed To Minimize Glycoprotein Adsorption

Additives	Glycoprotein	References
Neutral polymers:		
Propylen glycol	Recombinant human growth hormone	***40***
PEG	Mucins	***41***
HPMC	Serum proteins	***42***
Potassium salts:	Standard proteins	***20***
Zwitterionic buffers:		
Betaine, sarcosine		
Tricine + Tris	Lysozyme and α-chymotrypsinogen A	***21***
Tricine	Ricin	***43***
MES	Ovalbumin	***44***
	β- lactoglobulin A, B and C variants	***45***
	Ovalbumin	***44***
HEPES	Ovalbumin	***44***
MOPS	Ovalbumin	***44***
CAPS	Mucins	***41***
Amine modifiers:		
ω-amino acid buffer:		
ε-aminocaproic acid	rtPA	***46,37***
Diaminoalkanes:		
1, 4-Diaminobutane	Ovalbumin	***14,47***
	Recombinant factor VIIa (r-FVIIa)	***16***
	Recombinant human Erythropoietin (rhu-EPO)	***24***

(continued)

Table 1 *(continued)*
Additives Employed To Minimize Glycoprotein Adsorption

Additives	Glycoprotein	References
	Recombinant granulocyte colony stimulating factor (rhGGSF)	***48***
	Human transferrin	***13***
	Ovalbumin	***49***
	Human Transferrin	***50***
1, 3-Diaminobutane	Recombinant human interlukin-4	***12***
	Chorionic gonadotropin	***51***
Monovalent alkylamines:		
Triethylamine triethanolamine	Basic proteins	***52***
N,*N*-diethanolamine, *N*-ethydiethanolamine	Idem	***15***
N,*N*,*N*',*N*'-tetramethyl-1, 3-butanediamine	Idem	***53***
Ethanolamine	β-Lactoglobulin A, B, and C variants	***45***
Hydroxylamne, ethylamine	Ovalbumin	***47***
Polyamines:		
Spermifine, spermine	Ovalbumin	***47***
Chitosan	Basic Proteins	***54***
Polyethylenimine	Basi proteins	***54***
Tetraazamacrocycles	Basic proteins	***56***
Polybrene	Acidic and basic proteins	***54***

Surfactants:		
Nonionic surfactants:		
Brij 35	Recombinant granulocyte macrophage colony stimulating factor	***57***
	Recombinant human interlukin-3	***58***
	Human tumor necrosis factor	***59***
Tween 20	β-lactoglobulin A, B, and C variant	***45***
Tween 80	Recombinant human tissue plasminogen activator (rtPA)	***37***
Triton X-100	rt-PA	***46***
Cationic surfactants:		
CTAB	Apolipoproteins	***60***
Anionic detergents:		
SDS	Recombinant human interferon-γ (IFN-γ)	***28***
Zwitterionic surfactants:		
Dodecyldimethyl (3-sulfopropyl) ammonium hydroxide, hexadecyldimethyl(3-sulopropyl) ammomnium hydroxide, coco (amidopropyl)-hydroxydimethylsulfobetaine	Cationic proteins	***61***
N,-dodecyl-*N*,*N*-dimethyl-3-amino-1-propanesulfonate	Recombinant insulin-like growth factor I variants	***62***

Table 2
Typical Coated Capillaries Commercially Available for CE of Glycoproteins

Supplier	Nature of the coating	pH range	References
Bio-Rad	BioCaP–Polyacrylamide		***36,63–65***
Beckman	Polyacrylamide	2.0–10.0	***66–71***
	Polyvinyl alcohol	2.0–9	***72***
	Amine, positively charged	2.0–7	***26***
Hewlett Packard	Polyvinyl alcohol	2.0–9.0, 5.0	***40,65***
J&W Scientific	Polyethylene glycols		***27,73***
	DB-17		***3,34,68,74,75***
	µ-Sil DB-1 (dimethylpolysiloxane)		***34,69***
	DB-WAX		***34***
Supelco	C1 (CElect H50)	2.0–10.0	***4***
	C8 (CElect H150)		***3,34,36,57,76***
	C18 (CElect H250)		***3,76,77***
	CElect-P150		***34***

Adapted from **ref. *4***.

electrophoretic profile. The main peaks correspond to the glycoforms having oligosaccharides differing in size (d.p. 7–11).

5. Between-runs rinse method: Rinse with 15-column volume of 100 m*M* hydrochloric acid, 3-column volume of water and 15-column volume of the running buffer in this order.

3.1.1.2. Separation of the Recombinant Human Erythropoietin (rhuEPO) Glycoforms ***(24)***

1. Prepare an uncoated fused silica capillary (57 cm in total length), 50 cm in effective length, 75 µm in i.d.).
2. Dissolve rhEPO (1 mg/mL) in water (*see* **Note 9**).
3. For the protein analysis, the capillary is equilibrated for 3 min with the running buffer (10 m*M* tricine/10 m*M* sodium chloride/2.5 m*M* 1,4-diaminobutane–7 *M* urea, pH 6.2).
4. Separation conditions: injection, hydrodynamic (5 s at low pressure); column temperature, 28°C; applied voltage, 10 kV; detection, 214 nm. **Figure 2** displays the electrophoretic profile obtained for rhuEPO. The six major glycoforms of rhuEPO were resolved and migrated according to the number of the sialic acid residues present. The addition of urea was critical for this separation and considerably improved the peak shape and the resolution.
5. Between-runs rinse method: Rinse with 100 m*M* sodium hydroxide for 3 min, followed by water for 3 min.

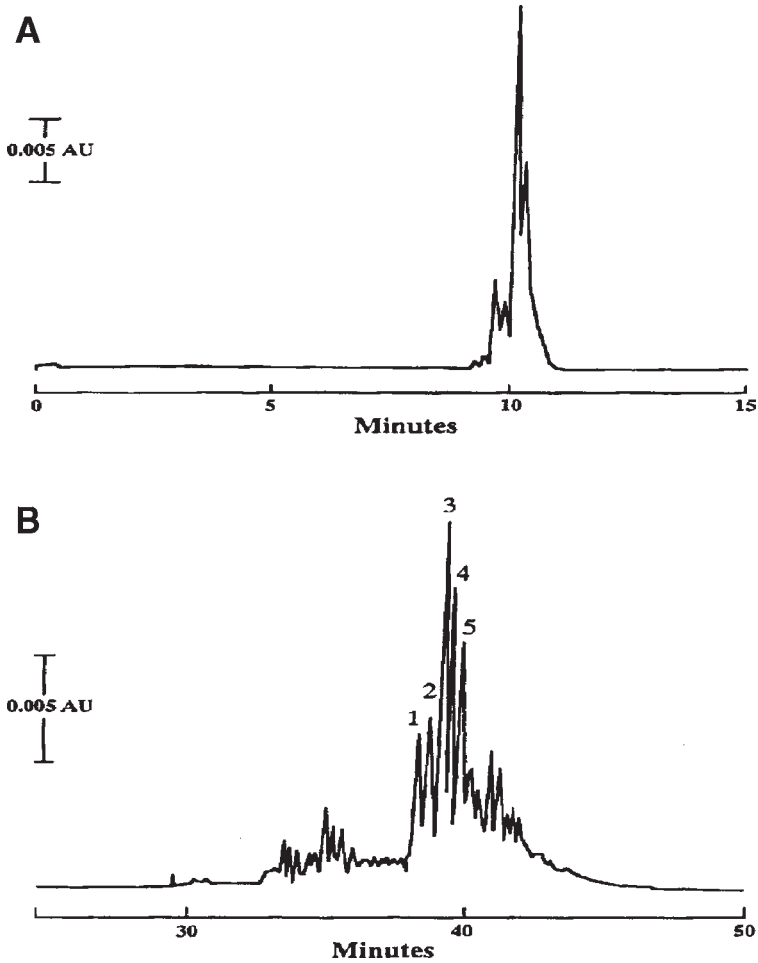

Fig. 1. Effect of the addition of 1 m*M* 1,4-diaminobutane on the separation of ovalbumin glycoforms by CZE in an uncoated capillary. Analytical conditions: capillary, uncoated fused cilica (75 µm i.d., 87 cm); running buffer, 100 m*M* borate buffer, pH 8.5 not containing **(A)** or containing **(B)** 1,4-diaminobutane (1 m*M*); applied voltage, 25 kV. (Reproduced from **ref.** ***14*** with permission).

3.1.2. CZE in Coated Capillaries

Coated capillaries allow a reduction of the electroosmotic flow while minimizing protein adsorption to the capillary surface. In addition, coatings offer the advantage that proteins may be separated at pHs close to their pIs at which the sub species within a single population can be better resolved. Generally,

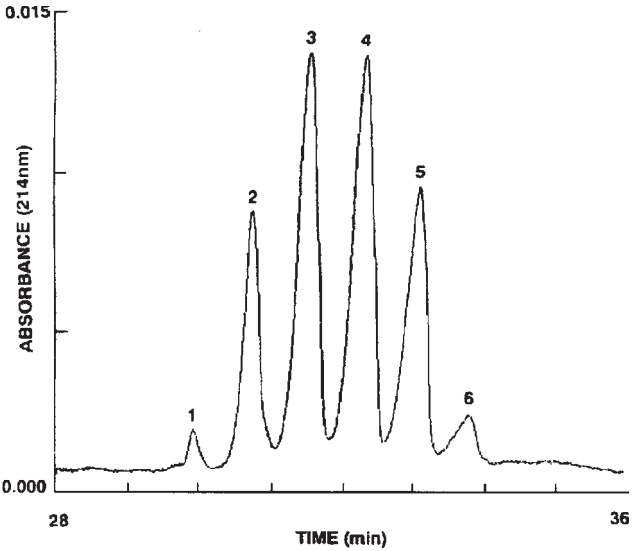

Fig. 2. Capillary zone elctrophoretic separation of recombinant human erythropoietin glycoforms (1 mg/mL). Analytical conditions: capillary, uncoated fused silica (75 μm i.d., 50 cm); running buffer, a buffer solution containing 10 m*M* tricine, 10 m*M* sodium chloride, 2.5 m*M* 1, 4-diaminobutane, and 7 *M* urea, pH 6.2; applied voltage, 10 kV. (Reproduced from **ref. *24*** with permission).

neutral and hydrophilic coatings with such materials as PAA and PVA are preferred to avoid hydrophobic interaction of proteins with the capillary surface and to give better recovery of basic glycoproteins. When adsorption still occurs, non ionic surfactants such as Tween 80 may be added to the separation buffer. A disadvantage of coated capillaries is the poor stability of the coatings at the extreme pHs. Separation are generally performed between pH 2.0 and 9.0.

3.1.2.1. Separation of Recombinant Human Bone Morphogenetic Protein 2 (rhBMP-2) Glycoforms ***(25)***

1. Dissolve the rhBMP-2 glycoprotein in 10 m*M* phosphoric acid
2. Prepare a polyacrylamide-coated capillary (50 cm in total length, 50 μm in i.d.).
3. Inject the sample electrophoretically at 6–12 kV for 4–8 s.
4. Separation conditions: Running buffer 100 m*M* phosphate buffer, pH 2.5; column temperature, 20°C; applied voltage, 12 kV; detection, 200 nm. The electropherogram (**Fig. 3**) shows nine peaks corresponding to glycoforms having 10–18 mannose residues attached to the oligomannose type structure linked to the Asn 56 glycosylation site. Glycoforms with the same number of the mannose residues were not separated and only 9 of the 15 possible glycoforms were separated. However, glycoforms having the same charge and differing only 0.5% in molecular mass could be separated with no additives to the background electrolyte.

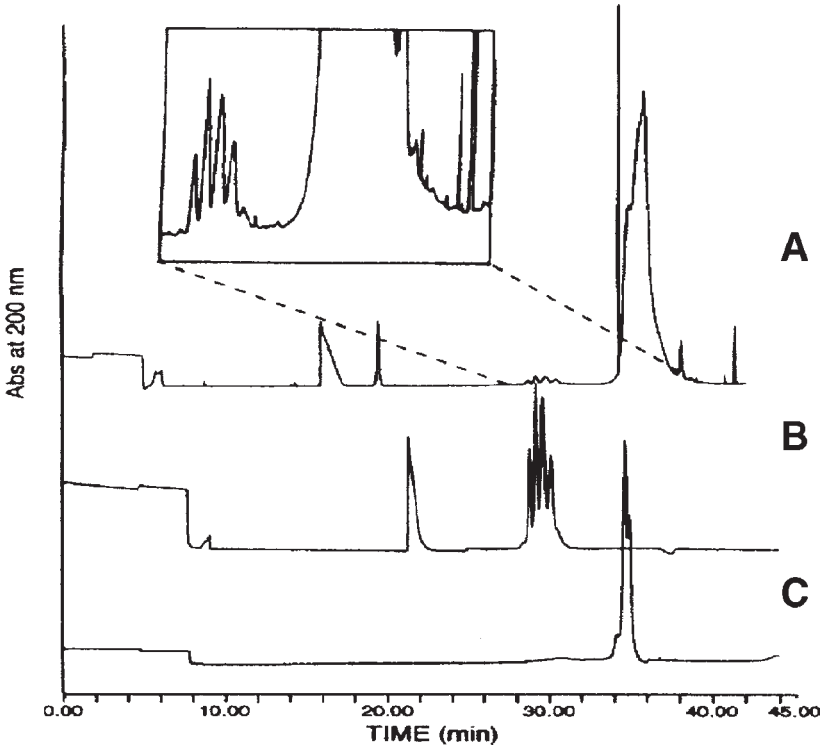

Fig. 3. Separation of **(A)**, rhEPO formulation containing human serum albumin (HSA), **(B)** bulk rhEPO, and **(C)** HSA. Analytical conditions: capillary, eCAP amine capillary (50 µm i.d., 47 cm); capillary temperature, 25°C; running buffer, 200 m*M* sodium phosphate containing 1 m*M* nickel chloride, pH 4.0; applied voltage, 15 kV. Metal ions were added to increase the electrophoretic mobility of HSA affording a complete separation of HSA and rhEPO without affecting the glycoform resolution pattern of rheEPO. (Reproduced from **ref. *25*** with permission).

3.1.2.2. Separation of Recombinant Human Erythropoietin Glycoforms ***(26)***

1. Dissolve rhuEPO in MilliQ water at 0.3 mg/mL
2. Prepare an eCAP amine capillary (47 cm in total length, 40 cm in effective length, 50 µm in i.d.) (*see* **Note 10**).
3. Inject the glycoprotein solution hydrodynamically using 0.5 psi of nitrogen for 8 s.
4. Separation conditions: running buffer, 200 m*M* phosphate buffer, pH 4.0; column temperature, 20°C; injection, from the cathode; applied voltage, 8 kV; detection, 200 nm. As in **Fig. 2**, the profile obtained under these conditions displayed four major peaks accompanied with a number of unresolved minor peaks. The polyamine-coated inner wall, positively charged, reverses the EOF, and in contrast to the conditions of **Subheading 3.1.1.2.**, glycoforms migrated in the order of decreasing number of sialic acid residue (**Fig. 4**).

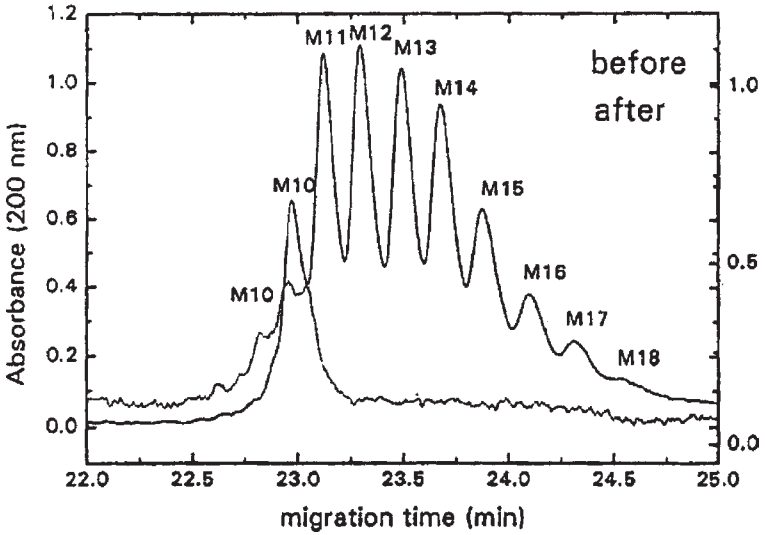

Fig. 4. Overlay of the CZE profiles of intact rhBMP-2 and α-(1,2)-mannosidase-digested rhBMP2. *Full line:* intact rhBMP-2; *dotted line*: digested to oligomannose 10, at an enzyme-protein ratio of 50 mU/mg in 1 mL of sodium acetate, pH 5.0, at 37°C for 48 h. Analytical conditions: capillary, precoated capillary (40 µm i.d., 50 cm); running buffer, 100 m*M* phosphate buffer; sample introduction, electromigration; applied voltage, 5–12 kV; detection, UV absorption (200 nm). (Reproduced from **ref. *26*** with permission).

3.1.2.3. Separation of α-Acid Glycoprotein (α-AG) Glycoforms *(27)*

1. Dissolve α-AG in water at a concentration of 1.2–1.5 mg/mL.
2. Prepare a coated capillary (DB-WAX) (80 cm in total length, 62 cm in effective length).
3. Inject the solution electrokinetically by applying –30 kV for 0.5 min.
4. Separation conditions: running buffer, 50 m*M* MES–Tris buffer, pH 5.6, containing urea (8 *M*); column temperature, 25°C; aplied voltage, –30 kV; detection, 210 nm. **Figure 5** compares the profiles obtained with and without the addition of urea to the running buffer. So far no equivalent separation of the glycoforms of this heavily glycosylated glycoprotein has been obtained.

3.2. Micellar Electrokinetic Capillary Chromatography (MEKC)

Large proteins are not able to penetrate into micelles formed by addition of a surfactant to the running buffer at a concentration above its CMC. The proteins are believed to associate with micelles due to hydrophobic, hydrophilic or electrostatic interactions. For instance, differences in charge to mass ratio of the glycoforms are often leveled out by the surfactant-protein interaction, and selectivity between glycoforms by MEKC may be limited (*see* **Note 11**). How-

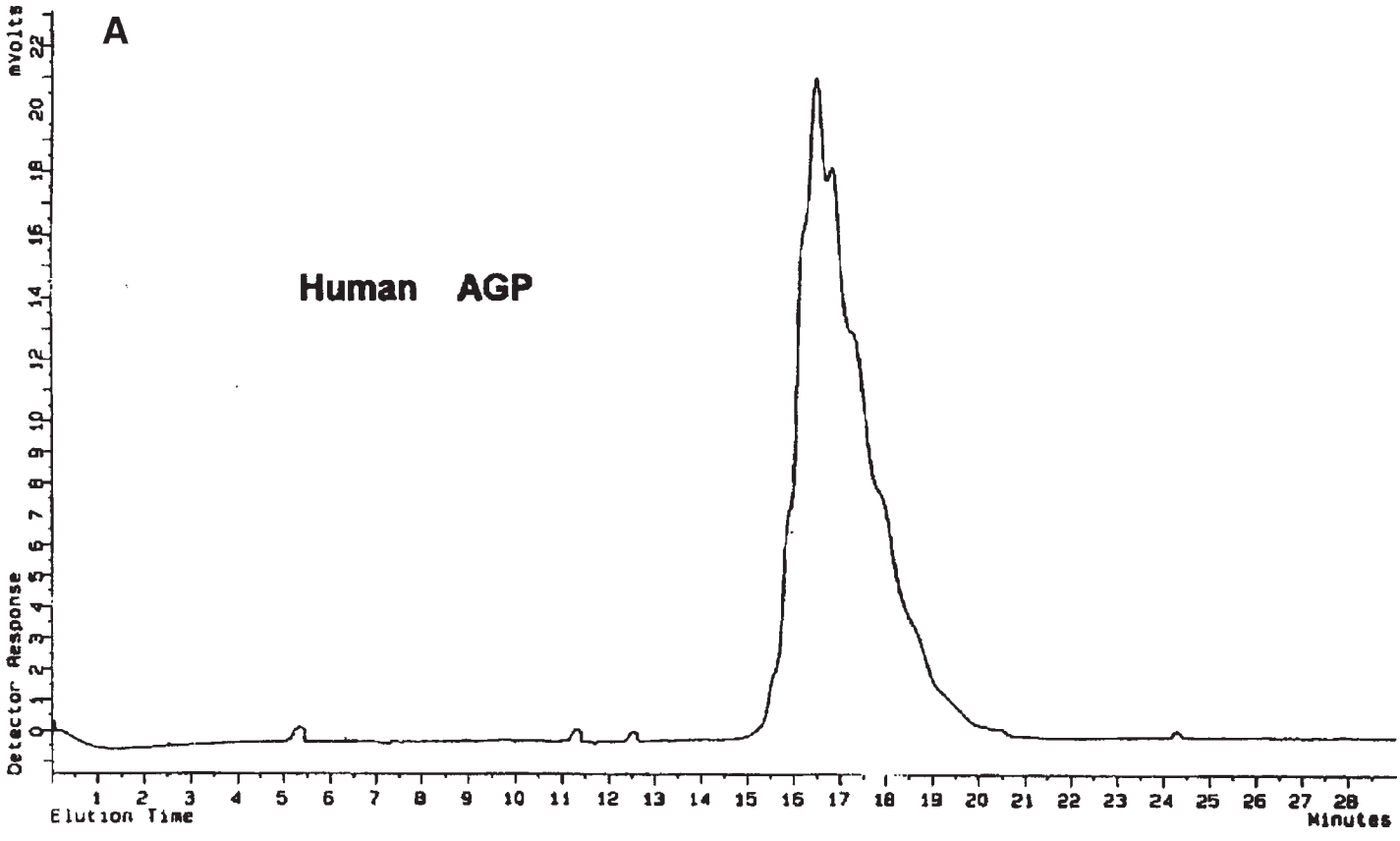

Fig. 5A. CZE of α-AG in a PEG-coated capillary. Analytical consition: capilary, PEG-coated capillary (50 µm i.d., 62 cm); capillary temperature, 25°C; running buffer, 25 m*M* MES-Tris, pH 5.6, not containing **(A)** or containing **(B)** 8 *M* urea; applied voltage, –30 kV; detection, UV absorption (210 nm). (Reproduced from **ref. *27*** with permission).

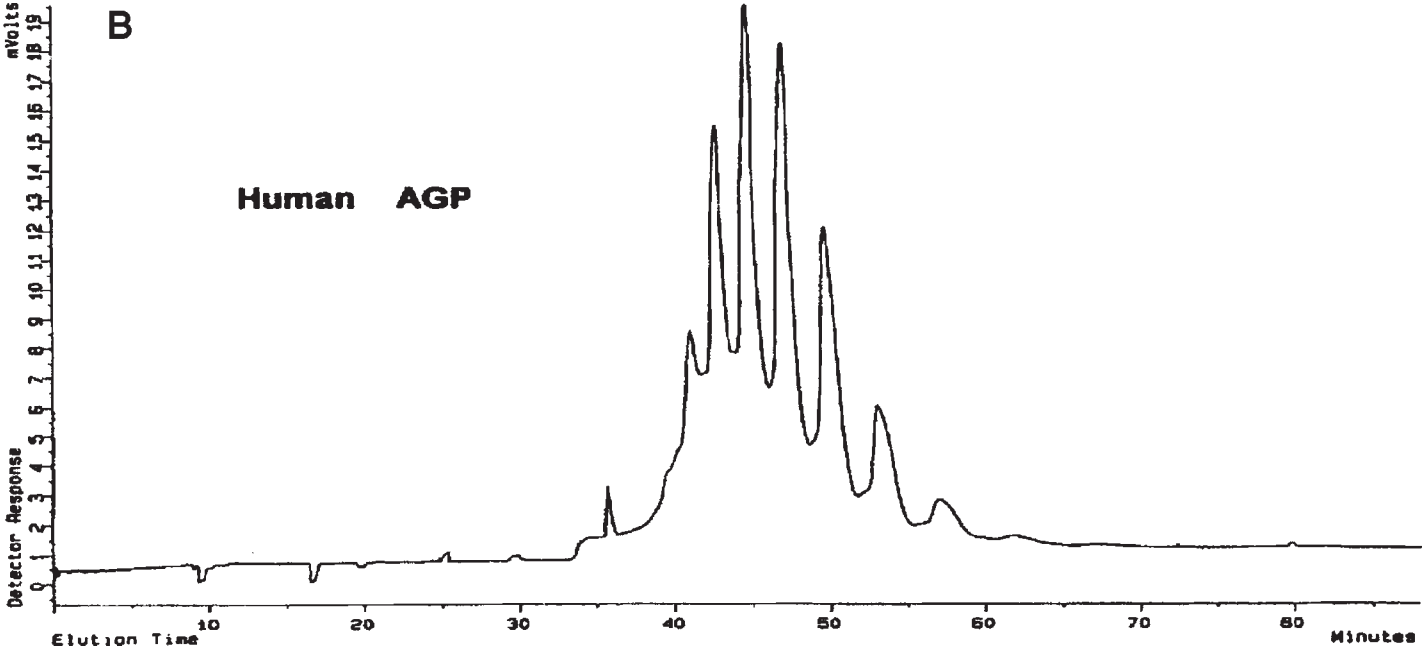

Fig. 5B. (*Continued.*)

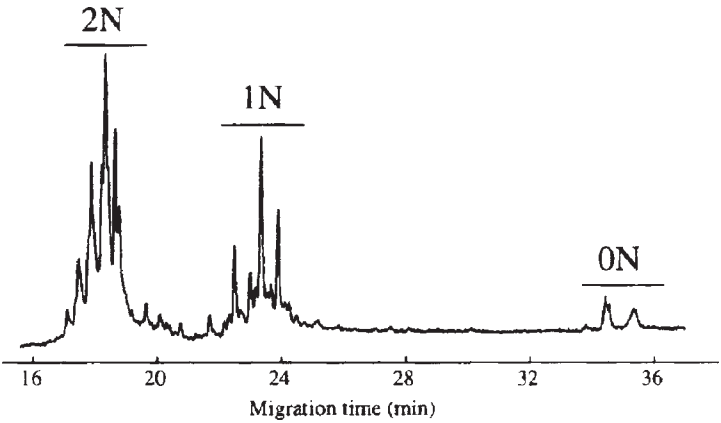

Fig. 6. MEKC of recombinant human IFN-γ. Peak groups represent IFN variants with two ASN sites occupied (2N), one site occupied (1N), or no site occupied (0N). Analytical conditions: capillary, uncoated fused silica (50 μm i.d., 57 cm); capillary temperature, 25°C; running buffer, 400 m*M* borate buffer containing 100 m*M* SDS, pH 8.5; applied voltage, 22 kV. (Reproduced from **ref. *29*** with permission).

ever, this mode may sometimes be successful for the glycoform separation of large and hydrophobic glycoproteins which tend to self aggregate or to adsorb to the capillary wall. This mode of separation is, however, particularly useful in the case of glycoproteins which exist as the dimers or as a polymeric forms in which subunits are held together through hydrogen bonding or hydrophobic interactions. In addition, the presence of surfactant may increase the solubility of the analyzed glycoproteins ***(10)***.

3.2.1. Separation of Human Recombinant Interferon (rhuIFN-γ Glycoforms) **(28,29)**

1. Prepare a new uncoated capillary (57 cm in total length, 50 cm in effective length, 50 μm in i.d.) by successive rinsing with 100 m*M* sodium hydroxide for 10 min, water for 5 min, 100 m*M* borate buffer, pH 8.5, for 1 h, 100 m*M* sodium hydroxide, and water for 10 min , in this order.
2. Dissolve rhuIF*N*-γ in 50 m*M* borate buffer, pH 8.5, containing SDS (50 m*M*) to make a sample solution of 1 mg/mL.
3. Inject the sample by hydrodynamic injection for 5 s.
4. Conditions for separation: running buffer, 400 m*M* borate buffer, pH 8.5., containing SDS (100 m*M*); column temperature, 25°C; applied voltage, 22 kV; detection, 200 nm. More than 30 species were resolved using a combination of these high concentrations of borate and SDS. Three main peak goups were evidenced from the glycoform pattern of IFN corrersponding to variants with two Asn sites (2N), one site occupied (1N) or no glycosylation (0N) (**Fig. 6**).

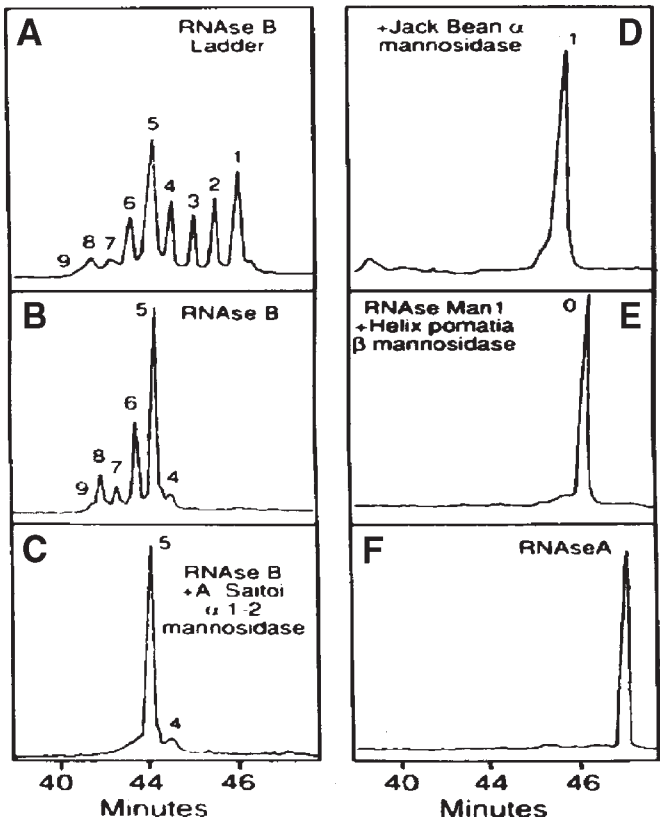

Fig. 7. MEKC separation of **(A)** RNase B ladder used to assign structures, **(B)** bovine pancreatic glycoforms of RNase B, **(C–E)** RNase B digested with mannosidases of different specificities and **(F)** RNase A. Analytical conditions: capillary, uncoated fused silica (50 µm i.d., 100 cm); capillary temperature, 30°C; running buffer, a solution containing 20 m*M* sodium phosphate, 5 m*M* sodum tetraborate, and 50 m*M* SDS, pH 7.2; applied voltage, 1 kV for 1 min followed by 20 kV; detection, UV absorption (200 nm). (Reprodued from **ref. *30*** with permission).

5. Between each separation, the capillary is rinsed with 100 m*M* sodium hydroxide, water and the running buffer for 5 min each.

3.2.2. Separation of Ribonuclease B (RNase B) Glycoforms **(30,31)**

1. Prepare an uncoated capillary (100 cm in total lengtth, 50 µm in i.d.).
2. Dissolve bovine pancreatic RNase B in the running buffer.
3. Inject the sample by pressure for 1.5 s.
4. Conditions for separation: running buffer, 20 m*M* sodium phosphate, containing sodium tetraborate (5 m*M*), and sodium dodecyl sulfate (50 m*M*), pH 7.2; applied voltage, 1 kV for 1min and 20 kV for 47 min; detection, 200 nm. The glycopro-

tein was resolved into five populations of glycoforms, each characterized by one of the oligomannose structures Man9 to Man5. RNase B was digested to a single population of RNase B with the incubation in the presence of α-mannosidases of different specificities (**Fig. 7**).

3.3. Capillary Isoelectric Focusing (CIEF)

Capillary isoelectric focusing is one of the most efficient mode of CE for glycoform separation. Glycoforms often exhibit similar masses and can be separated on the basis of the difference in isoelectric point . Glycoforms varying in degrees of sialylation, phosphorylation and/or sulfatation may be resolved by CIEF. Resolution of proteins with a pI difference of as little as 0.05 pH unit has been reported.

Precipitation of proteins at pHs close to their pIs that may occur during the focalization step is a particular concern in cIEF (*see* **Note 12**). To address this problem different strategies may be employed : reducing protein concentration, increasing the percentage of the ampholyte in the mixture or adding different classes of solubilizers to the protein-ampholyte mixture. Amongst the most efficient solubilizers are zwitterionic buffers such as CAPS, tricine or betain, non ionic surfactants such as reduced Triton X-100; nonidet (at concentrations below their CMC). Chaotropes, such as urea (6–8 *M*) or polypropylene glycol have also been reported to enhance efficiently protein solubility during cIEF ***(7,32,33)***. **Table 3** describes the various solubilizers reported to enhance solubility and overcome protein precipitation in CIEF separation of (glyco)proteins. CIEF of glycoproteins has also been discussed in Chapter 6 of this volume.

3.3.1. Two-Step CIEF

CIEF may be performed in two steps ("two-step approach") in which the focalization and mobilization are carried out sequentially. EOF has to be completely suppressed, using neutral hydrophilic coated capillaries, such as PAA- or PVA-coated capillaries (*see* **Note 10**). In addition polymers such as cellulose derivatives are useful to stabilize pH gradient and to fully suppress EOF. Typical concentrations of these polymers range from 0.1% to 0.5%. Mobilization of the focused glycoproteins may be accomplished either hydrodynamically (using pressure or vaccum) or chemically by the addition of salts or by incorporating zwitterions in the anolyte or catholyte solutions, resulting in alteration of the pH gradient. This latter procedure produces nonlinear relationships between the migration times of pI markers and their pI values.

3.3.1.1. Separation of a Mouse Monoclonal Antibody ***(3)***

1. Prepare a μSIL DB-1-coated capillary (27 cm in length).
2. Place the anolyte solution (10 m*M* phosphoric acid in 0.4% methyl cellulose) and the catholyte solution (20 m*M* sodium hydroxide) at the anode and cathode, respectively.

Table 3
Different Solubilizers Employed in CIEF of Glycoproteins

Class of solubilizer	Concentration range	Glycoprotein analyzed	References
Urea	4–8 *M*	Recombinant human tissue plasminogen activator (rtPA)	***34,37,46, 71,78***
		Human recombinant Erythropoietin (rhEPO)	***78***
		Monoclonal antibody actinavidin	***79***
		Murine monoclonal antibody	***68***
Nonionic surfactants:			
Reduced triton X-100	1%	Globulins	***80***
	0.2%	Monoclonal antibody HER2	***65***
	0.001%	Recombinant antithrombin III (r-AT III)	***81***
Zwiterrionic salts:			
CAPS	0.09 *M*	HIV-1 recombinant envelope glycoprotein (rgp 160)	***72***
CHAPS	2%	rtPA	***46***
Taurine	0.1 *M*	Thermamylase	***33***
Bicine	1 *M*	L-aspartate oxidase	***33***
Nondetergent Sulfobetaines	0.5 *M*	L-aspartate oxidase	***33***
	1 *M*	Alcalase	***33***
Sugars:			
Sucrose	20%	Thermamylase	***33***
	6%	rgp 160	***72***
Sorbitol	20%	Thermamylase, alcalase	***33***
n-Octylglucoside	1%	Scrapie prion protein	***70***
Polyols:			
Glycerol	20%	Alcalase	***33***

3. Desalt the mouse monoclonal antibody using a microcon 10 (molecular cut off of 10 kDa, Amicon Inc, Beverly, MA) (*see* **Note 13**).
4. Prepare a solution containing the desalted monoclonal antibody at a concentration of 500 ng/µL and the protein markers at 50 ng/µL. Prepare also a carrier ampholyte solution containing Pharmalyte, (pH 3.0–10.0), at 4%, TEMED at 1%, and methyl cellulose at 0.8%.
5. Mix the protein solution and the carrier ampholyte solution at a 1:1 ratio.
6. Rinse the capillary with the anolyte for 2 min and then fill the capillary with the proteins/carrier ampholyte mixture for 5 min.

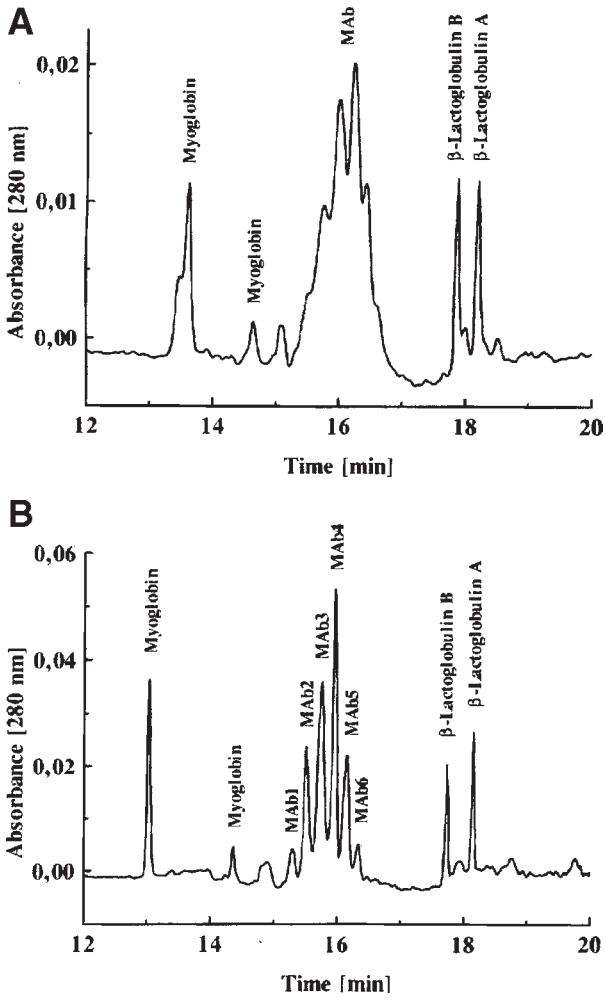

Fig. 8. Two-step CIEF of a mouse monoclonal antibody using low pressure mobilization (0.5 psi). Analytical conditions: capillary, μSIL DB-1 (27 cm); carrier ampholyte solution, a solution containing Pharmalyte (pH range 3.0–10.0, 4%), TEMED (1%), and methyl cellulose (0.8%). Monoclonal antibody concentration, 500 ng/μL; protein marker concentrtion, 50 ng/μL, focusing, 2 min at 10 kV **(A)** mobilization with 10 kV; **(B)** mobilization with 20 kV. (Reproduced from **ref. *3*** with permission).

7. Focus the proteins for 2 min at 10 kV and 25°C (*see* **Note 14**).
8. Mobilize the focalized zones by applying a low pressure (0.5 psi) and a voltage of 20 kV. The various forms of the monoclonal antibody are detected at 280 nm (*see* **Note 15**). **Figure 8** illustrates the influence of the voltage applied during the mobilization step on the resolution of the separated species.

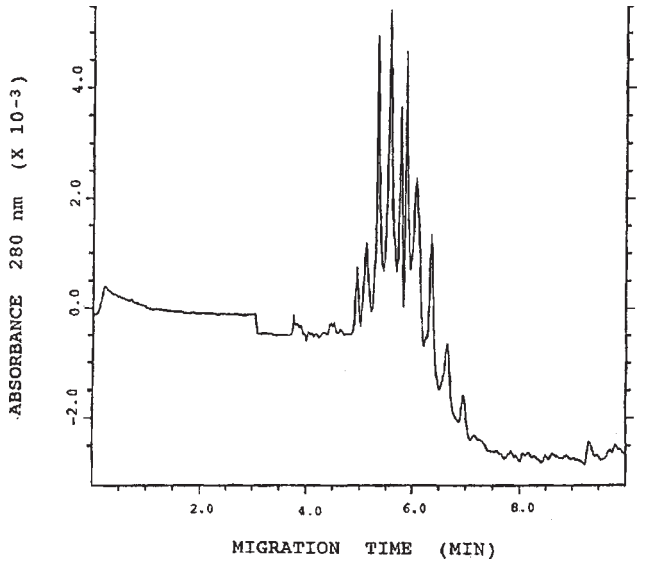

Fig. 9. On-step CIEF of rtPA glycoforms. Analytical conditions: capillary eCAP neutral capillary (50 µm i.d., 27 cm); rtPA sample was diluted to give a final concentration of 125–250 µg/mL. The ampholyte solution contained 4 *M* urea, 0.14% HPMC, 7.5% TEMED, Pharmalyte (pH range 3.0–10.0, 3%) and Pharmalyte (pH range 5.0–8.0, 3%). Voltage (reverse polarity), 500 V/cm; detection, (280 nm). (Reproduced from **ref. *34*** with permission).

3.3.2. One-Step CIEF

An alternative method is the "one-step approach" in which focusing and mobilization take place simultaneously. The mobilization is accomplished by EOF which is maintained constant by the addition of polymers in solution.

3.3.2.1. Separation of Recombinant Tissue Plasminogen Activator (rtPA) Glycoforms ***(34,35)***

1. Prepare an eCAp neutral capillary (27cm in total length, 50 µm in i.d.).
2. Place the anolyte solution (10 m*M* phosphoric acid) and the catholyte solution (20 m*M* sodium hydroxide) at the anode and cathode, respectively.
3. Dissolve rtPA at a concentration of 125–250 µg/mL in a solution containing urea (4 *M*) (*see* **Note 16**), HPMC (0.1%), TEMED (7.5%, by volume), and a 1:1 mixture of Ampholytes pH 3.0–10.0 and pH 5.0–8.0 (3% each) (*see* **Notes 17** and **18**).
4. Rinse the capillary for 1 min with water, then fill the capillary with this solution using a pressure for 2 min.
5. Focus the glycoforms using reverse polarity for 10 min at 500 V/cm at 20°C and detect the glycoforms passing the detection window at 280 nm. Charge heterogeneity of rtPA can be detected by a series of approx 10 peaks with increasing

migration times corresponding to increasing acidic isoelectric points of the individual charged species. The apparent pIs of rtPA glycoforms are in the range of pH 6.5–7.5 (**Fig. 9**).

6. After each run, rinse the capillary with 10 m*M* phosphoric acid for 2 min followed by water for 1 min.

3.4. Capillary Gel Electrophoresis

Capillary gel electrophoresis (CGE) of glycoforms is generally performed with the addition of SDS to produce SDS-glycoform complexes having the same density of charge. This mode is relatively less efficient to separate closely related glycoforms than the other CE modes. Indeed, SDS tends to mask the intrinsic charge of the different glycoforms and this mode will therefore separate the glycoforms only on the basis of mass difference. Successful separation will be obtained only for small glycoproteins or for glycoproteins consisting in glycoform mixtures having a large difference in their extent of glycosylation such as the presence or the absence of glycans attached to one glycosylation site. Rarely a full separation of glycoforms from a highly glycosylated and high mass glycoprotein will be achieved using this mode.

CGE is generally employed rather for molecular mass determination of glycoproteins or to detect aggregation or fragmentation of the glycoproteins. The basis of size separation in SDS gel electrophoresis is the constant binding ratio between a protein and SDS (*see* **Note 19**). CGE separation of glycoforms may be carried out under reducing conditions with the addition of dithiothreitol or mercaptoethanol that eliminates the dissulfide bridges. The technique of CGE is currently available through different kits from Beckman and Bio-Rad which include the sieving media, the separation conditions and molecular weight markers.

3.4.1. Separation of Antithrombin IIIα (AT IIIα) and Antithrombin IIIβ (AT IIIβ) (36)

1. Prepare an uncoated silica fused capillary (25 cm in total length, 21 cm in effective length, 50 µm in i.d.).
2. Prepare AT III solutions at 0.5–1 mg/mL.
3. Dilute each AT III solution to a twofold volume with the CE-SDS buffer (Bio-Rad), and add 2-mercaptoethanol and the internal standard at a final concentration of 5%.
4. Boil the solution for 10 min.
5. Inject the solution under a pressure of 100 psi/s and separate the AT III glycoforms at 15 kV using the Bio-Rad running buffer. The electrophoretic profiles obtained for ATIII samples purified using different commercially available heparin resins are presented in **Fig. 10**. Two peaks corresponding to ATIIIβ and ATIIIα were fully resolved and their ratio depended on the type of affinity gel used for the purification. ATIIIβ is lacking glycosylation at Asp135. An AT III sample with a molecular mass of 5.5 kDa migrated more slowly than expected, probably because of the presence of oligosaccharides (*see* **Note 19**).

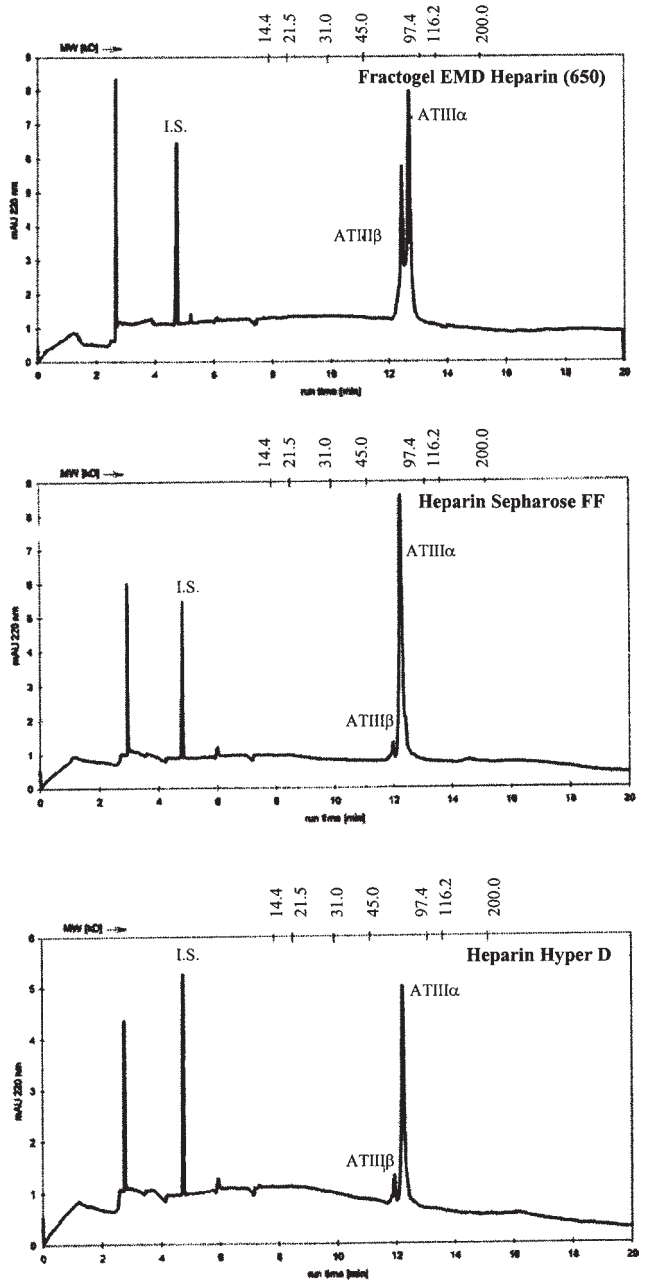

Fig. 10. SDS-CGE analysis of antithrombin III (ATIII) purified from human pkasma using different heparin resins. Analytical conditions: Uncoated capillary (50 μm i.d., 25 cm); sample ATIII at 0.5–1 mg/mL diluted 1:3 in mercaptoethanol. Running buffer, polymer buffer from Bio-Rad; applied voltage, 15 kV; detection, UV absorption (220 nm). (Reproduced from **ref.** ***36*** with permission).

3.4.2. Separation of Plasminogen-Treated rtPA Variants **(37)**

1. Prepare an eCAP neutral capillary (27 cm in effective length, 100 μm in i.d.).
2. Combine 100 μL of the sample buffer (supplied in the kit) with 85 μL of the sample, 5 μL of mercaptoethanol and 10 μL of the internal standard (Orange G). The final concentration of rtPA should be 0.425 mg/mL.
3. Mix the above materials, boil the mixture for 10 min, then cool it on ice for 3 min.
4. Inject the solution by applying a low pressure for 30 s.
5. Separate the glycoforms using the polymer separation buffer supplied in the kit and by applying a voltage of 300 V/cm in the reverse polarity mode. Detect the glycoform zones at 214 nm.
6. Plasminogen-treated rtPA is separated into three polypeptide chains, one B chain and two A chains. Only one peak was obtained for A chain in the case of the variant rtPA type I, while three peaks were obtained for the rtPA type II, which differs from the type I variant by the absence of glycosylation at the Asn184 site.

3.5. Selection of the CE Mode

When the analysis of an unknown glycoprotein is required, the first mode that should be tried to separate the different glycoforms is CZE in uncoated capillaries. This represents the simplest mode to be carried out, and with a reasonable number of parameters to optimize. Not all the (glyco)proteins adsorb to the silica surface and sometimes a separation of the glycoforms may be obtained quite easily only by selecting the appropriate pH and buffer conditions. It is therefore recommended to test first extreme pHs such as pH 2.5 or pH 9.0 to ensure that at least one peak can be visualized. Once this preliminary control is made, improvement of the glycoform separation is accomplished by testing pHs closer to the isoelectric point of the protein where differences in their charge to mass ratio is more pronounced. When glycoforms differ by only slight variations in their glycosylation, reduction of the electroosmotic flow may be necessary to achieve a complete resolution of the glycoforms. This can be accomplished by adding alkylamines to the separation buffer. In addition glycoproteins having only uncharged oligosaccharides (e.g., mannose-type oligosaccharides) can also be separated by complexation with the borate ion.

When adsorption of proteins occurs, permanently or dynamically coated capillaries should be preferred. The latter ones offer the advantages to be cheaper, more compatible with the diode array detection and more stable.

For proteins exhibiting a limited solubility in aqueous solutions or an hydrophobic character (for example membrane glycoproteins), MEKC may assist their solubilization through the use of surfactants such as sodium dodecyl sulfate, although in most cases, the resolution of glycoforms using MEKC is inferior to that obtained using CZE. This mode is suitable for glycoproteins bearing mainly neutral oligosaccharides and is therefore not recommended for very acidic glycoproteins whose negative charges prevent their interaction with the anionic surfactant.

CIEF can be employed either to further improve the resolution or to achieve separations that could not be possible using the two previous modes. However, as the separation of glycoforms is based on differences in pI, this mode is rather employed for glycoproteins whose glycoforms exhibit different degrees of sialylation, sulfatation or phosphorylation. This mode of separation is particularly suitable for separation of glycoproteins bearing complex type oligosaccharides and exhibiting a good solubility in water. Parameters to optimize in CIEF are quite numerous and to avoid an extensive optimization stage, the one-step approach may be preferred, as the mobilization is accomplished through the electroosmotic flow itself. However, both one-step and two-step approaches can be investigated using ampholyte having a wide pH range at the first stage to estimate the pI range of the glycoforms analyzed. Once this range is determined, optimization of the method will rely on the choice of appropriate mixtures of ampholyte having wide and narrow pH ranges, and on the eventual addition of solubilizers to avoid precipitation of the glycoforms during the focusing step (which occurs at pHs close to their pIs). When the separation of the glycoforms is desired for a routine control analysis, the two-step approach is more suitable due to the mobilization independent of EOF, which produces a higher reproducibility of migration time. The simplest mobilization method for the two-step approach is by using pressure. However, if complete resolution of the glycoforms is not attained, it may arise from the pressure mobilization that causes band broadening. In this case, chemical mobilization using either a salt or a zwitterion has to be tested. The direction of the mobilization (either anodic or cathodic mobilization) is chosen according to the acidic character of the analyzed glycoprotein. Faster separations are obtained using the one-step approach. The stability of the coating is of prime concern, as it will influence the velocity of mobilization and thereby the reproducibility of migration time.

CGE is a mode quite useful to separate glycoforms having a high variation in their glycosylation and those having a molecular mass range of 15–150 kDa. This mode of separation can provide useful information on the molecular weight of an unknown protein. Generally, CGE is employed to check the purity of glycoproteins rather than to separate different glycoforms. For example this represents the method of choice to detect either fragmentation of the starting glycoproteins that may occurs during the purification processes and storage at elevated temperature, or aggregation of the glycoproteins.

4. Notes

1. The quality of the water† employed to prepare the running buffers is critical: double distilled or ultrapure deionized water, filtered through a 0.22-μm membrane filter should be employed.

2. Running buffers: All buffers and rinse solutions used for CE must be filtered through a 0.22-µm membrane prior to use. Stock solutions may be prepared and stored in a refrigerator for less than one week.
3. When sodium dodecyl sulfate is added to a buffer (MEKC), the solutions should be rather stored at ambient temperature to avoid precipitation of SDS.
4. According to the pI of the analysed (glyco)protein, this concentration may be adapted. For instance, acidic glycoproteins may require concentration up to 100 m*M* of phosphoric acid as the anolyte.
5. Adsorption of proteins is generally evidenced by low efficiencies, asymetric peaks, lack of reproducibility of the migration times as well as of the peak areas and also by a progressive drift of EOF. In this case, it is recommended to change the rinse protocol between runs and add for example a rinsing step with 0.1 m*M* hydrochloric acid.
6. To prepare the detection window on the capillary, a portion of a few millimeters the external polyimide layer is removed by burning the capillary at a distance of 7 cm from the outlet. This distance may vary according to the instrument used.
7. Concentration of 1.4-diaminobutane (putrescine) may be optimized between 1 and 10 m*M* depending on the glycoprotein analyzed. Other alkylamines such as 1.3-diaminopropane may replace 1.4-diamniobutane.
8. Capillary thermostating is of particular importance in protein analyses as proteins undergo denaturation at elevated temperatures.
9. Protein solutions should be at high concentrations (more than 0.5 mg/mL) to compensate for the small volume injected typically from 5 to 20 nL. Diluted protein preparations should be concentrated (either by dialysis at a low temperature to avoid degradation, ultrafiltration or lyophilization) and then dissolved each time, if possible, in water. When the glycoprotein is not soluble in water, small amounts of salts may be added to the sample at a concentration 10-fold lower than that of the running buffer. This kind of sample solution preparation favors the stacking effect responsible for an increase in the detection sensitivity.
10. eCap amine capillaries may not be rinsed with strong alkaline solutions, as they may deteriorate the coating. In general, when using coated capillaries care has to be taken to avoid removal of the coating, which occurs mainly when strong acidic or alkaline solutions are employed to rinse the capillary. When a residual EOF is still present in a coated capillary, it is preferable to measure it when using the capillary for the first time and control the stability of the coating by periodic controls of the EOF value.
11. SDS which is known to bind proteins with a constant charge to mass ratio will convert proteins to negatively charged macromolecules with a similar density of negative charges. Glycoforms often differ slightly in their physicochemical properties. This is why selectivity in MEKC of glycoprotein is often limited. The selectivity may also be deteriorated due to the denaturation of the glycoproteins in the presence of surfactants.
12. Precipitation of proteins during CIEF may be evidenced by the apparition of non-reproducible spikes in the glycoform profile and may cause clogging of the

capillaries. This may be overcome by decreasing the concentration of the protein analyzed or by adding a solubilizer to the ampholyte/sample mixture (*see* **Table 3**). Spikes may also be provoked by the presence of bubbles in the ampholyte/gel mixture and can be removed by centrifugation (5000*g* for 5–10 min).

13. For CIEF, a particular care must be taken to remove salts and other contaminants from the glycoprotein preparations that may perturb the electric field and the pH gradient inside the capillary. Alternatively, it is possible to desalt on-column the samples before the focusing step by applying a low voltage (3–8 kV) for 2 min. Since the electrophoretic mobilities of salts are very high , they will elute out of the capillary during this preliminary step.
14. During focusing an initial current between 10 and 25 μA is observed. If acidic proteins are to be analyzed, higher currents may be generated because higher voltages have to be applied. During the focalization the current decreases until the proteins and ampholytes reach the zone in the pH gradient where they have no more charge. The completion of focalization is evidenced by the steady state of the current at a very low value (typically <2 μA).
15. UV detectors should be preferred over diode array detectors because of the noisy baseline obtained with the latter ones. This baseline perturbation may arise from a degradation of either the capillary coating or the ampholyte solutions.
16. Urea causes denaturation of proteins leading to a shift of their apparent pIs.
17. The optimum percentage of TEMED that has to be added depends on the volume of the capillary after the detection window (one-step CIEF). One simple way to determine this percentage is to analyse a mixture of two standard proteins having for the first one a pI just above the lowest pH of the range of pH gradient and for the second one just below the highest pH of the same range of pH gradient. The percentage of TEMED has to be increased until both the proteins can be detected.
18. Ampholytes of the same pH range but from different commercial sources may be compared, as they might produce slightly different patterns. Transparency at 280 nm of ampholytes can vary significantly both among manufacturers and from lot to lot ***(38)***. A mixture of ampholytes with a different pH range, narrow and wide, may be used to improve resolution among glycoforms having close pIs.
19. Glycoproteins often exhibit a marginal behavior in SDS-CGE which leads to inaccurate determination of molecular masses. To overcome this problem, the Ferguson method, in which CGE is carried out using increasing concentrations of the sieving polymers in the buffer, needs to be employed. The relative migration times of glycoproteins are measured as a function of gel concentration. The logarithm of a protein's mobility varies linearly as a function of the gel concentration employed. The slope of this line yields the retardation coefficient (Kr) which is proportional to the protein molecular weight ***(39)***.

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12

Separation of Glycoproteins by Capillary Isoelectric Focusing

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1. Introduction

Capillary isoelectric focusing (cIEF) has now become a somewhat routine, accepted mode of operation in capillary electrophoresis (CE). We and others have published several reviews of cIEF ***(1–14)***. More and more applications become evident every year, especially from the biotechnology industry ***(7,15–24)***. cIEF has become a variation of flat-bed isoelectric focusing (IEF), which is a fairly old, standard method of protein separations. Flat-bed IEF is a form of conventional electrophoresis, which is still often used, especially as part of a two-dimensional (2-D) arrangement involving cIEF-polyacrylamide gel electrophoresis (PAGE) ***(25–32)***. Proteomics now employs this 2-D arrangement for complex protein and peptide mixtures, often with immobilized pH gradients ***(28)***. In this, we use the terms protein and peptide interchangeably, although they are technically not the same terms. cIEF has some significant advantages over conventional IEF, in that it can be routinely run in a conventional, commercial CE instrument, whether from Bio-Rad, Agilent Technologies, Beckman Coulter, Waters, or others ***(7,9)***. It has become a completely automated, analytical method, fully microprocessor controlled, from sample introduction to data readout ***(9,19–22)***. A variety of detection methods are possible, such as fixed wavelength ultraviolet (UV), laser-induced fluorescence (LIF), and mass spectrometry (MS) ***(6,8,16,33–37)***. It is even possible to utilize a whole column detection approach with UV, and a commercial instrument is now available for such applications ***(38,39)***.

Because of the extremely high peak capacity (the number of peaks that can be separated in one capillary length) of cIEF, it has become one of the more preferred methods for the analysis of complex protein mixtures, such as in

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proteomics studies ***(16,37)***. As a function of the nature of the particular ampholyte used in cIEF, species with pIs (isoelectric point) that differ by <0.05 pH unit can now be resolved. The analytical performance of this technique can enable the resolution of hundreds of proteins per run. Thus, cIEF has begun to be adopted and adapted by a large number of pharmaceutical and biotechnology firms, especially in the analysis of recombinant proteins, antibodies, fusion proteins, peptide mixtures, and for proteomics studies. It is also very useful for performing affinity-cIEF, wherein the antibody or antigen to a protein of interest is added to the sample buffer (ampholyte mixture) and changes in the original analyte's peak shape and mobility are effected and observed ***(6,8,33,40,41)***. In this manner, active vs inactive proteins can be detected and quantitated, if the recognition counter species is available (antibody, antigen, receptor protein, and so forth). It is even possible to use cIEF for combinatorial library screening, to determine which members of a library are recognized by a specific biological receptor or recognition element ***(40)***. Proteins and peptides are identified in cIEF on the basis of their determined pI values, using an internal standard calibration plot with markers of known pI (peptide markers, commercially available) ***(7,9,33)***.

1.1. Mechanism of Separation in cIEF

In contrast to other forms of CE, such as capillary zone electrophoresis (CZE), in which separations occur on the basis of differences among the proteins in charge-to-mass ratios, in cIEF differences in isoelectric points (pIs) do account for the observed resolutions. Thus, cIEF really only works for those species that have a distinct pI value, perhaps acidic, perhaps basic, perhaps neutral (pI = 7). If there is no pI inherent in the protein or charged polymer, then these species cannot be separated by cIEF. Even if there are very small differences in these pI values, a judicious choice of the ampholytes to be used can often resolve these proteins. In addition, because cIEF involves an initial focusing step, in which the proteins come to rest within the ampholyte mixture and no longer migrate but rather are fully or almost fully focused, there is a high degree of preconcentration of the protein sample before mobilization and then detection. Thus, in cIEF several steps are really involved: (1) placement of the sample into the ampholyte/buffer in the capillary; (2) focusing of the ampholyte and proteins in the capillary under an applied voltage (0–30 kV); (3) mobilization of the now-focused proteins toward the detector; and (4) detection of the focused and mobilized proteins past the detector for identification and quantitation. Alhough, at times, two of these steps (focusing and mobilization) can be condensed into a single step (one-step method, *see* **Subheading 1.2.**), maximum preconcentration and focusing usually occur in the two-step mode, in which the proteins have the maximum amount of time to

completely focus, before they are mobilized past the detection window and out the capillary toward the cathode.

In essence, as in flat-bed IEF, the heart of all cIEF operations and separations lies within the nature of the ampholytes. Since the ampholytes used today in cIEF were originally developed for flat-bed IEF, they were never designed for direct, through the capillary, UV detection (or LIF or MS, for that matter). Ampholytes consist, in general, in the commercial varieties (Bio-Rad, Pharmacia, Beckman, Sigma, Fluka, and others), of polyamino acids and polyamino and polycarboxylate groups on a polymer chain. They can be mixtures of peptides or synthetic mixtures of charged polymers. They are available in certain pH ranges, termed wide (e.g., pH 3.0–10.0) or narrow (e.g., pH 3.0–4.0, 4.0–5.0, 6.0–8.0, and so forth) gradients. Usually, narrow ranges provide higher resolution for a given separation, as the pH range is divided over the entire capillary length (normally about 60 cm). Hence, the pH gradient defines the pH range of the ampholyte mixture over the total length (anode to cathode) of the capillary. However, as shown in **Fig. 1**, if the detection window is placed before the end of the capillary, normally the cathodic end, then some of the pH gradient will focus, on application of the external voltage, between the window and the cathodic (normal polarity mode) end of the capillary. Hence, if proteins were to focus within that region, which could be perhaps 10–20 cm, instrument dependent, they would not be detected, as they will focus past the detector and then migrate to the cathode, never seen by the detector itself. Hence, people have placed a base extender, usually a polyamine, such as tetraethylmethyl-enediamine (TEMED), in the buffer mixture, prior to focusing, which will focus (it is hoped) between the cathode and the detection window, thereby forcing the ampholyte mixture to focus between the detection window and anode. The proteins, if they have a pI below that of the base extender, will then be forced to focus between the anode and the detection window, and then they will all migrate past the detection window and be detected.

Thus, a major goal in all of cIEF is to ensure that all the analytes of interest will focus before the detection window, and then migrate toward the cathode in the normal polarity mode and be detected. The peak capacity and resolution possible in cIEF, as in flat bed IEF, depend entirely on the nature of the ampholytes being used. If a very smooth gradient is present in that ampholyte mixture, then one will resolve a larger number of proteins and increase the peak capacity. Also, if one uses a narrower pH range, one will be able to resolve species having more similar pI values than with a wider range mixture. Thus, it is a matter of finding the correct, optimal ampholyte mixture and range for a given sample composition and pI differences that will ensure a best chance for complete resolution and maximum peak capacity. The

nature of the ampholytes becomes critical, and everyone uses one or more commercial ampholyte mixtures. However, there are problems in so doing. These ampholytes were made for flat-bed IEF, in which iodine-125 labeling or Coomassie Blue staining have been the dominant detection methods. Hence, using such materials in cIEF causes some wavelength-dependent problems, in baseline shifts, which can vary from ampholyte to ampholyte range and vendor to vendor. These ampholytes were also never intended for MS compatibility, and they were usually removed before proteins separated in flat-bed IEF were detected by MS. It is now becoming clear that only some commercial ampholytes will be UV and MS friendly, and one has to actually evaluate each ampholyte sample in a blank run before it is safe to assume no interferences. At times, it is possible to pass the ampholytes through a short column of charcoal, remove the most UV absorbing materials, and the remaining ampholytes then cause much less interference in cIEF-UV/photodiode array spectroscopy (PDA) ***(42,43)***.

At times, it may be beneficial to use other additives in the buffer, besides the ampholytes and a base extender. Numerous reports exist on the use of cellulose materials, hydroxymethylcellulose, hydroxypropyl cellulose, and so forth, of varying molecular weight distributions and percent composition in the buffer (e.g., 0.5–2.5%). In the one-step method, as described in **Subheading 1.2.**, often it is desirable to slow down the electroosmotic flow (EOF), so that the proteins will have maximal opportunity to focus before they are automatically carried past the detection window. The EOF is dependant on the buffer pH and the extent to which free silanol groups are present on the capillary wall. In most, if not all, cIEF analyses, especially for those using a one-step approach, the separations are conducted using coated or uncoated capillaries and reduced EOF. The changes in EOF with the pH of the ampholyte are highest at the basic end and slowest in the acidic range. The cellulose additives are thus used to partially coat the capillary walls and reduce EOF, but they are also used to increase the viscosity of the buffer medium, again slowing the migration (and diffusion or remixing) of the proteins as they are focusing toward the detector and improving resolution and peak capacity. It may also be the case that the cellulose additives form a thin layer on the capillary wall, and thus prevent the sample proteins from being adsorbed, temporarily, on the capillary walls and changing EOF, peak shapes, focusing times, and/or resolutions. Finally, it is imperative to keep the proteins in solution once focused, as high concentrations of some proteins may result in their precipitation. The use of additives, such as urea or guanidine, can improve the solubility of the focused proteins and prevent spikes, which are actually the presence of precipitated proteins as they move past the detection window. Surfactants are not recommended as part of the buffer medium, as they may change the true pI of any protein, and thus not allow for accurate determination of some species.

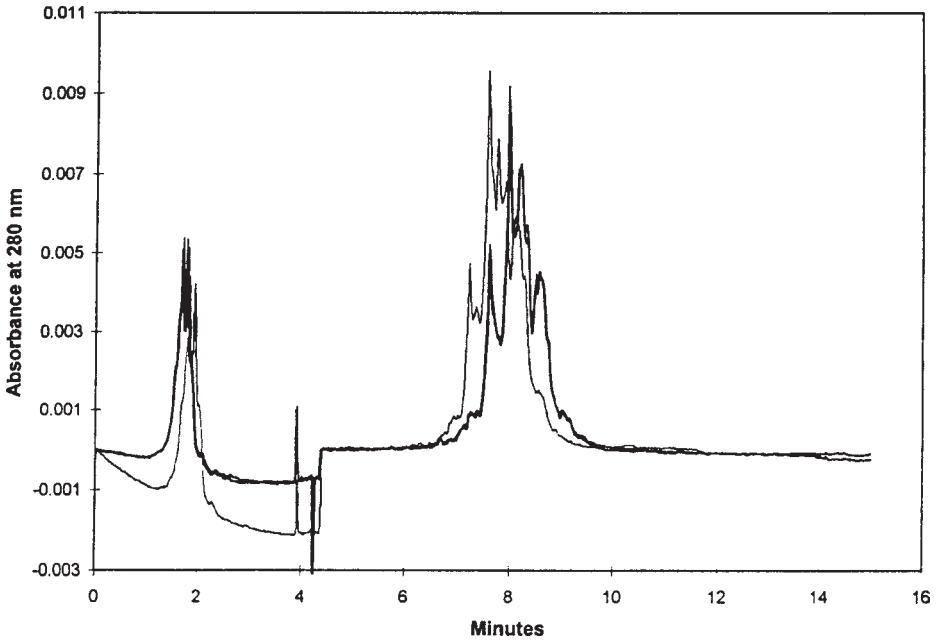

Fig. 1. Reproducibility of cIEF analyses of human transferrin. The dark, black (*to the right*) trace is an overlay of runs 15 and 16, and the gray trace (*lighter, to the left*) is an overlay of run 500. Three separate runs are represented here. Ampholytes covering a range of pH 3.0–8.0 were used. A zwitterionic spacer (pI = 8.7) was added to block the blind (cathodic) side of the capillary, run in the normal polarity mode. Analysis conditions: capillary 24 cm × 25 µm, coated with a linear polyacrylamide; focusing: 4 min at 15 kV; detection was done at 280 nm. Sample and capillary were thermostated at 20°C. (Reprinted with permission of the copyright holder and Elsevier Science Publishers, Inc. ***[45]***).

There are certain other caveats for optimal, reproducible applications of cIEF for glycoproteins. It is important to preserve the nature and stability of the coating on the capillary, as this will improve overall performance and overall reproducibility, which can be quite good. For example, **Fig. 1** illustrates three electropherograms of human transferrin superimposed to show the overall reproducibility of the pattern possible by cIEF ***(45)***. In this particular example of the two-step method for cIEF, a very large number of runs of the glycoprotein were performed (500), with the same capillary. The darker traces (those to the right in **Fig. 1**) represent two consecutive runs (nos. 15 and 16), and these electropherograms overlap almost perfectly. As indicated in **Subheadings 2.2.** and **3.2.**, all reagents must be replaced (buffer replenishment) after a few runs (e.g., every five runs) to achieve maximum reproducibility. It is also very

important to replace the catholyte (in this case, 40 m*M* NaOH) and mobilizer, because they lack buffering capacity, and their pH changes continuously during the analyses. If multiple analyses are performed from the very same sample vial, this solution can become contaminated by carryover of solutes on the electrode and capillary external surfaces. When this occurs, the migration times during mobilization are affected, decreasing reproducibility. It is possible, that even with the above precautions, after several hundred runs (left, lighter trace in **Fig. 1**), the peaks may have shifted toward the detector (cathodic drift), with shorter migration times. But still, the overall reproducibility of the separation profile is excellent ***(45)***. In this particular application of cIEF, the two-step method was applied: a separate focusing and then mobilization step.

1.2. Method of Operation of cIEF, One-Step vs Two-Step Methods

cIEF consists of filling a capillary, as used in conventional CZE, coated or uncoated, with a mixture of ampholytes, of varying pH ranges or a single, fixed range, together with other buffer additives, and then introducing the sample. The sample can be introduced by injection syringe or by electrokinetic means or by pressure applied at the sample vial end. Samples can be first mixed with the buffer components (ampholytes, base extender, cellulose derivatives, and so forth) prior to introduction into the capillary, or they can be introduced after the capillary is first filled with the run buffer and ampholytes ***(2–9)***. It is also possible to partially fill the capillary, inject the sample, and then completely fill the rest of the capillary with ampholyte, before application of any voltage. Different modes of cIEF operations are possible whether these involve one-step or two-step methods.

In the one-step method, which was used before the technique was commercially perfected, the capillary is filled with the run buffer and sample, and the voltage is applied until the current decreases to zero (ampholyte and proteins have focused) or to a low and stable minimum current. At this point the proteins are mobilized by the residual EOF toward the detection window and each peak appears in sequence, in a pH-dependent fashion ***(1–8)***. If the cIEF is operated in the normal polarity mode, with the proteins focused (fixed in place) with the acidic species nearest to the anode, then the proteins will migrate past the detection window with the most basic proteins eluting first. Once the proteins are focused and reach their respective pI values, they stop migrating in the run buffer, and are ready to be mobilized. In all cIEF modes including the one-step approach, both focusing and mobilization periods are required. Sometimes, as in the one-step method, these two processes occur almost at the same time, although it is obviously desirable to have the proteins first fully focused before they are mobilized. In the one-step approach, there is no separate mobilization step, no change of electrolyte buffer, no pressurization or vacuum

applied, and the focused proteins naturally migrate past the detection window, usually toward the cathode.

In the two-step cIEF approach, which was perfected by a variety of workers and then commercialized by Beckman and other firms, the capillary is first filled with ampholyte, the sample is then introduced, voltage is applied, the proteins are focused to their respective pIs, and then a variety of techniques can be used to mobilize the focused proteins. In some approaches, the cathodic buffer (catholyte) is changed from basic to acidic while the separation voltage is maintained, to force the focused proteins to migrate toward the cathode and past the detection window. Of course, one can also change the anodic buffer, or add a mobilization buffer to the cathodic anolyte, which again drives the proteins toward the detection window and the cathode. There is also salt mobilization, in which the catholyte is usually changed to a neutral salt, again causing mobilization toward the cathode. In some instruments it is also possible to apply a low pressure at the anode to drive the focused proteins toward the cathode, or to apply a vacuum at the cathode end and draw the proteins out past the detection window. Other approaches have used hydrostatic mobilization, in which the anode end of the capillary is physically elevated, and the focused proteins elute toward the cathode and past the detection window by capillary action. Still other approaches described in the literature, but not yet commercialized, have moved the normally fixed detector along the capillary length, toward the anode, thus detecting each protein while focused. In all of these mobilization schemes, it is important to maintain the optimum applied voltage, so that the proteins remain focused and do not diffuse as a result of the mobilization step (especially important in pressure/vacuum/hydrostatic mobilization methods, in which parabolic flow profile might ensue).

Figure 2 illustrates a typical CE/cIEF arrangement, with an indication of the anode, cathode, power supply, detection window, and so forth. Of course, in the one-step method, the sample is usually mixed with the ampholyte and buffer components first, before the solution is introduced in the capillary (syringe, pressure, vacuum). Hence for this method, it is not necessary to move the capillary anode end from the buffer reservoir to the sample and back, to introduce the sample although this is required for the two-step approach.

Most of cIEF is done in what has come to be termed the normal polarity mode. In this case, the direction of the EOF is from the anode to the cathode. Hence, the most acidic species focus closest to the anode and the most basic ones closest to the cathode. Under these conditions, a longer time period might be required for focusing and mobilization compared to a reversed polarity operation in which the anode and cathode are reversed. Now, the anode is closer to the detection window, and with the proper amount of base extender in place, all of the proteins present in the sample can be forced to focus between the

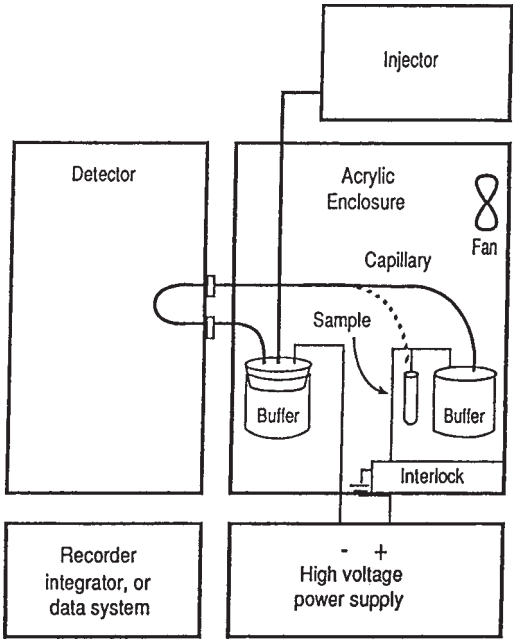

Fig. 2. Capillary electrophoresis instrumentation, schematic.

anode and the detection window. This is achieved using a shorter piece of capillary length, typically 20 cm compared with the normal anode to detection window length of 40 cm. Hence, the total analysis time can be drastically reduced, to less than typically 5–7 min, although this results in poorer resolution and peak capacity compared to the normal polarity operation mode. Again, one must ensure in the reverse polarity mode that all of the species present in the sample are indeed being focused between the anode and the detection window, so that they can be detected. This is not an issue with whole capillary detection, as the entire capillary serves as the detection zone. The real purpose of using the reversed polarity mode is to reduce the total analysis time and to force the proteins to separate and migrate in the shortest time possible.

To determine the absolute pI value of a protein present in the sample, one has to generate, as described below, a valid calibration plot using known, accurate pI marker peptides. These are, by and large, commercially available from a variety of vendors (Sigma, Bio-Rad, Fluka, and others), in high purity, and as single species. However, not all commercial protein/peptide standards are 100% pure, and cIEF can often show multiple components from a purified standard, thus leading to some ambiguity in peak assignment. For the determination of protein pI values it is customary to use known concentrations of at

least three different pI markers to bracket the expected or known pI range of the proteins present. It is not valid to utilize an external standard set of pI markers, because the presence of high concentrations of both standards and sample proteins can influence the ampholyte makeup and resultant pH range.

One thus has to first estimate the approximate pI values of the proteins, using a broad range pH ampholyte mixture. A cocktail of ampholytes can also be used to improve overall resolutions when compared to a single ampholyte mixture (e.g., pH 3.0–10.0 combined with pH 6.0–7.0 or 7.0–8.0, and so forth). When the correct pI markers are used, to bracket the lowest and highest pI values of the proteins present in the sample, the corresponding migration times vs pIs are then used to generate a calibration plot ***(7,9,33)***. The pI of the unknown protein species is deduced from the internal standard calibration plot. Naturally, higher pI accuracy can be obtained by using additional internal markers. It is recommended to use at least three distinct pI markers, one at a pI higher than that expected for any proteins in the sample, another in the middle of the pIs expected, and the third at the other extreme end of the pI range expected. The application of pI calibration plots is illustrated below (*see* **Fig. 5** and **Note 14**) with an actual example. cIEF can be used to identify an unknown protein based on its determined pI value, and it can also be used to determine percent composition or peak area ratios for a mixture of species, in a semiquantitative approach.

1.3. Problems Encountered in cIEF Operation

A frequent problem observed in cIEF is protein precipitation, which results in unwanted spikes, ambiguities in peak identification, and difficulty in the determination of pI values. This can be alleviated, to a large degree, by reducing the concentration of the sample, adding a solubilizing agent, such as urea or guanidine (urea can, at times, add to a protein or create variants, which then leads to artifactual peaks not coming from the original sample), adding an organic solvent (methanol or ethanol), or even heating the capillary to increase solubilization.

Baseline drift is yet another problem as this leads to difficulties in peak assignment and shifts in migration times vs pI value, thus preventing a reliable calibration plot. Baseline drift is generally caused by the ampholytes used and UV absorbing components. This problem can be alleviated by changing the source of ampholyte, or by using proper cleanup procedures, as suggested previously.

Peak spikes can also be caused by a high concentration of salt in the sam ple. Salts also precipitate when they are focused, especially zwitterionic salts. Desalting the sample by dialysis or ultrafiltration–microconcentration is often recommended prior to performing any cIEF.

As in other modes of CE operation, heat buildup can lead to convection currents in the capillary, excess diffusion, broad peaks, voltage fluctuation, and lack of reproducibility. Thus, heat control using Peltier cooling or a convection fan, and a narrow capillary inner diameter, are recommended when running high voltage cIEF (>100 V/cm). The heat buildup is proportional to the applied voltage, during either the focusing or mobilization stages, and can be prevented using the aforementioned precautions.

Another problem in cIEF is related to the length of the analysis, often exceeding 30–40 min (*see* **Note 1**). As mentioned earlier, this can be reduced by using the reversed polarity mode of operation or by shortening the total capillary length. However, reducing the capillary length will also decrease peak capacity, peak efficiency, and the overall resolution. Thus, a reasonable compromise is needed to attain proper resolution and analysis time. This can be achieved by the judicious choice of capillary length and coating, applied voltage, focusing time, mobilization method and time, buffer viscosity, ampholyte range, and buffer additives.

Anodic or cathodic drift is a phenomenon that also occurs in flat-bed IEF, although it is more significant in cIEF. This causes peak broadening, reduced resolution, and a nonlinear calibration plot of the pI markers. At times, this can be overcome by replenishing the electrolytes or changing their concentrations, or even by replacing the ampholytes to diverge from the pH of the electrode buffers. If one end of the ampholyte pH range approaches that of either electrode buffer, then anodic/cathodic drift is likely to occur.

2. One-Step Approach

2.1. Materials

1. Proteins, including horse cytochrome *c*, bovine pancreas α-chymotrypsinogen A, horse heart myoglobin, β-lactoglobulins A and B, ovalbumin, bovine pancreas ribonuclease, and bovine serum albumin (all highest grades available from Sigma Chemical Co.).
2. Pharmalyte pH 3.0–10.0 (Sigma, IEF grade).
3. TEMED, reagent grade (Sigma).
4. Methylcellulose (mol wt 86,000, viscosity of a 2% solution = 4000 cP) (Sigma).
5. Phosphoric acid (85% solution) (Sigma).
6. NaOH, reagent grade, 50% solution (Sigma).
7. An uncoated, 50-µm inner diameter (i.d.), 360-µm outer diamter (o.d.) fused silica capillary (Polymicro Technologies, Phoenix, AZ); a 50-µm i.d. CElect-H150-coated capillary (Supelco, Inc., Bellefonte, PA).
8. CE instrumentation consisted of an Isco Model 3850 capillary electropherograph (Isco, Inc., Lincoln, NE), with either the 50-µm i.d. uncoated capillary or the 50-µm i.d. coated capillary, 60-cm total length, either 40 or 20 cm anode to detection (normal or reversed polarity modes).

2.2. Methods

1. Anolyte solution: 10 or 20 m*M* phosphoric acid, prepared fresh daily by dilution of an 85% stock phosphoric acid solution. Catholyte solution: 20 m*M* NaOH, and the 100 m*M* NaOH solution used to rinse the uncoated capillary in between each run, were prepared daily. All acid and base solutions were filtered daily (0.45 μm) and degassed just before use.
2. Stock cIEF solution/buffer: 5% (v/v) Pharmalyte 3–10 and 0.1% methylcellulose (w/v), prepared in 50-mL quantities and stored at 40°C. This solution is stable for at least 2 wk.
3. TEMED (1.4%, v/v) was added to the sample just prior to performing the separations, and the sample was then filtered through a 0.45-μm 1-mL syringe filter.
4. A standard protein mixture consisting of horse cytochrome *c*, pI 9.3; α-chymotrypsinogen A, pI 8.7; horse heart myoglobin, pI 7.4 and 7.0; β-lactoglobulins A and B, pI 5.1 and 5.3; and ovalbumin, pI 4.7, each dissolved in the cIEF solution at a concentration of 0.5 mg/mL. Ribonuclease and bovine serum albumin were dissolved at the same concentration (*see* **Notes 2–5**).
5. In actual operations, a field of + or - (reversed polarity) 400 V/cm was applied across the length of the capillary.
6. Detection was at 280 nm; detection below this wavelength is not recommended owing to absorbance of the carrier ampholytes.
7. The uncoated capillary was rinsed with a 0.1 *N* NaOH solution and deionized water before being loaded with the protein–ampholyte sample mixture. The coated capillary was rinsed with only water between runs. It should not be treated with NaOH or acid rinses.
8. Data were collected with a CompuAdd 810 PC (CompuAdd, Austin, TX), but any modern laboratory computer (PC or Mac) could be used for data acquisition and compilation/readout.
9. With the instrument used here, a syringe flush port is used to rinse the capillaries and load samples for performing cIEF. However, with more modern CE instruments, which do not have a syringe flush port, rinsing and loading of the capillaries can be done automatically, as in the two-step approach described in **Subheading 3.** With a syringe-loaded injection port instrument, it is required that the entire length of the capillary be filled, as it is very difficult to selectively and reproducibly load a portion of the capillary each time (*see* **Note 6**).
10. Once the capillary is loaded with the sample–ampholyte mixture, the field is turned on. For fields of 400 V/cm in 50-μm i.d. capillaries, the initial current should be about 16–22 μA (*see* **Notes 7–9**). As focusing proceeds, the current should decay in an exponential fashion. Final currents should not be much lower than 2–3 μA. This current should remain stable during the entire mobilization step also, the time dependent on the specific capillary surface and EOF present.
11. The cIEF run is completed when all of the proteins in the sample have migrated past the detection window (*see* **Notes 10–13**). The detector output, whether fixed wavelength UV, PDA, LIF, or MS, is collected on a CE dedicated computer with appropriate chromatographic software for data handling, electropherogram print-

out, peak area/height calculations, migration time determinations, and integration for quantitation purposes.

12. pI determinations for individual proteins in a sample are made using a calibration plot derived from the peptide or protein markers (standards) added to the sample, after the sample alone is first run/analyzed.

3. Two-Step Approach

3.1. Materials

1. Separations are performed on a the P/ACE MDQ System (Beckman Coulter, Inc., Fullerton, CA).
2. An eCap neutral, coated capillary (Beckman) was employed, with dimensions of 50-µm i.d. × 31 cm (20 cm to the detector), to eliminate all EOF.
3. Sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), hydroxypropylmethylcellulose (HPMC), and TEMED were from Sigma.
4. Anolyte, catholyte, and mobilizer were from Bio-Rad Laboratories (Hercules, CA).
5. The Pharmalyte 8–10.5 was from Pharmacia Biotech (Piscataway, NJ), and Bio-Lyte 3–10 was from Bio-Rad Laboratories.
6. All recombinant human immunoglobulin Gs (IgGs) were from BASF Bioresearch Corporation (Worcester, MA). These were initially diluted to a concentration of 0.25 mg/mL for the cIEF analyses.

3.2. Methods

1. cIEF was performed with "normal" polarity (cathode nearest the detector).
2. Detection was set at 280 nm.
3. The anolyte, catholyte, and mobilizer were 20 m*M* phosphoric acid, 40 m*M* NaOH, and a zwitterionic solution (proprietary), respectively.
4. The ampholyte solution was a mixture of diluted Pharmacia pH 8.0–10.0, Bio-Lyte, pH 7.0–9.0, and Bio-Lyte pH 3.0–10.0. This also contained 0.4% TEMED (v/v) and 0.2% HPMC (w/v). The dilution factor was 20. The mixing ratio (v/v) was 8:1:1.
5. The pI standard markers (pI 10.1, 8.4, and 7.9) were diluted 1:20 with HPLC-grade water.
6. Samples for cIEF consisted of 70 µL of 0.25 mg/mL monoclonal antibody (mAb) with 100 µL ampholyte solution plus 4 µL of each pI standard marker, as above (*see* **Notes 15–17**).
7. The cIEF capillary was preconditioned by first rinsing with HPLC-grade water for 2 min at 20 psi, followed by a rinse with 20 m*M* H_3PO_4 for 2 min at 20 psi, and then another water rinse for 2 min.
8. The sample was introduced into the capillary by filling the entire capillary using positive pressure at 20 psi. Alternatively, the sample was introduced into the capillary by performing a 1-min rinse (20 psi) from sample vial to waste vial ***(44)***.
9. The focusing electric field was 580 V/cm for 8 min, followed by a mobilization field of 645 V/cm for 28 min or until all of the proteins of interest and standard

markers had migrated past the detector. Alternatively, the focusing electric field was 500 V/cm for 2 min, followed by the application of a low-pressure (0.5 psi) rinse mode with a field strength of 500 V/cm from anolyte to catholyte ***(44)*** (*see* **Notes 18** and **19**).

4. Notes

1. The reversed polarity mode allows a shorter separation distance (20 cm vs 40 cm) and faster run times, at the expense of lower resolution (see the preceding discussion).
2. To ensure that any minor protein bands will be detected, the concentration of the major protein should be at least 0.1 mg/mL.
3. When proteins in solution are to be analyzed, the solution should be diluted at least 10-fold with the cIEF solution (run buffer).
4. The final concentration of salt in the sample should be <10 m*M*.
5. In fully loading a capillary using this CE instrument, with a 50-μm i.d. capillary of 60 cm length, with an internal volume of about 1.2 μL, a sample of 4–5 μL should be forced through the capillary to ensure that it has been effectively loaded with sample.
6. With modern CE instruments, having precise vacuum or pressure injection devices for rinsing and loading the capillary, it is possible to reproducibly load only certain sections of the capillary with sample/ampholyte. Thus, loading with sample could be done in that section of the capillary prior to the detection point. This would avoid the problem with the instrument used here (Isco) of having sample loaded past the detection point and perhaps being lost.
7. If the initial current seen when the voltage is first applied is higher than 16–22 μA, this may be due to too much TEMED or salt in the sample. For lower values of current generated, one should immediately suspect improperly prepared buffers or not enough TEMED. For 75-μm i.d. capillaries, the initial current should be about 40–50 μA.
8. During the focusing stage, if the current does not decay exponentially, or if the current ever drops below 2 μA, one should immediately suspect bubble formation during the run or improperly prepared buffers.
9. Bubble formation requires one starting over the entire procedure, so that the capillary has to again be rinsed, refilled with sample/ampholyte, and the entire run needs to be repeated. Bubbles arise from localized heating due to salt buildup or a high preconcentration of a protein, and often efficient cooling of the capillary during the actual run can prevent bubble formation altogether. Adequate degassing of the sample and ampholyte mixture, just before introduction to the capillary, can also help prevent bubble formation during a run.
10. Uncoated capillaries tend to have changing EOF with time of the run, as the more acidic pH range of the ampholyte runs through the capillary. EOF is highest at basic pH and lowest at acidic ranges. Thus, the EOF will change with time during mobilization using an uncoated capillary. With a coated capillary, there is usually a very constant EOF, independent of the particular pH range moving through the capillary during the mobilization step.

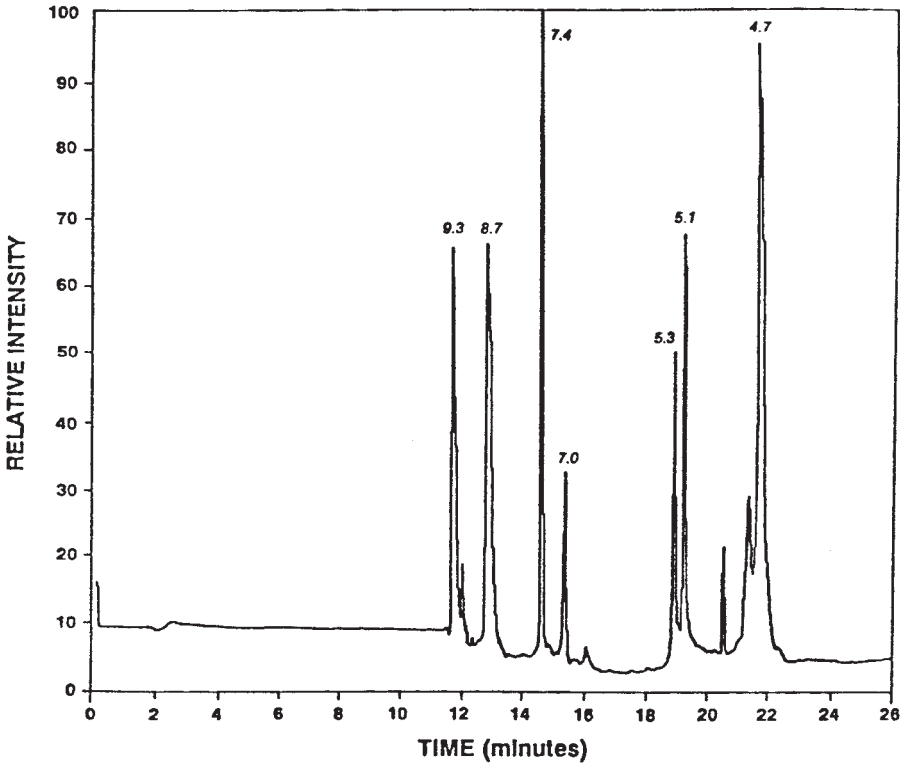

Fig. 3. cIEF of standard proteins in a C-8 coated capillary, 20-cm separation distance, reversed polarity mode. Conditions: 50-µm i.d., 60 cm total length, 400 V/cm, UV detection at 280 nm, 0.05 AUFS. Anolyte: 20 m*M* phosphoric acid; catholyte: 20 m*M* NaOH. Proteins dissolved at concentrations of 0.5 mg/mL in 5% Pharmalyte 3-10, 0.1% methylcellulose, 1.4% TEMED. Peak identification by isoelectric point and injection of each standard protein separately. (Reprinted with permission of the copyright holder and Academic Press, Inc. *[2]*).

11. To ensure complete detection of all species in the sample having unique pI values, one needs an idea of how many proteins may be present in a given sample, their approximate pI range, and the approximate concentrations of all species present. Low concentrations of proteins makes detection problematic and/or impossible.
12. Standard protein or peptide markers should be run prior to running any samples, to ensure that these can be resolved in the ampholyte range being used. **Figure 3** indicates a typical cIEF for standard proteins in a C-8 coated capillary, using a 20-cm separation distance and reversed polarity mode of operation. Other conditions are as indicated in the caption to **Fig. 3**.

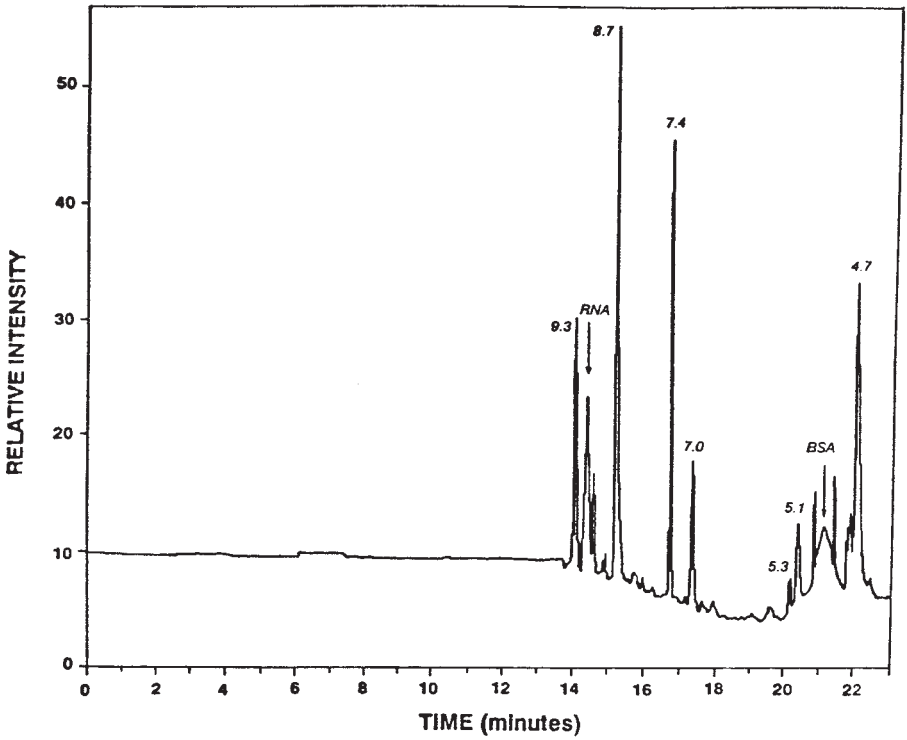

Fig. 4. cIEF of ribonuclease and bovine serum albumin in a C-8 coated capillary, 20 cm separation distance. Other conditions as in **Fig. 3**. Peaks are identified by their pIs, except for ribonuclease (RNA) and bovine serum albumin (BSA), the unknown proteins in the sample. (Reprinted with permission of the copyright holder and Academic Press, Inc. *[2]*).

13. Known pI markers should be added to the sample–ampholyte mixture when pI determinations of unknown or suspected samples is to be performed. At least three different pI markers should be used, usually peptides of known amino acid composition, at the same approximate concentration as for the proteins in the actual sample. It is essential, of course, that the pI markers focus differently than the sample proteins, or these will be lost to detection and identification. **Figure 4** illustrates a typical cIEF analysis for two proteins, ribonuclease and bovine serum albumin, along with standard proteins.
14. In deriving the correct pI value for an unknown protein in the sample, ideally three or four individual markers are added to the sample, and a linear calibration plot is derived (**Fig. 5**). At times, especially with an uncoated capillary, two separate calibration plots may prove more accurate in predicting pI values, especially if a sample contains proteins with very high and very low pIs (**Fig. 5**). An exter-

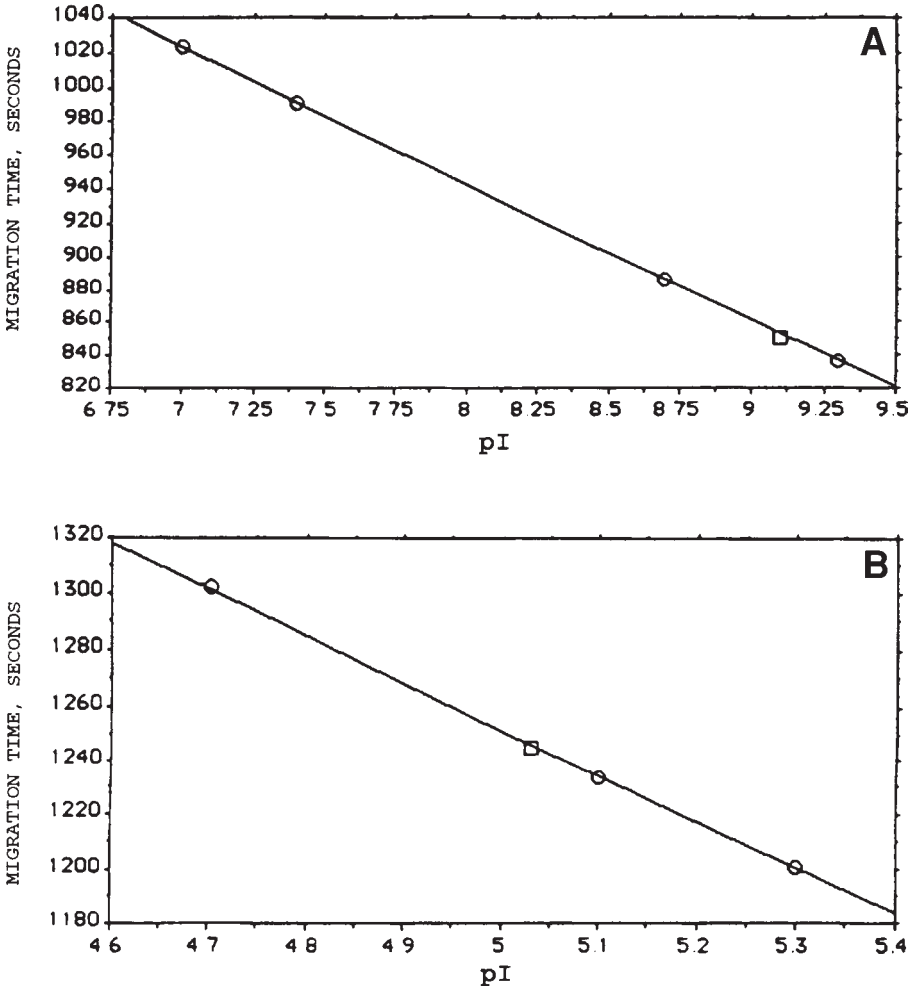

Fig. 5. **(A)** Plot of migration time vs pI for basic and neutral standards in Fig. 3. Ribonuclease identified by a *square*, standards by *circles*. **(B)** Plot of migration time vs pI for acidic standards in **Fig. 3**. Bovine serum albumin identified by a *square*, standards by *circles*. (Reprinted with permission of the copyright holder and Academic Press, Inc., *[2]*).

nal standard calibration plot should never be used to determine pIs of unknown or suspected proteins in a sample, this is not accurate or useful. It is important to recognize that the addition of marker proteins, and even concentration-depen-

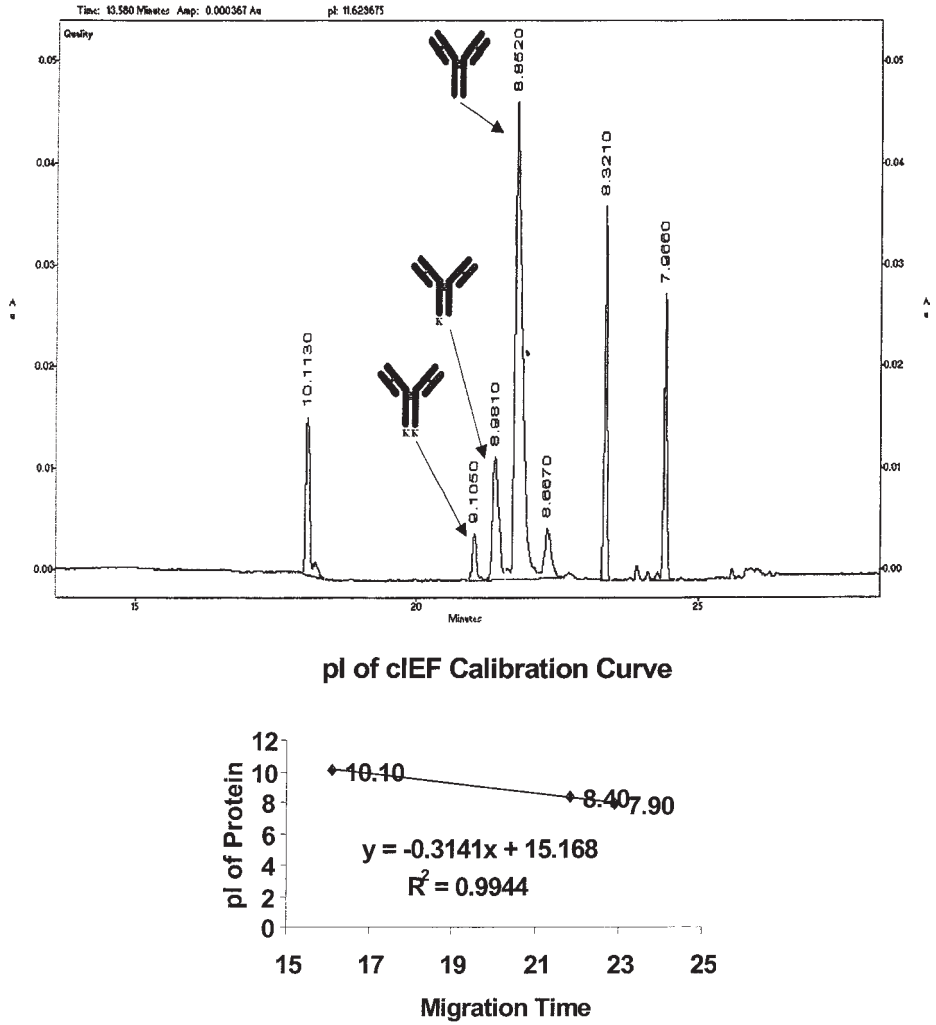

Fig. 6. pIs of mAb isoforms calculated by an internal standard calibration curve (*inset*, *bottom*). The pI calibration curve from three standards, with the equation of the straight line and the correlation coefficient, R_2. (Reprinted with permission of the copyright holder and Academic Press, Inc. *[7]*).

dent sample proteins, may change the nature of the ampholyte mixture. This can cause changes in migration patterns and elution times, and thus only internal standards should ever be used to derive pI values *(2)*.

15. Under the above conditions (**Fig. 6**), the cIEF electropherogram of the Chinese hamster ovary (CHO) mAb had three peaks, plus the internal standard markers. These three peaks were due to the presence of different lysine (Lys) variants present in the antibody sample. As indicated in **Fig. 6**, the earliest eluting peak was the most basic of the variants, eluting about 21 min, shown to contain the 2-Lys variant on the Fc portion of the mAb. The other two major peaks, eluting later than this variant, contained 1-Lys and 0-Lys, thus being less basic or more acidic, and eluting later in the electropherogram.
16. cIEF is also highly quantitative, unlike flat-bed IEF, which is only semiquantitative. Quantitative analysis of the three isoforms here, based on peak area percent, were: 91.9% 0-Lys, 2.1% 1-Lys, and 7.1% 2-Lys ($n = 8$).
17. Further studies are possible in cIEF using papain, enzymatic digestion of the intact mAb, in order to form the Fab and Fc species, so that the specific location of the Lys residues can be easily determined. Thus, carboxypeptidase B (CPB) has been used to digest the intact mAb, remove all C-terminal Lys residues, and changes in the three peaks of **Fig. 6** became evident (three peaks collapsed to a single peak, having the same elution time as the original 0-Lys species) ***(7,9)***. Other enzymatic digestions are always possible prior to cIEF of the newly formed species, such as peptide mapping ***(2)***.
18. As with the one-step method, the standard markers act as internal calibrators to allow one to automatically calculate the pIs of the isoforms in this mAb (**Fig. 6**, inset). Thus, the pI for the 0-Lys was determined to be pH 8.85, for the 1-Lys pH 8.98, and for the 2-Lys, pH 9.11.
19. The precision of the above determination of pI values was excellent, with same-day reproducibility of the pI values typically being 0.1% relative standard deviation (RSD) and day-to-day and lot-to-lot reproducibility less than 1% RSD .

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13

Glycoprotein Analysis by Capillary Zone Electrophoresis–Electrospray Mass Spectrometry

Kevin P. Bateman, John F. Kelly, Pierre Thibault, Louis Ramaley, and Robert L. White

1. Introduction

The study of glycoproteins as intact molecules or as smaller fragments generated by chemical or enzymatic digestion is well suited to the technique of capillary zone electrophoresis–electrospray mass spectrometry (CZE–ESMS). Separation of glycoform populations of intact glycoproteins or protein digests is possible using buffers compatible with mass spectrometric detection ***(1–8)***. CZE–ESMS analysis of intact proteins can provide semiquantitative information about the degree of heterogeneity of the glycoprotein ***(1,6,7)***. Enzymatic or chemical digestion of the glycoprotein, followed by CZE–ESMS, gives a direct measure of individual sites of heterogeneity ***(1–5)***. Combined CZE-collision induced dissociation (CID) mass spectrometric experiments (CZE–MS–MS) of digests enables the characterization of oligosaccharide structures ***(1–5)***. In particular, CID of glycopeptides is characterized by fragment ions corresponding to cleavage at each glycosidic bond. The occurrence of specific carbohydrate residues such as hexose (Man, Glc, Gal), *N*-acetylhexosamine (GlcNAc, GalNAc), or *N*-acetylneuraminic acid (NeuNAc) can be monitored by the observation of characteristic oxonium ions at *m/z* 163, 204, and 292, respectively. More recently, CZE-front-end CID–MS–MS has been shown to be a powerful tool for peptide sequencing of glycopeptides using subpicomole quantities of injected glycoprotein ***(3–5)***.

The poor concentration detection limits associated with CZE can be improved by using mass spectrometric detection. The sensitivity of this technique is further enhanced by improving the electrospray interface ***(2,5,8–14)***.

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Further improvements are achievable by using CZE with online solid-phase preconcentration coupled with electrospray ionization ***(4,15–18)***.

This chapter discusses the methodology for interfacing CZE with ESMS using micro- and nanoelectrospray ionization to give maximum sensitivity. The use of on-line preconcentration to further enhance glycopeptide detection is also addressed. Examples of the analysis of intact glycoproteins are demonstrated with reference to data display and interpretation. Methods for the specific identification of glycopeptides from glycoprotein digests by CZE–ESMS, CZE–ESMS–MS, and CZE-front end CID–MS–MS are also presented. An example of the improved sensitivity provided by on-line preconcentration is presented for a glycoprotein lectin. It should be noted that there are many different approaches for interfacing CZE with mass spectrometry, some easier than others. Also, electrospray tips are now commercially available from several suppliers which may simplify the experimental method.

2. Materials

2.1. Cationic Coating of Capillary

1. Fused silica capillary (50 µm inner diameter [i.d.] × 360 µm outer diameter [o.d.])
2. 1 *M* NaOH; deionized water; methanol; acetic acid.
3. Hexadimethrine bromide (polybrene); ethylene glycol (Aldrich).
4. Silane reagent: 7-oct-1-enyltrimethoxysilane (Hüls America Inc., Bristol, PA).
5. Cationic acrylamide and associated reagents: [(acryloylamino)propyl]trimethylammonium chloride (Chemische Fabrik Stockhausen, Krefeld, Germany) or [3-(methacryloylamino)propyl]trimethylammonium chloride (Aldrich), ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED).
6. GC column cleaning kit (Supelco).

2.2. Electrospray Interface for CZE–ESMS

1. Cationically coated fused silica capillary (50 µm i.d. × 360 µm o.d.) cut in 10-cm lengths.
2. Microtorch and small weight (15 g).
3. 48% HF solution; syringe pump.
4. Edwards 306A (or similar) high vacuum coater with gold target electrode.
5. Silver conductive paint.
6. Gold plating solution, gold counter electrode, and power supply.
7. Capillary butt connector (Supelco, Oakville, ON, Canada).
8. 10 µg/mL Leu-enkephalin in 0.1 *M* formic acid.

2.3. On-Line Preconcentrator

1. C_{18} packing material (>40 µm in size); methanol.
2. Teflon® tubing, 300 µm i.d. (LC Packings, San Francisco, CA).

2.4. Proteolytic Digestion

1. Glycoproteins and lectins (Sigma).
2. Digestion buffer: 0.1 *M* ammonium bicarbonate, pH 8.1.
3. TPCK-trypsin and endoproteinase Glu-C (Promega, Madison, WI) with dissolution buffer.

2.5. Separation of Glycoproteins and Glycoprotein Digests by CZE–ESMS

1. CE instrument model 310 (Thermo Bioanalysis Corp., Santa Fe, NM) (approx 90 cm length).
2. Rinsing electrolyte: Formic acid, acetonitrile.
3. Separation electrolyte: Formic acid, acetonitrile.
4. Sciex API-III$^+$ mass spectrometer (Perkin Elmer/Sciex, Concord, Ont.).

3. Methods

3.1. Capillary Coatings

3.1.1. Polybrene Coating (See **Notes 1**, **2** and **Figs. 1–3**)

1. Sequentially rinse capillary (cut to desired length) for 20 min each with 1 *M* NaOH, deionized water, and polybrene (5% w/v)–ethylene glycol (2% v/v) in deionized water.
2. Rinse capillary with separation electrolyte for 10 min.
3. Recondition capillary between runs with 1.0 *M* NaOH (1 min), deionized water (1 min), polybrene solution (4 min), and separation electrolyte (4 min).

3.1.2. Covalent Cationic Coating (See **Notes 2–4** and **Figs. 4–6**)

1. Filter all buffers through a 0.45-µm filter (Millipore) to prevent capillary blockage.
2. Rinse capillary (5 m) with 1 *M* NaOH, deionized water, and methanol, each for 1 h at 20 psi, using the GC column cleaning kit attached to a nitrogen gas cylinder.
3. Rinse a solution of 20 µL of 7-oct-1-enyltrimethoxysilane and 20 µL of glacial acetic acid in 4 mL of methanol through the column overnight (8–12 h) at 20 psi.
4. Rinse with methanol and deionized water (1 h each, 20 psi).
5. Make a solution of 8 µL of TEMED, 56 µL of aqueous ammonium persulfate (15% w/v), and acrylamide reagent (2% v/v) in 4 mL of deionized water. Immediately rinse through the column for 8 h (or overnight) at 20 psi.
6. Flush the capillary with deionized water for 1 h and store until needed. Prior to use, cut to desired length and flush with CZE buffer for 5–10 min.

3.2. Interface Construction

1. Taper the capillary by suspending the weight from one end of the capillary and melting the fused silica with the flame from a microwelding torch.
2. Trim the thinnest portion of the tapered column to 2–3 cm, and insert the shortened tip into a larger i.d. capillary (100 µm i.d.), and bend until the tapered end snaps.

3. Further sharpen the tip by etching in a stirred solution of 48% HF for 15 min. During this process use a syringe pump to rinse the internal volume of the capillary with filtered water (6 µL/min) to prevent the acid from etching the inner surface. After etching, rinse the capillary surface with water and purge with N_2 to remove any traces of HF.
4. Insert 20–30 tips into a 1.5-cm thick piece of foam at a 45° angle. Position the support foam with the tips 5 cm from the gold target electrode on the stage of the high vacuum coater. Sputter-coat the tips with gold for 15–20 min by maintaining a voltage and current across the electrodes of 1.0 kV and 20 mA, respectively.
5. Cover the gold-coated portion of the tip with silver conductive paint to within 1 cm of the tapered tip to prevent the stirred and heated (60°C) gold plating solution from removing the sputter-coated gold. To prevent blockage continuously rinse the tips with deionized water (6 µL/min) during the plating process. The plating current was maintained at 0.75 mA for 30 min.
6. Connect the tip to an appropriate length of capillary using a Supelco connector.
7. Load capillary with tip in the CZE instrument and mount tip on interface of the mass spectrometer. Infuse the Leu-enkephalin at approx 200 nL/min and apply an electrospray potential of +2.5 kV. Maximize the signal intensity by varying the position of the sprayer and the electrospray voltage.

3.3. On-Line Preconcentrator Construction (See *Note 5, Figs. 5 and 7)*

1. Suspend several milligrams of C18 packing material in methanol.
2. Attach a 2-cm piece of Teflon tubing to a short (5 cm) piece of fused silica capillary.
3. Using standard fittings attach the fused silica to a syringe and draw the packing material suspension into the Teflon tubing. Several pieces of tubing can be packed from this one suspension and used as needed.
4. Verify that a bed of 1 mm is formed using a light microscope.
5. Attach the Teflon tubing with C_{18} material to 15- and 80-cm pieces of covalently coated capillary that serve as the inlet and the outlet end, respectively. The outer diameter of the capillary (360 µm) is appropriate for a push-fit attachment to the Teflon tubing and does not require epoxy or other sealant to prevent leakage.

3.4. Proteolytic Digestions

1. The enzyme of interest is dissolved in the digestion buffer and trypsin or Glu-C is added at a 30:1 or 50:1 (w/w) substrate-to-enzyme ratio, respectively.
2. Digestion is carried out overnight (10–12 h) at 37°C.
3. For Glu-C/trypsin sequential digestions, boil the digest briefly to inhibit the Glu-C. Dry the digest and resuspend it in digestion buffer. Add trypsin at a 30:1 ratio and digest as described in **step 2**.
4. Evaporate the solution to dryness and resuspend in 10 m*M* acetic acid to neutralize remaining digestion buffer. Use a small volume to maintain a high concentration of peptides in the sample.

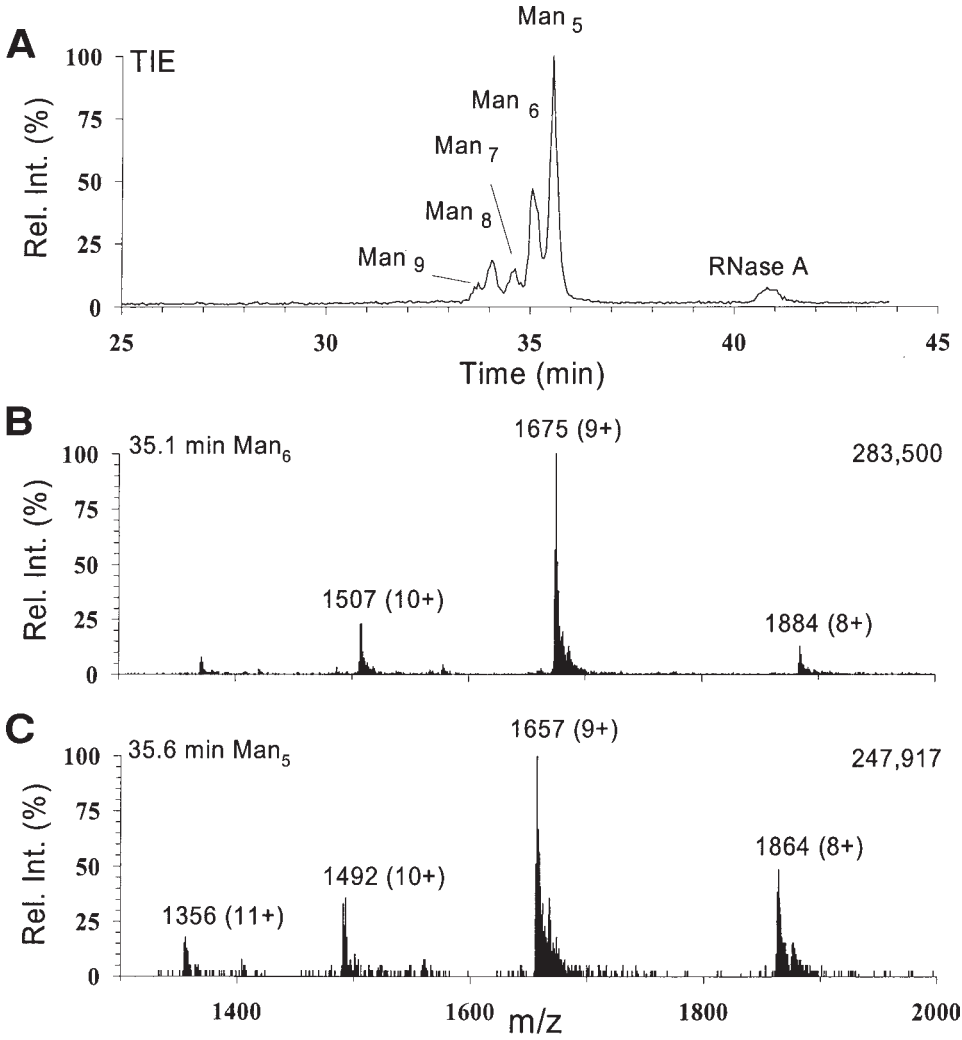

Fig. 1. CZE-ESMS analysis in full-scan acquisition mode of ribonuclease B using 2.0 *M* formic acid on a polybrene coated capillary. Approximately 90 ng of protein was injected on column. Total ion electropherogram for *m/z* 1300 to 2000 **(A)**, and extracted mass spectra for the $GlcNAc_2Man_6$ **(B)**, and $GlcNAc_2Man_5$ **(C)** glycoforms.

3.5. Glycoprotein Separations (See Notes 1, 2 and Figs. 1–4)

1. Resolution of protein glycoforms is improved by using increased ionic strength of formic acid and the addition of acetonitrile *(1)*. Typically 0.1 *M* formic acid is a good starting point, but up to 2 *M* formic acid with 25% acetonitrile (v/v) can be used to enhance separation.

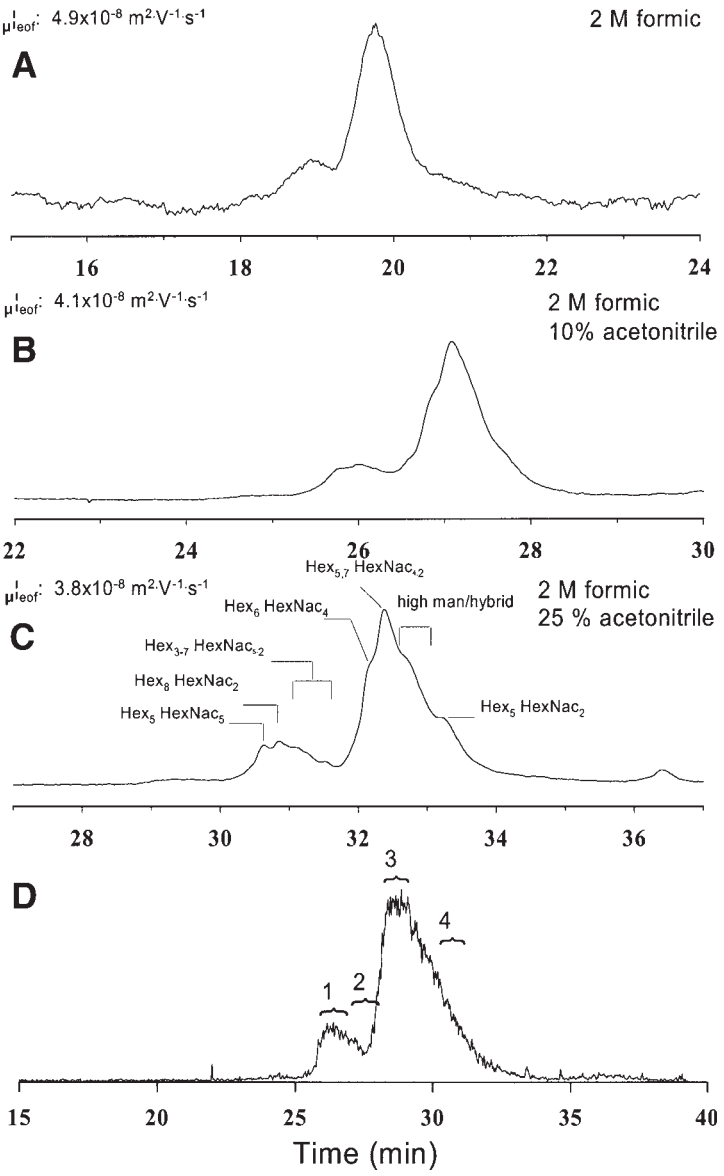

Fig. 2. Influence of acetonitrile concentration on the separation of avidin glycoforms using CZE–UV and CZE–ESMS. CZE–UV separation was obtained using 2.0 *M* formic acid with **(A)** 0%, **(B)** 10%, and **(C)** 25% acetonitrile. The corresponding CZE–ESMS analysis of avidin (TIE for *m/z* 900–1300) using 2.0 *M* formic acid in 25% acetonitrile (v/v) is shown in **(D)**. All separations were obtained using a polybrene coated capillary, and approx 180 ng of protein was injected on to the column. μ_{eof}. Mobility of electrosmotic flow.

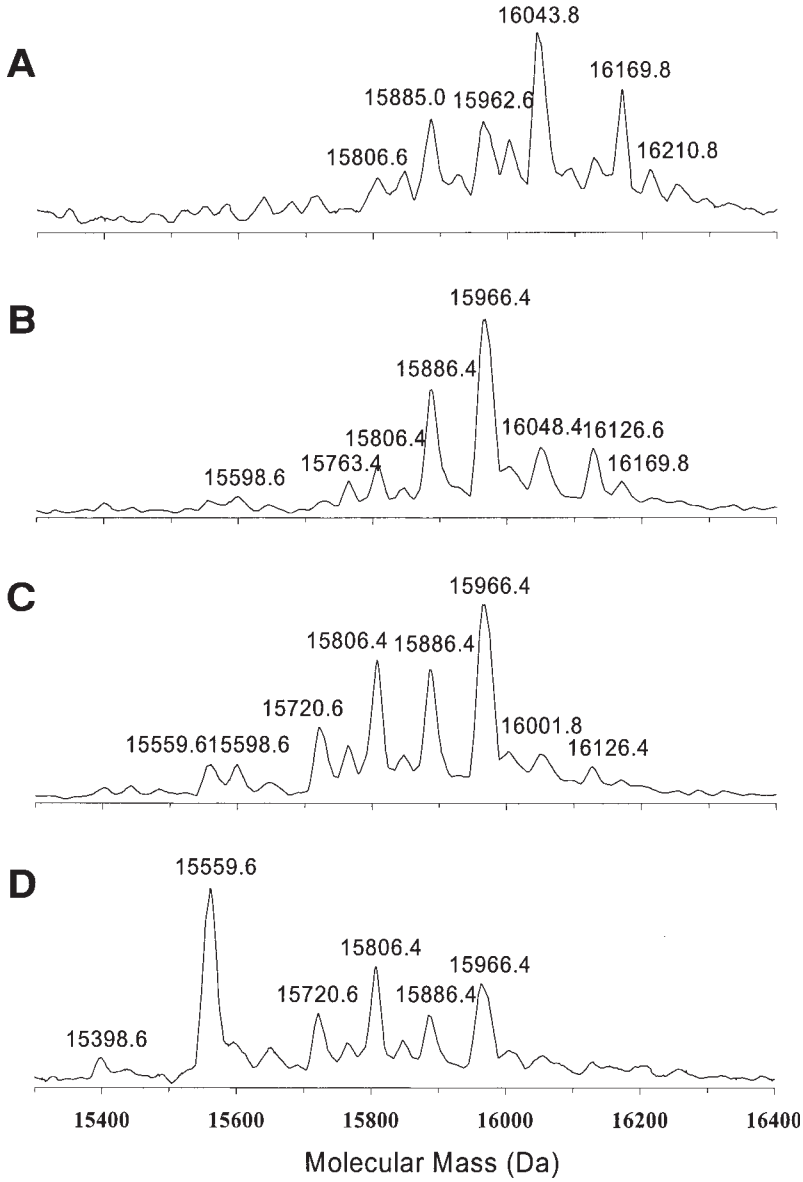

Fig. 3. Reconstructed molecular mass profiles produced using extracted mass spectra for the CZE–ESMS analysis of avidin. Combined mass spectra for peaks 1 **(A)**, 2 **(B)**, 3 **(C)**, and 4 **(D)** identified in **Fig. 2D**.

2. Using selected concentration of electrolyte condition capillary for 5 min.
3. Inject sample, 100 mbar for 0.1 min results in ~30 nL injection volume.

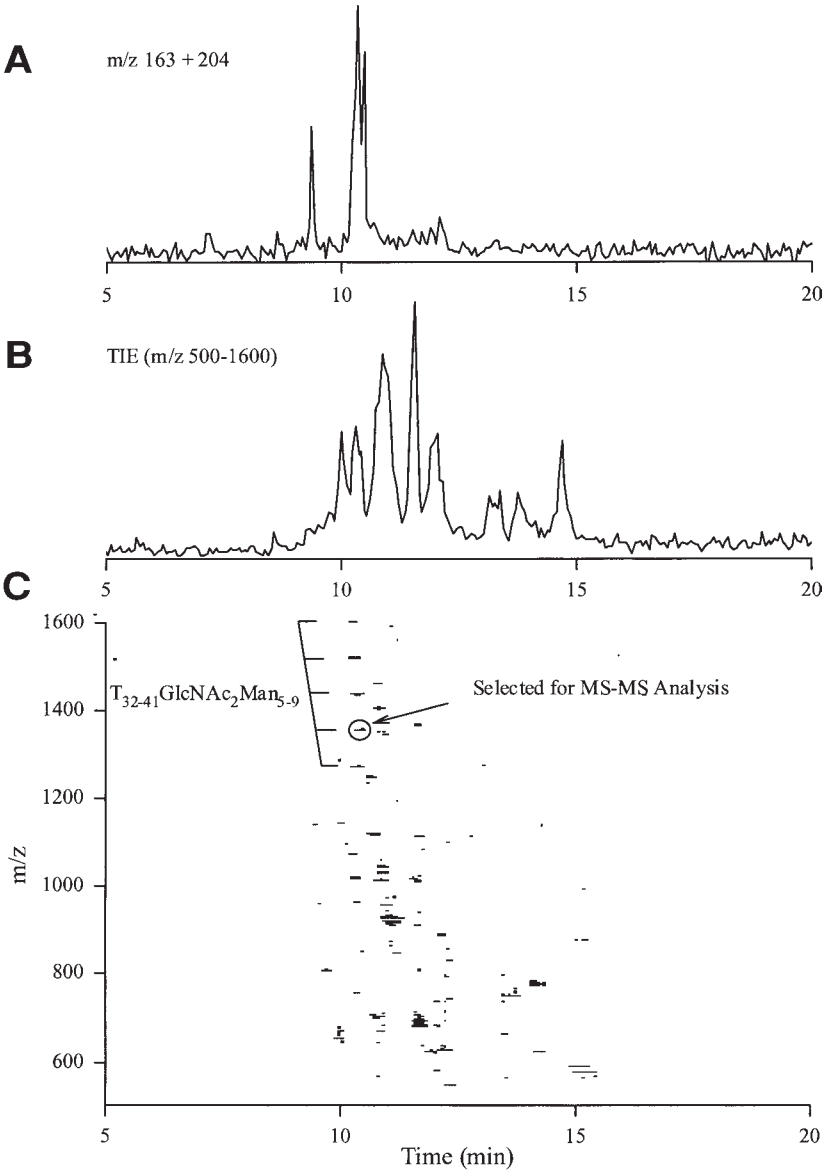

Fig. 4. CZE–ESMS analysis of the tryptic digest of *Phaseolus vulgaris* lectin. Separation performed using 1.0 *M* formic acid on a covalently coated capillary with an applied voltage of –23 kV. Approximately 800 fmol of lectin digest was injected. **(A)** Monitoring of oxonium fragment ions of hexose (*m/z* 163) and HexNAc (*m/z* 204) using an orifice voltage of 100 V. **(B)** Full-scan analysis using an orifice voltage of 50 V. **(C)** Contour profile from full-scan analysis showing the separated glycopeptides (*diagonal line*).

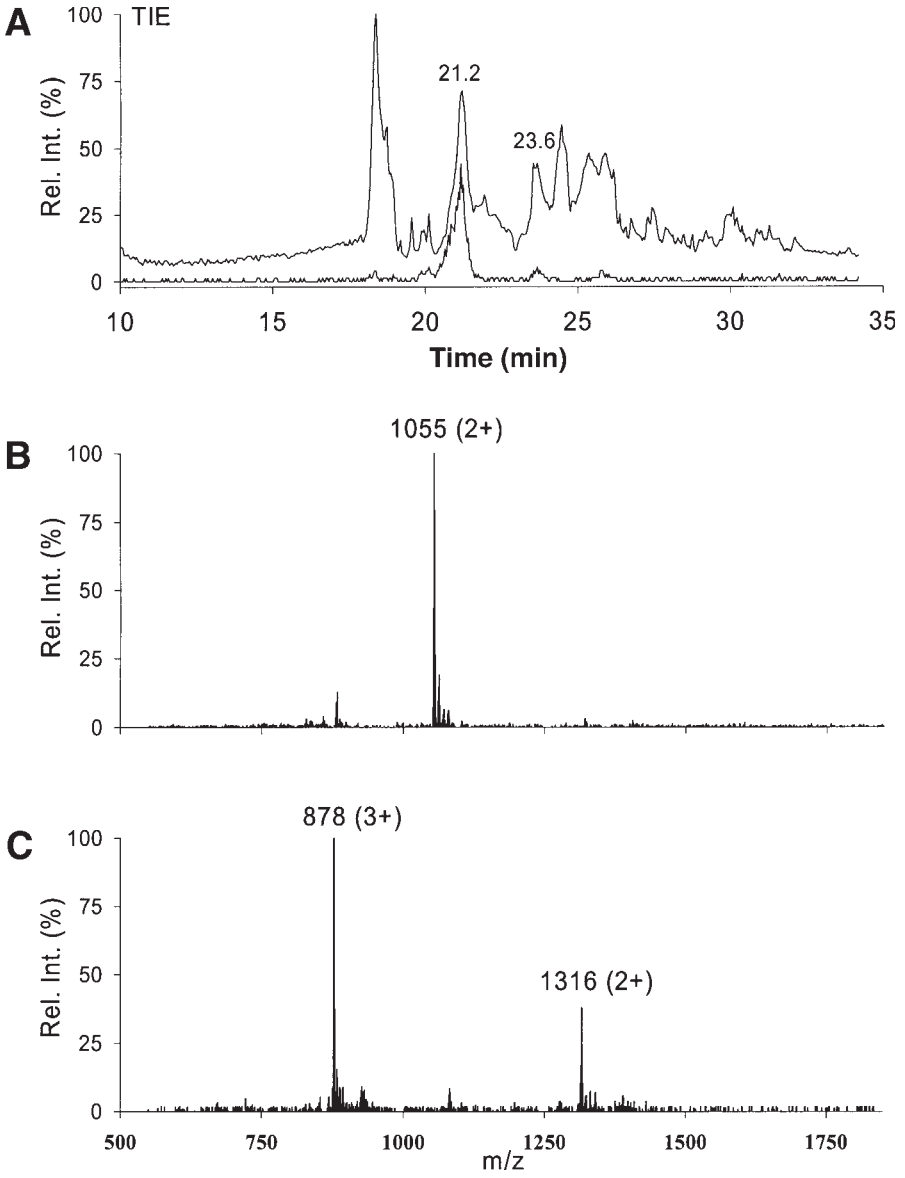

Fig. 5. Preconcentration-CZE–ESMS analysis of the tryptic digest of *Bauhinia purpurea* seed lectin. Four microliters of digest (0.25 pmol) were loaded on the C_{18} preconcentrator. Following rinsing and elution, the peptides were separated by applying a voltage of –22 kV across the 1-m covalently coated capillary. **(A)** Total ion electropherogram for *m/z* 550–1850 using an orifice voltage of 50 V and for *m/z* 366 using an orifice voltage of 120 V. Extracted mass spectra for peaks observed at **(B)** 21.2 min, and **(C)** 23.6 min show excellent signal-to-noise ratio for this low level of analysis.

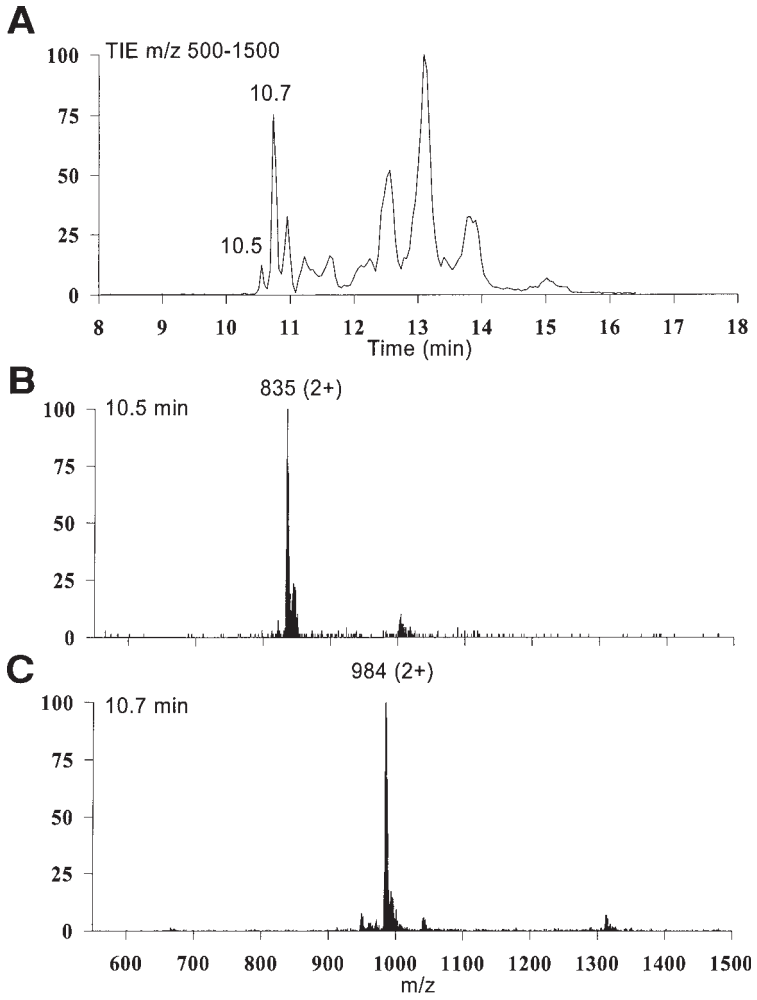

Fig. 6. Analysis of sialylated glycopeptides from κ-casein Glu-C digest using CZE–MS–MS precursor ion scanning for *m/z* 274. **(A)** Total ion electropherogram (*m/z* 500–1500) for the precursor ion , and extracted mass spectra at **(B)** 10.5, and **(C)** 10.7. For this analysis, 5 pmol of digest was injected on a covalently coated capillary and 0.1 *M* formic acid was used as separation buffer. A collision energy of 60 eV and an orifice voltage of 50 V were used.

4. Apply a voltage of –200 V/cm at the inlet end and the optimized electrospray voltage at the tip.
5. Acquire data using appropriate scanning method for up to 40 min.

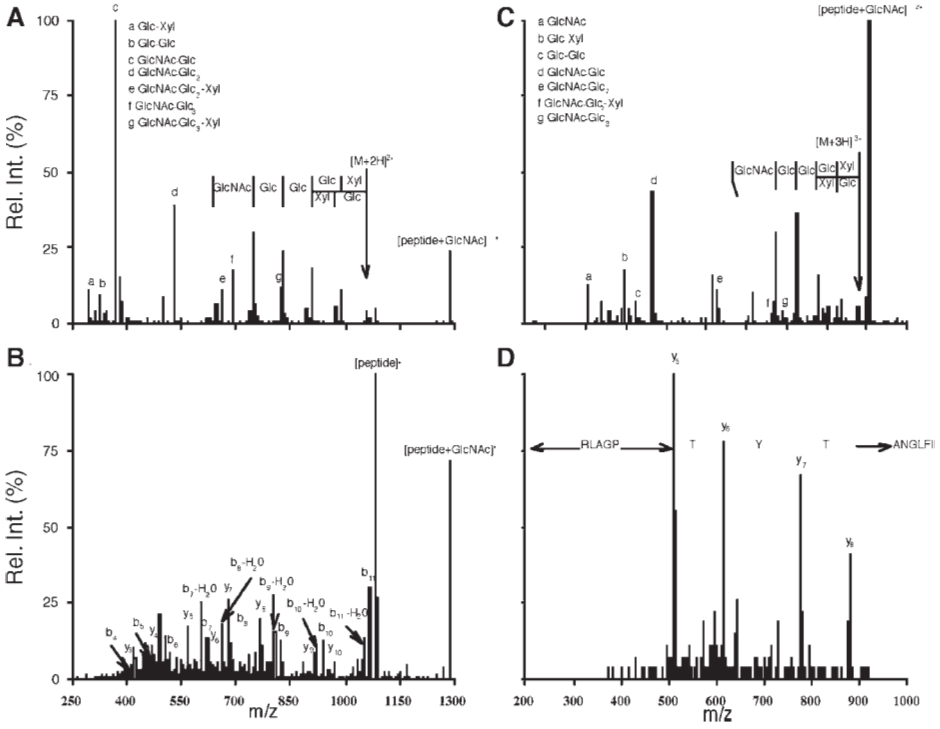

Fig. 7. Preconcentration-CZE–MS–MS and Preconcentration-CZE-front-end CID–MS–MS analysis of the tryptic digest of *B. purpurea* seed lectin. Four microliters of digest (0.25 pmol) were loaded on the preconcentrator. Following rinsing and elution, the peptides were separated by applying a voltage of –22 kV across the 1-m covalently coated capillary. For panels **(A)** and **(C)** the orifice voltage was 50 V and a collision energy of 60 eV was used to fragment the intact glycopeptides at *m/z* 1055 **(A)** and 878 **(C)**. For panels **(C)** and **(D)** the orifice voltage was set to 120 V to induce front-end CID. A collision energy of 50 eV was used for the ions at *m/z* 1288 **(B)** and 906 **(D)**. Lowercase letter correspond to fragment ions whereas capital letters indicate the amino acids. Carbohydrate residues are indicated with the three-letter code acronyms.

*3.6. Preconcentration CZE-ESMS (*See *Note 5, Figs. 5 and 7)*

1. Mount the capillary with preconcentrator in the CZE instrument.
2. Rinse with two column volumes of elution solvent (90:10 acetonitrile–1% HCl) followed by five column volumes of separation electrolyte (0.1 *M* formic acid).
3. Inject the sample at 1000 mbar for 4–8 min. This results in an injection volume of 4–8 µL.
4. Rinse the capillary with four column volumes of separation electrolyte.

5. Elute the sample from the stationary phase using a small plug of elution solvent (100 mbar, 2 min), followed by a brief rinse (1000 mbar, 0.1 min) with separation electrolyte to push the elution solvent through the stationary phase, preventing readsorption of the analyte.
6. Apply separation (–200 V/cm) and electrospray voltages and start the acquisition.

3.7. Scanning Techniques for Glycopeptides

3.7.1. Inact Glycoproteins

1. Full-scan acquisition over an appropriate mass range, as determined by prior direct infusion of the protein.

3.7.2. Glycoprotein Digests

3.7.2.1. Identification of Glycopeptides (*See* **Notes 5–9** and **Figs. 4–6**)

1. Use a mixed scan function whereby a narrow and wide mass range acquisition are obtained within two sequential scanning periods.
2. The high mass range acquisition (*m/z* 500–1800) is obtained at a normal orifice potential (50 V) with a 3 s/scan period.
3. The low mass range (*m/z* 130–400) is acquired during the second period using a high orifice potential (120 V) typically 1 s/scan period on appropriate instrument. A selected ion monitoring function for specific oxonium ions can be substitute for the low mass range scan.

3.7.2.2. Oligosaccharide Structural Features (*See* **Notes 4–9** and **Figs. 7–9**)

1. From the previous experiment, identify the mass of the glycopeptide of interest.
2. Set up a fragment ion scanning experiment for the selected precursor ion.
3. Product ion spectrum is obtained using CZE–MS–MS.

3.7.2.3. Glycopeptide Sequence (*See* **Notes 4–9** and **Figs. 7**, **10**, and **11**)

1. Scan the high mass range using a high orifice potential (120 V).
2. From the known migration times of the glycopeptide(s) (*see* **Subheading 3.7.2.1.**) extract the mass spectrum of the glycopeptide(s). Find the mass of the ion that corresponds to the peptide–HexNAc generated by the high orifice fragmentation of the glycopeptide.
3. Acquire a second generation of fragment ion for the selected precursor ion using CZE–MS–MS.

4. Notes

1. The most straightforward approach to glycoprotein analysis by CZE–ESMS is to look at the intact glycoprotein. In ideal cases where the protein is small, and contains a single site of glycosylation and little heterogeneity, excellent separation of the glycoforms can be achieved (**Fig. 1**). The protein is detected as a series of multiply charged ions and it is important to select an appropriate scan range to maximize the signal intensity. For a larger glycoprotein with a highly heteroge-

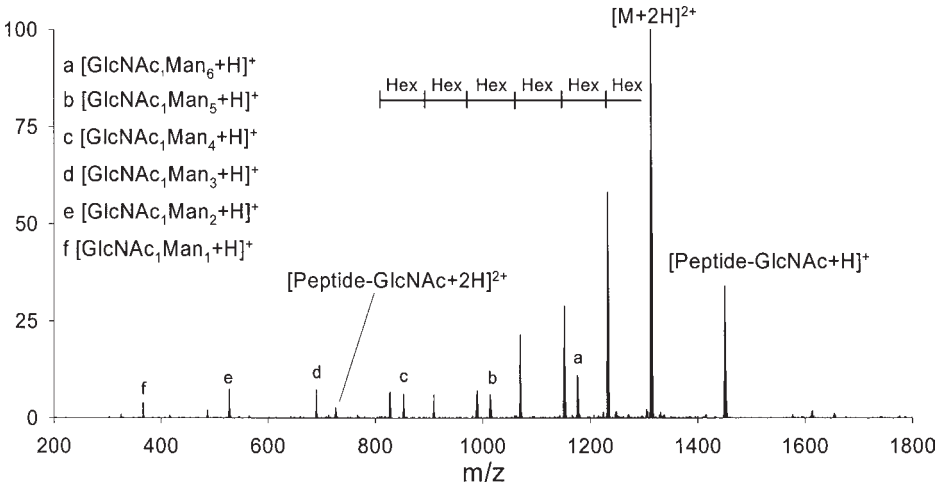

Fig. 8. CZE–MS–MS analysis of the glycopeptide at *m/z* 1314 from the tryptic digest of *P. vulgaris* lectin. Conditions as for **Fig. 4**, except that the separation voltage was decreased to –13 kV at 9 min The mass spectrometer was set up with Q1 transmitting *m/z* 1314 and Q3 scanning from m/z 200 to 1800 with 0.5 step size and a dwell of 2 ms. The collision energy was 30 eV and the orifice voltage was set to 50 V.

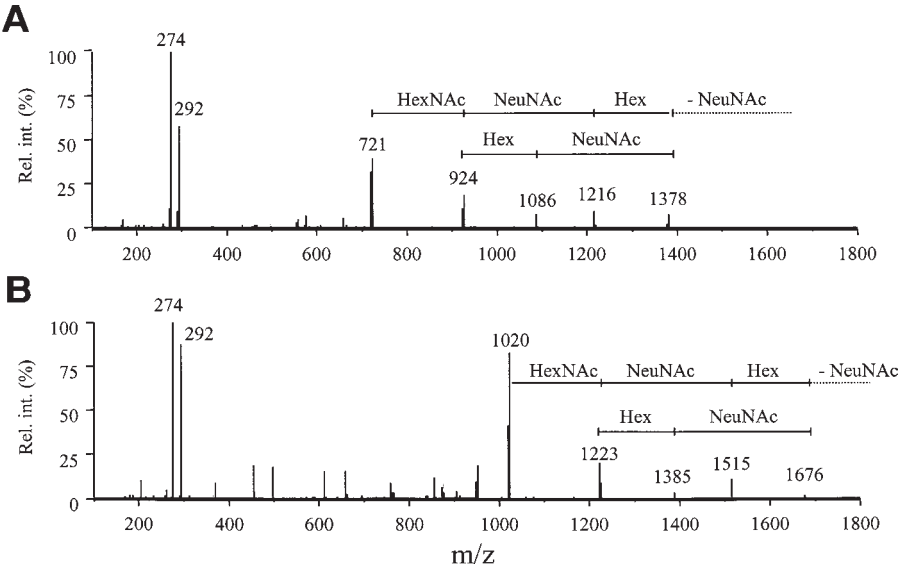

Fig. 9. CZE–MS–MS analysis of *O*-linked glycopeptides from the Glu-C digest of κ-casein. **(A)** Product ion scan of *m/z* 835 and **(B)** product ion scan of *m/z* 984. Conditions as for **Fig. 6** except that the separation voltage was decreased from –20 kV to –5 kV at 10 min.

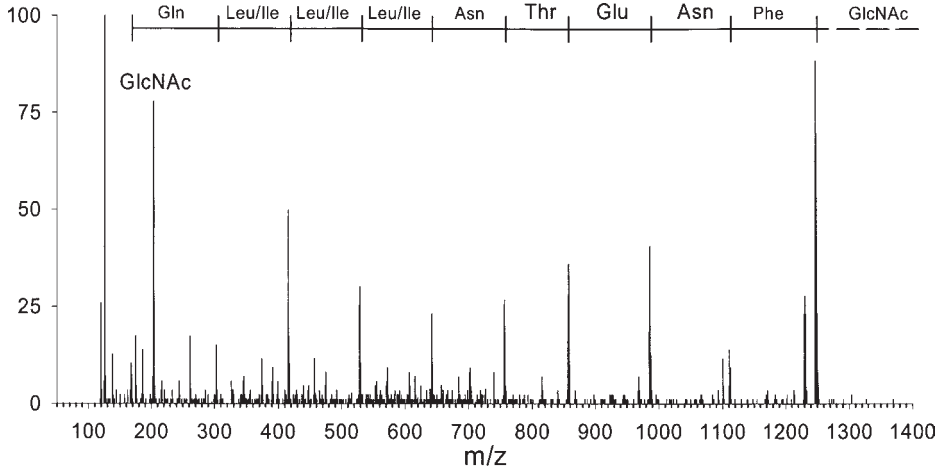

Fig. 10. CZE-front-end CID–MS–MS analysis of the glycopeptide at *m/z* 726 from the tryptic digest of *P. vulgaris* lectin. Conditions as for **Fig. 8**, except that the mass spectrometer was set up with Q1 transmitting *m/z* 726 and Q3 scanning from *m/z* 100 to 1400 with 0.5 step size and a dwell of 2 ms. The collision energy was 30 eV and the orifice voltage was set to 100 V.

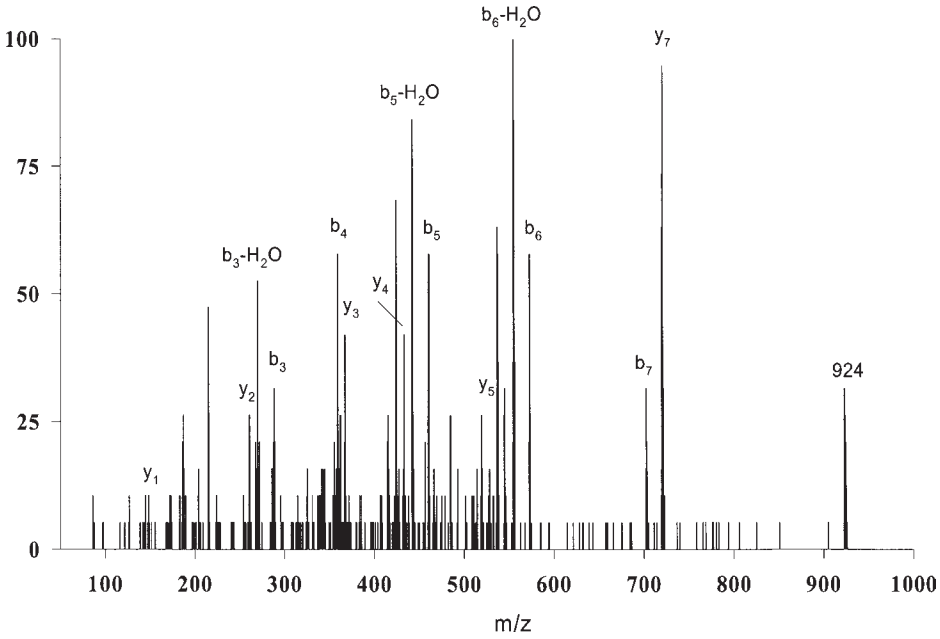

Fig. 11. Product ion spectrum of precursor *m/z* 924 corresponding to [peptide-HexNAc+H]$^+$ ion identified in **Fig. 9A**. The separation and MS–MS conditions were the same as for **Fig. 9** except the orifice voltage was increased to 100 V and the collision energy was 32 eV. Peak labeling as for **Fig. 7**.

Table 1
Tentative Assignment of the Glycoforms of Avidin Observed in the Reconstructed Molecular Mass Profile (Fig. 3)

Time (min)	$Mr_{Obs.}$ (Da)	$Mr_{Calc.}$ (Da)[a]	Proposed composition[b]
26.8	15806.6	15807.5	Hex_4 $HexNAc_2$ Pen_2 $Dhex_1$
26.8	15846.8	15846.8 or 15844.6	Hex_3 $HexNAc_3$ Pen_2 $Dhex_1$ or Hex_3 $HexNAc_5$
26.7	15885.0	15883.6	Hex_7 $HexNAc_2$
26.7	15923.6	15924.7	Hex_6 $HexNAc_3$
26.5	15962.6	15965.7	Hex_5 $HexNAc_4$
26.5	16001.8	15999.6	Hex_6 $HexNAc_2$ Pen_1 $Dhex_1$
26.2	16043.8	16045.7	Hex_8 $HexNAc_2$
26.4	16086.0	16086.8	Hex_7 $HexNAc_3$
26.2	16169.8	16168.9	Hex_5 $HexNAc_5$
26.1	16210.8	16209.9	Hex_4 $HexNAc_6$
28.2	15398.6	15397.2	$HexM_4$ $HexNAc_2$
29.1	15440.6	15438.2	Hex_3 $HexNAc_3$
30.5	15559.6	15559.3	Hex_5 $HexNAc_2$
29.6	15598.6	15600.4	Hex_4 $HexNAc_3$
28.9	15645.0	15645.4	Hex_3 $HexNAc_2$ Pen_2 $Dhex_1$
29.6	15690.0	15689.4 or 15691.4	Hex_4 $HexNAc_2$ $Dhex_2$ or Hex_5 $HexNAc_2$ Pen_1
29.3	15720.6	15721.4	Hex_6 $HexNAc_2$
28.9	15763.4	15762.5	Hex_6 $HexNAc_3$
29.2	15806.4	15807.5 or 15803.5	Hex_4 $HexNAc_2$ Pen_1 $Dhex_1$ or Hex_4 $HexNAc_4$
28.9	15846.6	15848.6 or 15844.6	Hex_3 $HexNAc_3$ $Pen2_1$ $Dhex_1$ or Hex_3 $HexNAc_5$
29.1	15866.4	15883.6	Hex_7 $HexNAc_2$
28.7	15966.4	15965.7	Hex_5 $HexNAc_4$
28.5	16001.8	15999.7	Hex_6 $HexNAc_2$ Pen_1 $Dhex_1$
28.2	16048.4	16047.8	Hex_3 $HexNAc_6$
28.2	16126.4	16127.8	Hex_6 $HexNAc_4$
28.2	16169.8	16168.9	Hex_5 $HexNAc_5$
28.0	16207.8	16207.9	Hex_9 $HexNAc_2$
28.1	16214.8	16213.9	Hex_3 $HexNAc_4$ Pen_2 $Dhex_1$

[a]Average molecular mass of unglycosylated protein = 14342.2 Da.
[b]Oligosaccharides are attached to Asn_{17}.
Hex, hexose; HexNAc, *N*-acetylhexosamine; Pen, pentose; Dhex, deoxyhexose.

neous site of glycosylation, the separation of individual glycoforms is less apparent in the total ion electropherogram (**Fig. 2A**). The use of contour plots (*m/z* vs time) can assist the data interpretation (**Fig. 2B**). The most suitable method for data dis-

play in this case is to use reconstructed molecular mass profiles from the extracted mass spectra (**Fig. 3**). The size of the oligosaccharides can be calculated based on the observed masses and the reported sequence. Tentative structures can then be assigned based on known compositions of oligosaccharides (**Table 1**).

2. The same conditions used for intact glycoproteins can be used for glycoprotein digests. However, the concentration of formic acid used in the separation electrolyte can be reduced from 2.0 *M* to 0.1 *M*. This usually results in better sensitivity, but is accompanied by a small decrease in peak resolution. The important aspect of this experiment is to set up the proper scan function to assist the identification of the glycopeptides ***(19–21)***. To set the experiment up on the Sciex API-III$^+$, selected ion monitoring (SIM) is used for different masses with a large window or small window and different orifice voltage for each mass. For example, the high mass range is obtained by setting the mass to m/z 1050 with a window of 1100 amu and a dwell time of 3.5 s. The low mass oxonium fragment ions (**Table 2**) are analyzed by setting the individual masses (i.e., 163, 204, 366) with no width and a dwell time of 0.2 s. The orifice voltage for the high mass range is set to 50 V and for the low mass ions to 100 V. By scanning the low mass ions when the orifice voltage is high, detection of the oxonium fragments generated by front end CID of the intact glycopeptides is possible. More than one oxonium ion should be monitored as false positives can arise from peptide bond fragmentation. An example of the type of data generated by this technique is shown in **Fig. 4**. The bottom panel of **Fig. 4** shows the contour plot with the labeled glycopeptides. The diagonal line corresponding to the concurrent change of molecular masses and electrophoretic mobilities is the signature of glycopeptide heterogeneity; in this particular example the separation between ions indicates a high-mannose oligosaccharide series.
3. Structural characterization of the oligosaccharide portion of the glycopeptides identified using the method described in **Note 2** can be achieved by using CZE–MS–MS. The same separation conditions are employed. However, the mass spectrometer is set to perform tandem mass spectrometry on the *m/z* value identified in the previous experiment. To obtain several MS–MS spectra across the sharp peak, the separation voltage can be decreased just before the peak migrates out of the capillary ***(22)***. This serves to broaden the peak slightly and allows for the acquisition of several scans across the peak. Typically, a collision energy of 30 eV (laboratory frame of reference) is used for doubly charged ions. The CZE–MS–MS of one of the high mannose glycopeptides identified in **Fig. 4** is shown in **Fig. 8**. Low energy MS–MS spectra of glycopeptides are dominated by cleavages of glycosidic bonds from the oligosaccharide side chain, with no fragmentation of the peptide amide bonds. From this information the sugar content of the oligosaccharide can be elucidated; however, no information about the nature of the glycosidic linkages is obtained.
4. To obtain sequence information on the peptide to which the oligosaccharide is attached, one or two further experiments are required. The conditions used are identical to those described in **Note 1** with a couple of modifications. Most importantly are (1) the change in orifice voltage and (2) selection of parent ion

Table 2
Diagnostic Oxonium Ions for Glycopeptides

m/z	Carbohydrate residue
133	Pentose (arabinose [Ara], ribose [Rib], xylose [Xyl])
147	Deoxyhexose (fucose [Fuc])
162	Hexosamine (galactosamine [GalN], glucosamine [GlcN])
163	Hexose (glucose [Glu], galactose [Gal], mannose [Man])
204	*N*-acetylhexosamine (*N*-acetylgalactosamine [GalNAc], *N*-acetylglucosamine [GlcNAc])
274	*N*-acetylneuramic acid - H_2O (NeuNAc- H2)
292	*N*-acetylneuramic acid (NeuNAc)
366	HexNAc-Hex
407	HexNAc-HexNAc

for the MS–MS experiment. The orifice voltage is increased so that the oligosaccharide is cleaved by front-end CID, resulting in production of the peptide plus the core sugar fragment ion (peptide–HexNAc). This is similar to using front-end CID to generate the oxonium ions detected to locate the migration times of the oligopeptides (**Note 2**). The generation of a product ion spectrum exempt of oligosaccharide fragment ions provides suitable information for peptide sequencing. The *m/z* value of the peptide plus a GlcNAc residue can be identified from the MS–MS spectrum of the intact glycopeptide (**Fig. 8**). An alternative approach is to conduct the full-scan acquisition using a high orifice voltage setting. The advantage is that ions generated by the front-end CID of the glycopeptides are readily observed. The interpretation is thus significantly facilitated by using the MS–MS data with the high orifice full-scan. Also the selection of the most abundant peptide ion is possible for the next MS–MS experiment. When the amount of sample is not a limiting factor, several runs can be carried out at various orifice voltages to optimize the intensity of the peptide–HexNAc ion. The spectrum generated using this method allows for the sequence determination of the peptide and the carbohydrate chain (**Fig. 10**). In the case of *N*-linked glycoproteins, the site of oligosaccharide can be unambiguously assigned based on the known *N*-X-S/T consensus motif ***(23)***. The use of trypsin as the proteolytic cleavage agent is important in this experiment, as it results in peptides with charged residues at both ends which leads to favorable fragmentation.

5. The examples shown in **Notes 2–4** required a relatively high concentration of protein digest (150 µ*M* or 4.5 µg/µL) to carry out the experiments. The use of online preconcentration as described in **Subheadings 2.** and **3.** permits the analysis of glycoprotein digests at a much lower level. The data shown in **Figs. 5** and **7** were obtained from a glycoprotein digest at a concentration of 65 n*M* (~2 ng/µL). By using the preconcentration technique, several microliters can be injected as opposed to nanoliters for conventional CZE injection. In this case the sequence

of the protein was known *a priori*, and inspection of the sequence revealed that digestion with trypsin or Glu-C would result in glycopeptides too large for MS–MS sequencing. Therefore a sequential digestion was carried out to generate peptides of a suitable size for sequencing by tandem mass spectrometry. This digestion resulted in a glycopeptide with a glutamic acid at the C-terminal of the peptide. Tandem mass spectrometry of this peptide generated a much more complex fragmentation pattern compared to that of a tryptic peptide (**Fig. 7B** vs **7D**). The limited sequence data sometimes generated by this technique (**Fig. 7D**) can still be sufficient to identify the protein by database searching.

6. The analysis of *O*-linked glycoproteins can be carried out using the same methods described previously for *N*-linked glycoproteins. However, as there is no known consensus sequence for the position of *O*-linked oligosaccharides, the assignment of glycosylation site may be ambiguous as discussed in **Note 9**.
7. An alternative approach to the selective identification of the glycopeptides is to use precursor ion scanning. In this method, a normal orifice voltage is used and the peptides are fragmented in the radio frequency (RF) only quadrupole collision cell of the mass spectrometer. The third quadrupole is set to transmit only the *m/z* value of the oxonium ion of interest. The first quadrupole is scanned over the mass range of interest (*m/z* 500–1500), and the corresponding spectra display only precursor ions for the selected transition. The total ion electropherogram (TIE) for the analysis of the κ-casein Glu-C digest using the precursor ion scanning for *m/z* 274 (NeuNAc oxinium ion -H_2O) is shown in **Fig. 6**. The peaks in the precursor-ion scan contain information on the molecular masses of the glycopeptides without interference from other nonglycosylated peptides of this digest. Examples of the mass spectra extracted from the first three peaks are shown in **Figs. 6B** and **C**. The later peaks are due to higher molecular weight components resulting from incomplete digestion of the glycoprotein.
8. The composition of the carbohydrate component of the O-linked glycopeptide can be obtained using CZE–MS–MS as discussed previously. These experiments were carried out on the digested *O*-linked glycoprotein, using *m/z* 835 and 984 as precursors. The resulting fragment ion spectra are presented in **Fig. 9** and clearly show abundant fragment ions at *m/z* 274 and 292, corresponding to NeuNAc. These spectra also show that the initial cleavage of a NeuNAc residue is followed by loss of either a hexose or another NeuNAc residue. This observation is consistent with the presence of a HexNAc-Hex disaccharide (presumably GalNAc-Gal) to which a NeuNAc is attached to each of these two residues. If both Neu5Ac sugars were attached to the hexose the ions at *m/z* 1216 and *m/z* 1515 in **Fig. 9A** and **9B**, respectively, would not be observed. Also evident in these spectra are the fragment ions for the naked peptides at *m/z* 721 and 1020, in **Figs. 9A** and **9B**, respectively. From the MS–MS data, information about both composition and branching can be obtained from these relatively simple experiments using picomoles or less of material.
9. As mentioned for *N*-linked glycopeptides, the formation of first-generation fragment ions to produce the peptide–HexNAc ion, which are subsequently analyzed

by MS–MS, can be used to provide additional information on the peptide sequence. This is demonstrated in **Fig. 11** for the tandem mass spectrum of *m/z* 924 (**Fig. 9A**) obtained from combined CZE–MS–MS. Fragmentation of the peptide bond produces the well-defined series of y and b ions corresponding to fragment with charge retention on the C- and *N*-terminus, respectively. Loss of water from the b-series ions is also a common fragmentation pathway when an acidic residue (glutamic acid) is located at the C-terminus of the peptide. The sequence of this peptide was determined to be STVATLE, which has three possible sites of *O*-glycosylation. The b_2 and y_6 ions are absent from the spectrum, indicating that the site of glycosylation could be on the first threonine. However, the corresponding ions containing the HexNAc are also absent, so no firm conclusion can be made. This is typical for *O*-linked peptides and alternative methods of ascertaining the site of glycosylation are necessary.

Loss of water from the b series ions is also a common fragmentation pathway when an acidic residue (glutamic acid) is located at the C-terminus of the peptide. The sequence of this peptide from the k-casein Glu-C digest is STVATLE, with three possible sites of O-glycosylation. Because the core HexNAc fragments before the peptide bonds, the exact location of the glycosylation site cannot be determined unambiguously using this method.

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14

Analysis of Bacterial Glycolipids by Capillary Electrophoresis–Electrospray Mass Spectrometry

Haemophilus influenzae *and* Neisseria meningitidis *Lipopolysaccharides*

Pierre Thibault, Adèle Martin, Michel Gilbert, Warren W. Wakarchuk, and James C. Richards

1. Introduction

A number of invasive diseases are caused by human mucosal pathogens such as those of the genera *Haemophilus*, *Neisseria*, *Moraxella*, *Campylobacter*, and *Bordetella*. The bacterium *N. gonorrhoeae* infects mucosal surfaces of the genital tract, and can enter the bloodstream, survive, and cause secondary infections if protective antibodies are not raised in time ***(1)***. Both *N. gonorrhoea* and *H. ducreyi* are highly pathogenic, causing sexually transmitted diseases such as gonorrrhea and genital ulcers, respectively ***(2)***. The pathogens *H. influenzae* and *N. meningitidis* are uniquely adapted to colonize the human respiratory tracts and can lead to disseminated infections including otitis, and bacterial meningitis in young children ***(3,4)***. Through evolution, a number of these Gram-negative bacteria have elaborated surface antigens that mimic those found in human glycosphingolipids, thereby providing a mechanism for evading the innate immune system and enhancing their survival in the challenging environmental conditions of the host mucosa. While the exact mechanisms of colonization and invasion of *H. influenzae* and *N. meningitidis* are still poorly understood, it is generally recognized that lipopolysaccharides (LPS) play an important role in the virulence and pathogenicity of these organisms ***(5,6)*** and can associate with mucus and damaged epithelium of the human nasopharyngeal tissue ***(7)***.

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LPS are important components of the outer membrane of the bacterial cell walls and are composed of complex hydrophilic oligosaccharides bound to a membrane anchoring a lipid A moiety. The LPS of *H. influenzae* and *N. meningitidis* contrast with those of other Gram-negative bacteria in that they lack the *O*-repeating antigens characteristic of many enteric pathogens (e.g., *Escherichia coli*, Salmonella spp.). These short chain LPS consist of two parts of different properties: a hydrophilic carbohydrate component containing acidic (3-deoxy-D-mann*O*-2-octulosonic acid, KDO) and neutral residues (glucose, galactose, heptose) bonded to an hydrophobic lipid A component comprising a glucosamine (GlcN) disaccharide to which are attached *O*- and *N*-linked fatty acids ***(8)***.

A structural model was proposed earlier for *H. influenzae* LPS (**Fig. 1A**) in which a conserved heptose-containing inner core trisaccharide is attached to a single KDO 4-phosphate residue ***(9)***. Significant molecular diversity to the outer core oligosaccharide can be observed through chain elongation at each heptose (Hep) within this triad. Furthermore, structural variability can also be observed in the extent to which are appended functional groups such as phosphates (P), pyrophosphates (PPs), phosphoethanolamine (PE), and phosphocholine (PC). In some cases, the presence of specific residues or functional groups has been related to the virulence and invasiveness of particular strains. For example, the PC substitution was found to be phase variable and its occurrence was paralleled with the persistence of *H. influenzae* in the human respiratory tract ***(10)***.

The LPS of *N. meningitidis* is structurally related to that of *H. influenzae*, but exhibit a different inner core comprising two KDO residues, one of which being extended by a Hep disaccharide and a *N*-acetylglucosamine (GlcNAc) residue (**Fig. 1B**). Here again, chain elongation can take place at the Hep residues (predominantly Hep I) and extension of these glycoforms is controlled by a number of glycosyltransferases.The genes involved in the sequential addition of the sugars to the outer core oligosaccharide of *N. meningitidis* and *N. gonorrhoeae* LPS have been identified and correlated with defined glycosyltransferase activities ***(11–13)*** . Some of these glycoforms are found to mimic blood group antigens such as P^k epitope (α-Gal [1–4]-β-Gal [1–4]-Glc) and paragloboside (β-Gal [1–4]-β-GalNAc [1–3]-β-Gal [1–4]-Glc), and are potential acceptors for sialyl transferases ***(14)***. The biological importance of *N*- acetylneuraminic acid (NeuNAc) containing LPS in the pathogenicity and virulence of both gonococci and meningococci was reviewed recently ***(15)***. LPS sialylation was found to affect the pathogenicity of meningococci, although to a lesser extent to that observed in gonococci. A number of serotype groups of meningococci (B, C, W, and Y) have the ability to synthesize the cytidine monophosphate NeuNAc substrate, and to incorporate NeuNAc into capsular polysaccharides and terminal galactose residues of LPS ***(16)***.

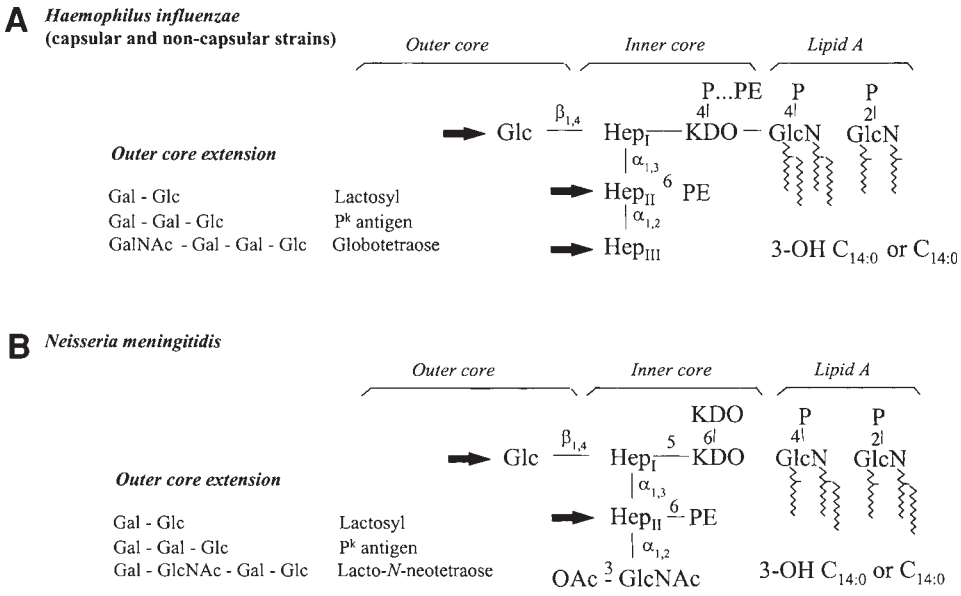

Fig. 1. Structures of the conserved core oligosaccharide from **(A)** *H. influenzae* (adapted from **ref.** ***18***) and **(B)** *N. meningitidis*. Possible oligosaccharide extension at the Hep residues are shown as an *inset*.

Further understanding of bacterial pathogenesis and identification of key virulence factors rely on sensitive and specific analytical methods that can probe the subtle changes in LPS structures. Over the past decade, electrospray mass spectrometry (ESMS) has played a pivotal role in the characterization of these complex glycolipids, and applications of this technique have been demonstrated for the structural characterization of *O*-deacylated LPS from *Haemophilus* and *Neisseria* strains ***(9,17–20)***. More recently, the coupling of high-resolution separation techniques such as capillary electrophoresis to ESMS (CE–ESMS) has provided unique resolution and identification of glycoform populations and substituted phosphorylated functionalities present in LPS ***(21–23)***. In an effort to enhance the sensitivity of this technique for trace level analysis of *O*-deacylated LPS, adsorptio*N*- preconcentration approaches for on-line enrichment of glycolipids prior to CE–ESMS was developed and enabled the identification of LPS surface antigens from as few as five bacterial colonies of *H. influenzae* strain Eagan ***(24)***.

Our investigations on the analytical potentials of CE–ESMS have outlined the unparalleled capabilities of this technique for separating closely related glycoform and isoform families in *O*-deacylated LPS based on their characteristic band patterns observed in the contour profile of *m/z* vs time. This type of

data representation aids significantly the assignment of glycolipids differing only by the location of phosphorylated groups or hexose residues. Selective mass spectral functions using mixed scans and selected ion monitoring can be integrated to identify important immunodeterminants such as PC and NeuNAc moieties present at trace levels in extracts of *O*-deacylated LPS. More detailed structural analyses on specific branching point or oligosaccharide sequence can be obtained using on-line tandem mass spectrometry. Some of the analytical merits of CE–ESMS for the identification of complex bacterial glycolipids and for the monitoring of glycosyltransferase reaction products are described in this chapter.

2. Materials

2.1. Growth of Bacterial Strains

1. Bacterial strains: *H. influenzae* strains; RM 7004 *lic2B*, and 319. *N. meningitidis* strains L1, L3, and L8 (*see* **Note 1**).
2. Bacterial plates: Chocolate agar plates from Quelab of Montreal, Canada; 5% sheep blood agar plates.
3. Liquid growth media: 3.7% (w/v) Brain heart infusion (BHI), 10 mg/L of hemin, 2 mg/L of nicotinamide adenine dinucleotide (NAD); Difco bacto Todd Hewitt (DBTH) broth from Difco.
4. Phosphate-buffered saline (PBS): 6.7 m*M* potassium phosphate, pH 7.4, containing 150 m*M* NaCl and 0.02% (w/v) NaN_3.
5. Bacterial killing solution: 0.5% (w/v) Phenol in PBS.
6. IF-75 fermenter from New Brunswick Scientific.

2.2. Extraction of Cell-Wall LPS

1. Phenol.
2. Water bath.
3. Omni mixer with stainless steel container.
4. Refrigerated centrifuge.
5. Ultracentrifuge.

2.3. Preparation of O-Deacylated LPS

1. LPS preparation from *H. influenzae* and *N. meningitidis* (*see* **Subheading 3.2.**).
2. *O*-Deacylation reagent: anhydrous hydrazine.
3. Acetone.
4. Eppendorf centrifuge.

2.4. Preparation of Core Oligosaccharides

1. LPS preparation from *N. meningitidis* L1 (*see* **Subheading 3.2.**)
2. Aqueous acetic acid (1% v/v).
3. Reacti bloc for reaction at 100°C.
4. Refrigerated centrifuge.

5. Ultracentrifuge.
6. Bio-Gel P2 column (2.6 cm × 140 cm, 200–400 mesh, Bio-Rad).
7. Elution buffer: 0.05 *M* pyridinium acetate, pH 4.5.

2.5. Preparation of BCQ-Coated Columns

1. Conditioning solutions: 1 *M* NaOH, deionized water, methanol.
2. Derivatization agent: 50 µL of 7-oct-1-enyltrimethoxysilane (Hüls America Inc., Bristol, PA) and 50 µL of glacial acetic acid in 10 mL of methanol.
3. Coating solution: 20 µL of *N*,*N*,*N*'*N*'-Tetramethylethyldiamine (TEMED) and 140 µL of 10% (w/v) aqueous ammonium persulfate added to a 2% (v:v) solution of BCQ ([acryloylamino]propyl) trimethylammonium chloride, Chemische Fabrik Stockhausen, Krefeld, Germany), in 10 mL of deionized water.

2.6. Profiling the Distribution of LPS Glycoforms by On-Line CE–ESMS

1. All buffer solutions were prepared with deionized water from an Elga water filtration system and filtered through a Millipore 0.45 µm filter.
2. CE separation buffers: 30 m*M* Morpholine–acetate, pH 9.0, containing 5% (v/v) methanol (for negative ion detection); 0.1 *M* aqueous formic acid (for positive ion detection).
3. Capillary columns: 90 cm length × 50 µm inner diameter (i.d.) bare fused silica (for negative ion detection); BCQ-coated column (for positive ion detection).
4. A Crystal model 310 CE instrument (ATI Unicam Boston, MA, USA) was coupled to the mass spectrometer via a coaxial sheath-flow interface as shown in **Fig. 2** ***(21–25)***. Mass spectral analyses were conducted using either an API 300 triple quadrupole mass spectrometer (Perkin Elmer/Sciex, Concord, ON, Canada) for analyses involving orifice stepping functions (*see* **Subheadings 3.5**, **3.6.**, and **3.9.**) or a Q-TOF (Micromass, Manchester, UK) hybrid quadrupole/time-of-flight instrument ***(26)*** for high-resolution experiments.

2.7. Monitoring Sialylation of Core Oligosaccharides Using CE–ESMS

1. Core oligosaccharides from *N. meningitidis* L1 LPS.
2. Recombinant *N. meningitidis* α-2,3-sialyltransferase (*see* **Note 8**).
3. Incubation buffer, 10 m*M* 2-morpholinoethanesulfonic acid (MES), pH 6.0, 5 m*M* $MgCl_2$.
4. Incubator for enzymatic incubation at 37°C.
5. Donor: 2 m*M* CMP-NeuNAc.

3. Methods

3.1. Growth of Bacterial Strains (See Note 2)

1. Resuscitate bacterial strains from frozen stocks on chocolate agar plates (*H. influenzae*) or 5% sheep blood agar plates (*N. meningitidis*) and incubate overnight at 37°C.

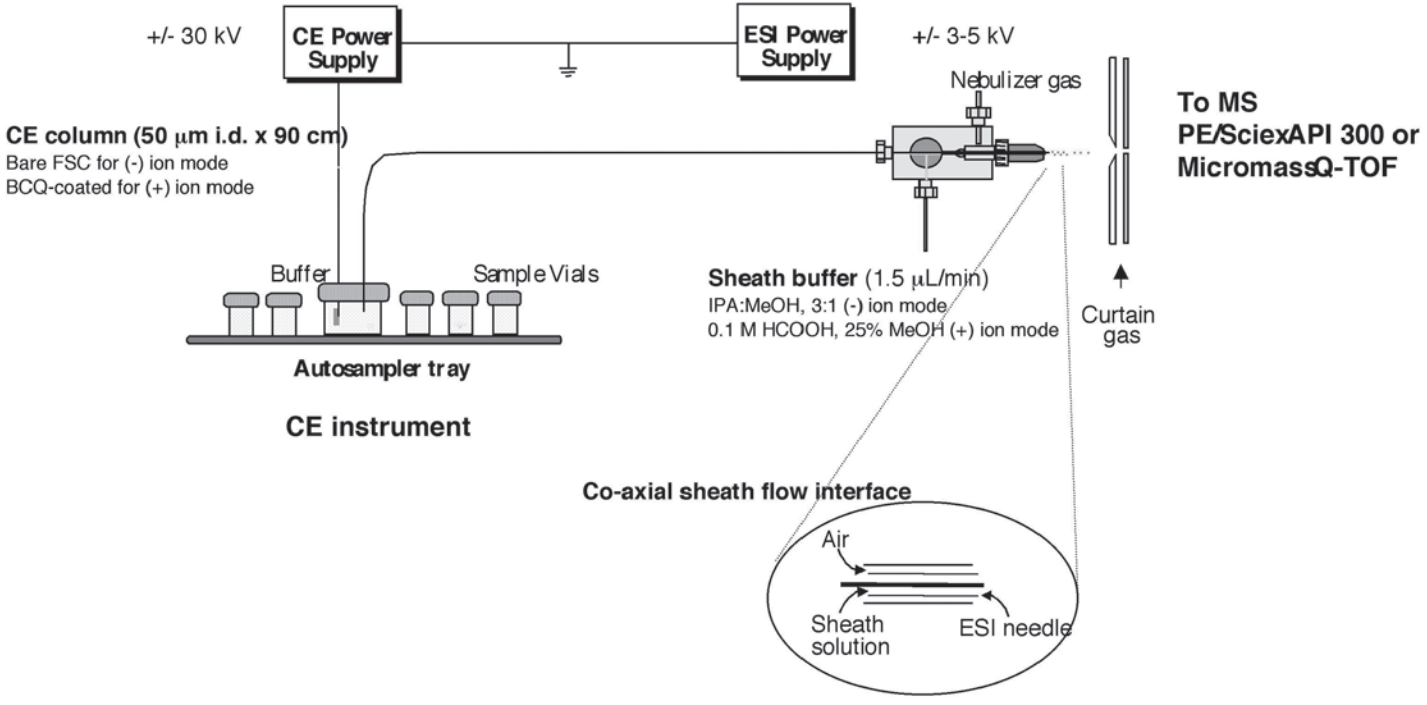

Fig. 2. Schematic representation of the coaxial CE–ESMS interface. An electrospray needle (27-gage) is butted against a low dead volume tee, thereby providing delivery of the sheath solutions to the end of the capillary column. Conditions: Fused silica capillary or BCQ-coated column of 90 cm length × 50 μm i.d. (180 μm o.d.). Electrolyte: 30 m*M* morpholine, pH 9.0 (negative ion detection), 0.1 *M* HCOOH (positive ion detection). Separation voltage: +30 kV (normal mode) or –25 kV (anodal electroosmotic conditions when using BCQ column).

2. Select colonies from plates and cultivate in 10-L batches of BHI broth supplemented with haemin and NAD at 37°C for 20 h ***(18)*** or in a 60-L fermenter batch of DBTH broth ***(13)***.
3. Harvest cells by low-speed centrifugation (5000*g*).
4. Decant centrifugate.
5. Resuspend cell pellet in bacterial killing solution and stir for 16 h.
6. Collect bacterial cell mass by centrifugation (5000*g*).

3.2. Extraction of Cell-Wall LPS (See Note 3)

1. Prepare a 90% phenol solution by adding 50 mL of water to 500*g* commercial grade phenol. Heat on high for 3–4 min in a microwave oven to liquefy.
2. Preheat deionized water to 65–70°C in a water bath.
3. Add bacterial cell mass (approx 20 g wet weight) to a stainless steel container. Add 50 mL each of preheated water and phenol solution.
4. Stir vigorously by mechanical mixing for 20–30 min.
5. Place stainless steel container in ice bucket to cool mixture to below 10°C.
6. Separate aqueous and phenol phases by low-speed centrifugation (5000*g*) at 5°C for 30 min. Collect water phase.
7. Add 50 mL warm water (65–70°C) to phenol phase and repeat **steps 4–6**.
8. Combine water phase extracts (approx 100 mL) and collect LPS by following **steps 9–10** or **steps 11–12**.
9. Dialyze against running tap water for 2–3 d.
10. Collect LPS by lyophylization.
11. Precipitate LPS from water phase (**step 8**) by addition of four volumes of ethanol.
12. Collect LPS by low-speed centrifugation and lyophylization.
13. Dissolve LPS in water to a final concentration of 1–2%, and ultracentrifuge (105,000*g*) at 4°C for 5 h.
14. Decant water and repeat **step 13** (twice).
15. Collect purified LPS by suspending in water and lyophilization.

3.3. Preparation of O-Deacylated LPS (See Note 4)

1. Suspend 0.5–1.0 mg of purifed LPS in 200 µL of anhydrous hydrazine and incubate at 37°C for 1 h with constant stirring.
2. Cool the eaction mixture in ice (0°C) and slowly add 600 µL of cold acetone to destroy excess hydrazine.
3. Obtain the precipitated product by centrigugation.
4. Wash the pellet with 600 µL of acetone (twice), followed with 500 µL of a mixture of acetone–water (4:1, v/v).
5. Dissolve pellet in water and lyophilize to obtain *O*-deacylated LPS as a solid.

3.4. Profiling the Microheterogeneity of O-Deacylated LPS Glycoforms and Isoforms Using CE–ESMS (See Note 5, Figs. 2 and 3)

1. Condition bare fused silica column by rinsing sequentially 1 *M* NaOH (15 min), deionized water (20 min), and the CE separation buffer (15 min).

2. Remove a 3-mm portion of the polyimide coating from the electrospray emitter end of the capillary to ensure proper wetting of the tip with the sheath liquid. This is achieved by gently burning the coating with an open flame and removing the char with a tissue impregnated with methanol.
3. Insert column in the coaxial CE–ESMS interface from the emitter end and cut a 1-cm portion of the capillary from the injector end to avoid blockage.
4. A sheath buffer (isopropanol–methanol, 3:1) is introduced to the back tee of the CE–ESMS interface as indicated in **Fig. 2**.
5. Optimization of the mass spectrometer (emitter position, nebulizer and sheath solution flow rates) is achieved by electrokinetically infusing a solution of angiotensin I (10 µg/mL in the separation buffer) at a voltage of 30 kV (4 µA current) and maximizing the signal of the doubly deprotonated ion at *m/z* 647.
6. Redissolve *O*-deacylated LPS in water to give a concentration of approx 100 µg/mL.
7. Inject approx 40 nL (head pressure 300 mbar for 0.1 min) of the sample on the column using the CE instrument.
8. Record mass spectra by acquiring mass spectrum over the range *m/z* 500–2000 with a duty cycle of 3 s/scan. The orifice voltage is set to a minimal setting (typically 50 V) to avoid in-source fragmentation.

3.5. Identification of Phosphocholine Containing LPS Using Polarity Switching, and High/Low Orifice Stepping on a Quadrupole Mass Spectrometer (See Note 6 and Fig. 4)

1. Equilibrate column as described in **Subheading 3.4.**
2. Optimization of the mass spectrometer in both negative and positive ion mode is achieved by electrokinetically infusing a solution of angiotensin I (10 µg/mL in the separation buffer) at a voltage of 30 kV (4 µA current) and maximizing the analyte signal (*m/z* 647 and 649 in negative and positive ion mode, respectively). More emphasis should be placed on proper sensitivity in negative ion mode.
3. Redissolve *O*-deacylated LPS in water to give a concentration of approx 100 µg/mL.
4. Inject approx 40 nL (head pressure 300 mbar for 0.1 min) of the sample on the column using the CE instrument.
5. Record mass spectra by incorporating two periods for a given scan cycle; selected ion monitoring for *m/z* 328 for 200 ms, orifice voltage 150 V (positive ion mode) and full-scan acquisition for *m/z* 500–1500, 3 s/scan, orifice voltage 50 V (negative ion mode).

3.6. Identification of NeuNAc Containing O-Deacylated LPS in CE–ESMS Experiments Using High/Low Orifice Stepping (See Note 7, Figs. 5 and 6)

1. Equilibrate the column and optimize the mass spectrometer as described in **Subheading 3.4.**
2. Redissolve *O*-deacylated LPS in water to give a concentration of approx 100 µg/mL.

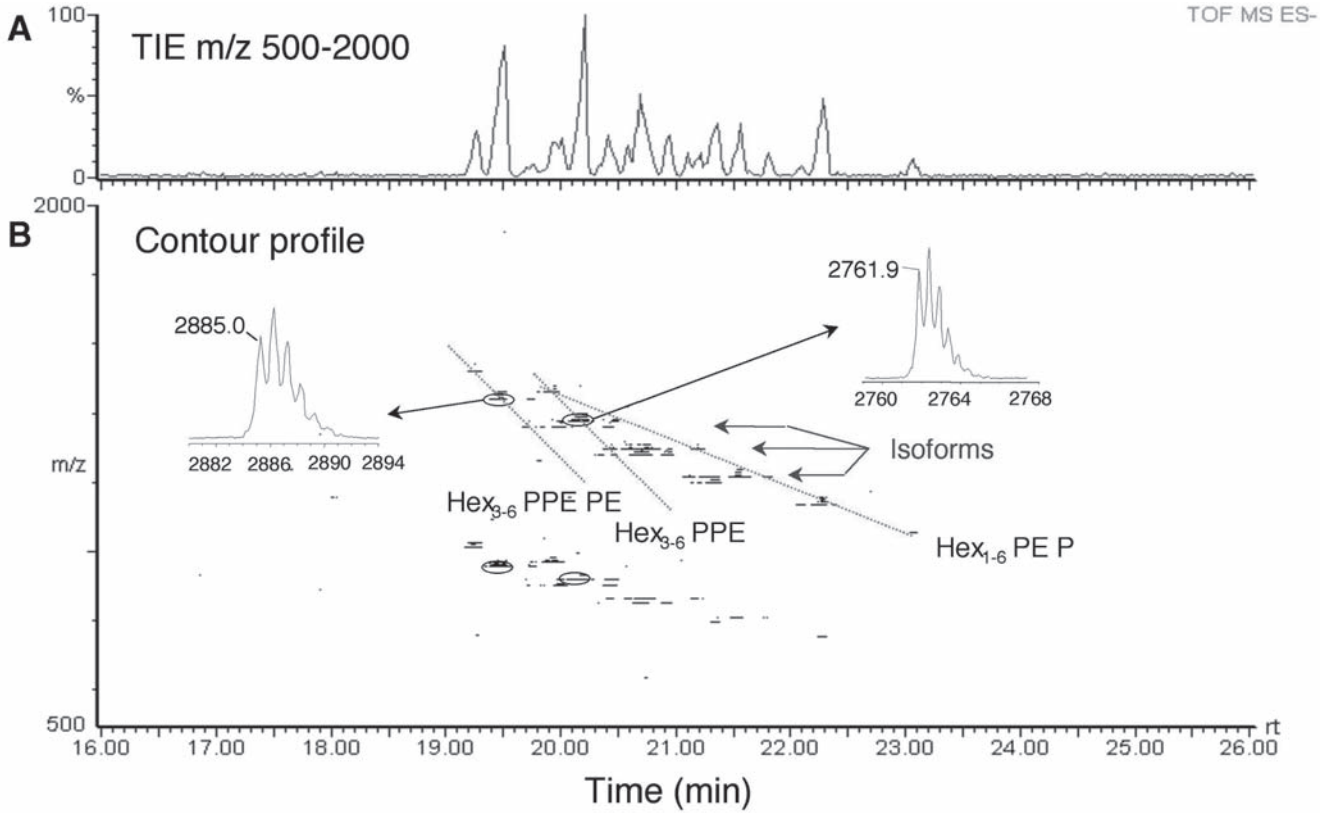

Fig. 3. CE–ESMS analysis of *O*-deacylated LPS from *H. influenzae* strain 7004 *lic2B*. **(A)** Total ion electropherogram (*m/z* 500-2000). **(B)** Contour profile of *m/z* vs time. *Dotted lines* indicate family of closely related glycoforms generally composed of sequential addition of Hex residues, whereas isoforms are isomeric glycolipids corresponding to substitution of a functional group. Examples of reconstructed molecular mass profiles are shown as *insets*. Mass spectral acquisition obtained on a Micromass Q-TOF instrument.

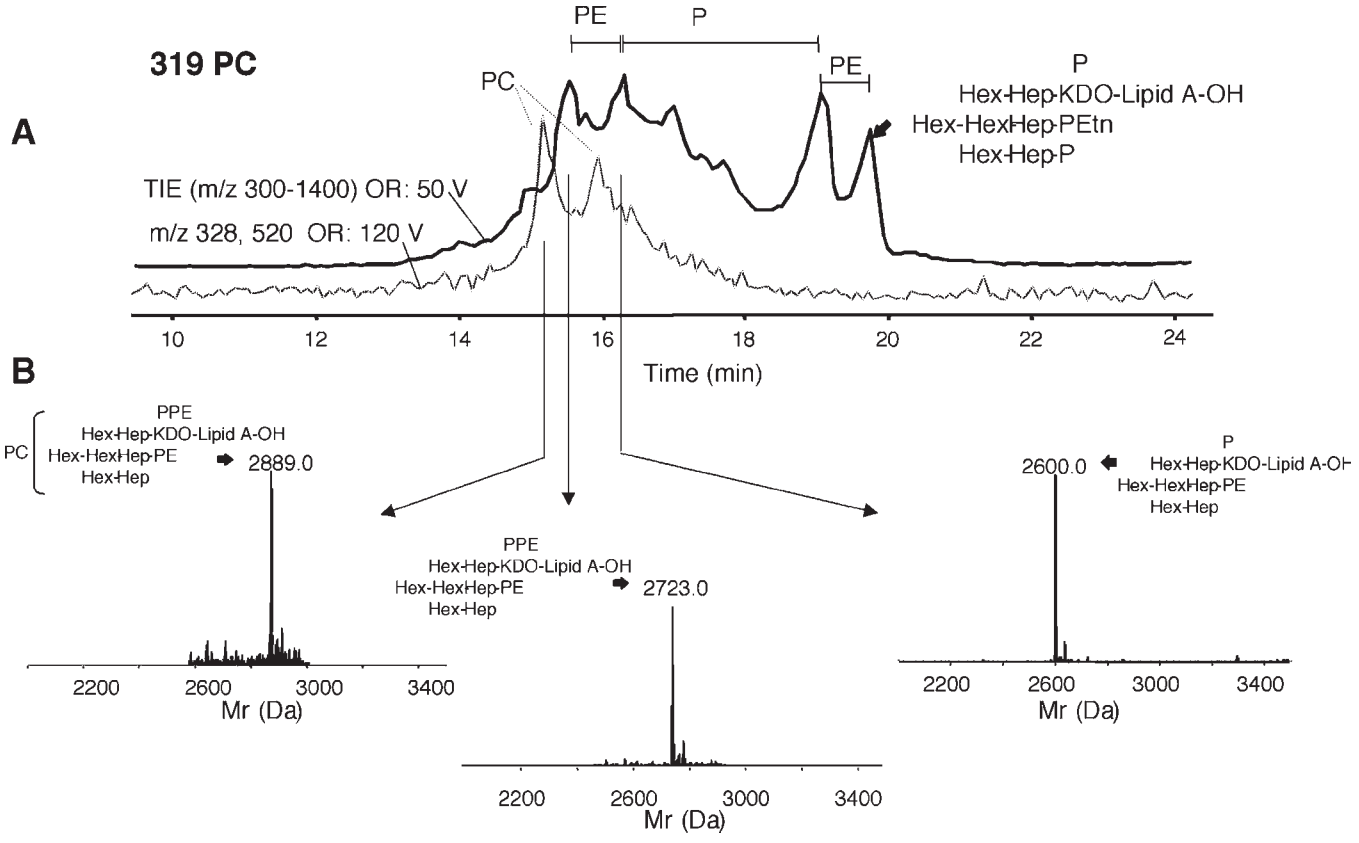

Fig. 4. Identification of PC containing *O*-deacylated LPS from *H. influenzae* strain 319 using polarity switching. **(A)** Total ion electropherogram, negative ion (*m/z* 300–1400) OR: 50 V, and ion profile for *m/z* 328, positive ion OR 120 V. **(B)** Reconstructed mass profiles for selected glycoforms are indicated for peaks at 15.0, 15.3, and 16.3 min. The data was obtained by injection of 20 ng of total *O*-deacylated LPS on a triple quadrupole instrument.

3. Inject approximately 40 nL (head pressure 300 mbar for 0.1 min) of the sample on the column using the CE instrument.
4. Record mass spectra by incorporating two periods for a given scan cycle; selected ion monitoring for *m/z* 290 for 200 ms, orifice voltage 120 V (negative ion mode) and full-scan acquisition for *m/z* 500–1500, 3 s/scan, orifice voltage 50 V (negative ion mode).

3.7. Preparation of Core Oligosaccharides

1. Suspend 5.0 mg of purifed LPS in 1 mL of 1% aqueous acetic acid and incubate at 100°C for 2–3 h.
2. Cool the reaction mixture in ice (0°C) and centrifuge to remove the insoluble lipid A component.
3. Lyophilize core oligosaccharide containing supernatant solution.
4. Separate core oligosaccharide mixture on a Bio-Gel P2 column using a pyridinium acetate (0.05 *M*, pH 4.5) elution buffer. Monitor refractive index to profile elution of core oligosaccharides
5. Collect and lyophilize fractions containing neutral sugars.

3.8. Following the Incorporation of NeuNAc in Core Oligosaccharides Using Positive Ion CE–ESMS Experiments and High/Low Orifice Stepping (See Note 8, Figs. 7 and 8)

1. Incubate 40 µg of core oligosaccharide with 6 mU of α-2,3-sialyltransferase at 37°C for 4 h. Dissolve to a concentration of approx 1 mg/mL of substrate in 10 m*M* MES, pH 6. Add CMP-NeuNAc (2 m*M* final).
2. Prepare BCQ-coated column as follows ***(30)***: The bare fused silica column is rinsed sequentially with 1 *M* NaOH, deionized water, and methanol, each for 1 h at 20 psi. The derivatization agent is passed through the column overnight (8–12 h) at 20 psi. The capillary is subsequently rinsed with methanol and deionized water (1 h each). All solutions are filtered before use through a 0.22-µm Ultrafree MC centrifuge filter unit (Millipore Corp., Bedford, MA). The freshly prepared coating solution is rinsed through the column for 8 h (or overnight) at 20 psi and flushed with deionized water for 1 h, purged with N_2, and stored.
3. Equilibrate column with 0.1 *M* HCOOH for 30 min and optimize the mass spectrometer in postive ion mode using a solution of angiotensin I (10 µg/mL in 0.1 *M* HCOOH) electroinfused at a voltage of –25 kV. (Note anodal electroosmotic conditions).
4. Inject approx 40 nL (head pressure 300 mbar for 0.1 min) of the sample onto the column using the CE instrument.
5. Record mass spectra by incorporating two periods for a given scan cycle; selected ion monitoring (SIM) for *m/z* 292, 200 ms, orifice voltage 100 V (positive ion mode) and full-scan acquisition for *m/z* 500–1500, 3 s/scan, orifice voltage 50 V (positive ion mode).
6. On-line MS–MS spectra are acquired using the same electrophoretic conditions as described in **Subheading 3.4.** except that precursor ions are selected by the

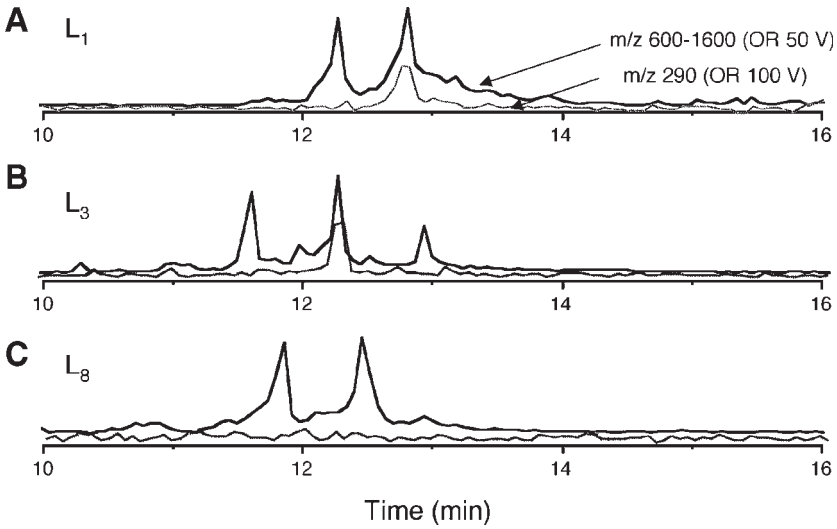

Fig. 5. Identification of sialylated *O*-deacylated LPS for *N. meningitidis* immunotypes L1 **(A)**, L3 **(B)**, and L8 **(C)**. The total ion electropherogram *m/z* 600–1600 (OR 50 V) is superimposed on the reconstructed ion profiles for fragment ion *m/z* 290 (OR: 100 V) obtained using orifice voltage stepping. The data were obtained in negative ion detection by injecting 20 ng of *O*-deacylated LPS on a triple quadrupole instrument.

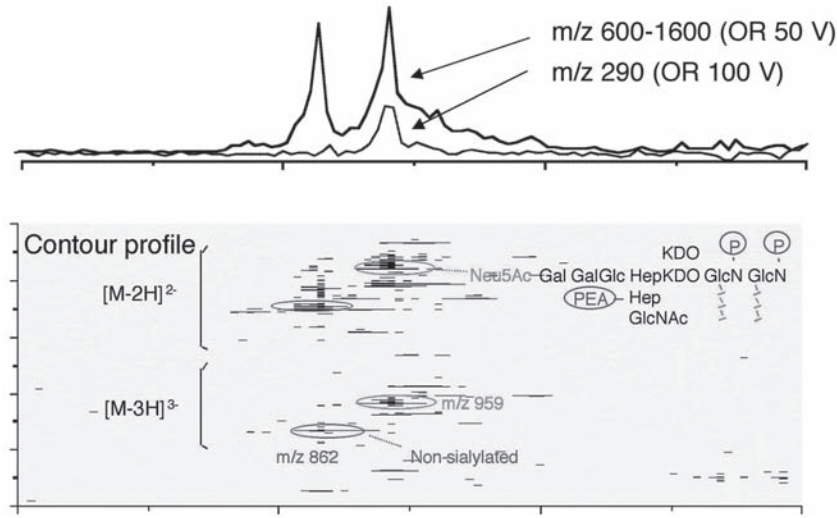

Fig. 6. CE–ESMS analysis (negative ion) of LOS arising from *O*-deacylation of LPS from *N. meningitidis* using mild hydrazinolysis treatment. **(A)** The total ion electropherogram *m/z* 600–1600 (OR 50 V) is superimposed on the reconstructed ion profiles for fragment ion *m/z* 290 (OR: 100 V) obtained using orifice voltage stepping. **(B)** Contour profile of *m/z* vs time. The sialylated and non-sialylated glycoform of the major LPS product is shown as an *inset*.

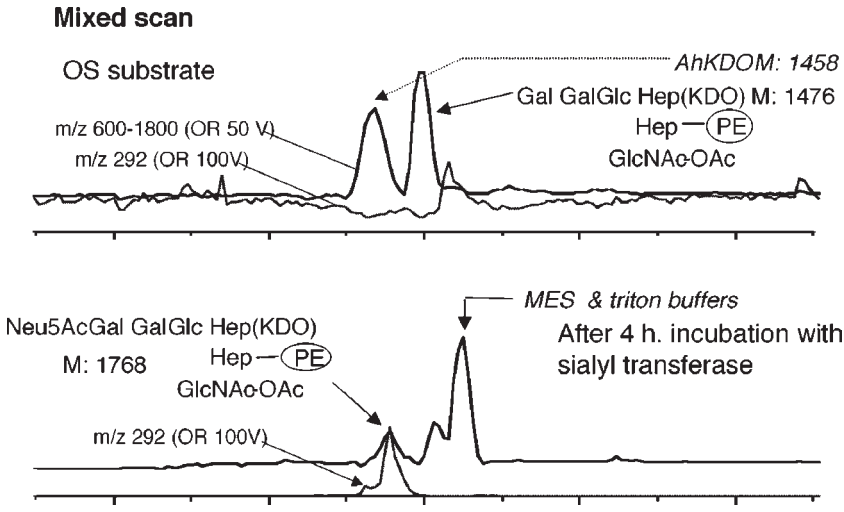

Fig. 7. CE–ESMS analysis (positive ion) of core oligosaccharide from mild acid hydrolysis of LPS from *N. meningitidis* immunotype L1 before **(A)** and after **(B)** incubation with α-2,3-sialyltransferase. The total ion electropherogram *m/z* 600–1800 (OR 50 V) is superimposed on the reconstructed ion profiles for fragment ion *m/z* 292 (OR: 100 V) obtained using orifice voltage stepping. The data were obtained in positive ion detection using a triple quadrupole instrument.

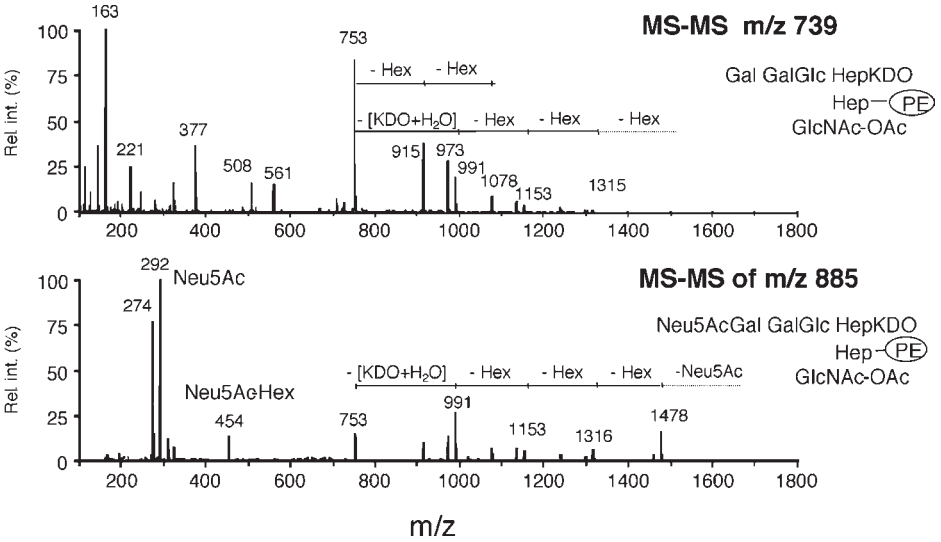

Fig. 8. CE–MS–MS analysis of core oligosaccharide from *N. meningitidis* immunotype L1. Product ion spectra of the doubly protonated ions **(A)** *m/z* 739 (substrate) and **(B)** *m/z* 885 (following incubation with α-2,3-sialyltransferase).

first quadrupole while the third quadrupole is scanned to transmit fragment ions formed in the radio frequency (RF) only collision cell. Collisional activation is conducted at typically 50 eV (laboratory frame of reference) using nitrogen as target gas.

4. Notes

1. A mutant strain referred to as RM 7004 *lic2B* was obtained from the culture collection of Professor E. R. Moxon (Oxford University, UK) and was produced by site-directed mutagenesis of encapsulated bacterium RM 7004 originally isolated from the cerebrospinal fluid of children with meningitis ***(27)***. *H. influenzae* strain 319 is a Hex4 phase variant of strain Eagan, obtained from Dr. J. Weiser (University of Pennsylvania). The *N. meningitidis* immunotypes L1 strain 126E (NRCC 4010), L3 strain 406Y (NRCC 4030), and L8 strain M978 (NRCC 4724) were from NRC's Institute for Biological Sciences (Ottawa, ON, Canada) bacterial culture collection.
2. Growth and manipulation of *N. meningitidis* and *H. influenzae* bacteria need to be conducted under level II containment to ensure proper biosafety. Once bacteria are killed by stirring cells with a phenol-containing solution (bacterial killing solution), LPS can be extracted using the precautions normally followed in the analytical chemistry laboratory.
3. LPS preparation material is obtained by the hot aqueous phenol extraction procedure ***(28)***. We have found that high yields of LPS can be achieved when the bacterial cell mass is dried before extraction with phenol–water, a procedure that is described elsewhere ***(29)***. LPS are obtained from the aqueous phase following extensive dialysis against running tap water or by precipitation with ethanol and, subsequently purified by ultracentrifugation ***(18)***. Although lower yields are obtained by the ethanol precipitation procedure, these LPS preparations have been found to contain less RNA as determined by sugar analysis. In the core of *H. influenzae* LPS, the latter preparations contain higher populations of LPS in which the KDO residues are substituted by PPE groups instead of P at the 0–4 position ***(18)***.
4. By using the mild *O*-deacylation described in **Subheading 3.3.**, anhydrous hydrazine provides an excellent method for solubilizing short-chain LPS samples through the removal of ester-linked fatty acids from the lipid A. However, this procedure also removes *O*-acetyl substituents that can be present in the core region of the molecule ***(30)***. The mild hydrazinolysis procedure is now well established for probing the heterogeneity of LPS from *H. influenzae* and the resulting anionic *O*-deacylated LPS can be analyzed directly by CE–ESMS (*see* **Note 5**).
5. CE–ESMS analyses are conducted using a coaxial sheath flow interface (*see* **Fig. 2**). Enhanced sensitivity and spray stability can be obtained by tapering the end of the CE capillary to 15 μm i.d. using a microtorch or a laser capillary puller ***(31)***. All buffers must then be filtered to avoid blockage of the capillary. Superior resolution of anionic *O*-deacylated LPS was found using a morpholine–acetate buffer compared to ammonium acetate or ammonium formate, presumably due

to buffer–analyte complexation. In addition, this buffer provides a low separation current and low conductivity, thereby preventing corona discharge. Methanol (5% v/v) is added to the separation buffer to reduce analyte adsorption on the capillary wall. Under the present operating conditions, a detection limit of 10 µg/mL is typically achievable in full mass scan acquisition using a triple quadrupole mass spectrometer. Separation requiring faster and more sensitive mass spectral acquisition (<1 s/scan) can be achieved using a time-of-flight instrument that also enables isotopic resolution of closely related glycolipids. **Figure 3** shows the analysis of *O*-deacylated LPS from a mutant strain RM 7004 where site-directed mutagenesis was targeted toward the expression of the *lic2B* gene. The contour profile (**Fig. 3B**) shows a series of doubly and triply deprotonated molecules from which the molecular mass profile can be reconstructed. The peak at 20.2 min gave molecular mass (M_{obs}) of 2761.9 Da, consistent with a glycolipid composed of PPE Hex_5 Hep_3 KDO $GlcN_2$ with two *N*- linked 3-OH myristic acid ($C_{14}H_{27}O_2$) and two phosphate groups ($M_{calc.}$: 2761.9 Da). The contour representation (**Fig. 3B**) enables the identification of closely related families of glycolipids based on the appearance of diagonal lines resulting from a regular increase of molecular mass and a concurrent decrease in electrophoretic mobility. For example, the *O*-deacylated LPS having a molecular mass of 2761.9 Da is a member of a glycoform family extending from Hex_3 to Hex_6, all of which comprise a single PPE group presumably on the KDO residue. A second set of glycoform having the same glycan distribution but having an additional PE group is observed in **Fig. 3B** as a parallel diagonal line shifted to earlier migration time. A third glycan series isomeric to the $Hex_{3\text{-}6}$ PPE family but of higher mobility (isoforms) is explained by the presence of a phosph*O*-KDO while the remaining PE group is located on the second Hep residue ***(18)***.

6. The monitoring of minor glycoforms substituted with PC functionalities is best achieved using high/low orifice stepping to promote the formation of a cationic fragment ion at *m/z* 328 characteristic of the Hex-PC residue (**Fig. 4**). Alternatively precursor ion scanning for *m/z* 328 can be used to probe this unusual functionality ***(32)***. Expression of PC on *H. influenzae* LPS is phase variable and can vary among different strains ***(10)***. Trace level detection of Hex-PC in *O*-deacylated LPS from *H. influenzae* strain 319 was achieved using polarity switching with a mixed scan function whereby the positive ion *m/z* 328 was acquired under SIM and full-scan acquisition with negative ion detection. Glycoform assignments are indicated in **Fig. 4**. This method can also be employed in the negative ion mode for the detection of PPE groups (characteristic fragment ion at *m/z* 220) and terminal NeuNAc groups (*m/z* 290) ***(23)***. The use of morpholine is not completely compatible with positive ion detection owing to the propensity of morpholine to form multimolecular adducts with multiply protonated species ***(23)***. This problem can be overcome by using ammonium acetate buffers, although this reduces electrophoretic resolution.
7. The high/low orifice stepping can be used to identify terminal NeuNAc residues in *O*-deacylated LPS using negative ion detection. **Figure 5** illustrates this appli-

cation for different immunotypes of *N. meningitidis*. Glycoforms containing NeuNAc (L1 and L3) are highlighted in the *m/z* 290 ion profile and contrasted against a negative control from *N. meningitidis* L8 immunotype. The CZE-ESMS analysis of *O*-deacylated LPS derived from *N. meningitidis* L1 immunotype is presented in **Fig. 6** and shows two well-resolved peaks at 12.2 and 12.8 min. The extracted mass spectrum of the early migrating component presented abundant multiply charged ions at *m/z* 647, 862, and 1294 (**Fig. 6B**) giving a molecular mass of 2590 ± 1 Da consistent with the expected value for the monoisotopic mass ($M_{calc.}$: 2591.02 Da) for an *O*-deacylated LPS containing a Pk epitope (**Fig. 1B**). The second peak of higher intensity ($M_{obs.}$: 2881 ± 1 Da) corresponded exactly to a further addition of a NeuNAc residue.

8. An extract of a recombinant α-2,3-sialyltransferase ***(33)*** stabilized in 0.2% Triton X-100 was obtained from Drs. W. Wakarchuk and M. Gilbert (IBS, Ottawa). Core oligosaccharides obtained from mild acid hydrolysis of *N. meningitidis* L1 LPS were incubated with a purified extract of sialyl transferase to monitor the enzymatic incorporation of NeuNAc on the terminal Gal residue of this immunotype. In addition to the cleavage of the KDO–GlcN bond, the mild acid treatment of sialylated LPS generally results in the loss of terminal NeuNAc residues. These asialylated oligosaccharides were found to be convenient soluble substrates for the sialyl transferase. The absence of NeuNAc residue was confirmed by CE–ESMS in a manner similar to that described previously (*see* **Note 7**) except that the posititve oxonium ion of NeuNAc at *m/z* 292 was monitored instead (*see* **Fig. 7**). Separation of these closely related glycoforms was best effected in positive ion mode using a BCQ-coated column to prevent analyte adsorption when using acidic buffers. The major oligosaccharides found in the mild acid hydrolysis sample corresponded to the core asialylated L1 saccharide comprising an anhydro side chain KDO, an *O*-acetyl GlcNAc, and a Hep-PE (M_{obs}: 1476 Da) as shown in **Fig. 7A**. CE–ESMS analysis of oligosaccharides following incubation for 4 h with sialyl transferase indicated partial incorporation of NeuNAc to the parent substrate (**Fig. 7B**). Unambiguous identification of sialylated oligosaccharide was confirmed by conducting on-line MS–MS analyses of the same sample (**Fig. 8**). Fragment ions result from the consecutive cleavage of glycosidic bonds. The presence of a terminal NeuNAc is substantiated by the observation of abundant fragment ions at *m/z* 292 and 274 corresponding to the oxonium ion and its dehydrated form, respectively.

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15

Capillary Electrophoresis as an Assay Method for Monitoring Glycosyltransferase Activity

Warren W. Wakarchuk and Anna-Maria Cunningham

1. Introduction

Glycobiology has rapidly developed into an important area of research devoted to the understanding and analysis of the interactions that carbohydrates have with other biomolecules. To accompany the rapid development of this field, better techniques for the analysis of the biosynthesis of complex carbohydrates are required. To investigate interactions between proteins and carbohydrates, an adequate supply of carbohydrate ligand must be obtained. Isolation of biologically active carbohydrates is possible, but only in small amounts. Recent efforts in the cloning of glycosyltransferase genes from a variety of sources has opened the possibility of enzymatic synthesis or chemienzymatic synthesis of a larger number of relevant oligosaccharides (***1–4***, http://www.vei.co.uk/tgn/gt_guide.htm).

To utilize glycosyltransferases for large-scale synthesis, and for the discovery of new specificities, a simple, rapid, and sensitive assay technique is required. The traditional assay for glycosyltransferases has been to monitor incorporation of sugars from radiolabeled sugar donors into either endogenous carbohydrate or an exogenously added synthetic acceptor. The labeled products could then be captured by a resin, or recovered by precipitation as insoluble material ***(5,6)***. To determine the enzyme specificity the products are then analyzed by degradation with glycosidases specific for a particular linkage. Assays have also been developed using antibodies or lectins to detect the carbohydrate products, but one must have such an antibody to the oligosaccharide, and there are not always lectins that will bind the compound of interest ***(7,8)***. Assays using glycosyltransferase acceptors coupled to suitable chromophores have been described for use in assays using high-performance liquid chromatography (HPLC) ***(9,10)***, gel electrophoresis, or capillary electrophore-

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sis (CE) ***(11,12)***. Some of these methods involve a multistep synthesis of a sugar derivative that can be labeled with a chromophore or fluorophore to provide the acceptor for use in the glycosyltransferase assay ***(12–14)***. With the sensitive detection methods that have been described, better access to chromophore- and fluorophore-labeled sugar derivatives would facilitate the analysis of glycosyltransferases (and glycosidases) and many glycobiology researchers would no longer be dependent on specialist laboratories to synthesize these acceptor molecules.

Our laboratory has been investigating glycosyltransferases from several species of pathogenic bacteria that synthesize carbohydrate structures that are identical to those found in their respective hosts. For the identification and cloning of the important enzyme activities from these strains we have developed a CE-based assay, which has allowed us to perform activity- based screening of genomic libraries. We have developed a single-step synthesis and single step purification of carboxyfluorescein-labeled acceptor molecules using the commercially available compounds *p*-aminophenylglycosides and 6-(fluorescein-5-carboxamido) hexanoic acid succimidyl ester. The examination of these enzymes has been aided by a second type of acceptor made by the reductive amination of commercially available oligosaccharides with the fluorophore 8-aminopyrene-1,3,6-trisulfonate ***(15)***. The rationale for the development of these labeled acceptors was based on literature that describes CE of such derivatives as a rapid and extremely sensitive means to quantitate fluorescent sugar derivatives ***(16,17)***. Our work on the characterization of bacterial glycosyltransferases has shown these derivatives to be very useful for the detection of enzyme activity from crude cell extracts, the identification of enzymes in recombinant clones, and their characterization.

2. Materials

2.1. Synthesis of Fluorophore-Labeled Glycosyltransferase Acceptors

2.1.1. Derivatives of 6-(Fluorescein-5-Carboxamido) Hexanoic Acid Succimidyl Ester (FCHASE)

1. Most of our work has used the following glycosides: *p*-aminophenyl-β-2-deoxy-2-acetamido-glucosamine, *p*-aminophenyl-β-galactose, *p*-aminophenyl-β-glucose, *p*-aminophenyl-β-lactose (Sigma).
2. 6-(Fluorescein-5-carboxamido)hexanoic acid succimidyl ester, as a single isomer (FCHASE, Molecular Probes).
3. Reaction buffer: 20 m*M* $Na_4B_2O_7$, pH 8.6, with methanol to solubilize the FCHASE.
4. Silica-60 thin-layer chromatography (TLC) plates, 0.2 mm and 1 mm. Solvent: Ethyl acetate–methanol–water–acetic acid (7:2:1:0.1).
5. Sep-Pak C_{18} cartridges from Waters, with 50% acetonitrile–50% water for elution.

2.1.2. Derivatives of 8-Aminopyrene-1,3,6-Trisulfonate (APTS)

1. Lacto-*N*-neotetraose or other reducing oligosaccharide.
2. 8-Aminopyrene-1,3,6-trisulfonate (Molecular Probes).
3. 10% Acetic acid in water, tetrahydrofuran (THF).
4. Sodium cyanoborohydride.
5. TSK-HW40S gel permeation column with NH_4HCO_3 or water as an eluant.

2.2. Glycosyltransferase Reactions

1. Sugar nucleotide donors, UDP-Gal and CMP-*N*-acetylneuraminic acid (Neu5Ac).
2. 0.5 *M N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES) buffer, pH 7.5.
3. Sodium acetate buffer, 0.5 *M*, pH 6.0.
4. 100 m*M* $MnCl_2$.

2.3. Detection of Enzyme Activity Using CE-LIF

1. Beckman CE system equipped with LIF detector.
2. CE system equipped with an ultraviolet–visible spectrum (UV–VIS) or diode array detector.
3. Scanning spectrophotometer.

3. Methods

3.1. Synthesis of FCHASE-Labeled Aminophenylglycosides (See Note 1 and Fig. 1)

1. Dissolve10 mg of *p*-aminophenylglycoside (Sigma) in 0.5 mL of 20 m*M* sodium tetraborate, pH 8.6.
2. Dissolve 5 mg of 6-(fluorescein-5-carboxamido)hexanoic acid succimidyl ester (single isomer, Molecular Probes) in 0.5 mL of methanol, and add to the aminophenylglycoside solution.
3. Stir the mixture in the dark for 3 h at room temperature. Evaporate the mixture to dryness in a Savant SpeedVac.
4. Resuspend the dry mixture in 100–200 µL of 50% acetonitrile and apply the suspension on a 1 mm thick, 20 × 20 cm silica 60 TLC plate. Develop the TLC with the following solvent system: ethyl acetate–methanol–water–acetic acid (7:2:1:0.1). Depending on the starting glycoside, the R_f of the product is between 0.2 and 0.6.
5. After air drying in a fume hood, scrape the bright yellow product off the plate and elute it 5× with 10 mL of distilled water. Pool the water eluates and concentrate and desalt by binding to a Sep-pak C_{18} reverse-phase cartridge. After washing the cartridge with 20 mL of water, elute the product in 1–3 mL of 50% acetonitrile. Quantitate the product by spectrophotometry with $\varepsilon_{494} = 68{,}000\ M^{-1}\ cm^{-1}$.

3.2. Synthesis of APTS-Labeled Lacto-N-neotetraose (See Note 2 and Fig. 1)

1. Dissolve 1.5 mg (2 µmol) of lacto-*N*-neotetraose in 25 µL of water.

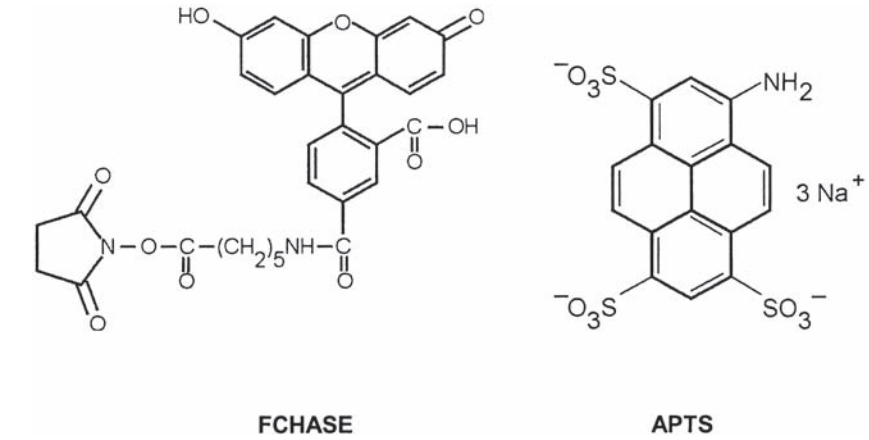

Fig. 1. Reagents for the labeling of *p*-aminophenylglycosides, and reductive amination of reducing sugars. FCHASE is 6-(fluorescein-5-carboxamido)hexanoic acid *N*-hydroxysuccimidyl ester (single isomer) (Molecular Probes, cat. no. F-6106). APTS is 8-aminopyrene-1,3,6-trisulfonate (Molecular Probes, cat. no. A-6257).

2. Dissolve 2.0 mg (4 µmol) of APTS in 10 µL of 10% acetic acid.
3. Dissolve 1 mg (15 µmol) of sodium cyanoborohydride in 25 µL of THF.
4. Mix the ingredients together in a screw-cap microcentrifuge tube and incubate at 37°C for 12–16 h.
5. Evaporate the reaction mixture to dryness in a Savant SpeedVac, and then redissolve in 0.3 mL of water.
6. Apply the redissolved material to a 1.5 × 20 cm column of TSK HW40F resin, equilibrated in 50 m*M* ammonium bicarbonate. Monitor the eluant at 254 nm. The recovered peak can be quantitated by using $\varepsilon_{455} = 17{,}150\ M^{-1}\ cm^{-1}$.

3.3. Analysis of Fluorophore- and Chromophore-Labeled Acceptors (See Note 3 and Fig. 2)

1. Perform capillary electrophoresis with a Beckman P/ACE 5510 equipped with an argon ion LIF detector, $\lambda = 488$ nm. For UV detection, perform the separation on a P/ACE 5000 equipped with a diode array detector.
2. Use a standard 50 µm × 50 cm bare silica capillary, with the detector window at 47 cm. Condition the capillary before each run by washing it with 0.2 *M* NaOH for 2 min; water for 2 min; then 20 m*M* sodium phosphate–25 m*M* sodium tetra-borate, pH 9.4, for 2 min. For some separations the buffer was 10 m*M* sodium tetraborate, 20 m*M* sodium dodecyl sulfate (SDS), 60 m*M* sodium phosphate. For APTS-labeled compounds use 20 m*M* sodium phosphate buffer, pH 7.4.
3. Introduce samples by pressure injection for 2–5 s, and perform the separation at 18–22 kV, 50–70 µA. Conduct peak integration with the P/ACE station software package (Beckman-Coulter).

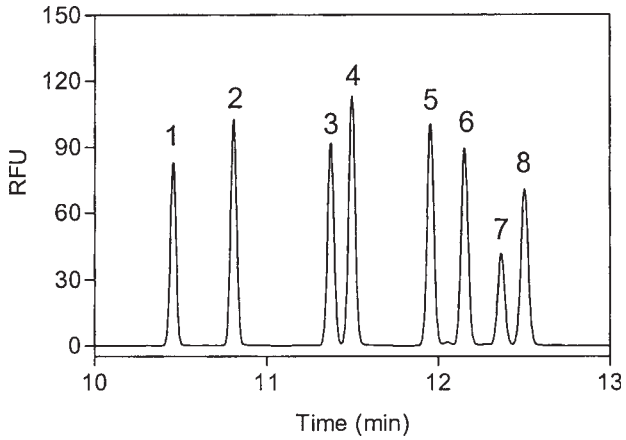

Fig. 2. CE separation of FCHASE-aminophenylglycosides. The structures for the carbohydrate portions of these conjugates are: GlcNAc-β-1,3-Gal-β-1,4-Glc-(1); Gal-α-1,4-Gal-β-1,4-Glc-(2); Gal-β-1,4-GlcNAc-(3); Gal-β-1,4-Glc-(4); GlcNAc-β-(5), α-2,3-Neu5Ac-α-2,3-Gal-β-1,4-GlcNAc-(6); Neu5Ac-α-2,3-Gal-β-1,4-Glc-(7), and Gal (8). The running buffer for this separation was 10 m*M* sodium tetraborate, 20 m*M* SDS, 60 m*M* sodium phosphate.

3.4. Detection of Enzyme Activity in Bacterial Extracts (See *Note 4* and *Fig. 3*)

3.4.1. From Nonrecombinant Sources

1. Scrap a colony, or loopful, of bacteria from a fresh plate, and disperse it in a small amount of buffer. For single-colony assays, 10 µL of buffer is sufficient.
2. A more efficient extraction may be desired if enzyme activity cannot be detected in permeabilized cells. To obtain a small-scale extract, the cells should be first frozen in a microcentrifuge tube, then an equal volume of glass beads (100–110 µ*M*) should be added and the mixture ground for 1–2 min with a small tapered pestle, which fits down into the narrow part of the tube. To this paste add 50–100 µL of 50 m*M* HEPES buffer, pH 7.0, and continue grinding for 1 min. A second portion of buffer can be added, then clarify the mixture by a slow-speed centrifugation (2000*g*).
3. Use portion of this extract in a transferase assay.

3.4.2. From Recombinant Libraries (See *Note 5* and *Fig. 4*)

1. Plate a λZAPII genomic library of *N. meningitidis* MC58 at low density and pick 3600 well isolated plaques in pools of 100 (*see* **ref. *1*** for more details). Make phage suspensions and used them to infect 1.5-mL cultures of *E. coli* XL1-Blue (in LB medium with 0.2% maltose, 10 m*M* $MgSO_4$ and 2 m*M* isopropyl-β-D-1-thiogalactopyranoside [IPTG]) grown at 37°C for 4.5 h.

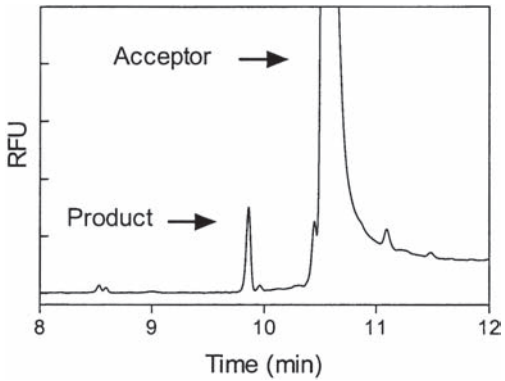

Fig. 3. Single-colony assay for α-galactosyltransferase activity from *Moraxella catarrhalis*, where the acceptor was Gal-β-FCHASE. The assay was performed by taking one colony from a plate and dispursing it in a 20-μL transferase reaction mixture. The reaction mixture contained 50 m*M* MES-NaOH, pH 6.5, 10 m*M* $MnCl_2$; 5 m*M* dithiothreitol; and 1 m*M* UDP-Gal. This reaction solution was incubated for 1 h at 37°C, and then the cells were removed by centrifugation and the mixture analyzed by CE. The area of product peak corresponds to ~10^6 molecules as calculated from a standard curve. In this electropherogram the acceptor peak height was ~1000 RFU, while the product peak height was ~1.5 RFU.

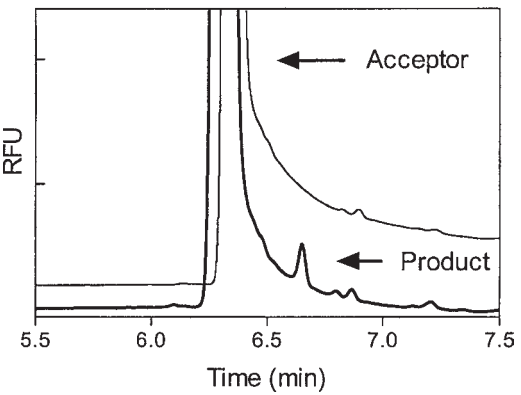

Fig. 4. Activity screening of recombinant libraries to identify glycosyltransferases. Using a λ phage library with DNA from *N. meningitidis*, we started screening pools of 1000 plaques. The thinner line was an electropherogram from a reaction for which no product was formed, the thicker line was from an electropherogram from a reaction in which α-2,3-sialyltransferase was present. The electropherograms were deliberately offset to make the visual comparison easier. For these running conditions the sialylated product migrated after the acceptor, as the overall charge of the molecule has increased, which slows its migration.

2. Collect the cells by centrifugation, and then resuspend and sonicate them in 0.5 mL of buffer. The reaction solutions contain 13 µL of unclarified sonicated extract; 50 m*M* 2-morpholinoethanesulfonic acid (MES), pH 6.0; 10 m*M* $MgCl_2$; 0.4 m*M* CMP-Neu5Ac; and 0.5 m*M* LacNAc-FCHASE. Incubate the solutions overnight at 32°C.
3. A similar approach can be used with colonies, and we have used pools of 100 for the initial sceen as well. With colonies the growth prior to screening is as follows: The colonies are picked and dispersed into 1 mL of media with 50% glycerol. For screening, a 1.5-mL culture is started with 20 µL from the 1-mL glycerol stock. The culture is grown for 1–2 h and then IPTG is added to 1 m*M* and the growth continued for 4 h at 37°C. The remainder of the glycerol stock is stored at –20°C.

3.5. Measurement of Kinetic Parameters of an α-2,3-Sialyltransferase with the CE-Based Assay (See Note 6 and Fig. 5)

1. For measurement of K_m and k_{cat} values, perform assays with the APTS-labeled acceptor at room temperature. Monitoring of products should be carried out for all acceptor concentrations to ensure that no more than 10% conversion to product occurred, and that the reaction rate was linear. Determine empirically the ranges of acceptor and donor concentrations, then use a range spanning 0.2 K_m up to 5 K_m to obtain the values. Examine the data using the Grafit™ 3.0 software package (Erithacus Software, London, UK).
2. Purify the enzyme used in this analysis by immobilized metal affinity chromatography as described by Gilbert et al. ***(2)***. A series of test reactions are required to determine what concentration of enzyme would give the desired level of conversion.

4. Notes

1. For the labeling with FCHASE we normally use a two-fold excess of the *p*-aminophenylglycoside, as the FCHASE compound is much more expensive than the glycoside. For analysis by CE it is critical to use the isomer-free FCHASE material, as the isomers migrate differently, which gives rise to complex electropherograms after enzyme reactions. We have also used the fluroescein-5-EX reagent (Molecular Probes, cat. no. F-6130) for labeling but do not recommend it because it appears to isomerize readily after the labeled *p*-aminophenylglycosides have been isolated. We do not know the nature of this isomerization, but we have observed a peak with the same mass but different mobility by both TLC and CE. The two isomers peaks are equally reactive in the assay, which makes it difficult to do quantitive analysis when there are isomer peaks in the electropherogram. Initially the reagent we used for labeling the *p*-aminophenylglycosides was fluorescein isothiocyanate (FITC), but we have found this to make less stable linkages that degrade over a few months. Compounds made with FCHASE are stable for at least 1 yr when stored in 50% acetonitrile at –20°C. The yield of labeled material is usually between 65 and 90% based on the FCHASE compound.

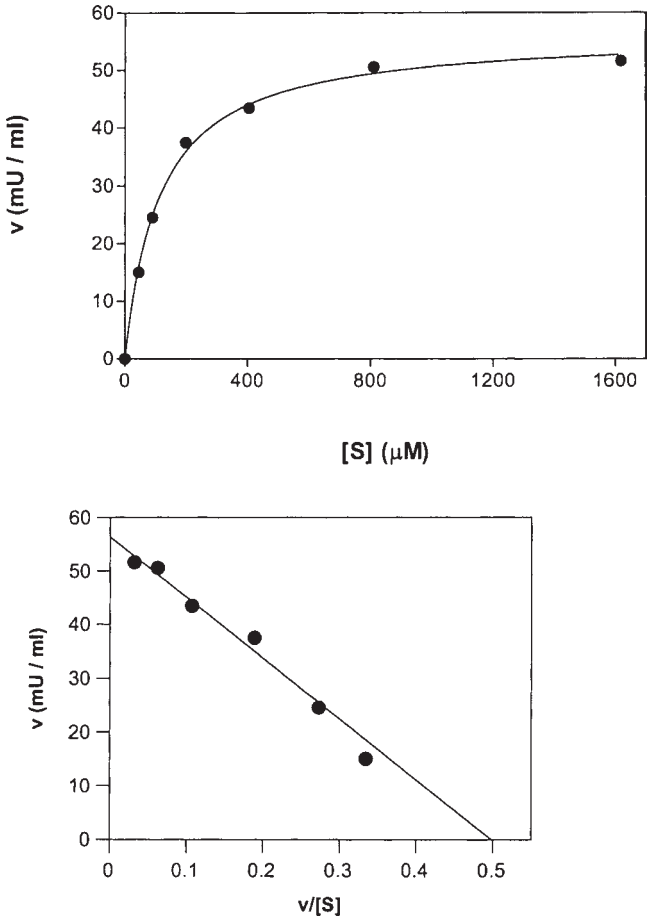

Fig. 5. Progress curve and Eadie–Hofstee plot to determine the K_m(app) and k_{cat}(app) for the APTS conjugate of lacto-*N*-tetraose with the first protein from *N. meningitidis*. The donor concentration was 200 μ*M* CMP-Neu5Ac and the sialyltransferase was used at 54 μg/mL. The activity data that were plotted were converted to mU/mL. From this data the K_m(pp) was 113 μ*M* ± 9 with a V_{max} of 56 ± 1 mU/mL, which gives a k_{cat}(app) value of 2.3 min^{-1}.

Because not all the *p*-aminophenylglycosides we needed to use were commercially available, we have used *p*-nitrophenylglycosides, which are available for more sugars than are the aminophenyl compounds. These were then reduced to the aminophenyl compound by catalytic hydrogenation, and then labeled and purified as described.

We have also taken FCHASE- and APTS-labeled materials and used enzymatic synthesis to extend the structure to have a broader spectrum of acceptors for

enzyme assays. An excellent reference on preparative enzymatic synthesis is from Dr. Monica Palcic of the University of Alberta ***(18)***. Using published reaction conditions ***(4,18)***, we synthesized LacNAc-FCHASE, a very good acceptor for the *CMPN. meningitidis* α-2,3-sialyltransferase, from GlcNAc-FCHASE using either commercial bovine β-1,4-galactosyltransferase or a bacterial enzyme of the same specificity ***(4)***. These reactions go essentially to completion, which means they require only binding and elution from the SepPak cartridge for purification.

2. The APTS-labeled oligosaccharides are very water soluble and highly charged. This has meant we have been unable to develop a TLC solvent that would adequately separate enzymatic reaction products. It is possible to separate unreacted APTS from the labeled saccharide using *n*-propanol–ethanol–water (5:3:2). It is not recommended to use this for preparative TLC, as the subsequent concentration of APTS-labeled oligosaccharides is not as simple as FCHASE, as APTS-labeled material does not adsorb to the SepPak cartridges. For this reason we recommend the gel permeation step described in **Subheading 3.2.** The yield of labeled material based on the initial oligosaccharide was approx 80%. The major advantage of APTS-labeled saccharides over the FCHASE-labeled material is their higher solubility. For kinetic analysis some of the FCHASE-monosaccharide acceptors were not soluble at the concentrations required for a complete acceptor range (>6 m*M*). In addition, it was easier to label the tetrasaccharides, lacto-*N*-tetraose and lacto-*N*-neotetraose, with APTS, than it would have been to enzymatically build up the equivalent from Lac-FCHASE. For general use we recommend the FCHASE-labeled saccharides as they have a more intense fluorescence than the APTS-labeled saccharides, and the FCHASE material is easy to analyze and purify by TLC, whereas the APTS-labeled saccharides are not.
3. For most of the separations with FCHASE- and APTS-labeled glycosides, 20 m*M* sodium phosphate–25 m*M* sodium tetraborate, pH 9.4, as running buffer is adequate. There are some FCHASE glycosides that do not give baseline separation with this buffer. Such pairs we have seen are Glc-FCHASE/Lac-FCHASE and Lac-FCHASE/Gal-α-1,3-Lac-FCHASE. For these separations a suitable running buffer was 10 m*M* sodium tetraborate, 20 m*M* SDS, and 60 m*M* sodium phosphate with 5% methanol.

   Prior to CE analysis, it is essential that the FCHASE-labeled samples be diluted to lower the ionic strength to ensure sharp peaks. In addition the samples have to be diluted to a concentration of 2–5 μ*M*, which makes the unreacted acceptor FCHASE-saccharide peak about 300–700 relative fluorescent units (RFU). For accurate quantitation we use a percent conversion based on the acceptor peak. This method then does not depend on the injection of exactly the same amount of material, as it is a relative measure. For increased sensitivity to detect very small amounts of product we have used FCHASE concentrations as high as 50 μ*M*. At concentrations higher than 10–20 μ*M* peak tailing becomes apparent, and the acceptor peak is >1000 RFU, which is off scale, making calculation of the percentage conversion very inaccurate.

4. It is not necessary to make extracts to observe glycosyltransferase activity from certain bacterial species. We have been able to take colonies from a freshly grown plate and simply disperse the colony in an enzyme reaction mixture. Another technique that is useful is to simply treat a cell suspension with 1% toluene to permeabilize them prior to the assay. Sonication can also be used, but care must be taken with small samples not to overheat them. When a small-scale extract is made either by sonication or grinding with glass beads the mixture should not be clarified by centrifugation at high speed, as the enzymes are frequently in the particulate fraction. The levels of enzyme activity observed are generally higher when a technique is used to disrupt the cells prior to the assay.
5. We have previously published the use of the CE-based assay to screen recombinant libraries for α-2,3-sialyltransferase ***(1)***. The genomic library was made from the *N. meningitidis* strain MC58; this strain contains an α-2,3-sialyltransferase for which we had developed the CE-based assay using LacNAc-FCHASE. The LacNAc-FCHASE was synthesized enzymatically from GlcNAc-FCHASE as we have previously described ***(1)***. This screening technique has been used with both λ phage and plasmid-derived genomic libraries. We were able to see enzyme activity in pools as large as 5000 λ clones. For these screening reactions is essential to run a control reaction without the glycosyltransferase donor substrate, so that a very small product peak can clearly be indentified from the background.
6. Because it is not possible to do continuous assays with these substrates, we had to do single-point assays from the linear portion of the reaction. With these acceptors the linear range of the reaction is up to 40% conversion; however, we kept the level of conversion lower than 10% for all kinetic analysis. We were able to ensure that the reaction was performed with excess donor by empirically determining the saturating concentrations for each substrate.
   The kinetic parameters were also determined for the donor CMP-Neu5Ac, using the same technique as for the acceptor. The problem with using the CE method for determining donor kinetic parameters is that at very low donor concentrations the amount of product formed in the reaction becomes very small. For example, if the acceptor concentration is 500 μ*M*, and the donor concentration is 10 μ*M*, for the reaction to be 10% conversion of the donor, this is only 1 μ*M* product (0.2% conversion of the acceptor). These very small amounts of product are close to values that cannot be accurately integrated. Another method we have used is to make a standard curve for the product and then simply use the peak area to calculate the amount of product. This method is not as accurate as using the percentage of conversion considering that the reproducibility of the volume injected into the capillary introduces some error.

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16

Determination of Association Constant of Carbohydrate–Protein Interaction

Atsushi Taga and Susumu Honda

1. Introduction

The high capabilities of capillary electrophoresis (CE) in separation and detection have been well recognized, and most chapters of this book are devoted to carbohydrate analysis utilizing these capabilities. CE has other inherent advantages. One is that it allows real-time observation of chemical as well as physical reactions occurring in a capillary. We have previously published a few articles on various techniques of in-capillary chemical derivatization and demonstrated the usefulness of these techniques for the analysis of biological substances ***(1–3)*** and now discuss physical interactions in a capillary.

When a protein is introduced to an electrophoretic solution containing a ligand in the electric field, the protein migrates under the influence of the ligand, resulting in a change of migration velocity. Mathematical treatment of this velocity change, or migration time change, gives important information on the magnitude of the interaction. There are two systems for such binding studies; one is the normal system composed of a protein as sample and a ligand as additive and the other is the reversed system composed of a ligand as sample and a protein as additive. Each system has its own characteristic features.

This chapter focuses on the observation of carbohydrate–protein interactions. In the normal system a protein sample is introduced to a capillary filled with an electrophoretic solution containing a carbohydrate ligand as additive. The carbohydrate must induce enough mobility change of the protein to a measurable extent, because the magnitude of interaction is estimated from this mobility change. If the carbohydrate under investigation has an electric

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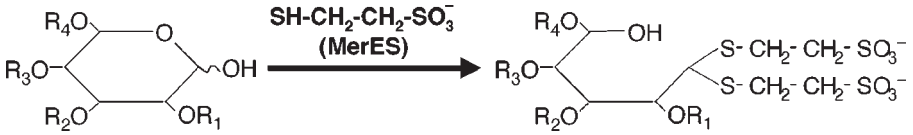

Fig. 1. Introduction of the sulfonate tags to a reducing carbohydrate by dithioacetalation with MerES.

charge, as in the case of lactobionic acid ***(4)*** adopted as a model ligand in the first example of this series of our studies, it can be a direct object of this interaction experiment. However, as most carbohydrates have no electric charge under the conditions employed in the CE analysis for such binding studies, prior chemical derivatization to ionic species is required. The dithioacetal method described in this chapter ***(5)*** is convenient for this purpose. Two 2-mercaptoethanesulfonate (MerES) groups are introduced to each reducing carbohydrate by a simple procedure using immersion for a short time in a trifluoroacetic acid solution of MerES (**Fig. 1**).

As the introduced sulfonate group gives negative charge and high mobility to the derivative under neutral conditions, the derivative can be used as the substitute for the original carbohydrate.

The principle of the estimation of association constant was described in the aforementioned first paper ***(4)***, as outlined below.

As illustrated in **Fig. 2**, the interaction between a protein and a carbohydrate in the electric field is an equilibrium reaction and the association constant (K_a) of the protein to the carbohydrate can be written as **Eq. 1**:

$$K_a = [PC]\,[P]^{-1}\,[C]^{-1} \tag{1}$$

where [P], [C], and [PC] are the concentrations of the protein, the carbohydrate, and the complex thereof, respectively. On the other hand the molar fraction of the complex to the total protein (α) is

$$\alpha = [PC]\,([P] + [PC])^{-1} \tag{2}$$

Therefore, **Eq. 3** is derived from **Eqs. 1** and **2**.

$$K_a^{-1}\,[C]^{-1} + 1 = \alpha^{-1} \tag{3}$$

Apparent migration velocities of the protein in the absence (V_o) and the presence (V_P) of the carbohydrate are given by **Eqs. 4** and **5**, respectively,

$$V_o = V_{eo} - v_P \tag{4}$$

$$V_P = V_{eo} - \{\alpha v_{PC} + (1 - \alpha)\, v_P\} \tag{5}$$

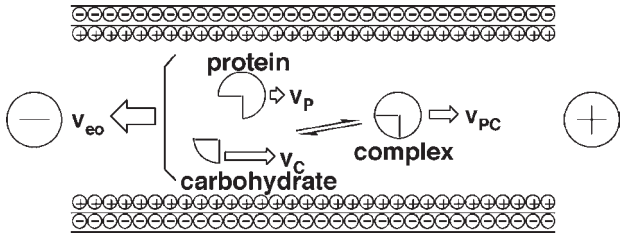

Fig. 2. Carbohydrate–protein interaction in a capillary in the electric field.

where V_{eo} is the velocity of electroosmotic flow (EOF), and v_P and v_{PC} are the velocities of electrophoretic migration of the protein and the complex, respectively.

Subtraction of **Eq. 5** from **Eq. 4** gives **Eq. 6**:

$$V_o - V_P = \alpha(v_{PC} - v_p) \tag{6}$$

and substitution of α from **Eq. 3**) into **Eq. 6** gives

$$K_a^{-1}\,[C]^{-1} + 1 = (v_{PC} - v_p)\,(V_o - V_P)^{-1} \tag{7}$$

Here,

$$V_o = l\,t_1^{-1} \text{ and } V_P = l\,t^{-1}$$

and

$$v_{PC} - v_P = l\,[(t_0^{-1} - t_2^{-1}) - (t_0^{-1} - t_1^{-1})] = l\,(t_2 - t_1)\,t_1^{-1}\,t_2^{-1} \tag{8}$$

where t_0, t_1 and t_2 are the migration times of the neutral marker, the protein, and the complex, respectively, and l is the effective length of a capillary. The term t_1 is the migration time of the protein in the absence of the carbohydrate and t_2 can be approximated as the time when further addition of the carbohydrate does not change migration time of the protein any more.

Therefore, an important **Eq. 9** is derived **Eqs. 6–8** as follows,

$$(t - t_1)^{-1} = t_1^{-1}\,t_2\,(t_2 - t_1)^{-1}\,K_a^{-1}\,[C]^{-1} + (t_2 - t_1)^{-1} \tag{9}$$

In **Eq. 9** $(t - t_1)^{-1}$ is a first-order function of $[C]^{-1}$, and gives a straight line with a slope $t_1^{-1}\,t_2\,(t_2 - t_1)^{-1}\,K_a^{-1}$ (A) and a Y-intercept $(t_2 - t_1)^{-1}$ (B). Consequently K_a can be obtained as follows:

$$K_a = (Bt_1 + 1)\,A^{-1}\,t_1^{-1} \tag{10}$$

This principle is based on the estimation of mobility difference, that is, migration time difference, of a protein sample between the absence and the presence of a carbohydrate ligand. Because this change is small (usually within

1 min) and the migration time may fluctuate owing to the variation of the velocity of EOF, migration time should be measured as accurately as possible. Correction for the variation of EOF is also important to obtain a reliable value of association constant. With careful measurement of migration times and reasonable correction, however, relative standard deviation of K_a can be reduced to as low as approx 3%.

It should be stressed that this method allows the observation of the interaction between a protein and a carbohydrate under circumstances very similar to physiological conditions. It does not require immobilization of one of this pair, unlike in affinity chromatography and surface plasmon resonance methods for the same purpose. The CE method might usually involve derivatization of the carbohydrate with MerES, but the introduced MerES group does not have a significant influence on the estimation of association constant.

In addition this method has an advantage in that it requires only small amounts of a carbohydrate ligand and a protein sample, especially the latter, although the lower limits depend on the magnitude of interaction. A further advantage is that the amount of a protein; accordingly its sample concentration does not need to be exactly known, as the final **Eq. 9** does not contain such a term.

Figure 3 shows the change of migration time of *Ricinus communis* agglutinin, 60 kDa (RCA_{60}), observed when the MerES derivative of lactose was added to the electrophoretic solution at various concentrations. In every electropherogram the faster moving peak is that of cinnnamyl alcohol as a neutral marker. The RCA_{60} peak was retarded more and more strongly as the concentration of MerES-lactose increased. A plot of corrected migration time (t) vs MerES-lactose concentration (C) showed a convex parabolic curve (**Fig. 4**). Conversion of the t vs C plot to $(t - t_1)^{-1}$ vs C^{-1} plot gave an almost straight line as in **Fig. 5**, demonstrating the correctness of the inference mentioned above. The obtained value of K_a was 2.47×10^3 L mol^{-1}, close to the reported K_a value for the RCA_{60}-lactobionic acid interaction ($3.3. \times 10^3$ L, mol^{-1}) ***(5)***.

The association constants of maltooligosaccharides as the MerES derivatives of *Lens calinaris* agglutinin (LCA) were estimated in a similar manner. **Table 1** gives the K_a values, together with the A and B values, for maltose, maltotriose, and maltotetraose, respectively ***(4)***.

The observed increase of association constant with degree of polymerization is informative for the elucidation of binding site in this oligosaccharide–lectin interaction.

In this chapter a protocol is proposed for the estimation of the association constant between a neutral carbohydrate and a protein by the normal system involving prior conversion of the carbohydrate to the MerES derivative. An emphasis is placed on the correction of migration times for the variation of the velocity of EOF to obtain a reliable value of association constant.

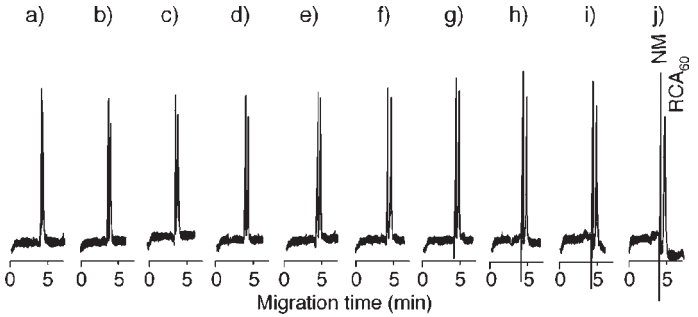

Fig. 3. Electropherograms of RCA_{60} in the absence and presence of MerES-Lac. Analytical conditions: Capillary, fused silica (50 µm i.d., 50 cm); electrophoretic solution, 50 mmol/L phosphate buffer, pH 6.8, containing MerES-Lac at concentrations of 0 **(a)**, 0.2 **(b)**, 0.4 **(c)**, 0.6 **(d)**, 0.8 **(e)**, 1.0 **(f)**, 2.0 **(g)**, 3.0 **(h)**, 4.0 **(i)** or 5.0 mmol/L **(j)**; applied voltage, 15 kV; detection, UV absorption at 220 nm; capillary temperature, 30°C. (Reproduced with permission from **ref. *5***).

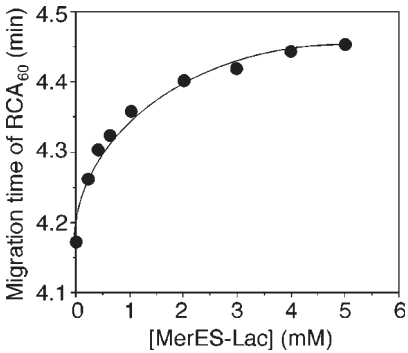

Fig. 4. Plot of *t* vs [MerES-Lac] for the RCA_{60}–MerES-Lac interaction. (Reproduced with permission from **ref. *5***).

Analysis by the reverse system is useful for simultaneous determination of the association constants to a protein, although the capillary has to be coated to prevent heavy adsorption of the protein as additive ***(6,7)***. A protocol for this system will appear elsewhere.

2. Materials

2. 1. Derivatization of Carbohydrates with MerES

1. Carbohydrates as additives.
2. Trifluoroacetic acid (TFA): Reagent grade.
3. 2-Mercaptoethanesulfonic acid sodium salt (MerES-Na): Reagent grade.

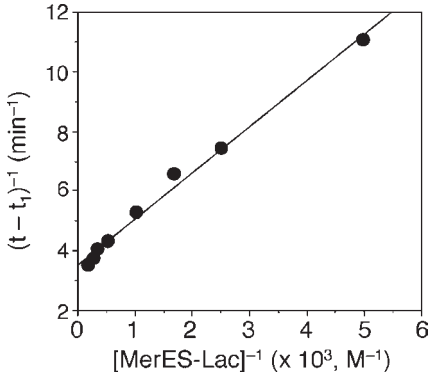

Fig. 5. Plot of $(t - t_1)^{-1}$ vs [MerES-Lac]-1 for RCA_{60}–MerES-Lac interaction. (Reproduced with permission from **ref. *5***).

Table 1
Association Constants of LCA to Maltooligosccharides

Oligosaccharide	Slope (A)	Y-intercept (B)	K_a (M^{-1})
Maltose	1.24	0.761	6.67×10^2
Maltotriose	1.45	1.00	9.64×10^2
Maltopentaose	1.67	2.22	13.66×10^2

4. The MerES reagent solution: Prepare it by dissolving MerES in TFA to a concentration of 4 w/v%. It should be prepared fresh before use.
5. Water purified by deionization, distillation, and filtration through a membrane (pore size 0.45 μm) (DDFW).
6. A Sephadex G-10 column (10 mm inner diamter [i.d.], 100 cm).
7. The sulfuric acid reagent: 3 v/v% Sulfuric acid in ethanol as a spray reagent.
8. A thin-layer plate: Silica gel coated glass plate for thin layer chromatography (TLC).
9. An 0.1 mol/L sodium hydroxide solution in DDFW.

2.2. Capillary Electrophoresis

1. Phosphate buffer (PB): Prepare it by adding a 50 mmol/L disodium hydrogen phosphate solution in DDFW to a 50 mmol/L sodium dihydrogen phosphate solution in DDFW to pH 6.8 with constant stirring.
2. Electrophoretic solutions containing MerES derivatives: Prepare each of them by dissolving a specified amount of the neutralized product of MerES derivative from a carbohydrate (**Subheading 3.1.**) in PB (400 μL).
3. 1 mol/L Sodium hydroxide.
4. Protein sample stock solutions: Prepare each by dissolving a protein sample in PB to a concentration of 100 μg/100 μL.

5. Protein sample solutions for the estimation of association constant: Prepare each of them by diluting a sample stock solution with PB containing cinnamyl alcohol (neutral marker) to make relevant concentrations of the protein and cinnamyl alcohol (*see* **Note 1**).
6. Apparutus: A commercial apparatus for CE equipped with a constant voltage power supply, a sample introducing system, a capillary temperature control system, and a UV detector having high sensitivity at around 200 nm (*see* **Note 2**).
7. A capillary role: made of fused silica, 50 µm i.d. (*see* **Note 3**).

3. Methods

3.1. Derivatization of Carbohydrates with MerES

1. Lyophilize a 40-µL portion of a 100 m*M* solution of a carbohydrate in DDFW (*see* **Note 4**).
2. Dissolve the residue in 600 µL of the MerES reagent solution.
3. Allow the solution to stand for 5 min at room temperature.
4. Evaporate the reaction mixture to dryness in an evacuated desiccator containing pellets of sodium hydroxide (*see* **Note 5**).
5. Dissolve the residue in a minimum volume of DDFW and apply the solution to a Sephadex G-10 column.
6. Elute the column with DDFW and fractionate every 50 drops.
7. Apply a small portion of each fraction on a thin-layer plate in a circle spot with a diameter of 5 mm. After drying spray the plate with the sulfuric acid reagent and heat the plate for visualization of the carbohydrate-containing substances (*see* **Note 6**).
8. Collect the carbohydrate-positive fractions giving brownish color and lyophilize the combined fractions.

3.2. Capillary Electrophoresis

1. Cut out a 72-cm portion of a capillary from a role, and make a detector window at the 50-cm position from the inlet (*see* **Note 7**).
2. Install the capillary in the CE apparatus (*see* **Note 8**).
3. Rinse the capillary with 1 *M* of sodium hydroxide for 3 min, followed by PB for 10 min (*see* **Note 9**).
4. Introduce a protein sample in PB containing cinnamyl alcohol to the anodic end of the capillary to make a short (a few millimeters) plug (*see* **Note 10**).
5. Soak both ends of the capillary into PB in reservoirs (*see* **Note 11**).
6. Apply a specified voltage between both ends to start CE and maintain the voltage until the peaks of the neutral marker and the protein sample were detected.
7. Repeat **steps 3–6** at least two more times for averaging t_1.
8. Analyze the protein sample in PB containing various concentrations of the MerES derivative of a carbohydrate in a similar manner as in **steps 3–6**.
9. Correct each migration time for EOF (*see* **Note 12**).
10. Repeat **step 8** and correct the migration time for EOF, if an irregular value deviating from the straight line of the $(t - t_1)^{-1}$ vs $[C]^{-1}$ plot is obtained (*see* **Note 13**).

3.3. Estimation of Association Constant

1. Average the migration times of the neutral marker (t_0) and the protein in PB (in the absence of the carbohydrate ligand) (t_1) obtained by repeated measurements by CE.
2. Plot the $(t - t_1)^{-1}$ against $[C]^{-1}$, using the mean value of t_1 (*see* **step 7** in **Subheading 3.2.**) and the individual values of t obtained for the electrophoretic solutions containing various concentrations of the MerES derivative of a carbohydrate (*see* **Note 14**).
3. If a plot deviates from the $(t - t_1)^{-1}$ vs $[C]^{-1}$ line, measure the migration time of the protein sample again at this ligand concentration and replace the deviating plot by the new one.
4. Measure the slope (A) and the y-intercept (B).
5. Calculate K_a from A and B using **Eq. 10**.

4. Notes

1. The concentration of the protein apparently does not affect the accuracy of the estimation of K_a, because such a term is not contained in **Eq. 9**. It should be as low as possible, provided the protein is detectable. The same is to cinnamyl alcohol.
2. CE apparatus: Any high-voltage supplier capable of supplying ~30 kV can be used. Sample solutions can be introduced by pressure drop utilizing either compression, suction, or siphoning. Introduction by electromigration may cause introduction of the component ions in a sample at different velocities. There are two types of UV detectors, one designed for a particular wavelength using a special lamp and an interference filter and another for multiple wavelengths using a deuterium or tungsten lamp and a grating spectroscope. The detector of the former type equipped with a zinc lamp, which emits most abundantly at 214 nm, can be used for the present experiment. The detectors of the latter type are also usable. Because the interaction between substances is generally dependent on temperature, the control of capillary temperature is important in binding studies.
3. Capillary: The velocity of EOF is varied among the material of the capillary, because it has an influence on the zeta potential between the capillary inner wall and the electrophoretic solution in the capillary. The authors use the products of Polymicro Technologies (Phoenix, AZ) as the standards.
4. The carbohydrates to be derivatized with MerES should be anhydrous to prevent incomplete reaction and/or hydrolysis during reaction and/or cleanup of the products. Therefore, the drying process should be done as completely as possible.
5. TFA should be removed as completely as possible to ensure quantitative yields of the products by preventing hydrolysis in the cleanup process.
6. Carbohydrate detection in Sephadex G-10 fractions: A number of other methods are available for the detection of carbohydrates, but charring on a heated thin-layer plate is the simplest.
7. A detection window can be made by burning out an approx 1-mm portion of the polyimide coating on a capillary by a thin flame.
8. Installation of the capillary: The detector window should be positioned at the center of the UV light to obtain the highest sensitivity.

9. Conditioning of the capillary: Rinsing with alkali is used to clean the inner wall of the capillary. Subsequent rinsing with PB is to facilitate equilibration with the electrophoretic solution to be used.
10. Sample plug: A 1-mm plug in a capillary with i.d. 50 μm corresponds to a volume of approx 2 nL. Shorter plugs are desirable, but the sensitivity must be enough to detect the sample. A length of a few millimeters is an average in CE.
11. Reduction of the amount of the ligand: Usually CE is performed using the same medium containing the ligand for both the electrode and electrophoretic solutions, but this procedure requires a large amount of the ligand. Introduction of the electrophoretic solution containing an MerES derivative of a carbohydrate in PB only to the capillary and replacement of the electrode solutions by PB not containing the MerES derivative can greatly reduce the amount of the ligand to be used.
12. Correction of migration time for EOF: Fluctuation of migration time is caused mainly by variation of the velocity of EOF. However, the velocity of electrophoretic migration is almost unchanged under the given conditions. The apparent velocity of a protein in the absence of a ligand (V_o) is the algebric sum of the velocities of EOF (V_{eo}) and electrophoretic migration (V_{ep}), as shown:

$$V_o = V_{eo} + V_{ep} \tag{11}$$

If in another run V_o and V_{eo} change to V_o' and V_{eo}', respectively, by fluctuation,

$$V_o' = V_{eo}' + V_{ep} \tag{12}$$

Subtraction of **Eq. 12** from **Eq. 11** gives

$$V_o - V_o' = V_{eo} - V_{eo}' \tag{13}$$

Conversion of the velocity terms to migration time terms leads to

$$l\,(t_1^{-1} - t_1'^{-1}) = l\,(t_0^{-1} - t_0'^{-1}) \tag{14}$$

Therefore,

$$t_1 = t_0\,t_0'\,t_1'(t_0\,t_0' + t_0'\,t_1' - t_0\,t_1')^{-1} \tag{15}$$

The term t_1 in **Eq. 15** indicates a corrected value of migration time from the observed migration time t_1'. The average values obtained by at least three measurements should be used for t_0 and t_1 in this equation (*see* **Note 14**). Similarly **Eq. 16** is applicable to the correction of t (migration time in the presence of the ligand)

$$t = t_0\,t_0'\,t'(t_0\,t_0' + t_0'\,t' - t_0\,t')^{-1} \tag{16}$$

13. Revision of a deviating plot: It is wise to plot the corrected $(t - t_1)^{-1}$ against $[C]^{-1}$, immediately after the migration times are measured. If a plot deviates from the straight line, it should be reestimated by repeating CE for this particular value of [C].
14. Repetition of the measurement of the t_1 value: Since t_1 (the migration time of the protein at [C] = 0) is common to all plots, this is especially important. Repeat the measurement at least three times and use the average value for t_1.

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APPENDIX

Structures of Carbohydrates Found in Animals and Bacteria

Shigeo Suzuki, Malcolm Perry, Pierre Thibault, and Susumu Honda

1. Introduction

Oligo- and polysaccharides are widely distributed in animal tissues as well as body fluids in conjunction with macromolecular substances such as proteins and sphingolipids. The glycoconjugates formed by covalent bonding of an oligosaccharide(s) to a protein are called glycoproteins, whereas the group of substances coming from acidic polysaccharides and a protein are called proteoglycans. The glycoconjugates formed from an oligosaccharide and a sphingolipid are designated glyco(sphingo)lipids. The carbohydrates in these glycoconjugates are biologically important and their analyses are frequently required to obtain the basic information on structure–activity correlation. There are other groups of immunologically active substances containing oligo- or polysaccharides in bacterial cell walls. Because such substances induce pathological disorders in humans as well as animals, the analysis of the constituent carbohydrates is also important for diagnosis of bacterial infection.

As a variety of oligo- and polysaccharides, as well as their monosaccharide constituents, are described in many chapters of this volume, this appendix briefly summarizes the structural features of such carbohydrates of animal and bacterial origins. The description of the carbohydrates of plant origin is omitted, as this subject is outside the scope of this volume.

2. Monosaccharides

The oligo- and polysaccharides in animal glycoconjugates are composed of a relatively small number of monosaccharide species, but bacterial carbohydrates contain a wide variety of monosaccharides. The following are the struc-

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tures of the monosaccharides found as components of these carbohydrates. Unless otherwise stated, monosaccharides are shown in their D-configurations, and the abbreviations in parentheses are used to indicate oligo- and polysaccharide structures in this appendix.

2.1. Monosaccharides in Glycoproteins (Fig. 1)

Asparagine-linked oligosaccharides (*N*-glycans) commonly have the mannose and glucosamine (as *N*-acetyl) residues in the core structure at the innermost position, whereas serine- or threonine-linked oligosaccharides (*O*-glycans) have the galactosamine residue (as *N*-acetyl) at the carbohydrate–protein junction. There are also *O*-glycans linked to the hydroxyproline residue through the xylose residue, although the occurrence of this type of *O*-glycan is relatively rare. Some *N*-glycans have additional monosaccharide units such as the galactose and/or L-fucose residues (*see* **Subheading 3.1.**), while *O*-glycans can contain additional glucosamine and/or galactose residues. Neuraminic acid is widely distributed in *N*- and *O*-glycans as the *N*-acetyl or the glycolyl derivative. **Figure 1** gives the structures of the monosaccharides in *N*- and *O*-glycans.

2.2. Monosaccharides in Proteoglycans (Fig. 2)

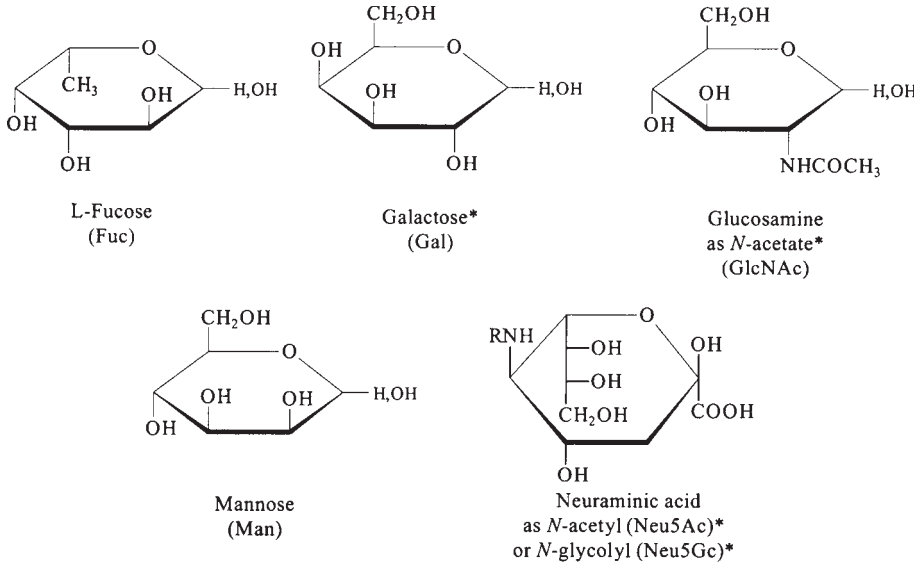
The backbone of polysaccharide chains of proteoglycans is composed of uronic acid and hexosamine residues alternately linked to each other. The uronic acid in each polysaccharide chain is either glucuronic acid or a combination of glucuronic and L-iduronic acids. The hexosamine is either glucosamine or galactosamine. In most proteoglycans these hexosamines are *N*-acetylated, but in specific proteoglycans such as heparin, most of the hexosamine residues are *N*-sulfated. In some proteoglycans xylose and galactose are also found at the innermost position of the polysaccharide chain. **Figure 2** shows the structures of monosaccharides found in proteoglycans.

2.3. Monosaccharides in Glycosphingolipids (Fig. 1)

The glycosphingolipids generally consist of a lactosyl disaccharide (galactose and glucose) linked to ceramide. However, this saccharide can be further modified by monosaccharide residues such as the glucosamine (as the *N*-acetyl) and neuraminic acid (as the *N*-acetyl or the *N*-glycolyl) residues. The resulting glycosphingolipids can be classified into gangliosides and glycolipids of the lacto-, neolacto-, globo-, and isoglobo-series. The structures of these monosaccharides are given in **Fig. 1**.

2.4. Monosaccharides in Bacterial Cell Walls (Fig. 3)

Bacteria comprising a cell wall are classified as Gram-positive or Gram-negative based on their ability to take up a stain in which heat-fixed cells are



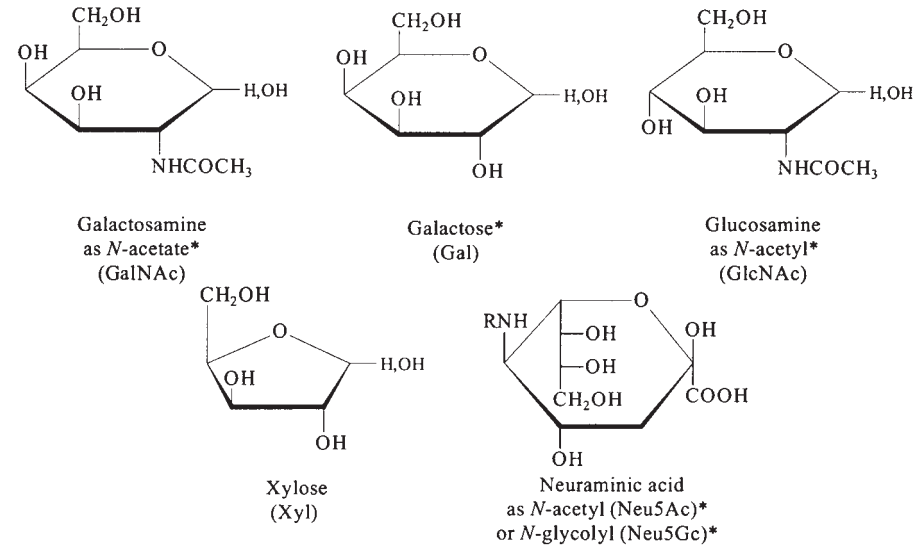


Fig. 1. Monosaccharides in glycoproteins. (The asterisk indicates monosaccharides are also found in glycosphigolipids.)

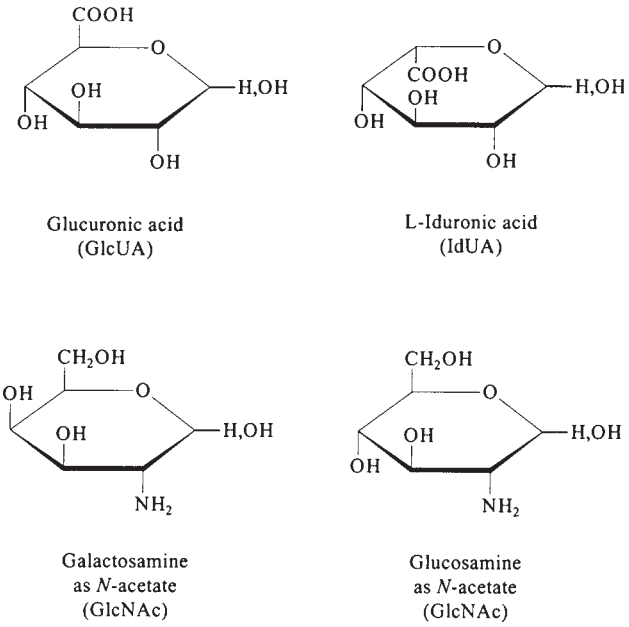


Fig. 2. Monosaccharides in proteoglycans.

successively treated with the dye crystal violet and iodine and then destained in ethanol or acetone. Gram-positive bacteria are composed of a thick monolayered cell wall (200–800 Å), whereas Gram-negative bacteria have a thin cell wall (20–30 Å) covered by an outer membrane composed of complex lipopolysaccharides (LPS), proteins, and phospholipids. The outer surface of Gram-negative bacteria are frequently coated with complex polysaccharides referred to as *O*-antigens, and by polysaccharide capsules (LPS)

The cell walls of bacteria have a peptidoglycan structure that can comprise repeating units of *N*-acetylglucosamine–*N*-acetylmuramic acid disaccharide whose lactyl side chain forms an amide bound with a short peptide (e.g., D-alanine-L-lysine-isoglutamate-L-alanine tetrapeptide in *Staphylococcus aureus*). These repeating units are linked to each other typically with pentaglycine bridges. A significant proportion of the cell wall of Gram-positive bacteria contains teichoic acids, a complex biopolymer of glycerol or ribitol linked by phosphodiester bridges. The hydroxyl groups of this sugar-phosphate chain are substituted by D-alanine residues and monosaccharides such as glucose or *N*-acetylglucosamine. Teichoic acids are then linked to proteoglycan chains via phosphodiester bonds to the hydroxyl group of *N*-acetylglucosamine. *Saccharomyces cerevisiae* contains different types of

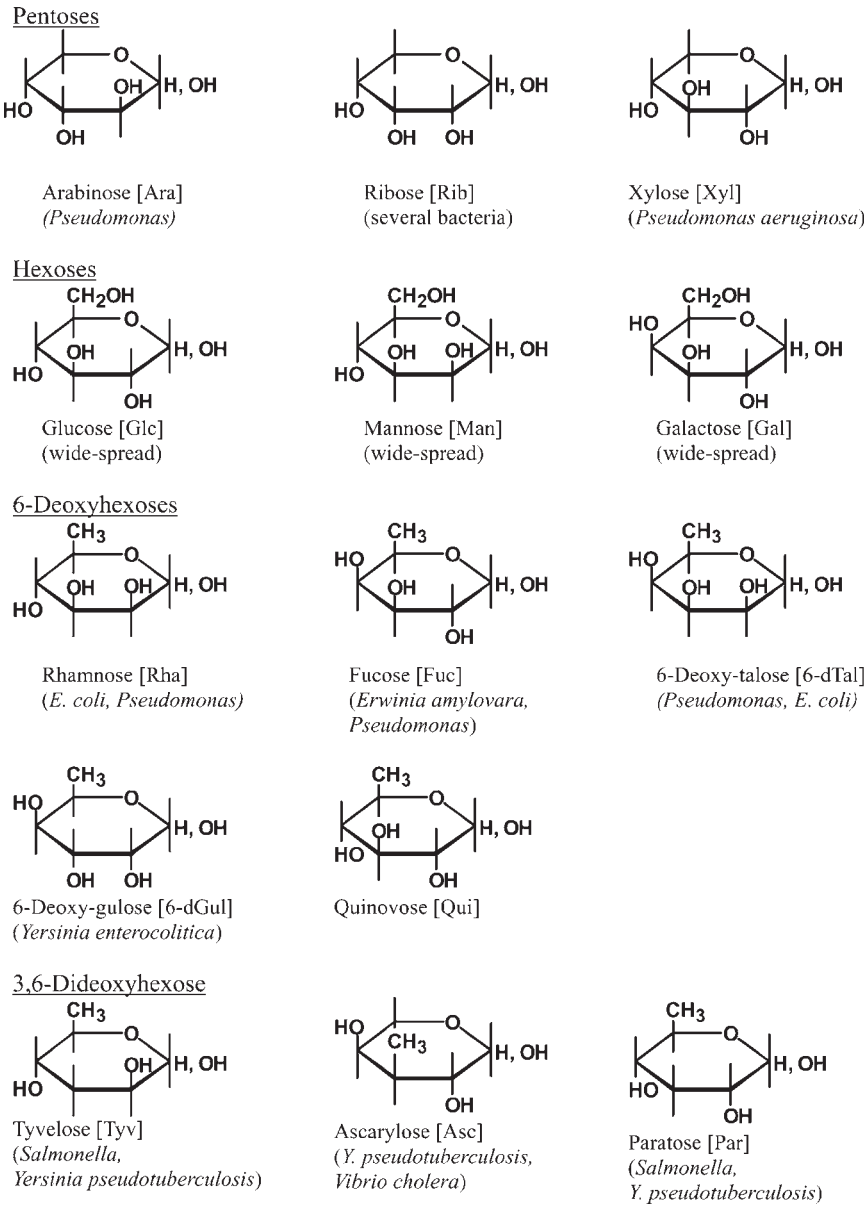
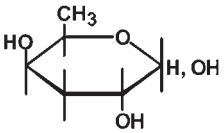
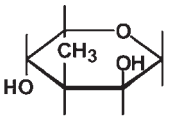


Fig. 3. (*continued on next page*) Monosaccharides found in bacterial cell walls, *O*-antigens, and LPS. For convenience, all monosaccharides are represented in the D-pyranose form which is one of a number of biologically active configurations that each residue can adopt. The occurrence of residue in bacteria LPS is indicated in parentheses.

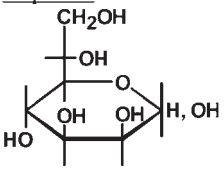


Abequose [Abe]
(*Salmonella, Citrobacter freundii*)

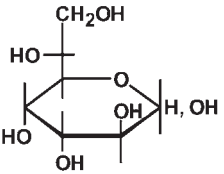


Colitose [Col]
(*Salmonella, Y. pseudotuberculosis*)

Heptoses

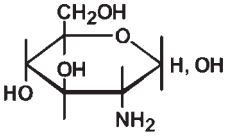


L-*glycero*-D-mannoheptose [man-Hep]
(wide-spread)

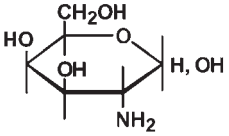


D-*glycero*-D-altroheptose [alt-Hep]
(*Campylobacter jejuni*)

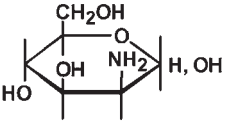
2-Amino-2-deoxyhexoses



Glucosamine [GlcN]
(wide-spread)

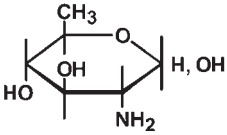


Galactosamine [GalN]
(wide-spread)

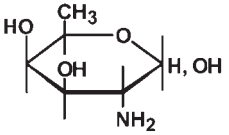


Mannosamine [ManN]
(wide-spread)

2-Amino-2,6-dideoxyhexoses

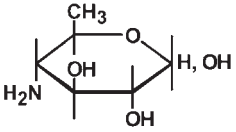


Quinovosamine [QuiN]
(several bacteria)

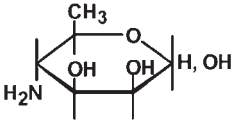


Fucosamine [FucN]
(*P. fluorescens, P. aeruginosa*)

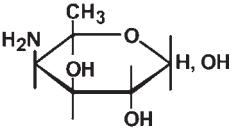
4-Amino-4,6-dideoxyhexoses



Viosamine [Qui4N]
(*Shigella dysenteria, E. coli*)



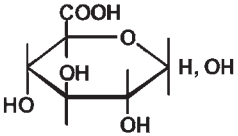
Perosamine [Rha4N]
(*Salmonella, E. coli, Y. enterocolitica*)



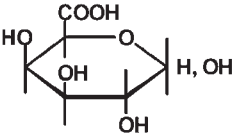
Tomosamine [Fuc4N]
(*E. coli, P. fluorescens*)

Fig. 3. *(continued)*

Hexuronic acids

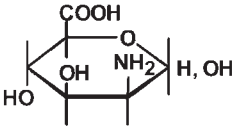


Glucuronic acid [GlcA]
(several bacteria)

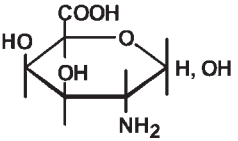


Galacturonic acid [GalA]
(several bacteria)

2-Amino-2-deoxy-hexuronic acids

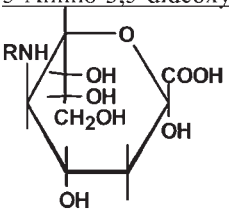


2-Amino-mannuronic acid [ManNA]
(*V. cholera*)

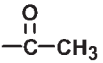


2-Amino-galacturonic acid [GalNA]
(*S. arizonae, Shigella dysenteria*)

5-Amino-3,5-dideoxynonulosonic acids



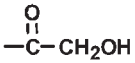
R:



N-Acetylneuraminic acid [Neu5Ac]

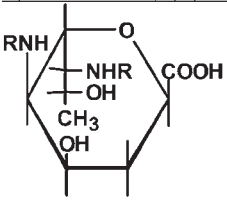
H

Neuraminic acid [Neu] (*S. arizona, C. freundii, E. coli, V. cholera*)



N-glycolylneuraminic acid [Neu5Gc]

5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid



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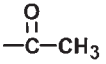
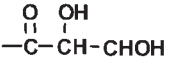


N-acetylpseudaminic acid

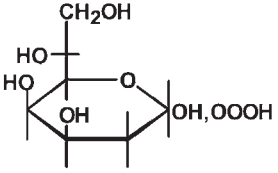
H

Pseudaminic acid (*P. aeruginosa*)



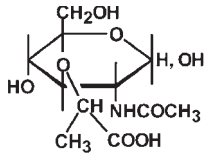
N-glyceropseudamimic acid (*V. alginolyticus*)

Mannoctulosonic acids



3-Deoxy-D-manno-zulosonic acid
(wide-spread)

Fig. 3. *(continued)*



N-Acetylmuramic acid [MurNAc]

Fig. 3. *(continued from previous pages.)*

polymannosyl chains attached to asparagine and serine/threonine residues of a polypeptide core. Although they structurally resemble proteoglycans, they are usually called mannoproteins.

The outer membrane components of Gram-negative bacteria display extensive heterogeneity in the length and structures of glycans found as part of the capsule, and LPS, *O*-antigens. LPS, for example, consists of two parts with different physical properties: a hydrophilic carbohydrate component containing acidic (3-deoxy-D-*manno*-2-octulosonic acid, KDO) and neutral residues (e.g., glucose, galactose, L-*glycero*-mannoheptose, etc.) bonded to a hydrophobic lipid A component typically composed of a glucosamine disaccharide to which are attached *O*- and *N*-linked fatty acids. The LPS of many enteric pathogens is also extended by *O*-repeating oligosaccharide unit antigens, which confers additional complexity. The LPS of bacterial pathogens are also strain characteristic and the correlation of their structure–immnological relationship is an area of intense research activity. **Figure 3** shows the structures of numerous monosaccharide residues found in bacterial *O*-specific antigens of LPS. A more comprehensive description of monosaccharide residues in *O*-specific antigens can be found in a review by Knirel and Kochetkov ***(1)***. For simplicity, all monosaccharides are represented in the D-pyranose form which is one of a number of biologically active configurations that each residue can adopt.

3. Oligosaccharides in Glycoproteins

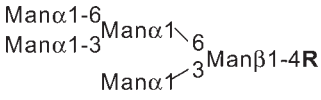
The oligosaccharides in glycoproteins can be classified into three groups: *N*-linked glycans, *O*-linked glycans, and glycosylphosphatidylinositol (GPI)-membrane anchors. *N*-linked glycans are attached to the peptide backbone by a β-*N*-glycosidic bond to an asparagine residue forming the sequon triad Asn-X-Ser/Thr, where X is any amino acid except proline. *O*-linked glycans are linked to serine or threonine residues via the α-*O*-glycosidic bond, or to 5-hydroxylysine as for collagen. GPI anchors are attached to their peptide chains through an amide bond between mannose-6-phosphoethanolamine and the C-terminal carboxyl group.

It is well established that the *N*-glycans are formed by transfer of the nonamannosylchitobiose block from dolicol diphosphate to an asparagine residue in the polypeptide core. Dolichol apparently anchors the growing oligosaccharide to the endoplasmic reticulum membrane. The introduced nonamannosylchitobiose blocks then sequentially loses the mannose residues by the action of processing enzymes and is modified by other monosaccharide residues, such as the galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and neuraminic acid (Neu) residues, by the action of glycosyl-transferases. The magnitude of modification is varied in such a wide range that the number of *N*-glycan species hitherto reported is as many as afew hundred.

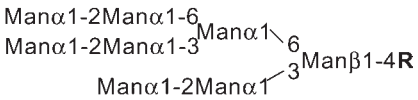
3.1. N-*Glycans*

3.1.1. High-Mannose Type N-*Glycans* ***(Fig. 4)***

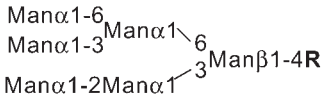
A series of *N*-glycans having various numbers of mannose residues but not yet modified by other monosaccharides are called high-mannose type *N*-glycans. Figure 4 shows the structures of high-mannose type *N*-glycans that have five to nine sequentially linked mannose residues attached to the *N,N'*-diacetylchitobiose group. Higher members may have positional isomers different from each other in the attaching position of the intermannosyl linkage. Tomiya and co-workers ***(2)*** proposed numbering of these high-mannose type *N*-glycans by the *Mn.x* system, where M refers to a high-mannose type *N*-glycan, *n* is the number of the mannose residue, and *x* the serial number to identify the positional isomer, as shown in **Fig. 4**. Suzuki and co-workers ***(3)*** proposed a nomenclature based on the serial description of (1) three-letter abbreviation of the oligosaccharide source, if it can be specified; (2) the total number of the monosaccharide residues following a hyphen; (3) the letter M, C, or H to indicate chain type, (M refers to the high-mannose type, C complex type, and H hybrid type) with the total number of the monosaccharide residues as a suffix; (4) a parenthetical description of the component monosaccharide species, each with its number as a suffix; and (5) a serial number followed by a hyphen. Thus, for example, the high-mannose *N*-glycan, Manα1–6(Manα1–3) Maα1–6(Maα1–3)Manβ1–4GlcNAcβ1–4GlcNAc can be called M5.1 and RNB7M(M_5GN_2)-1 by the Tomiya et al. and the Suzuki et al. systems, respectively. In the latter system RNB and GN are the abbreviations of ribonuclease B as its source and *N*-acetylglucosamine residue, respectively. Tomiya et al.'s system seems simpler in numbering high-mannose type *N*-glycans but the Suzuki et al.'s system gives straightforward information on the *N*-glycan structure and its source. More complex structures having a larger number of mannose residues have been found in invertase and *Saccharomyces* mannoproteins.



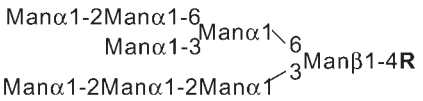
RNB7M(M_5GN_2)-1



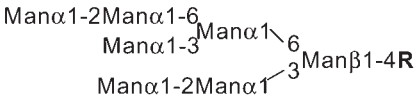
RNB10M(M_8GN_2)-1



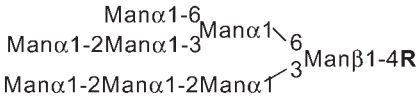
RNB8M(M_6GN_2)-1



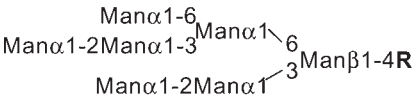
RNB10M(M_8GN_2)-2



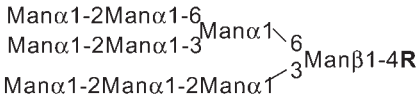
RNB9M(M_7GN_2)-1



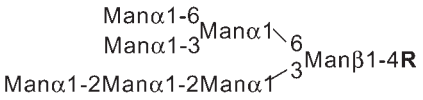
RNB10M(M_8GN_2)-3



RNB9M(M_7GN_2)-2



RNB11M(M_9GN_2)-1



RNB9M(M_7GN_2)-3

R= GlcNAcβ1-4GlcNAc

Fig. 4. High-mannose type *N*-glycans. Each *N*-glycan is named according to the Suzuki et al. system.

3.1.2. Complex Type N-*Glycans (**Figs. 5** and **6**)*

When the mannose residues in high-mannose type *N*-glycans are sequentially released by processing to the trimannosylated *N,N'*-diacetylchitobiose block, Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc and this pentasaccharide is modified by the action of glycosyltransferases with monosaccharide donors, various kinds of modifications including di-, tri-, tetra-, and pentaantennary structures are formed. Such modifications are called complex type *N*-glycans. The basic structures of these multiantennary modifications are shown in **Fig. 5**. Each

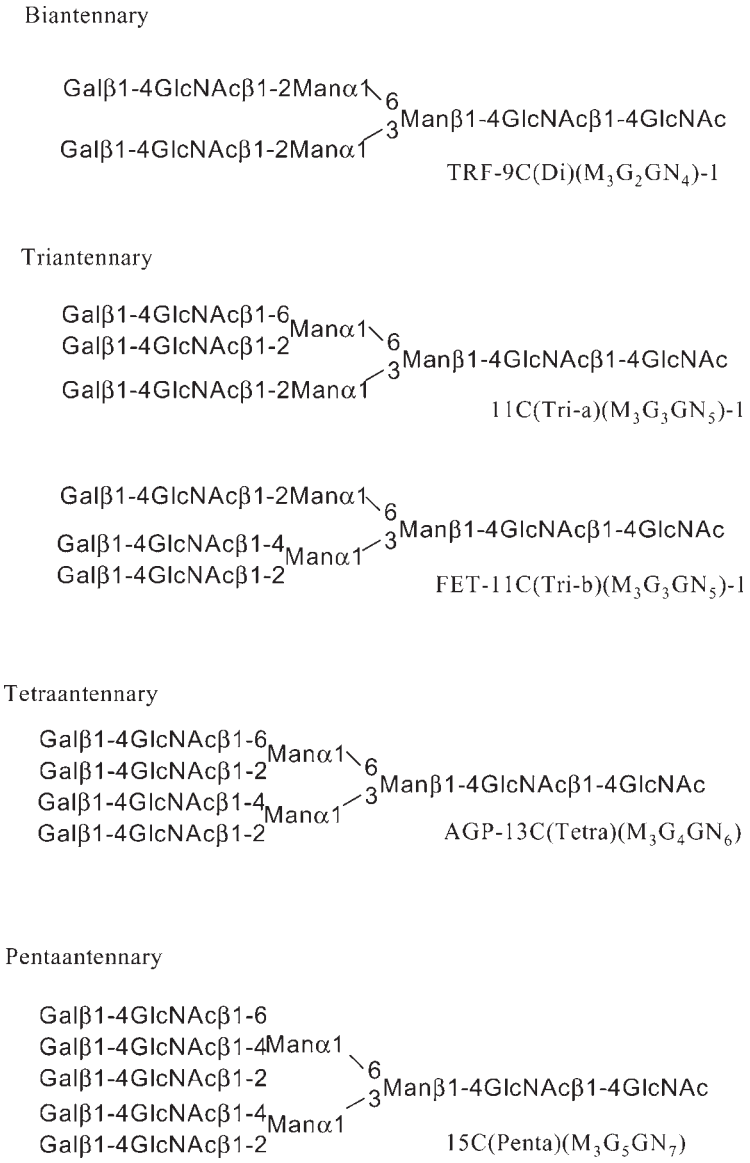


Fig. 5. Basic forms of complex type *N*-glycans in glycoproteins. Each *N*-glycan is named according to the Suzuki et al. system.

antenna contains a GlcNAcβ1–2Man or a Galβ1–3/4GlcNAcβ1–2Man chain. There are also complex type *N*-glycans in which the outermost GlcNAc residue is further *N*-acetylglucosaminylated or lactosaminylated. The Fuc and/or

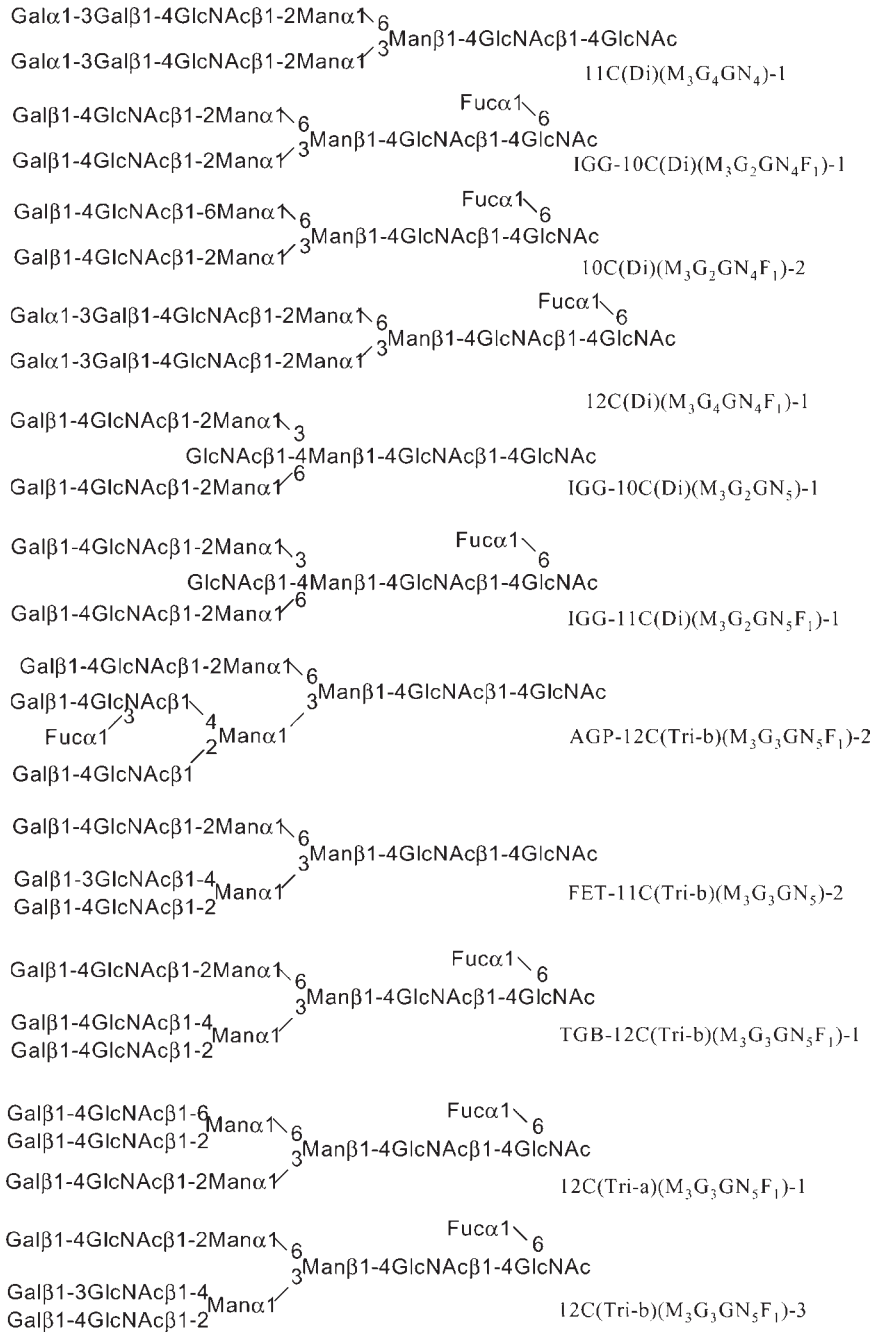


Fig. 6. *(Continued on next page)* Various modifications of complex type *N*-glycans in glycoproteins. Each *N*-glycan is named according to the Suzuki et al. system.

Fig. 6. *(continued from p. 296.)*

GlcNAc residues may be attached to the innermost GlcNAc residue and the Man residue adjacent to the *N,N'*-diacetylchitobiose group, respectively. Some of such variations are shown in **Fig. 6**.

The complex type *N*-glycans can also be numbered or named analogously to the high mannose type *N*-glycans. For example, the basic form of the biantennary structure (**Fig. 5**, top) can be numbered as 200.4 by the Tomiya et al. system, in which the first figure (2) represents the number of the antenna, the second figure (0) the absence of the Fuc residue at the reducing terminal GlcNAc residue, the third figure (0) the absence of the bisecting GlcNAc residue, and the fourth figure (4) the serial number. In the Suzuki et al. system this *N*-glycan can be called TRF-9C(Di)(M3G2GN4), which means that this transferrin-derived *N*-glycan is a nonasaccharide (9) of complex (C) type, having a diantennary (Di) structure composed of three Man (M_3), two Gal (G_2), and four GlcNAc (GN_4) residues.

The galactose residues in the peripheral positions are easily sialylated by the α2–3 or α2–6 linkage to give a negative charge to *N*-glycans. The sialic acid residues found in glycoproteins are generally *N*-acetylated or *N*-glycolylated, and the variability in the number of the sialic acid residue and in the linkage is enormous.

3.1.3. Hybrid Type N*-Glycans (**Fig. 7**)*

As described in **Subheading 3.1.1.**, the nonamannose block is composed of two oligomannose chains. In the process of complex type *N*-glycan formation, both oligomannose chains can be degraded and modified to give complex type *N*-glycans. However, when only one of the oligomannose chains is degraded and modification occurs only in the degraded chain, hybrid type *N*-glycans are formed. **Figure 7** shows typical examples of such hybrid type *N*-glycans.

3.2. O*-Glycans (Fig. 8)*

The mechanism of the biosynthesis of *O*-glycans is quite different from that of *N*-glycans. Typically an *N*-acetylgalactosamine is first added to either the serine or the threonine residue and this carbohydrate moiety is further modified by the action of glycosyltransferases. Therefore, almost all *O*-glycans have the GalNAc residue at the innermost position, but an exception exists that has the xylose–hydroxyproline linkage. **Figure 8** shows various examples of serine/threonine linked *O*-glycans. They can be named by the extension of the Suzuki et al.'s system for naming *N*-glycans. Thus, for example GlcNAcβ1–6(Galβ1–3)GalNAc 1-Ser/Thr can be named $3O(G_1GN_1GN_1)$-1, where O and *GN* designate an *O*-glycan and the *N*-acetylgalactosamine residue, respectively. The other abbreviations are the same as in *N*-glycans.

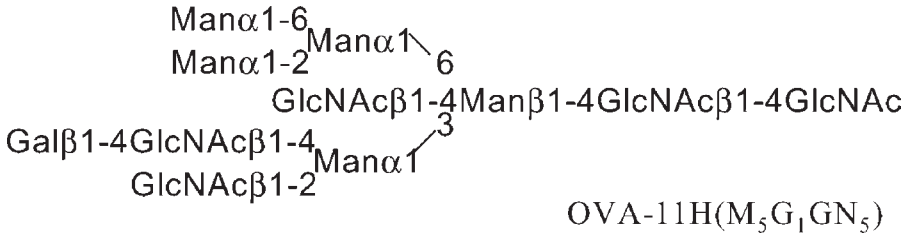


Fig. 7. A typical example of hybrid type *N*-glycans in glycoproteins.

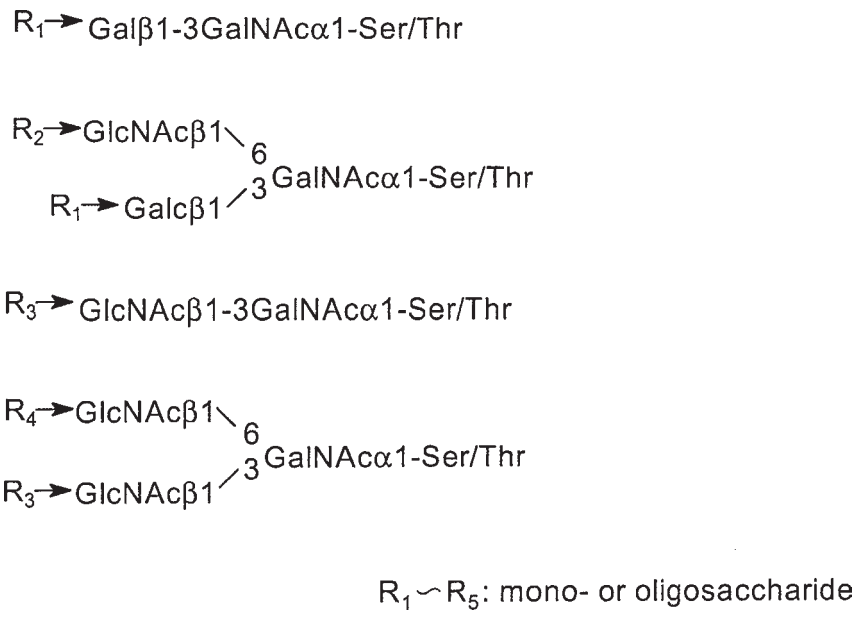
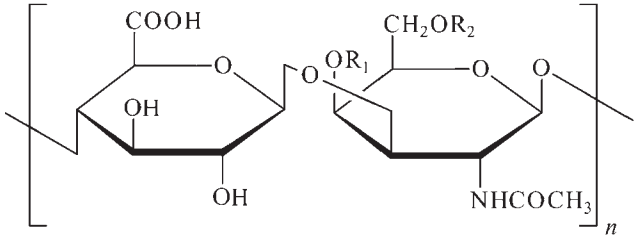


Fig. 8. Various Ser/Thr-linked *O*-glycans found in glycoproteins.

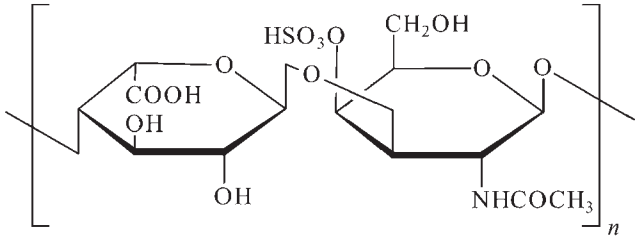
4. Oligosaccharides Released from Proteoglycans by Enzymatic Reaction (Fig. 9)

The polysaccharide (glycosaminoglycan) chains in a proteoglycan are composed of an uronic acid(s) and a hexosamine residues alternately linked to each other. The hexosamine residue is *N*-acetylated in most proteoglycans, but heparin is one of the exceptions where this residue is either *N*-acetylated or *N*-sulfated. The hydroxyl groups are partially sulfated, but there is heterogeneity in the location of sulfation. **Figure 9A** shows the core structures of the polysaccharide chains of proteoglycans.

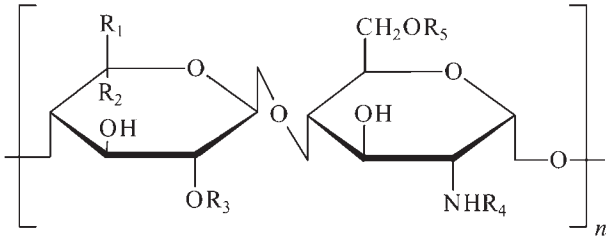
A



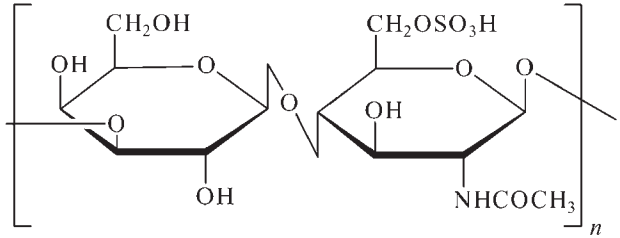
Chondroitin sulfate A (R_1=SO_3H, R_2=H)
Chondroitin sulfate C (R_2=SO_3H, R_1=H)



Chondroitin sulfate B (Dermatan sulfate)



Heparin or heparan sulfate
(R_1 or R_2 =COOH, R_3, R_5=H or SO_3H, R_4=SO_3H or CH_3CO)



Keratan sulfate

Fig. 9. **(A)** Proteoglycans.

B

ΔUAβ1-3GalNAc	(ΔDi-0S)
ΔUAβ1-3GalNAc4S	(ΔDi-4S)
ΔUAβ1-3GalNAc6S	(ΔDi-6S)
ΔUA2Sβ1-3GalNAc	(ΔDi-UA2S)
ΔUA2Sβ1-3GalNAc4S	(ΔDi-S_B)
ΔUA2Sβ1-3GalNAc6S	(ΔDi-S_D)
ΔUAβ1-3GalNAc4S,6S	(ΔDi-S_E)
ΔUA2Sβ1-3GalNAc4S,6S	(ΔDi-triS)
ΔUAβ1-3GlcNAc	(ΔDi-HA)

Fig. 9. (*continued*) **(B)** Different unsaturated disaccharides released from proteoglycans using lyase digestion.

The activity of proteoglycans is divergent, including blood anticoagulation, lipid clearing in blood, cell growth acceleration, cell adhesion inhibition, lubrication, and so forth. Although structure–function relationships have been well documented for a number of proteoglycans, especially for blood anticoagulation by heparin, the extensive variability and complexity of their structures has posed significant analytical challenges to further understanding of numerous biological systems. In view of the complexity of the glycan chains found in proteoglycans, their structural characterization has often been undertaken following chemical or enzymatic release from their native conjugates. However, chemical degradation using acidic hydrolysis and nitrous acid deamination can result in a complex mixture of fragments of additional heterogeneity, thereby rendering the elucidation of the original polysaccharide structure more challenging. On the other hand, enzymatic methods allow moderate cleavage and the released products can easily be identified and quantified. In particular, the use of lyases that cleave the hexosamine bonds to give 4,5-unsaturated uronic acid containing oligosaccharides can be used in the analysis of proteoglycans. Digestion with a lyase, for example, chondroitinase ABC, does not release the sulfate groups of the carbohydrate chains, thus resulting in various kinds of sulfated, unsaturated oligosaccharides. **Figure 9B** shows examples of such disaccharides from chondroitin sulfates. Heparin gives various *N*-sulfated/acetylated oligosaccharides having degree of polymerization (DP) 2, 4, 6, and

Galβ1-Cer	
Galβ1-4Glcβ1-Cer	
GalNAcβ1-4Galβ1-4Glcβ1-Cer	Ganglio series
Galβ1-3GalNAcβ1-4 Galβ1-4Glcβ1-Cer	Ganglio series
[Galβ1-3GlcNAcβ1-3]$_n$Galβ1-4Glcβ1-Cer	Lact1 type (Lacto series)
[Galβ1-4GlcNAcβ1-3]$_n$Galβ1-4Glcβ1-Cer	Lact2 type (Neolacto series)
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer	Globo series
Galα1-3Galβ1-4Glcβ1-Cer	Isoglobo series

Fig. 10. Various types of glycosphingolipids.

8 (structures not shown) under much more strictly controlled conditions. Heparin plays important biological functions, and the molar proportions of the oligosaccharides can be used to characterize the original proteoglycans.

5. Oligosaccharides in Glycosylsphingolipids (Figs. 10 and 11)

The carbohydrate moieties of glycosylsphingolipids are composed of various oligosaccharides attached to ceramide. They result from variations of substitution of lactose at the nonreducing galactose residue. The basic structures of various series of oligosaccharides in glycosylsphingolipids are illustrated in **Fig. 10**. **Figure 11** gives a number of gangliosides of gangliotriose (GalNAcβ1–4βGalβ1–4Glc1–1Cer) and gangliotetraose (Galβ1–3GalNAcβ1–4Galβ1–4Glc1–1Cer) series.

6. Carbohydrates in Bacterial Cell Walls (Fig. 12)

The polysaccharides from bacterial cell walls have structures of significant heterogeneity and complexity, and their complete structural characterization often necessitates a broad range of analytical techniques such as gel permeation and ion-exchange chromatography, gas chromatography, nuclear magnetic resonance spectroscopy, and mass spectrometry. Most structural characterization studies of complex carbohydrates from bacterial cell walls have involved chemical degradation methods to release oligosaccharides and glycolipids that are amenable to physical techniques. As an example of a bacterial cell wall component, **Fig. 12A** shows the conceptional structure of a mannoprotein from *Saccharomyces cerevisiae* ***(4)***.

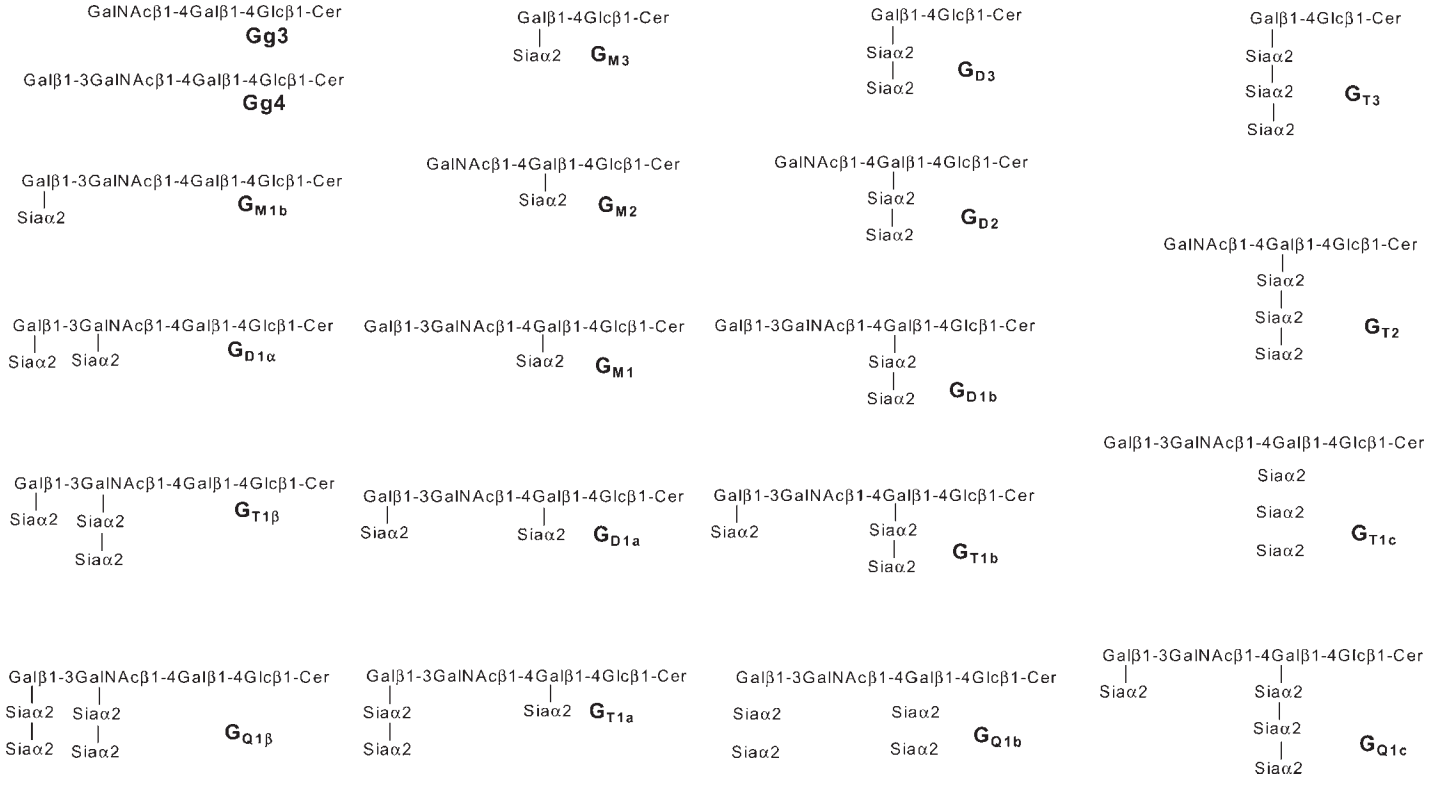


Fig. 11. Structures of different gangliosides.

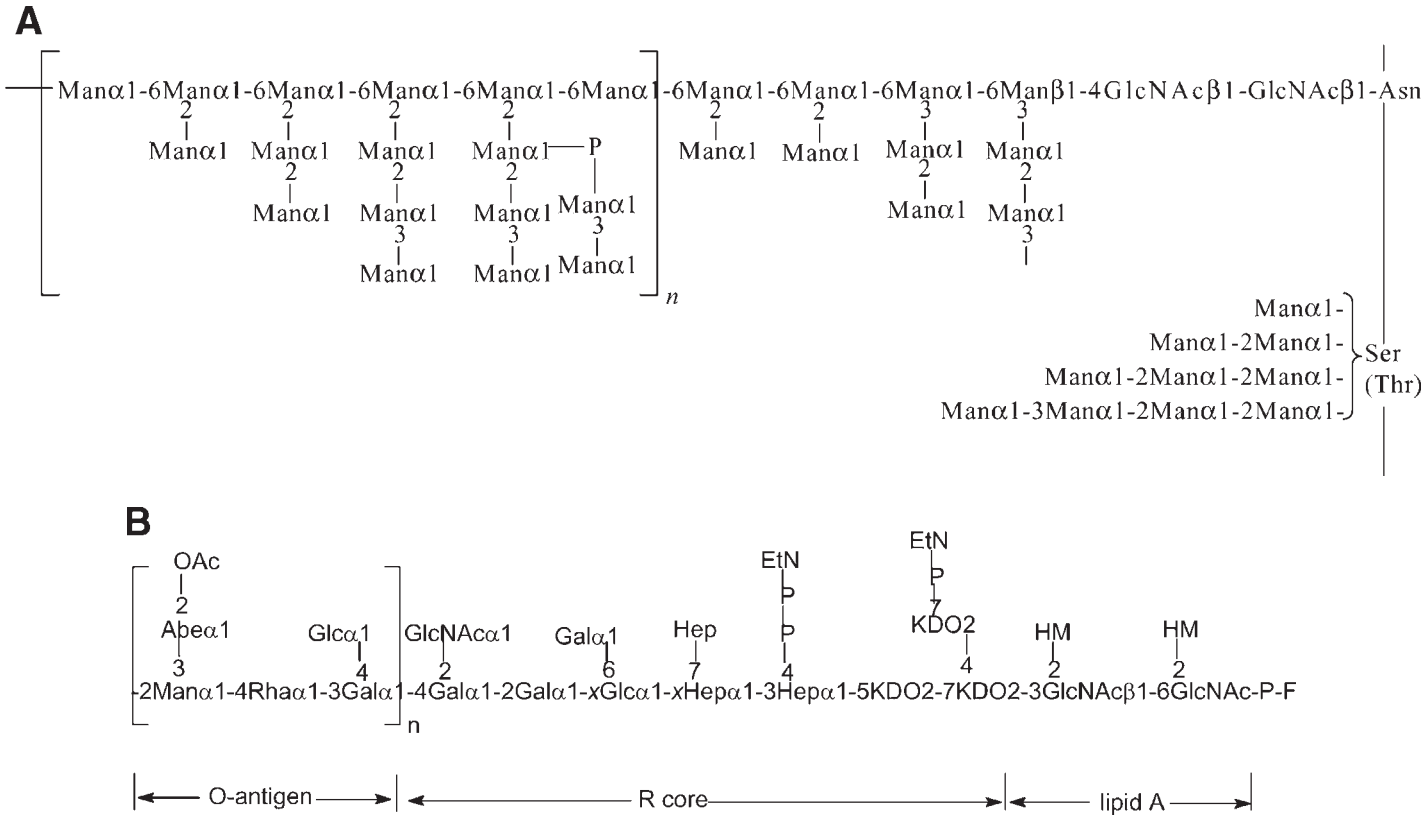


Fig. 12. **(A)** Mannoprotein in *Saccharomyces cerevisiae*. **(B)** Lipopolysaccharide from *Salmonella typhimurium*. FA, fatty acid; KDO: 2-keto-3-octonic acid; HM, β-hydroxymyristic acid; Hep, L-glycero-mannoheptose; EtN, ethanolamine; x, attaching position not determined.

Cell wall components can adopt very complex structures, an example of which is LPS of Gram-negative bacteria. As mentioned previously (**Subheading 2.4.**), the structure of LPS mainly consists of three distinct regions: the *O*-specific chain, the core, and the lipid A. The *O*-specific chains contain the immunodeterminant structures against which the antibodies formed during infection or immunization are directed. Both the *O*-specific chains and the core are comprised of long carbohydrate chains whereas the lipid A is formed of fatty acid, phosphate, and phosphoethanolamine substituents bonded to a central glucosamine disaccharide. In a large number of bacteria, the lipid A and the core are linked together through one or more acidic carbohydrates such as 3-deoxy-D-*manno*-2-octulosonic acid (KDO). The relatively labile nature of the glycosydic bond between KDO and the glucosamine of the lipid A has been exploited previously using mild acid hydrolysis to separate the hydrophylic carbohydrate from the insoluble lipid A. In some bacteria, the *O*-specific chain is either absent or truncated as a result of genetic mutation or as a given characteristic of the bacterial strains. **Figure 12B** shows the LPS of *Salmonella typhimurium (5)* which comprises the *O*-specific antigen, the core oligosaccharide, and lipid A. Other examples of LPS from *Haemophilus influenzae* and *Neisseria meningitidis* are given in Chapter 13.

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