Methods in Molecular Biology™

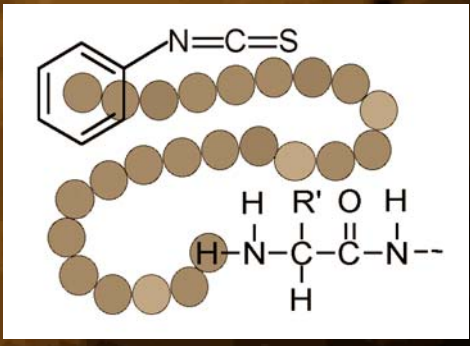
VOLUME 211

Protein Sequencing Protocols

Second Edition

Edited by

Bryan John Smith



HUMANA PRESS

1

Strategies for Handling Polypeptides on a Micro-Scale

Bryan John Smith and Paul Tempst

1. Introduction

Samples for sequence analysis frequently are in far from plentiful supply. Preparation of protein without loss, contamination or modification becomes more problematical as the amount of the sample decreases. The most successful approach is likely to include the minimum number of steps, at any of which a problem might arise. The strategy for preparation of a given protein will depend on its own particular properties, but several points of advice apply. These are:

- Minimize sample loss: *see* **Note 1**.
- Minimize contamination of the sample: *see* **Note 2**.
- Minimize artificial modification of the sample: *see* **Note 3**.

When it comes to sample purification, polyacrylamide gel electrophoresis is a common method of choice, since it is suited to sub-μg amounts of sample, entails minimal sample handling, is quick, and has high resolving power. Proteins may be fragmented while in the gel (*see* Chapters 5 and 6), or electroeluted from it using commercially available equipment. Commonly, however, proteins and peptides are transferred onto membranes prior to analysis by various strategies as described in Chapter 4. Capillary electrophoresis (Chapter 8) and high-performance liquid chromatography (HPLC) are alternative separation techniques. Capillary electrophoresis has sufficient sensitivity to be useful for few μg or sub- μg amounts of sample. For maximum sensitivity on HPLC, columns of 1 mm or less inside diameter (id) may be used, but for doing so there are considerations extra to those that apply to use of larger-bore columns. These are discussed below.

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

Although desirable to minimize the amount of handling of a sample, it is frequently necessary to manipulate the sample prior to further purification or analysis, in order to concentrate the sample or to change the buffer, for instance. Some examples of methods for the handling of small samples follow below. They do not form an exhaustive list, but illustrate the type of approach that it may be necessary to adopt.

2. Materials

2.1. Microbore HPLC

1. An HPLC system able to operate at low flow rates (of the order of 30 μL/min) while giving a steady chromatogram baseline, with minimal mixing and dilution of sample peaks in the postcolumn plumbing (notably at the flow cell) and with minimal volume between flow cell and outflow (to minimize time delay, so to ease collection of sample peaks).

   An example design is described by Elicone et al *(**1**)*. These authors used a 140B Solvent Delivery System from Applied Biosystems. The system was equipped with a 75 μL dynamic mixer and a precolumn filter with a 0.5 μm frit (Upchurch Scientific, Oak Harbor, WA) was plumbed between the mixer and a Rheodyne 7125 injector (from Rainin, Ridgefield, NJ) using two pieces (0.007 inch ID, 27 cm long [1 in. = 2.54 cm]) of PEEK tubing. The injector was fitted with a 50 μL loop and connected to the column inlet with PEEK tubing (0.005 inch × 30 cm). The outlet of the column was connected directly to a glass capillary (280 μm OD/ 75 cm ID × 20 cm; 0.88 μL), which is the leading portion of an U-Z view flow cell (35 nL volume, 8-mm path length; LC Packings, San Francisco, CA), fitted into an Applied Biosystems 783 detector. The trailing portion of the capillary cell was trimmed to a 15 cm length and threaded out of the detector head, resulting in a post flow cell volume of 0.66 μL and a collection delay of 1.3 s (at a flow rate of 30 μL/ min). Alternatively, various HPLC systems suitable for microbore work are available from commercial sources.
2. Clean glassware, syringe, and tubes for collection (polypropylene, such as the 0.5 μL or 1.5 μL Eppendorf type).
3. Solvents: use only HPLC-grade reagents (Fisons or other supplier), including distilled water (commercial HPLC-grade or Milli-Q water). A typical solvent system would be an increasing gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) in water. The TFA acts as an ion-pairing reagent, interacting with positive charges on the polypeptide and generally improving chromatography. If TFA is not added to the acetonitrile stock, the baseline will decrease (owing to decreasing overall content of TFA), which makes identification of sample peaks more difficult. A level baseline can be maintained by adding TFA to the acetonitrile stock, in sufficient concentration (usually about 0.09% v/v) to make its absorbency at 214 or 220 nm equal to that of the other gradient component, 0.1% TFA in water. Check this by spectrophotometry. The absorbency remains stable for days.

4. Microbore HPLC columns of internal diameter 2.1 mm, 1 mm or less, are available from various commercial sources.

2.2. Concentration and Desalting of Sample Solutions

1. HPLC system: not necessarily as described above for microbore HPLC, but capable of delivering a flow rate of a few hundred μL to 1 mL per min. Monitor elution at 220 nm or 214 nm.
2. Clean syringe, tubes, HPLC-grade solvents, and so on as described in **Subheadings 2.1.**, **steps 2** and **3**.
3. Reverse-phase HPLC column, of alkyl chain length C2 or C4. Since analysis and resolution of mixtures of polypeptides is not the aim here, relatively cheap HPLC columns may be used (and reused). The method described employs the 2.1 mm ID × 10 mm C2 guard column. (Brownlee, from Applied Biosystems), available in cartridge format.

2.3. Small Scale Sample Clean-Up Using Reverse-Phase "Micro-tips"

1. Pipet tip: Eppendorf "gel loader" tip (cat. no. 2235165-6, Brinkman, Westbury, NY).
2. Glass fiber, such as the TFA-washed glass fibre disks used in Applied Biosystems automated protein sequencers (Applied Biosystems, cat. no. 499379).
3. Reverse-phase chromatography matrix, such as Poros 50 R2 (PerSeptive Biosystems, Framingham, MA). Make as a slurry in ethanol, 4:1::ethanol:beads (v/v).
4. Wash buffer: formic acid (0.1%, v/v in water). Elution buffer: acetonitrile in 0.1% formic acid, e.g., 30% acetonitrile (v/v).
5. Argon gas supply, at about 10–15 psi pressure, with line suited to attach to the pipet tip.
6. Micro-tubes: small volume, capped, e.g., 0.2 mL (United Scientific Products, San Leandro CA, Cat. no. PCR-02).

3. Methods

3.1. Microbore HPLC (see Notes 4–13)

3.1.1. Establishment of Baseline (see **Notes 4** and **7**)

A flat baseline at high-sensitivity setting (e.g., 15 mAUFs at 214 mm) is required for optimal peak detection. The use of an optimized HPLC and clean and UV absorbency-balanced solvents should generate a level baseline with little noise and peaks of contamination. A small degree of baseline noise originates from the UV detector. Beware that this may get worse as the detector lamp ages. Some baseline fluctuation may arise from the action of pumps and/or solvent mixer. Slow flow rates seem to accentuate such problems that can go unnoticed at higher flows. Thorough sparging of solvents by helium may

reduce these problems. New or recently unused columns require thorough washing before a reliable baseline is obtained. To do this, run several gradients and then run the starting solvent mixture until the baseline settles (this may take an hour or more). Such problems are reduced if the column is used continuously, and to achieve this in between runs, an isocratic mixture of solvents (e.g., 60% acetonitrile) may be run at low flow rate (e.g., 10 μL/min). Check system performance by running standard samples (e.g., a tryptic digest of 5 pmole of cytochrome C).

*3.1.2. Identification of Sample Peaks (*see ***Notes 4, 7,*** *and* ***8)***

1. Peaks that do not derive from the sample protein(s), may arise from other sample constituents, such as added buffers or enzymes. To identify these contaminants, run controls lacking sample protein. Once the sample has been injected, run the system isocratically in the starting solvent mixture until the baseline is level and has returned to its pre-inject position. This can take up to 1 h in case of peptide mixtures that have been reacted with UV-absorbing chemicals (4-vinyl pyridine for example) before chromatography.
2. Peaks may be large enough to permit on-line spectroscopy where a diode array is available. Some analysis of amino acid content by second derivative spectroscopy may then be undertaken, identifying tryptophan-containing polypeptides, for instance, as described in Chapter 9.
3. Polypeptides containing tryptophan, tyrosine, or pyridylethylcysteine may be identified by monitoring elution at just three wavelengths (253, 277, 297 nm) in addition to 214 nm. Ratios of peak heights at these wavelengths indicate content of the polypeptides as described in **Note 8**. This approach can be used at the few pmole level.
4. Flow from the HPLC may be split and a small fraction diverted to an on-line electrospray mass spectrograph, so as to generate information on sample mass as well as possible identification of contaminants.

*3.1.3. Peak Collection (*see ***Notes 4, 9–12)***

1. While programmable fraction collectors are available, peak collection is most reliably and flexibly done by hand. This operation is best done with detection of peaks on a flatbed chart recorder in real time. The use of flatbed chart recorder allows notation of collected fractions on the chart recording for future reference. The delay between peak detection and peak emergence at the outflow must be accurately known (*see* **Note 5**).
2. When the beginning of a peak is observed, remove the forming droplet with a paper tissue. Collect the outflow by touching the end of the outflow tubing against the side of the collection tube, so that the liquid flows continuously into the tube and drops are not formed. Typical volumes of collected peaks are 40–60 μL (from a 2.1 mm ID column) and 15–30 μL (from a 1 mm ID column). *See* **Note 9**.
3. Cap tubes to prevent evaporation of solvent. Store collected fractions for a short term on ice, and transfer to freezer (–20°C or –70°C) for long-term storage (*see* **Notes 10** and **11**).

4. Retrieval of sample following storage in polypropylene tubes is improved by acidification of the thawed sample, by addition of neat TFA to a final TFA concentration of 10% (v/v).

*3.2. Concentration and Desalting of Sample Solutions (*see ***Notes 14–24)***

1. Equilibrate the C2 or C4 reverse-phase HPLC column in 1% acetonitrile (or other organic solvent of choice) in 0.1% TFA (v/v) in water, at a flow rate of 0.5 mL/min at ambient temperature.
2. Load the sample on to the column. If the sample is in organic solvent of concentration greater than 1% (v/v), dilute it with water or aqueous buffer (to ensure that the protein binds to the reverse-phase column) but do this just before loading (to minimize losses by adsorption from aqueous solution onto vessel walls). If the sample volume is greater than the HPLC loop size, simply repeat the loading process until the entire sample has been loaded.
3. Wash the column with isocratic 1% (v/v) acetonitrile in 0.1% TFA in water. Monitor elution of salts and/or other hydrophilic species that do not bind to the column. When absorbency at 220 nm has returned to baseline a gradient is applied to as to elute polypeptides from the column. The gradient is a simple, linear increase of acetonitrile content from the original 1% to 95%, flow rate 0.5mL/min, ambient temperature, over 20 min. Collect and store emerging peaks as described above (*see* **Subheading 3.1.2.** and *see* **Note 9**).
4. The column may be washed by isocratic 95% acetonitrile in 0.1% TFA in water, 0.5 mL/min, 5 min before being re-equilibrated to 1% acetonitrile for subsequent use.

*3.3. Small Scale Sample Clean-up Using "Micro-tips" (*see ***Notes 25–28).***

1. Using a pipet tip, core out a small disk from the glass-fiber disk. Push it down the inside of the gel-loader tip (containing 20 μL of ethanol), until it is stuck. Pipet onto this frit 10 μL of reverse-phase matrix slurry (equivalent to about 2 μL of packed beads). Apply argon gas to the top of the tip, to force liquid through the tip and pack the beads. Wash the beads by applying 3 lots of 20 μL of 0.1% formic acid, forcing the liquid through the micro-column with argon, but never allowing the column to run dry. Use a magnifying glass to check this, if necessary. Leave about 5 mm of final wash above the micro-column. The column is ready to use.
2. Apply the sample solution to the micro-column and wash with 3 lots of 20 μL 0.1% formic acid, leaving a minimum of the final wash solution above the micro-column. Pipet 3–4 μL (i.e., about 2 column volumes) of elution buffer into the micro-tip, leaving a bubble of air between the elution buffer and the micro-column in ash buffer. The elution buffer is then forced into the micro-column (but without mixing with the wash buffer, for clearly, this would alter the composition of the buffer and possibly adversely affect elution). Collect the buffer containing the eluted sample. If further elution steps are required, do not let the

micro-column dry out, and proceed as before by leaving a bubble of air between the fresh elution buffer and the preceding buffer. Collect and store eluted fractions as in **Subheading 3.1.2.** and *see* **Notes 9–12**.

4. Notes

1. Small amounts of polypeptide are difficult to monitor and may be easily lost, for instance, by adsorption to vessel walls. Minimize the number of handling maneuvers and transfers to new tubes.
2. Work in clean conditions with the cleanest possible reagents. Consider the possible effects of added components such as amine-containing buffer components such as glycine (which may interfere with Edman sequencing), detergents, protease inhibitors (especially proteinaceous ones such as soybean trypsin inhibitor), agents to assist in extraction procedures (such as lysozyme), and serum components (added to cell culture media).
3. Modification of the polypeptide sample can arise by reaction with reactive peroxide species that occur as trace contaminants in triton and other nonionic detergents *(**2**)*. The presence of these reactive contaminants is minimized by the use of fresh, specially purified detergent stored under nitrogen (such as is available from commercial sources, such as Pierce). Mixed bed resins, mixtures of strong cation and anion resins (available commercially from sources such as Pharmacia Biotech, BioRad, or BDH) can be used to remove trace ionic impurities from nonionic reagent solutions such as triton X100, urea, or acrylamide. Excess resin is merely mixed with the solution for an hour or so, and then removed by centrifugation or filtration. The supernatant or filtrate is then ready to use. Use while fresh in case contaminants reappear with time. In this way, for example, cyanate ions that might otherwise cause carbamylation of primary amines (and so block the N-terminus to Edman sequencing) may be removed from solutions of urea.

   Polypeptide modification may also occur in conditions of low pH; for instance, N-terminal glutaminyl residues may cyclize to produce the blocked pyroglutamyl residue, glutamine, and asparagine may become deamidated, or the polypeptide chain may be cleaved (as described in Chapter 6). Again, exposure of proteins to formic acid has been reported to result in formylation, detectable by mass spectrometry *(**3**)*. Problems of this sort are reduced by minimizing exposure of the sample to acid and substitution of formic acid by, say, acetic or trifluoroacetic acid (TFA) for the purposes of treatment with cyanogen bromide (*see* Chapter 6).

4.1. Microbore HPLC

4. When working with μg or sub- μg amounts of sample the problem of contamination is a serious one, not only adding to the background of amino acids and nonamino acid artifact peaks in the final sequence analysis, but also during sample preparation, generating artificial peaks, which may be analyzed mistakenly. To reduce this problem most effectively, for microbore HPLC or other technique, it is necessary to adopt the "semi-clean room" approach, whereby ingress of contaminating protein is minimized. Thus:

a. Dedicate space to the HPLC, sequencer and other associated equipment. As far as possible, set this apart from activities such as peptide synthesis, biochemistry, molecular biology, and microbiology.
b. Dedicate equipment and chemical supplies. This includes equipment such as pipets, freezers, and HPLC solvents.
c. Keep the area and equipment clean. Do not use materials from central glass washing or media preparation facilities. It is not uncommon to find traces of detergent or other residues on glass from central washing facilities, for instance. Remember that "sterile" does not necessarily mean protein-free!
d. Use powderless gloves and clean labcoats. Avoid coughing, sneezing and hair near samples. As with other labs, ban food and drink. Limit unnecessary traffic of other workers, visitors, and so on.
e. Limit the size of samples analyzed, or beware the problem of sample carryover. If a large sample has been chromatographed or otherwise analyzed, check with "blank" samples that no trace of it remains to appear in subsequent analyses.

5. Micro-preparation of peptides destined for chemical sequencing and mass spectrometric analysis often requires high performance reversed-phase LC systems, preferably operated with volatile solvents. Sensitivity of sample detection in HPLC is inversely proportional to the cross-sectional area of the HPLC column used, such that a 1 mm ID column potentially will give 17-fold greater sensitivity than a 4.6 mm ID column. Microbore HPLC tends to highlight shortcomings in an HPLC system, however, so to get optimal performance from a microbore system attention to design and operation is necessary, as indicated in Materials (**Subheading 2.**) and Methods (**Subheading 3.**).

   At the slow flow rates used in microbore HPLC, the delay between the detection of a peak and its appearance at the outflow may be significant, and must be known accurately for efficient peak collection. If the volume of the tubing between the UV detector cell and the outflow is known, the time delay (*t*) may be calculated:

$$t = \frac{\text{tubing volume, } \mu L}{\text{flow rate, } \mu L/\text{min}}$$

   where *t* is in minutes. The collection of any peak must be delayed by t minutes after first detection of the peak. The flow rate should be measured at the point of outflow - a nominal flow rate set on a pump controller may be faster than the actual flow rate due to the effect of back pressure in the system (e.g., by the column).

   Alternatively, *t* may be determined empirically as follows:

   a. Disconnect the column, replace it with a tubing connector.
   b. Set isocratic flow of 0.1% TFA in water at a rate equal to that when the column is in-line and check flow rate by measuring the outflow.
   c. Inject 50 µL of a suitable coloured solution, e.g., 0.1% (w/v) Ponceau S solution in 1% acetic acid (v/v).
   d. Collect outflow. To see eluted color readily, collect outflow as spots onto filter paper (e.g., Whatman 3MM).

e. Measure the time between first detection of the dye peak, and first appearance of color at the outflow. Repeat this process at the same or different flow rates sufficient to gain an accurate estimate, which may be used to calculate the tubing volume (see equation for *t*).

The slow flow rate has another consequence too, namely a delay of onset of a gradient. The volume of the system before the column may be significant and a gradient being generated from the solvent reservoirs has to work its way through this volume before reaching the column or UV detector. For instance, a pre-column system volume of 600 μL would generate a 20-min delay if the flow rate were 30 μL/min. If the length of this delay is unknown, it may be measured empirically as follows:

a. Leave the HPLC column connected to the system. Have one solvent (A) as a mixture, 5% (v/v) acetonitrile in 0.1% v/v TFA in water, and another solvent (B) as 95% (v/v) acetonitrile in 0.1% (v/v) TFA in water. (NOTE: solvents not balanced for UV absorption).
b. From one solvent inlet, run solvent mixture A isocratically at, say 30 μL/min, until the baseline is level.
c. Halt solvent flow, replace A with B and resume flow at same flow rate.
d. Measure time from resumption of flow to sudden change of UV absorption. This is the time required for a solvent front to reach the detector, with the column of interest in the system.

Remember to allow for this delay when programming gradients.

6. Reverse-phase columns are commonly used for polypeptide separations. Columns of various chain lengths up to C18 are available commercially in 2.1 or 1 mm ID. As for wider-bore HPLC, the best column for any particular purpose is best determined empirically, though the following may be stated: use larger-pore matrices for larger polypeptides; use shorter-length alkyl chain columns for chromatography of hydrophobic polypeptides. As an example of the latter point, human Tumor Necrosis Factor-α (TNF-α) is soluble in plasma and is biologically active as a homotrimer, but binds so tightly to a C18 reverse-phase column that 99% acetonitrile in 0.1% v/v TFA in water will not remove it. It can be eluted from C2 or C4 columns by increasing gradients of acetonitrile, however.

   Gradient systems used in microbore reverse-phase HPLC are also best determined empirically, but commonly would utilize an increasing gradient of acetonitrile (or other organic solvent) in 0.1% (v/v) TFA (or other ion-pairing agent, such as heptafluorobutyric acid) in water. Flow rates would be of the order of 30 μL/min for a 1 mm ID column, or 100 μL/min for a 2.1 mm ID column. Use ambient temperature if possible, to avoid the possibility of baseline fluctuation due to variation in temperature of solvent as it passes from heated column to cooler flow cell.
7. In the various forms of chromatography, elution of polypeptide sample is commonly monitored at 280 nm. However, not only may some polypeptides lack significant absorbency at 280 nm, but also detection is an order of magnitude less sensitive than at 220nm. Absorbency at the lower wavelengths is due to the pep-

tide bond (obviously present in all polypeptides). However, absorbency due to solvent and additives such as TFA and contaminants tends to be higher. This "background" absorbency becomes greater as wavelengths are reduced towards 200 nm and with it the problems of maintaining a stable baseline and detection of contaminants become greater. The trade-off between greater sensitivity and background absorbency is best made empirically with the user's own equipment. Detection at 214 nm or 220 nm is commonly used, with lower wavelengths being more problematical.

8. Sample peaks may be analyzed on-line by spectroscopy. With a diode array and enough sample to generate a reliable spectrum, second derivative spectroscopy may be used as described in Chapter 9. At the few pmole level, monitoring at 253 nm, 277 nm, and 297 nm may indicate peaks that may be of interest by virtue of containing tryptophan, tyrosine or pyridylethylcysteine. A peptide's content of tryptophan, tyrosine, and (pyridylethyl) cysteine may be judged from the ratios of absorbency at 253, 277, and 297 nm. Thus:
   a. Greatest absorbency at 253 nm with minimal absorbency at 297 nm indicates the presence of pyridylethylcysteine.
   b. Greatest absorbency at 277 nm with minimal absorbency at 297 nm indicates the presence of tyrosine.
   c. Greatest absorbency at 277 nm with moderate absorbency at 253 nm and 297 nm indicates the presence of tryptophan.

   If more than one of these three types of residue occur in one peptide, identification is more problematical since the residues' UV spectra overlap. However, comparison with results from model peptides assist analysis, as described by Erdjument-Bromage et al *(**4**)*, whose results are summarized in **Table 1**. The presence of tyrosine is the most difficult to determine, but combinations of tryptophan and pyridylethylcysteine may be identified. As Erdjument-Bromage et al. *(**5**)* point out, this analysis is only valid when the mobile phase is acidic (e.g., in 0.1% TFA in water and acetonitrile), for UV spectra of tryptophan and tyrosine change markedly with changes in pH. This type of analysis may be performed on 5–10 pmole of peptides.
9. Drops flowing from HPLC have a volume of the order of 25 µL. At the type of flow rate used for microbore HPLC, a drop of this size may take a minute to form and so may contain more than one peak. This is unacceptable. Collection of outflow down the inside wall of the collection tube inhibits droplet formation and allows interruption of the collection (changing to the next fraction) at any time.
10. Once peptides elute from a reverse-phase HPLC column, they are obtained as a dilute solution (1–2 pmoles per 5 µL) in 0.1% TFA/10–30% (v/v) acetonitrile, or similar solvent. At those concentrations and below, many peptides tend to "disappear" from the solutions. The problem of minute peptide losses during preparation, storage, and transfer has either not been fully recognized or has been blamed on unrelated factors, column losses for example. Actually, column effects are minimal *(**1**)*. Instead, it has been shown that losses primarily occur in test tubes and pipet tips *(**5**)*. At concentrations of 2.5–8 pmoles per 25 µL (amounts and volume repre-

Table 1
Reverse-Phase HPLC with Triple Wavelength Detection of Peptides Containing Trp (W), Tyr (Y), or pyridyl ethyl-Cys (pC)[a]

	Relative Peak Height (in %)			Number of Residents		
Peptide	A_{253}	A_{277}	A_{297}	W	Y	pC
pCPSPKTPVNFNNFQ	100	12	2	-	-	1
QNpCDQFEK	100	14	1	-	-	1
GNLWATGHF	45	100	28	1	-	-
ILLQKWE	43	100	26	1	-	-
YEVKMDAEF	33	100	3	-	1	-
TGQAPGFTYTDANK	38	100	2	-	1	-
YSLEPSSPSHWGOLPTP	45	100	21	1	1	-
GITWKEETLMEYLENPK	42	100	24	1	1	-
EDWKKYEKYR	40	100	23	1	2	-
YEDWKKYEKYR	37	100	19	1	3	-
Insulin beta chain / 4VP	100	39	4	-	2	2
Insulin alpha chain / 4VP	100	32	3	-	2	4
DST peptide (25 a.a.)	100	73	23	1	-	1
PepepII (27 a.a.)	100	100	20	1	1	1

[a]Peptides (20 picomoles each, or less) were chromatographed on a Vydac C4 (2.1 × 250 mm) column at a flow of 0.1 mL/min. Peak heights on chromatographs, produced by monitoring at different wavelengths, are expressed in %, relatively to the tallest peak. Total number of W, Y, or pC present in each peptide are listed. Sequences of bovine insulin alpha and beta chains are taken from SWISS and PIR database; PepepII, ISpCWAQIGKEPITFEHINYERVSDR; DST peptide, DLFNAAFVSpCWSELNEDQQDELIR. Insulin was reduced with 2-mercaptoethanol and reacted with 4-vinyl pyridine prior to HPLC. Reprinted with permission from **ref. *(4)***.

sentative for a typical microbore LC fraction), about 50% of the peptide is not recovered from storage in 0.1% TFA (from 1 min to 1 wk). When supplemented with 33% TFA, recoveries were 80% on the average. Best transfers, regardless of volume and duration of storage, were obtained in 10% TFA/30% acetonitrile. From those data it follows that, upon storage at –70°C for 24 h or more, up to 45% losses may be incurred for LC collected peptides. Although adding concentrated TFA prior to storage results in best recoveries (> 90%), it might degrade the peptides. Thus, it is best to store HPLC-collected peptides at –70°C and always add neat TFA in a 1 to 8 ratio (TFA: sample) after storage, just before loading on the sequencer disc. Additionally, coating the polypropylene with polyethylenimine may reduce this loss, as indicated by an observed improved retrieval of radiolabeled bradykinin from polypropylene tubes (increased from 30% to 65% yield). Tubes were coated by immersion in 0.5% polyethylenimine in water overnight, room temperature, followed by rinsing in distilled water and thorough drying in a glass-drying oven (Dr J. O'Connell, unpublished observation).

Having collected a sample in a mixture of solvents in which it is soluble, it is unwise to alter this mixture for the sample may then become insoluble. Thus, concentration under vacuum will remove organic solvent before removing the less-volatile water, as changing the solvent mixture. Again, if the sample contacts membranes such as used for concentration, filtration or dialysis it may become irreversibly bound. Complete drying down may also be a problem— redissolving the dried sample may be difficult, requiring glacial acetic acid or formic acid (70% v/v, or greater).

11. Repeated cycles of freezing and thawing may cause fragmentation of polypeptides eventually, this tending to increase adsorption losses. Beware that the temperature inside a (nominally) –20°C freezer may rise to close to 0°C during defrosting or while the door is left open while other samples are being retrieved, such that sample quality may suffer. Storage at –70°C is safer.
12. Another solution to the problem of storage of HPLC fractions, at least for subsequent sequencing by Edman chemistry, is immediate transfer to polyvinylidene difluoride (PVDF) membrane, on which medium (dried) polypeptides are stable for prolonged periods. This may be accomplished by use of the single use Prosorb device from Applied Biosystems. The sample solution is drawn by capillary action through a PVDF membrane, to which polypeptides bind. Addition of polybrene (Biobrene, Applied Biosystems) is recommended for sequencing of PVDF-bound peptides (see the literature that accompanies Biobrene for its method of use). For processing large numbers of samples, PVDF sheets may be used to trap the polypeptides. The membrane is placed in a Hybridot 96-well manifold (BRL), or similar, and the sample solutions are drawn slowly through the membrane. The location of the bound protein spots may be confirmed by staining of the wetted membrane for a few minutes in Ponceau S (Sigma), 0.1% (w/v) in acetic acid (1% v/v in water), followed by destaining in water.

    PVDF requires wetting with organic solvent prior to wetting by water. Dried PVDF membrane may be re-wetted with 20% methanol in water without significant loss of polypeptide sample. Many reverse-phase HPLC fractions (e.g., from a gradient of organic solvent in TFA-water) will likewise wet PVDF directly.
13. Various criteria can be applied to sample peaks in order to decide whether they are suitable for sequencing by Edman Chemistry, i.e., pure and in sufficient quantity. These are:
    a. The peak should not show signs of any shoulders indicative of underlying species.
    b. Spectra collected at multiple points through the peak should be identical-differences indicate multiple species present.
    c. If mass spectrometry is carried out on part of the sample peak, a single mass is a reasonably good indication of purity.

    If a sample peak appears not to be pure by such criteria, collected fractions may be prepared for chromatography on a second, different HPLC system as follows:
    a. Add neat TFA in the ratio 1:8::TFA: sample (v/v), in order to improve recovery (*see* **Subheading 4.1.**, **step 7**, above).

b. Dilute by addition of one volume of water or 0.1% TFA in water (v/v), just before injection. Recoveries after rechromatography are usually of the order of 40–60%.

4.2. Desalting/Concentration

14. The presence of salts and detergents can interfere with analysis by mass spectrometry or protein sequencing by Edman chemistry (if these reagents restrict access of chemicals to the sample, or generate artificial products). Again, if a sample solution is too dilute, analysis may be problematical.
    As an example of the HPLC method for concentration and desalting of sample solutions described in **Subheading 3.2.**, it has been used in preparation of human TNF-α, a hydrophobic protein that can absorb to membranes used for filtration as well as to C18 reverse-phase HPLC columns. TNF-α at as little as 2 ng/mL in 2 L cell culture medium containing 10% (v/v) fetal calf serum (FCS) was prepared at approx 100% yield as follows:
    a. Concentration approx fivefold on a 10 KDa cut-off membrane (using a Filtron miniultra-cassette, with losses of TNF-α being minimized by the presence of other proteins).
    b. Affinity chromatography on a solid-phase-linked, anti-human TNF-α antibody, the TNF-α eluting in 7.5 mL of a buffer of trizma-HCl, 50 m*M*, pH 7.6, magnesium chloride, 3 M.
    c. Final concentration and desalting by C2 HPLC as described in **Subheading 3.2.**, eluting from the column in 0.5 mL.
15. The concentrating/desalting method described is a basic one for separating hydrophilic and hydrophobic species, the former being salt and the latter being the TNF-α in the example above. The system may be modified in various ways for less hydrophobic polypeptides. Thus, replacement of the C2 HPLC column by a C4 or even C18 column may provide better discrimination between salts and hydrophilic polypeptides. Alternatively, the relatively cheap "guard" column used here may be replaced by an analytical column such that mixtures of polypeptides may be resolved on the column after salts have been removed.
16. Nonionic detergents may not be separated from polypeptide during concentration or desalting on reverse phase columns - Triton X 100 and Tween do not elute with hydrophilic species but do so in the subsequent acetonitrile gradient. A detergent can be removed by dialysis but requires extensive dilution to below the detergent's critical micelle concentration (CMC), followed by prolonged dialysis. *n*-Octyl-β-glucopyranoside is one of the better detergents in this respect, since it has a relatively high CMC of 20–25 m*M*. Alternatively, matrices such as Calbiosorb (Calbiochem) may be used to remove detergent chromatographically. Nonionic species may be removed from solutions of proteins by ion-exchange chromatography. One proviso is that the protein should bear charge, i.e., the solution pH should not be equal to the proteins pI. With that condition satisfied the protein may be bound to the ion exchange matrix while non-ionic species may be washed away. Protein may be removed subsequently, by altering pH or salt concentration.

17. The use of an ion exchange pre-column (DEAE-Toyopearl, 4 × 50mm) has been described for removal of SDS and Coomassie brilliant blue R-250 from gel extracts, prior to peptide separation by reverse phase HPLC *(6)*. Hydrophilic interaction chromatography on poly(2-hydroxyethyl-aspartimide)-coated silica (PolyLC Inc.) in n-propanol-formic acid solvent can also remove salts and contaminants that may occur in samples electroeluted form polyacrylamide gels, for example *(7)*.
18. Batch chromatography offers an alternative means of concentration and salt removal (*see* **Note 28**).
19. Sample peaks may be analyzed by on-line spectroscopy during concentration or desalting, as described in **Note 8**.
20. Reverse-phase HPLC may be interfaced with electrospray mass spectrometry, so the method described may, in such a coupled system, be used to desalt samples for analysis.

    To avoid build-up of salty deposits in the mass spectrometer the salt peak may be diverted to waste.

    A similar end may be achieved by using a gel filtration column in-line, ahead of the mass spectrometer, proteins emerging ahead of salts and other small species. Gel filtration dilutes rather than concentrate samples, however.
21. Various commercially available small scale devices offer alternatives to the HPLC method. For example, single use Ultrafree-MC filters (Millipore) are suitable for concentration of samples down to volumes of the order of 50–100 μL: the sample is placed in the device and then centrifuged, driving smaller species through the membrane while retaining larger species. The sample may be repeatedly topped up and centrifuged in order to process larger volumes. Similarly, the sample may be repeatedly concentrated and then diluted with water or alternative buffer for the purposes of buffer exchange. Small-volume dialysis devices are also available for exchange of buffers in samples as small as 10 μL (for instance the Slide-A-Lyzer units from Pierce). Generally these approaches are not suitable for small molecular-weight peptides, but Fierens et al. *(8)* have reported a means (albeit not suited to all circumstances) whereby peptides may be retained by filter membranes with nominal cutoffs greater than the size of the peptides. This involves addition of albumin, to which the peptides may bind, and which does not pass through the filter membrane.

    Beware that buffer exchange and concentration procedures carry with them the danger of sample aggregation and precipitation, and the loss of sample solution that cannot be retrieved from the surfaces and corners of the devices used.
22. Salts may be removed from polypeptide solutions by transfer of the polypeptide to PVDF (*see* **Note 12**). Salts are not retained on PVDF, whereas polypeptides are. Remaining traces of salts or contaminating amino acids may be removed by washing of the membrane in a small volume of methanol, followed by drying in air. Thus samples applied in 100 m*M* Trizma buffer or in 1 *M* NaCl show the same initial and repetitive yields as samples applied in water, with no extra peaks of contamination.

The presence of detergent can interfere with polypeptide binding to PVDF (e.g., 0.1% v/v brij 35 reduces binding by 5- to 10-fold). Dilution of the sample overcomes this problem. Applied Biosystems recommend dilution of Triton X100 to 0.05% or less, and sodium dodecyl sulfate (SDS) to 0.2% to allow efficient binding of protein to ProBlott PVDF membrane. Similarly they recommend dilution of urea or guanidine hydrochloride to 2–3 *M*. Sample dilution is not a problem in so far as a large volume may be filtered through the PVDF (by repeatedly refilling sample well or ProSorb) but the following should be remembered: large volumes of diluent may introduce significant contamination; dilution of detergent may cause the sample to come out of solution of bind to vessel walls. To minimize the latter, make dilutions immediately before filtration.

23. Various stains are available for detection of proteins on PVDF membranes. This allows location of the protein and may allow approximate quantification. The fluorescent stain Sypro Ruby (Molecular Dynamics) has sensitivity approaching that of silver stains. It does not interfere with subsequent analysis of bound sample. Sypro Ruby may be used for quantification (detection under UV light and scanning in a Bio Rad FluorS scanning densitometer). Methods have also been described for quantification of Ponceau S-stained protein on membranes *(**9**,**10**)*. Beware that the handling involved in staining and scanning may introduce contamination.
24. Proteins may be removed from salty solution and concentrated by precipitation. This may be achieved by addition of 1/4 volume of 100% w/v trichloroacetic acid solution (i.e., 100 g TCA in 100 mL solution – beware the highly corrosive nature of this solution: wear protective clothing), thus giving a final TCA concentration of 20%. Stand the mixture on ice for 1 h or so and centrifuge. Discard the supernatant. Remove traces of acid by several washes in acetone and finally dry under vacuum. Other molecules than proteins may co-precipitate, for instance nucleic acids. Sauvé et al. have described a method for concentration of proteins from solutions of 10 ng/mL *(**11**)* to 100 ng/mL *(**12**)*. The proteins are extracted in water-saturated phenol, and then extracted from the phenol solution by ether, whence they are isolated by evaporation of the ether. Recoveries were determined to be of the order of 80%, better than about 50% achieved by the TCA precipitation method. Protein in solution with guanidine hydrochloride may be precipitated by addition of sodium deoxycholate and TCA *(**13**)*. With any precipitation procedure there is a potential problem of rendering the protein insoluble. Heating in SDS-PAGE sample buffer, for subsequent PAGE, overcomes this in most cases.

4.3. Small Scale Sample Clean-up

25. The method described is that of Erdjument-Bromage et al. *(**14**)*. The method was intended for processing of small samples prior to mass spectrometry, which can be adversely affected by salts, detergents, or other components in the sample. The method is essentially low-pressure reverse-phase chromatography, in which salts do not bind to the column and can therefore be removed. Like other forms of

chromatography, elution of bound material may be achieved by a single step from low to high concentration of organic solvent, or by a succession of smaller incremental steps of increasing solvent concentration. By using incremental steps, bound material may be fractionated. Erdjument-Bromage et al. ***(14)*** illustrated this with a sample of trypsin digests of 100 fmole of glucose-6-phosphate dehydrogenase in polyacrylamide gel. A two-step elution of the digested peptides from the micro-tip was achieved using 16% and 30% acetonitrile in 0.1% formic acid, and mass spectrometric analysis was subsequently successfully achieved.

The approach may be adapted to other cases. For example, the matrix may be changed to another to achieve a different chromatographic separation. For example, affinity purification of phosphopeptides may be carried out by use of immobilized metal affinity chromatography ***(15)***. Gallium (III) ions are immobilised on beads of chelating resin (Poros MC) by washing the beads in a solution of $GaCl_3$. A solution of mixed peptides is loaded onto the column and the phosphopeptides are selectively bound. After washing to remove unbound peptides, phosphopeptides are eluted in a buffer of pH 8.5 in the presence of phosphate, which displace the phosphopeptide. The micro-columns in this case were about 12 μL, and sample volumes were optimally less than 50% the volume of the column, namely about 5 μL. Beware the toxicity of gallium (III) chloride, and its violent reaction with water; wear protective clothing.

26. Versions of micro-tips are now available commercially, such as Zip-Tips from Millipore (similar to the micro-tips described earlier, and operable with a pipet rather than pressurized gas), or Supro-tips from AmiKa Corp. (Columbia, MD) where the matrix is bound to the tip. A variety of matrices is available, allowing desalting and concentration of polypeptides, step-wise fractionation, preparation of phosphopeptides and His-tagged polypeptides, and removal of detergents and other contaminants. Note that these come in fixed sizes, with fixed capacity, whereas the manually prepared version can be adapted and made larger if required.
27. Note that these columns are made small in order to deal with small volume samples (of the order of 10 μL or less and containing 1 μg of protein or less). Beware that the capacity of the small columns may be easily exceeded (for instance by contaminants as well as the desired sample itself), and this may adversely affect the purification.
28. Sample clean-up can be achieved by small-scale batch chromatography without use of micro-tips. This may be useful for larger volumes of sample than are convenient for micro-tips. In essence the approach is: incubate the sample with chromatography medium; centrifuge the mixture to separate supernatant from chromatography matrix; further analyse the supernatant or the chromatography matrix (if the desired molecule is bound). Elute the sample molecule from the chromatography matrix if desired. The choice of chromatography matrix, and conditions for sample binding and elution are dependent on the case in point. If it is necessary to check the pH of a small volume of solution prior to this batch chromatography, this may be done economically by use of the "dip-stick" type of pH indicator strips (e.g., from BDH, Poole, UK). For this, cut the strip into further,

smaller strips of less than 1 mm width. Each of these requires only 1 μL or so of solution to gain a colourimetric reading of pH. Detergents may be removed from samples by use of products such as BioBeads (Bio-Rad) in a similar batch mode.

This approach may be used to remove contaminants from solution. An example of this is the removal of albumin from plasma or cell culture medium, where it may be so abundant as to interfere with analysis of other proteins present. This is achieved by incubation of the sample with Cibacron Blue linked to sepaharose or agarose beads. The albumin binds to the Cibacron Blue and can be removed (totally or partly) on the beads. Rengarajan et al. *(**16**)* have described this approach for dealing with small serum samples. To 10 μL serum mixed with 240 μL phosphate buffer was added 160 mL slurry of Affi-Gel Blue (agarose-bound dye, Bio-Rad, Hercules, CA). This mixture was incubated for 30 min at room temperature prior to centrifugation. The Affi-Gel Blue beads were washed to retrieve supernatant trapped between the beads and the pooled supernatants concentrated prior to further analysis. Blue sepharose (Pharmacia Biotech) works in similar fashion. Beware that proteins other than albumin may bind to the Cibacron Blue dye moiety.

Alternatively, the molecule of interest may be bound to the beads. As an example, Gammabind Plus sepharose (Pharmacia Biotech) can be used to separate molecules containing an immunoglobulin Fc domain such as IgG itself, or proteins genetically fused to an Fc "tag." The sample, at neutral pH, is incubated with beads (with gentle mixing) and then centrifuged. The beads may be directly heated in SDS-PAGE sample buffer prior to electrophoresis. Alternatively, the bound molecules may be eluted for other analyses, by washing the beads in low pH buffer (pH 2.0 or 3.0). Qian et al. *(**17**)* have described analysis of multi-His-tagged peptides and proteins while they were still bound to an affinity matrix of immobilized metal ion beads. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) of the samples was possible directly on the polypeptide-loaded beads, and it was also possible to proteolyse the sample on the bead prior to mass spectrometric analysis.

Another example of batch chromatography may be useful for concentration of proteins from dilute solution (100 ng/mL), prior to SDS-PAGE *(**12**)*. The protein solution is incubated with Strataclean beads (from Stratagene, cat. no. 400714), with shaking at room temperature for 20 min or so. Any protein(s) present bind to the beads and can be pelleted on the beads. They are then released by heating in SDS PAGE sample buffer prior to electrophoresis. High ionic strength (2 *M* ammonium sulphate) and various detergents do not interfere with this process, though 1% deoxycholate did interfere with recovery of albumin and ovalbumin, at least.

Acknowledgments

We thank Dr. J. O'Connell of Celltech R and D. for allowing us to use his example of peptide loss and its remedy given in **Note 10**.

References

1. Elicone, C., Lui, M., Geromanos, S. S., Erdjument-Bromage, H., and Tempst, P. (1994) Microbore reversed-phase high performance liquid chromatographic purification of peptides for combined chemical sequencing/laser-desorption mass spectrometric analysis. *J. Chromatog.* **676**, 121–137.
2. Chang, H. W. and Bock, E. (1980) Pitfalls in the use of commercial nonionic detergents for the solubilisation of integral membrane proteins: sulfhydryl oxidising contaminants and their elimination. *Anal. Biochem.* **104**, 112–117.
3. Beavis, R. C. and Chait, B. T. (1990) Rapid, sensitive analysis of protein mixtures by mass spectrometry. *Proc. Natl. Acad. Sci. USA* **87**, 6873–6877.
4. Erdjument-Bromage, H., Lui, M., Sabatini, D. M., Snyder, S. H., and Tempst, P. (1994) High-sensitivity sequencing of large proteins: partial structure of the rapamycin-FKBP12 target. *Protein Sci.* **3**, 2435–2446.
5. Tempst, P., Geromanos, S., Elicone, C., and Erdjument-Bromage, H. (1994) Improvements in microsequencer performance for low picomole sequence analysis. *Methods: Comp. Methods Enzymol.* **6**, 248–261.
6. Kawasaki, H., Emori, Y., and Suzuki, K. (1990) Production and separation of peptides from proteins stained with Coomassie brilliant blue R-250 after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **191**, 332–336.
7. Jenö, P., Scherer, P. E., Manning-Krieg, U., and Horst, M. (1993) Desalting electroeluted proteins with hydrophilic interaction chromatography. *Anal. Biochem.* **215**, 292–298.
8. Fierens, C., Thienpont, L. M., Stockl, D., and De Leenheer, A. P. (2000) Overcoming practical limitations for the application of ultrafiltration in sample preparation for liquid chromatography/mass spectrometry of small peptides. *Anal. Biochem.* **285**, 168–169.
9. Morcol, T. and Subramanian, A. (1999) A red-dot-blot protein assay technique in the low nanogram range. *Anal. Biochem.* **270**, 75–82.
10. Bannur, S. V., Kulgood, S. V., Metkar, S. S., Mahajan, S. K., and Sainis, J. K. (1999) Protein determination by Ponceau S using digital color image analysis of protein spots on nitrocellulose membranes. *Anal. Biochem.* **267**, 382–389.
11. Sauvé, D. M., Ho, D. T., and Roberge, M. (1995) Concentration of dilute protein for gel electrophoresis. *Anal. Biochem.* **226**, 382–383.
12. Ziegler, J., Vogt, T., Miersch, O., and Strack, D. (1997) Concentration of dilute protein solutions prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **250**, 257–260.
13. Arnold, U. and Ulbrich-Hofmann, R. (1999) Quantitative protein precipitation from guanidine hydrpchloride-containing solutions by sodium deoxycholate/trichloroacetic acid. *Anal. Biochem.* **271**, 197–199.
14. Erdjument-Bromage, H., Lui, M., Lacomis, L., Grewal, A., Annan, R. S., McNulty, D. E., et al. (1998) Examination of micro-tip reverse-phase liquid chromatographic extraction of peptide pools for mass spectrometric analysis. *J. Chromatog. A* **826**, 176–181.

15. Posewitz, M. C. and Tempst, P. (1999) Immobilized Gallium(III) affinity chromatography of phosphopeptides. *Anal. Chem.* **71**, 2883–2892.
16. Rengarajan, K., de Smet, B., and Wiggert, B. (1996) Removal of albumin from multiple human serum samples. *BioTechniques* **20**, 30–32.
17. Qian, X., Zhou, W., Khaledi, M. G., and Tomer, K. B. (1999) Direct analysis of the products of sequential cleavages of peptides and proteins affinity-bound to immobilized metal ion beads by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.* **274**, 174–180.

2

SDS Polyacrylamide Gel Electrophoresis for N-Terminal Protein Sequencing

Bryan John Smith

1. Introduction

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) is a very common technique used for analysis of complex mixtures of polypeptides. It has great resolving powers, is rapid, and is suitable for proteins of either acidic or basic pI. The last is because the protein is reacted with SDS, which binds to the protein in the approximate ratio 1.4:1 (SDS:protein, w/w) and imparts a negative charge to the SDS-protein complex. The charged complexes move towards the anode when placed in an electric field, and are separated on the basis of differences in charge and size. SDS-PAGE is commonly used to estimate a protein's molecular weight, but estimates are approximate (being termed "apparent molecular weights") and sometimes prone to marked error. For instance, disproportionately large increases in apparent molecular weight may occur upon covalent phosphorylation of a protein *(**1**)*, or artificial entrapment of phosphoric acid *(**2**)*. Most designs of SDS-PAGE employ a "stacking gel." Such a system enables concentration of a sample from a comparatively large volume to a very small zone within the gel. The proteins within this zone are concentrated into very narrow bands, making them not only more easily detected but also better resolved from neighboring bands of other proteins. The principle involved in this protein concentration (or "stacking") is that of isotachophoresis. It is set up by making a stacking gel on top of the "separating gel," which is of a different pH. The sample is applied at the stacking gel and when the electric field is applied the negatively charged complexes and smaller ions move towards the anode. At the pH prevailing in the stacking gel, protein-SDS complexes have mobilities intermediate between the faster Cl^- ions (present throughout the electrophore-

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

sis system) and the slower glycinate ions (present in the cathode reservoir buffer). The protein-SDS complexes concentrate in the narrow zone between Cl^- and glycinate ions. When the moving zones reach the separating gel with its different pH their respective mobilities change and glycinate overtakes the protein-SDS complexes, which then move at rates governed by their size and charge in a uniformly buffered electric field. Isotachophoresis is described in more detail in the literature (e.g., **ref. *3***).

SDS-PAGE requires microgram to submicrogram amounts of each protein sample. That is similar to amounts required for analysis by automated protein sequencing and mass spectrometry. The achievement of interfacing SDS-PAGE with sequencing has brought a notable step forward in sample handling technique: small amounts of a complex mixture may be resolved suitably for sequencing in just a few hours. This is done by transferring or "blotting" proteins which have been resolved by SDS-PAGE to polyvinylidene difluoride (PVDF) or other similar support, as described in Chapter 4. This medium may also be used for other analyses such as characterization by use of specific antibodies (Western blotting), such that specific proteins (on sister blots) may be identified for further analysis by sequencing or by mass spectrometry. It is important to maximize yields of sequencable protein throughout the whole process, however, and conditions for transfer may require optimization to obtain significant amounts of sample bound to the PVDF. Prior to that, however, the conditions for SDS-PAGE need to be such that minimal protein N-terminal blockage occurs by reaction of the free amino group with species in the gel. Usually, these reactive species and the blocking groups that they produce remain unknown but ways to remove them at least partially have been developed empirically. These include electrophoresis of the gel before application of the sample, but this destroys the isotachophoretic stacking system described earlier. For some applications, the accompanying reduction of resolution may be undesirable, but Dunbar and Wilson ***(4)*** have described a method that minimizes this problem. Their approach to SDS-PAGE for preparation of polypeptide samples for sequencing is described in this chapter.

2. Materials

1. Apparatus for PAGE: Slab gels are used, so as to allow the blotting procedures that follow electrophoresis. There are many commercial suppliers of the units, glass plates, spacers and combs (*see* **Note 1**) that are required for PAGE. Should it be necessary to build apparatus from scratch, refer to the design of Studier ***(5)*** but, for safety reasons, ensure that access to electrodes or buffers is impossible whilst the apparatus is connected to a live power supply. The direct current power supply required to run gels may also be obtained from commercial sources. Again for safety, check that the power supply has a safety cutout.

2. Stock acrylamide solution: (*see* **Notes 2** and **3**).

   Total acrylamide concentration, %T = 30% w/v. Ratio of crosslinking agent, bis-acrylamide to acrylamide monomer, % C = 2.7% w/w.

   Dissolve 73 g acrylamide and 2 g of *bis*-acrylamide in distilled water (HPLC grade), and make to 250 mL. Filter to remove any particulate matter. Store in brown glass, stable for weeks at 4°C.

   To lessen the problem of protein derivatisation, use the purest reagents available. BioRad and Fluka are sources of suitable acrylamide and *bis*-acrylamide. Beware the irritant and neurotoxic nature of acrylamide monomer, and avoid its contact with skin by wearing gloves, safety glasses, and other protective clothing. Wash thoroughly after any contact. Be especially wary of handling the dry powder acrylamide - use a fume hood and facemask. Ready prepared acrylamide-*bis*-acrylamide solutions are available commercially that obviate the need to handle powders; for instance, "Protogel " (from National Diagnostics) gives satisfactory results.
3. Stock (4X concentrated) Separating Gel buffer Pre-electrophoresis lower reservoir buffer; A: 0.4% (w/v) SDS, 1.5 *M* Tris-HCl, pH 8.8.

   Dissolve 1.0 g SDS and 45.5 g Tris base (tris (hydroxymethyl) amino methane) in about 200 mL distilled water (high-performance liquid chromatography [HPLC] grade), adjust the pH to 8.8 with concentrated HCl, and make the volume to 250 mL with water. Filter and store at 4°C at which it is stable for months. Use Analar grade SDS and tris base (e.g., from Sigma).
4. Stock (4X concentrated) Pre-electrophoresis upper reservoir buffer/Stacking Gel buffer; B: 0.4% (w/v) SDS, 0.5 *M* Tris-HCl, pH 6.8.

   Dissolve 1.0 g SDS and 15.1 g tris base in about 200 mL distilled water (HPLC grade), adjust the pH to 6.8 with HCl, and make to 250 mL with water. Filter and store at 4°C, at which it is stable for months. Use Analar reagents (e.g., from Sigma).
5. Stock ammonium persulphate: (*see* **Note 4**): 10% (w/v) ammonium persulphate in water.

   Dissolve 1.0 g ammonium persulphate (Analar grade) in 10 mL distilled water (HPLC grade). Although apparently stable in the dark at 4°C for weeks, it is probably best practice to renew it every few days.
6. TEMED (N, N, N', N'-tetramethylethylenediamine): use as supplied (e.g., from BioRad, electrophoresis purity grade) (*see* **Note 4**).
7. Water-saturated butanol: In a glass vessel mix some n- or butan-2-ol with a lesser volume of water. Leave to stand. The upper layer is butanol saturated with water.
8. Reservoir buffer (for sample electrophoresis); C: 0.192 *M* glycine, 0.1% (w/v) SDS, 0.025 *M* Tris-HCl, pH 8.3.

   Dissolve 28.8 g glycine, 6.0 g Tris Base, and 2.0 g SDS in distilled water (HPLC grade) and make to 2 L with water. The pH should be about pH 8.3 without adjustment. Store at 4°C. Stable for days.
9. Stock (200X concentrated) Glutathione solution: 10 m*M* reduced glutathione in water.

Dissolve 30.7 mg reduced glutathione (Analar grade, e.g., from Sigma) in 10 mL distilled water (HPLC grade). Store frozen at –20°C or –70°C. Stable for weeks.

10. Stock (1000X concentrated) Sodium thioglycollate solution: 100 m*M* sodium thioglycollate in water.

    Dissolve 114 mg sodium thioglycollate (Analar grade, e.g., from Sigma) in 10 mL distilled water (HPLC grade). Stable for weeks, frozen to –20°C or –70°C.
11. Stock (2X strength) sample buffer: (*see* **Note 5**): 4.6% (w/v) SDS, 0.124 *M* Tris-HCl (pH 6.8), 10.0% (v/v) 2-mercaptoethanol, 20.0% (w/v) glycerol, 0.05% (w/v) bromophenol blue

    Dissolve the following in distilled water (HPLC grade) to volume less than 20 mL: 0.92 g SDS; 0.3 g Tris base; 4.0 g glycerol; 2 mL 2-mercaptoethanol; 2 mL bromophenol blue solution (0.1% w/v in water). Adjust pH to 6.8 with HCl, and make volume to 20 mL. Although stable at 4°C for days, over longer periods exposed to air, the reducing power of the 2-mercaptoethanol may wane. Aliquots of stock solution may be stored for longer periods (weeks to months) if frozen to –20°C or –70°C. Use Analar reagents (e.g., from Sigma).
12. Protein staining solution: (*see* **Notes 6–8**):
    Protein stain: Sigma, product number B-8772: Coomassie Brilliant Blue G (C.I. 42655) 0.04% w/v in 3.5% w/v perchloric acid (*see* **Notes 6–8**). Stable for months at room temperature, in the dark. Beware the low pH of this stain. Where protective clothing. Use fresh, undiluted stain, as supplied.
13. Destaining solution: Distilled water.

3. Methods (*see* Notes 1–11)

1. Take the glass plates, spacers, and comb appropriate to the gel apparatus to be used. Thoroughly clean them by washing in soapy water, rinse in distilled water, and then methanol. Allow to air-dry. Assemble plates and spacers as instructed by suppliers in preparation for making the gel.
2. Prepare the separating gel mixture. 30 mL of mixture will suffice for one gel of about 14 × 14 × 0.1 cm, or four gels of 8 × 9 × 0.1 cm. For gel(s) of 15% T, the mixture is made as follows. Mix 15 mL stock acrylamide solution and 7.5 mL of distilled water (HPLC grade); degas on a water vacuum pump; add 7.5 mL of separating gel buffer A, 45 µL stock ammonium persulphate solution and 15 µL of TEMED. Mix gently and use immediately, because polymerisation starts when the TEMED is added (*see* **Notes 2–4**).
3. Carefully pipette the freshly mixed gel mixture between the prepared gel plates, without trapping any air bubbles. Pour to about 1 cm below where the bottom of the well-forming comb will come when it is in position. Carefully overlayer the gel mixture with a few mm-deep layer of water-saturated butanol (to eliminate air, which would inhibit polymerization and to generate a flat top to the gel). Leave the gel until it is set (0.5–1.5 h).
4. Prepare the upper gel mixture. 5 mL will provide a gel layer 1–2 cm deep for one gel 14 cm wide, 0.1 cm thick, or four gels of 9 cm wide, 0.1 cm thick. A 5% T gel

is made as follows: mix 0.83 mL stock acrylamide solution A and 2.92 mL distilled water (HPLC grade); degas on a water vacuum pump; add 1.25 mL of stock separating gel buffer A, 15 μL stock ammonium persulphate, and 5μL TEMED. Mix gently and use immediately.

5. Pour off the butanol from the polymerised separating gel. Rinse the top of the gel with a little water, then a little upper gel mixture (from **step 4**). Fill the gap remaining above the gel with the upper gel mixture from **step 4** Insert the well-forming comb without trapping any air bubbles. Leave to polymerize (0.5–1.5 h).
6. When the gel has finally polymerized store it at 4°C overnight or longer (*see* **Note 9**).
7. When the gel is to be used, remove the comb and the bottom spacer in order to expose the top and bottom edges of the gel. Install in the gel apparatus. Dilute the stock separating gel/pre-electrophoresis lower reservoir buffer A, by mixing one volume of it with three volumes of distilled water (HPLC grade). Pour it into the lower (anode) reservoir of the apparatus. Dilute the stock pre-electrophoresis upper reservoir/stacking gel buffer B by mixing one volume of it with three volumes of distilled water (HPLC grade). Mix in the stock glutathione solution, diluting it 200-fold to a final concentration of 50 μ*M* glutathione. Pour this mixture into the top reservoir.
8. Add a few μL of sample solvent to one well and "pre-electrophorese" the gel by running it at low voltage (25–75v) according to the size of the gel). Continue this pre-electrophoresis until the blue band of bromophenol blue from the sample solvent reaches the boundary between upper and lower gels, and then switch off the power.
9. While pre-electrophoresis is in progress, prepare the sample(s) for electrophoresis as follows: Dissolve the sample in a small volume of water in a small polypropylene vial (e.g., Eppendorf) and mix in an equal volume of sample solvent. The volume of the sample solution should be small enough and the protein concentration great enough to enable sufficient protein to be loaded in a single well on the gel. Heat the sample in the capped vial at 100°C (i.e., in boiling water, or 100°–110°C in a heating block) for 2 min. Allow to cool and briefly centrifuge to bring any condensation to the bottom of the tube and to sediment any solid material present in the sample. The bromophenol blue dye indicates if the sample solution is acidic by turning yellow. If this occurs, add a few μL of NaOH solution, just sufficient to approximately neutralize the solution and turn it blue.
10. When the gel has been pre-electrophoresed (*see* **step 8**) remove the reservoir buffers and replace them with reservoir buffer for sample electrophoresis, C. Add stock sodium thioglycollate to the top (cathode) reservoir buffer, diluting it 1000-fold to a final concentration of 100 μ*M* sodium thioglycollate. Using a microsyringe or pipette, load the prepared samples (*see* **step 9**). Start electrophoresis by applying voltage of, e.g., 150v (or 30–40 mA) for a gel of 8 × 9 × 0.1 cm, and continue until the bromophenol blue dye front (which indicates the position of the smallest, fastest-migrating species present in the sample) reaches the bottom of the gel. Turn off the power and remove the gel from the apparatus.

11. At this stage the gel is ready to be blotted (as described in Chapter 4), or stained. For staining, wash the gel for a few minutes with several changes of water (*see* **Note 6**), then immerse the gel (with gentle shaking) in the Colloidal Coomassie Brilliant Blue G. This time varies with the gel type (e.g., 1.0–1.5 h for a 1–1.5 mm thick SDS polyacrylamide gel slab), but cannot really be overdone. Discard the stain after use, for its efficacy declines with use. At the end of the staining period, decolorize the background by immersion in distilled water, with agitation, and a change of water whenever it becomes colored. Background destaining is fairly rapid, giving a clear background after a few hours (*see* **Notes 6** and **7**). Gels may be likewise stained after blotting to visualize protein remaining there (*see* **Notes 8** and **10**).

4. Notes

1. A factor in improving final sequencing yields is minimization of the size of the band of protein of interest, i.e., minimization of the size of the piece of PVDF which bears the sample band and which is finally put into the sequencer. Thus, use narrow sample wells in the gel, and put as much sample as possible in a single well. In doing this, however, beware that overloading a track with sample may distort electrophoresis and spoil band resolution.
2. The system described is basically the traditional discontinuous SDS-PAGE system of Laemmli *(**6**)*, set up in the manner described by Dunbar and Wilson (4) in order to generate a stacking buffer system during the pre-electrophoresis. The system described has a separating gel of 15% T, 2.7% C, 0.1% (w/v) SDS, 0.375 *M* Tris-HCl, pH8.8. The upper, stacking gel is 5% T, 2.7% C, 0.1% (w/v) SDS 0.125 *M* Tris-HCl, pH6.8, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, 0.025% (w/v) bromophenol blue.

   This system may be modified in order to suit the needs of a particular application. Thus, gels of greater or lesser %T (acrylamide content) may be made by increasing or decreasing (respectively) the volume of stock acrylamide solution added to the mixture (*see* **Subheading 3.2.**) and adding proportionately less or more water (respectively). Gradient gels may be made by mixing two different % T mixtures as the gel is poured (e.g., *see* **ref. *7***).

   Another alternative is to vary the SDS content. For instance, Dunbar and Wilson *(**4**)* use 2% (w/v) SDS. Alternatively a "native" or nondenaturing gel may be made by deleting SDS entirely from all solutions, and also deleting the reducing agent, 2-mercaptoethanol from the sample solvent. In fact, a SDS-free gel may also be used for denaturing SDS-PAGE by inclusion of SDS in sample solvent and reservoir buffers, sufficient SDS deriving from these sources. Resolution of small proteins or peptides, below 5–10 kDa, may be problematical. Schagger and von Jagow *(**8**)* describe the use of tricine as trailing ion for improved resolution of polypeptides as small as 5 kDa or less.
3. In SDS-PAGE for the purposes of sequencing or mass spectrometry, the aim is to resolve mixtures of proteins while minimizing modification of the N-terminus or of side chains of the sample. Modifications are caused by reactive species in the

gel or sample solvent. As reviewed in **refs.** ***(9)*** and ***(10)***, several modifications have been recognized: the product of cysteinyl reacting with residual acrylamide monomer, cysteinyl-S-β-propionamide; addition of 2-mercaptoethanol, probably to cysteinyl; oxidation of methionyl to methionyl sulphoxide, possibly by persulphate. The procedures described in **Subheading 3.** have been found to minimise problems due to reactive species. Correia et al. ***(11)*** described other effects, namely cleavage at Asp-Pro bonds during heating in sample buffer, and formation of covalent lysine-dehydroalanine crosslinks.

To minimize sample modification, only the purest available reagents are used. Check that supplies of reagents, especially of acrylamide, are acceptable by making a gel and running standard proteins, blotting them, and sequencing them.

Secondly, the amounts of ammonium persulphate and TEMED used to initiate polymerization are lower than used in some laboratories. Also, it is recommended that prepared gels are stored at 4°C for some time (overnight at least). These two points are meant to minimize the presence of reactive species such as radicals, with the storage of the gel intended to allow complete dissipation of radicals and completion of polymerization.

Thirdly, the purpose of pre-electrophoresis is to run reduced glutathione into the gel so that it runs ahead of the sample and reacts with any reactive species remaining (this strategy, and the use of sodium thioglycollate, being described in **ref.** ***12***).

4. As stated in **Note 3**, amounts of ammonium persulphate and TEMED used are lower than those used in some laboratories. Oxygen can inhibit the polymerisation process, so degassing is used to reduce this problem. However, exhaustive degassing (e.g., prolonged degassing on a high vacuum pump) can result in a failure of the gel solution to polymerize evenly and completely. If polymerization fails, repeat the process with less degassing. Additionally, ensure that the ammonium persulphate solution is fresh. Do not increase amounts of persulphate or TEMED, for the reasons discussed in **Note 3**.
5. The sample buffer may be made as much as fourfold concentrated by simple alteration of the volumes given in **Subheading 2.**, **item 11**. The advantage of this is that it is necessary to add less to the sample, so the sample itself is diluted less, and a larger amount of sample may therefore be loaded onto the gel. The sample may be prepared in sample buffer as described, then frozen at –10°C for future use. Dithiothreitol (DTT) has a less unpleasant smell than 2-mercaptoethanol and may be used as the reducing agent at 50 m*M* in the final sample solution (as suggested by Invitrogen for their "NuPAGE" gels, *see* **Note 11**). Note that if DTT is to be held as a stock solution its oxidation in the presence of trace concentrations of metals such as Fe^{3+} or Ni^{2+} should be inhibited by inclusion of a chelator such as EGTA ***(13)***. For nonreduced SDS PAGE, omit the reducing agent. For nondenaturing PAGE, omit both reducing agent and SDS, and do not heat. If the sample is too dilute or contains too much salt (which may cause distortion of migration in the gel), this may be rectified by various concentration and buffer exchange strategies described in Chapter 1.

6. As an alternative to the commercial colloidal Coomassie Brilliant Blue G stain, it may be made from its separate components. Another commercially available alternative is the Gel Code blue stain reagent from Pierce (product number 24590 or 24592). Details of the stain components are not divulged, other than they also include Coomassie (G250), but the stain is used in the same way as described for the Sigma reagent, gives similar results, and costs approximately the same.

   The water wash that precedes staining by Coomassie Brilliant Blue G is intended to wash away at least some SDS from the gel, and so speed up destaining of the background. However, it should be remembered that proteins are not fixed in the gel until in the acidic stain mixture and consequently some loss of small polypeptides may occur in the wash step. Delete the wash step if this is of concern. A fixing step may be used immediately after electrophoresis (e.g., methanol:glacial acetic acid:water::50:7:43, (v/v/v) for 15–30 min, followed by water washing to remove the solvent and acid), though this may counter subsequent attempts to blot or otherwise extract the protein from the gel.

   Destaining of the background may be speeded up by frequent changes of the water, and further by inclusion in this wash of an agent that will absorb free dye. Various such agents are commercially available (e.g., Cozap, from Amika Corp.), but a cheap alternative is a plastic sponge of the sort used to plug flasks used for microbial culture. The agent absorbs the stain and is subsequently discarded. The background can be made clear by these means, and the stained bands remain stained while stored in water for weeks. They may be re-stained if necessary.

   Heavily loaded samples show up during staining with Coomassie Brilliant Blue G, but during destaining of the background the blue staining of the protein bands becomes accentuated. Bands of just a few tens of ng are visible on a 1 mm-thick gel (i.e., the lower limit of detection is less that 10 ng/mm^2). Variability may be experienced from gel to gel, however. For example, duplicate loadings of samples on separate gels, electrophoresed and stained in parallel, have differed in the degree of staining by a factor of 1.5, for unknown reasons. Furthermore, different proteins bind the dye to different extents: horse myoglobin may be stained twice as heavily as is bovine serum albumin (BSA), though this, too, is somewhat variable. While this formulation of Coomassie Brilliant Blue G is a good general protein stain, It is advisable to treat sample proteins on a case by case basis. This Coomassie stain may be used to quantify proteins in gels being quantitative, or nearly so, from about 10–20 ng/mm^2 up to about 1–5 μg/mm^2. The stoichiometry of dye binding is subject to some variation, such that standard curves may be either linear or slightly curved, but even the latter case is acceptable provided standards are run on the same gel as samples. Some reports claim that Coomassie blue staining may create problems in subsequent mass spectrometric analysis by virtue of adduct formation. In this case, alternative stains are available (*see* **Note 7**).
7. There is a variety of alternative stains to the Coomassie method described in **Note 6**. One of note is Sypro Ruby, available commercially from Molecular Probes (Eugene, OR, product number S-12000 or S-12001). The components

of the reagent are not revealed. Its cost is of the order of twice that of the Sigma Brilliant Blue G stain. To stain with this reagent, rinse the gel in water briefly, put it into a clean dish and then cover it with Sypro Ruby gel stain solution. Gently agitate until staining is completed, which may take up to 24 h or longer. Overstaining will not occur during prolonged stained. Do not let the stain dry up on the gel during long staining procedures. Discard the stain after use, for it becomes less efficacious with use. During the staining procedure the gel may be removed from the stain and inspected under UV light to monitor progress. If the staining is insufficient, the gel may be replaced in the stain for further incubation. Destain the background by washing the gel in a few changes of water for 15–30 min.

Generally, the Sypro Ruby method is more sensitive than the Coomassie Brilliant Blue G method, although for best sensitivity prolonged staining (24 h or more) may be required. Protein to protein variation can occur. For instance, horse myoglobin binds about 10-fold less dye than BSA does. Thus, in one experiment, the minimum amount of BSA detectable after Sypro Ruby staining was about 5 ng/mm^2 (about four- to fivefold more sensitive than samples stained in parallel by the Coomassie Brilliant Blue G method), whereas the minimum amount of horse myoglobin detectable was about 50 ng/mm^2 (similar to that detectable by the Coomassie Brilliant Blue G stain). Sypro Ruby requires UV irradiation for detection, but does not entail a fixation step. This is an advantage over traditional silver staining methods, whose sensitivity it approaches.

The silver staining method may be made compatible with subsequent analysis by in-gel proteolysis and mass spectrometry if the gel is fixed in 50% methanol, 5% acetic acid (v/v), but not by glutaraldehyde ***(14)***. Gharahdaghi et al. ***(15)*** recommend an additional step of removal of silver from the protein by washing in fresh reducing solution (15 m*M* potassium ferricyanide, 50 m*M* sodium thiosulfate), prior to in-gel proteolysis and matrix-assisted laser-desorption ionization-time of flight (MALDI-TOF) mass spectrometry of eluted peptides. A mass spectrometry-compatible silver stain is available commercially (SilverQuest from Invitrogen).

Negative stains stain the background while leaving the protein band unstained. Cohen and Chait ***(16)*** used either copper or imidazole-zinc negative stain prior to protein extraction from the gel for mass spectrometric analysis (*see* **Note 12**).

8. Do not stain the gel before blotting. Blot, and then stain the PVDF with suitable stain to detect bands. A suitable PVDF blot stain is Ponceau S (0.1% w/v in 1% v/v acetic acid in water), the blot being immersed for 1–2 min, then washed in water to destain the background (and with time, bands also). See Chapter 4 for further detail of blot staining. If, for some reason, the gel has been stained by Coomassie stain before it is decided to blot it for further analysis, it can still be blotted in just the same way as used for unstained gels. The stained protein will transfer to PVDF and can be subject to N-terminal sequencing procedures. The yield of sequencable protein is markedly less than if the gel is not stained, but may still be sufficient to provide sequence information.

9. If resolution of bands is not so critical, gels may be prepared with the discontinuous buffer system in place. For this, prepare the separating gel as described in **Subheadings 3.2.** and **3.3.** and prepare the stacking gel as described in **Subheading 3.4.** but substitute buffer B for buffer A. The gel may then be used for nonsequencing purposes, or pre-electrophoresed with 50 μ*M* glutathione in reservoir buffer C, to scavenge reactive species present. Gels with a discontinuous buffer system in place cannot be stored for much longer than a day, for the buffers diffuse into each other. The system described in Methods, with just one buffer (prior to pre-electrophoresis), may be conveniently stored for days, provided that the gel is sealed tightly in a plastic bag or in plastic wrapping such as laboratory sealing film.
10. The overall yield of PAGE, blotting and sequencing is heavily dependent not only on the PAGE stage, but also on the blotting stage. For further detail of the latter refer to Chapter 4.
11. The method described earlier is for preparation of SDS gels from scratch. Ready-made gels are available commercially from various sources, however. Ready-made gels from Daiichi, and Novex (Invitrogen) have proven suitable in our lab. They provide a viable alternative to scratch-made gels; while more expensive on materials, they reduce manpower costs and provide convenience.

    A discontinuous buffer system such as that described earlier (for the stacking gel system) is not stable during long-term storage. Commercial gels from Novex do not have such a system. Novex technical literature explains that at the pH 8.8 of the separating gel, the mobility of glycine is low enough to allow protein-SDS complexes to stack in a low % T gel as well as they would do with a pH 6.8 stacking gel, at least for proteins of about 70 KDa or less. No pre-electrophoresis of Novex gels is necessary, possibly because of the extended period of time between preparation of the gel and its use (*see* **Note 3**). In fact, if the pre-electrophoresis procedure (**Subheading 3**, **steps 7** and **8**) and inclusion of thioglycollate (**Subheading 3.10.**) are carried out in a Novex gel, band resolution is worsened.

    Invitrogen (Carlsbad, CA) market "NuPAGE" gels that may be stored for prolonged periods: a maximum of one year is recommended but older gels may still behave satisfactorily (performance did not deteriorate after 2 yr storage in our lab). The system operates at pH 7.0, using Bis-Tris instead of Tris-chloride buffers. The loading buffer, pH 8.5, uses lithium dodecyl sulphate, which has greater solubility than SDS, and either 2-mercaptoethanol or 0.05 *M* DTT as reducing agent (*see* **Note 5**). The buffers and other reagents supplied by Invitrogen are compatible with peptide sequencing. Use of an anti-oxidant in the upper buffer reservoir is recommended to inhibit (re)oxidation of protein during electrophoresis and so maintain band sharpness, but beware that its presence is sufficient to cause some reduction of at least some proteins in a nonreducing gel. The gels can successfully resolve proteins of about 5 kDa or less.
12. Proteins may be prepared from polyacrylamide gels by use of equipment available from various companies such as BioRad and AmiKa Corp. This may be useful if procedures such as peptide mapping are to follow. In essence, the protein is electrophoresed out of the piece of gel into solution. It is held in a small

volume of solution by a dialysis membrane of low nominal molecular-weight cut-off (e.g., 10 kDa). Unstained gel may be excised after being located by comparison with stained sister track(s). The yield of protein is in the range of 50–100%, and it is sequencable. Contaminants may emerge with the protein, causing it to look somewhat streaky upon re-electrophoresis, for example. Some sample clean-up may be necessary if these contaminants interfere with subsequent analysis.

Depending on the apparatus used, the final sample volume may be as much as 1 mL. If this is excessively large, the sample may be concentrated, and the buffer may be exchanged for another, as described in Chapter 1. If the sample band is small and it is necessary to minimize the final volume of the gel extract, the gel piece may simply be cut into small cubes and immersed in the minimal volume of a suitable buffer such as 100 m*M* Tris-HCl, pH 8.5, 0.1% SDS (w/v). Overnight incubation at room temperature, followed by brief centrifugation, may give sufficient protein in the supernatant to allow analysis. Beware that the rate of passive diffusion through and out of the gel decreases as the molecular weight of the sample protein and the degree of polyacrylamide crosslinking (%C) each increases. Cohen and Chait describe passive extraction of protein from polyacrylamide gel prior to mass spectrometric analysis ***(16)***. Protein is detected in the gel by negative staining (*see* **Note 7**) and the gel piece cut out, destained and then crushed. Amounts of 25 pmole or more of protein may be extracted by vigorous shaking at ambient temperature for 4 to 8 h in a mixture of water, acid and organic solvent, such as formic acid: water: 2-propanol::1:3:2 (v/v/v). After drying under vacuum the sample may be analysed by mass spectrometry. For samples of less than 25 pmole the crushed gel is shaken with a MALDI matrix solution (e.g., a saturated solution of 4-hydroxy-a-cyano-cinnamic acid in formic acid: water: 2-propanol::1:3:2, v/v/v) for 1–2 h at room temperature, then left exposed to air to allow crystallization of the 4-hydroxy-α-cyano-cinnamic acid. The suspension of crystals may be analyzed directly by MALDI MS.

13. The voltage or current used to run the gels is somewhat arbitrary. Low power will give slow migration of proteins. Higher voltage gives faster migration but generates more heat. The buffers can be cooled by circulating them through a bath of ice, for instance, but migration of bands is then slower. The voltage suggested in **Subheading 3.10.** will run the bromophenol dye to the bottom of the gel of 8 × 9 × 0.1 cm, in about 2 h. Whether the gel is run at constant current or wattage, set an upper limit to the voltage. This prevents the voltage rising to dangerously high levels in the event of a fault in the system causing high resistance. Alternatively, run at constant voltage.
14. Various chemical and enzymatic methods have been described for cleavage of proteins while still in polyacrylamide gel, prior to elution an further analysis by HPLC or mass spectrometry – *see* Chapters 5 and 6.

References

1. Hutchinson, K. A., Dalman, F. C., Hoeck, W., Groner, B., and Pratt, W. B. (1993) Localisation of the ~ 12kDa Mr discrepancy in gel migration of the mouse gluco-

corticoid receptor to the major phosphorylated cyanogen bromide fragment in the transactivating domain. *J. Steroid Biochem. Mol. Biol.* **46**, 681–688.
2. Fountoulakis, M., Vilbois, F., Oesterhelt, G., and Vetter , W. (1995) Phosphoric acid entrapment leads to apparent protein heterogeneity. *Biotechnology* **13**, 383–388.
3. Deyl, Z. (1979) *Electrophoresis: A Survey of Techniques and Applications. Part A*. Elsevier, Amsterdam.
4. Dunbar, B. and Wilson, S. B. (1994) A buffer exchange procedure giving enhanced resolution to polyacrylamide gels prerun for protein sequencing. *Anal. Biochem.* **216**, 227–228.
5. Studier, F. W. (1973) Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79**, 237–248.
6. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
7. Walker, J. M. (1994) Gradient SDS polyacrylamide gel electrophoresis of proteins, in *Methods in Molecular Biology*, vol. 32, *Basic Protein and Peptide Protocols* (Walker, J. M., ed.) Humana Press Inc., Totowa, NJ, pp. 35–38.
8. Schagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379.
9. Patterson, S. D. (1994) From electrophoretically separated protein to identification: strategies for sequence and mass analysis. *Anal. Biochem.* **221**, 1–15.
10. Patterson, S. D. and Aebersold, R. (1995) Mass spectrometric approaches for the identification of gel-separated proteins. *Electrophoresis* **16**, 1791–1814.
11. Correia, J. J., Lipscomb, L. D., and Lobert, S. (1993) Nondisulfide crosslinking and chemical cleavage of tubulin subunits: pH and temperature dependence. *Arch. Biochem. Biophys.* **300**, 105–114
12. Yuen, S., Hunkapiller, M. W., Wilson, K. J., and Yuan, P. M. (1986) *Appl. Biosys. User Bull. No 25*.
13. Getz, E. B., Xiao, M., Chakrabarty, T., Cooke, R., and Selvin, P. R. (1999) A comparison between the sulphydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in biochemistry. *Anal. Biochem.* **273**, 73–80.
14. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins from silver-stained gels. *Anal. Chem.* **68**, 850–858.
15. Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imal, B. S., and Mische, S. M. (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel; a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**, 601–605.
16. Cohen, S. L. and Chait, B. T. (1997) Mass spectrometry of whole proteins eluted from sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. *Anal. Biochem.* **247**, 257–267.

3

Two-Dimensional Polyacrylamide Gel Electrophoresis for the Separation of Proteins for Chemical Characterization

Michael J. Dunn

1. Introduction

The first complete genome, that of the bacterium *Hemophilus influenzae*, was published in 1995 ***(1)***. We now have the complete genomic sequences for more than 80 prokaryotic and eukaryotic organisms, and a major milestone has been reached recently with the completion of the human genome ***(2,3)***. A major challenge in the post-genome era will be to elucidate the biological function of the large number of novel gene products that have been revealed by the genome sequencing initiatives, to understand their role in health and disease, and to exploit this information to develop new therapeutic agents. The assignment of protein function will require detailed and direct analysis of the patterns of expression, interaction, localization, and structure of the proteins encoded by genomes; the area now known as "proteomics" ***(4)***.

The first requirement for proteome analysis is the separation of the complex mixtures containing as many as several thousand proteins obtained from whole cells, tissues, or organisms. Recently, progress has been made in the development of alternative methods of protein separation for proteomics, such as the use of chip-based technologies ***(5,6)***, the direct analysis of protein complexes using mass spectrometry ***(7)***, the use of affinity tags ***(8,9)***, and large-scale yeast two-hybrid screening ***(10)***. However, two-dimensional polyacrylamide gel electrophoresis (2-DE) remains the core technology of choice for separating complex protein mixtures in the majority of proteome projects ***(11)***. This is due to its unrivaled power to separate simultaneously thousands of proteins, the subsequent high-sensitivity visualization of the resulting 2-D separations ***(12)*** that are amenable to quantitative computer analysis to detect differentially

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

regulated proteins *(13)*, and the relative ease with which proteins from 2-D gels can be identified and characterized using highly sensitive microchemical methods *(14)*, particularly those based on mass spectrometry *(15)*.

2. Materials

Prepare all solution from analytical grade reagents (except where otherwise indicated) using deionized double-distilled water.

1. 18 cm Immobiline IPG DryStrip pH 3-10 NL gel strips (Amersham Pharmacia Biotech, Amersham, UK) (*see* **Notes 1** and **2**).
2. IPG Immobiline DryStrip reswelling tray (Amersham Pharmacia Biotech) (*see* **Note 3**).
3. Multiphor II horizontal flatbed electrophoresis unit (Amersham Pharmacia Biotech) (*see* **Note 4**).
4. Immobiline DryStrip kit for Multiphor II (Amersham Pharmacia Biotech) (*see* **Note 5**).
5. Power supply capable of providing an output of 3500 V (*see* **Note 6**).
6. MultiTemp III thermostatic circulator (Amersham Pharmacia Biotech).
7. IEF electrode strips (Amersham Pharmacia Biotech) cut to a length of 110 mm.
8. Urea (GibcoBRL Ultrapure, Life Technologies, Paisley, UK) (*see* **Note 7**).
9. Solution A: 9 *M* urea (100 mL): Dissolve 54.0 g of urea in 59.5 mL deionized water. Deionize the solution by adding 1 g Amberlite MB-1 monobed resin (Merck, Poole Dorset, UK) and stirring for 1 h. Filter the solution using a sintered glass filter.
10. Solution B: sample lysis buffer: 9 *M* urea, 2% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT), 0.8% (w/v) 2-D Pharmalyte pH 3.0–10.0 (Pharmacia, St. Albans, UK) (*see* **Note 8**). Add 2.0 g CHAPS (*see* **Note 9**), 1.0 g DTT and 2.0 mL of Pharmalyte pH 3.0–10.0 to 96.0 mL of solution A.
11. Solution C: 8 *M* urea solution (40 mL): Dissolve 19.2 g of urea in 25.6 mL deionized water. Deionize the solution by adding 1 g Amberlite MB-1 monobed resin (Merck, Poole Dorset, UK) and stirring for 1 h. Filter the solution using a sintered-glass filter.
12. Solution D: reswelling solution: 8 *M* urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, 0.2% (w/v) 2-D Pharmalyte 3-10. Add 60 mg DTT, 150 mg CHAPS, and 150 µL Pharmalyte 3-10 to 29.7 mL of solution C.
13. Silicon fluid, Dow Corning 200/10 cs (Merck, Poole, Dorset, UK).
14. Solution E: Electrolyte solution for both anode and cathode: Distilled water.
15. Solution F: equilibration buffer (100 mL): 6 *M* urea, 30% (w/v) glycerol, 2% (w/v) SDS, 50 m*M* Tris-HCl buffer, pH 6.8. Add 36 g urea, 30 g glycerol, and 2 g SDS to 3.3 mL 1.5 *M* Tris-HCl buffer, pH 6.8.
16. Solution G: DTT stock solution: Add 200 mg DTT to 1 mL deionized water. Prepare immediately before use.
17. Solution H: Bromophenol Blue solution: Add 30 mg Bromophenol Blue to 10 mL 1.5 *M* Tris-HCL buffer, pH 6.8.

3. Methods

1. Sample preparation: Samples of isolated cells can be prepared by suspension in a small volume of lysis buffer, followed by disruption by sonification in an ice bath. Samples of solid tissues should be homogenised while still frozen in a mortar cooled with liquid nitrogen. The resulting powder is then suspended in a small volume of lysis buffer. Plant cells and tissues often require additional treatment *(**18**)*. The final protein concentration of the samples should be about 10 mg/mL. Protein samples should be used immediately or stored frozen at –80°C.
2. Rehydration of IPG gel strips with the protein sample: Dilute an aliquot of each sample containing an appropriate amount of protein (*see* **Note 10**) with solution D to a total volume of 450 µL (*see* **Note 11**). Pipet each sample into one groove of the reswelling tray. Peel off the protective cover sheets from the IPG strips and insert the IPG strips (gel side down) into the grooves. Avoid trapping air bubbles. Cover the strips with 1 mL of silicon oil, close the lid and allow the strips to rehydrate overnight at room temperature
3. Preparation of IEF apparatus: Ensure that the strip tray, template for strip alignment, and electrodes are clean and dry. Set the thermostatic circulator at 20°C (*see* **Note 12**) and switch on at least 15 min prior to starting the IEF separation. Pipet a few drops of silicon fluid onto the cooling plate and position the strip tray on the plate. The film of silicon fluid, which has excellent thermal conductivity properties and a low viscosity, allows for good contact between the strip tray and the cooling plate. Pipette a few drops of silicon fluid into the tray and insert the IPG strip alignment guide.
4. After rehydration is complete, remove the IPG strips from the reswelling tray, rinse them briefly with deionized water and place them, gel side up, on a sheet of water-saturated filter paper. Wet a second sheet of filter paper with deionised water, blot it slightly to remove excess water and place on the surface of the IPG strips. Blot them gently for a few seconds to remove excess rehydration solution in order to prevent urea crystallization on the surface of the gel during IEF.
5. IPG IEF dimension: Place the IPG gel strips side-by-side in the grooves of the alignment guide of the strip tray, which will take up to 12 strips (*see* **Note 13**). The basic end of the IPG strips must be at the cathodic side of the apparatus. Wet the electrode wicks with about 0.5 mL of the electrode solution (solution E) and remove excess liquid with a tissue. Place the electrode wicks on top of the strips as near to the gel edges as possible. Position the electrodes and press them down onto the electrode wicks. Fill the strip tray with silicon oil to protect the IPG strips from the effects of the atmosphere.
6. IEF running conditions: Run the IPG IEF gels at 0.05 mA per strip, and 5 W limiting. For the higher protein loads used for micro-preparative runs it is recommended to limit the initial voltage to 150 V for 30 min (75 Vh) and then 300 V for 60 min (300 Vh). Continue IEF with maximum settings of 3500 V, 2 mA, and 5 W until constant focusing patterns are obtained. The precise running conditions required depend on the pH gradient, the separation distances used, and the type of sample being analysed (*see* **Note 14**).

7. After completion of IEF, remove the gel strips from the apparatus. Freeze the strips in plastic bags and store them at –80°C if they are not to be used immediately for the second dimension separation.
8. Equilibration of IPG gel strips: Equilibrate IPG gel strips with gentle shaking for 2 × 15 min in 10 mL equilibration buffer (solution F). Add 500 µL/10 mL DTT stock (solution G) and 30 µL/10 mL Bromophenol Blue stock (solution H) to the first equilibration solution. Add 500 mg iodoacetamide per 10 mL of the second equilibration solution (final concentration iodoacetamide 5% w/v).
9. SDS-PAGE dimension: The second-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation is carried out using a standard vertical SDS-PAGE system (*see* **Note 15**) of the normal Laemmli type *(**22**)* (*see* **Note 16**) as described in Chapter 2. The gels can be either of a suitable constant percentage concentration of polyacrylamide or of a linear or nonlinear polyacrylamide concentration gradient. We routinely use 1.5 mm-thick 12% T SDS-PAGE gels (26 cm × 20 cm). No stacking gel is used.
10. Rinse the equilibrated IPG gel strips with deionized water and blot them on filter paper to remove excess liquid.
11. Apply the IPG gel strips to the SDS-PAGE gels by filling the space in the cassette above the separation gel with upper reservoir buffer and gently slide the strips into place. Good contact between the tops of the SDS gels and the strips must be achieved and air bubbles must be avoided. Cement the strips in place with 1% (w/v) agarose in equilibration buffer.
12. The gels are run in a suitable vertical electrophoresis apparatus. We use the Ettan DALT II vertical system (Amersham Pharmacia Biotech) which allows up to 12 large-format (26 × 20 cm) second-dimension SDS-PAGE gels to be electrophoresed simultaneously. The gels are run at 5 W/gel at 28°C for 45 min and then at 200 W maximum overnight at 15°C until the Bromophenol Blue tracking dye reaches the bottom of the gels. This takes approx 5 h for a full set of 12 gels.
13. The gels can be subjected to any suitable procedure to detect the separated proteins (*see* **Note 17**) or electroblotted onto the appropriate type of membrane (*see* Chapter 4) for subsequent chemical characterisation. A typical separation of human myocardial proteins using this technique is shown in **Fig. 1**.

4. Notes

1. We routinely use IPG gels with an 18 cm pH gradient separation distance, but it is possible to use gels of other sizes (e.g., 7 cm, 11 cm, 13 cm, 24 cm pH gradient separation distance) *(**18**)*. Small format gels (e.g., 7 cm strips) are ideal for rapid screening purposes or where the amount of sample limited, whereas extended separation distances (e.g., 24 cm IPG strips) provide maximum resolution of complex protein patterns.
2. A wide-range, linear IPG 3.0–10.0 L pH gradient is often useful for the initial analysis of a new type of sample. However, for many samples this can result in loss of resolution in the region pH 4.0–7.0, in which the pI values of many proteins occur. This problem can be overcome to some extent with the use of a nonlinear IPG

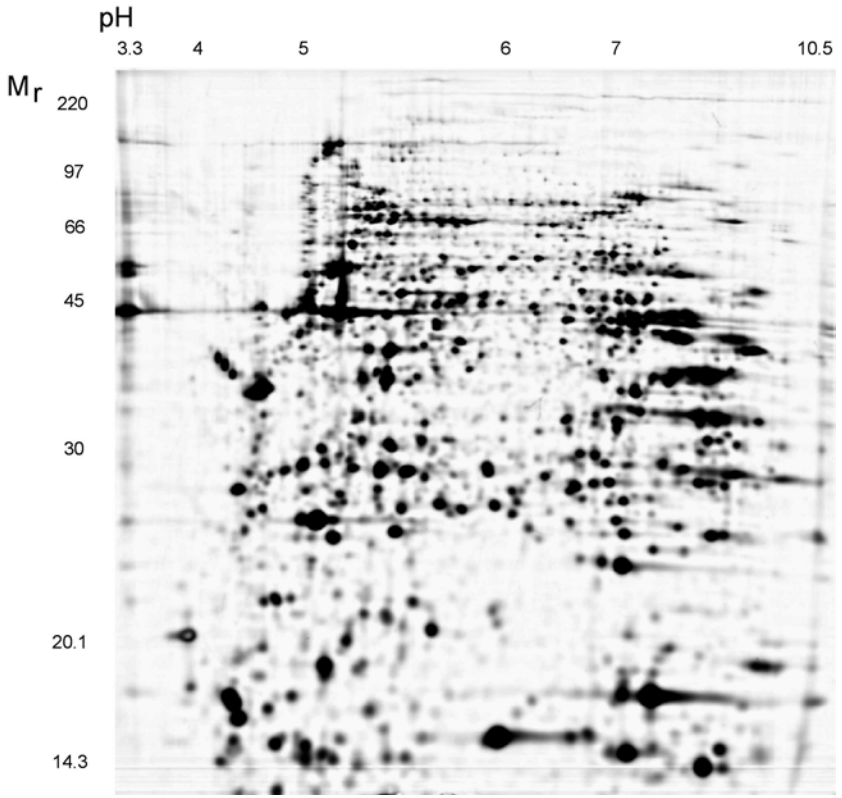


Fig. 1. Silver stained 2-D pattern of human myocardial proteins. A loading of 100 μg protein was used. The first dimension was pH 3.0–10.0 NL IPG IEF and the second dimension was 12% T SDS-PAGE.

3-10 NL pH gradient, in which the pH 4.0–7.0 region contains a much flatter gradient than in the more acidic and alkaline regions. This allows good separation in the pH 4.0–7.0 region while still resolving the majority of the more basic species. However, use of a pH 4.0–7.0 IPG IEF gel can result in even better protein separations within this range. With complex samples such as eukaryotic cell extracts, 2-DE on a single wide-range pH gradient reveals only a small percentage of the whole proteome because of insufficient spatial resolution and the difficulty of visualising low copy number proteins in the presence of the more abundant species. One approach to overcoming the problem is to use multiple, overlapping narrow range IPGs spanning 1–1.5 pH units; an approach that has become known as "zoom gels" ***(23)***, "composite gels," or "subproteomics" ***(24)***. Strongly alkaline proteins such as ribosomal and nuclear proteins with closely related pIs between 10.5 and 11.8 can be separated using narrow range pH 10.0–12.0 or pH 9.0–12.0 IPGs ***(19)***.

3. The IPG Immobiline DryStrip reswelling tray (Amersham Pharmacia Biotech) is a grooved plastic tray with a lid designed for the rehydration of IPG DryStrip gels of any length from 7–24 cm in the presence of the solubilised protein sample.
4. We use the Multiphor II horizontal flatbed electrophoresis unit (Amersham Pharmacia Biotech). Any horizontal flat-bed IEF apparatus can be used for IPG IEF, but the Immobiline DryStrip kit (*see* **Note 5**) is designed to fit the Multiphor II. Another alternative is to use the IPGphor (Amersham Pharmacia Biotech), an integrated system dedicated to first-dimension IEF using IPG DryStrip with built-in temperature control unit and power supply.
5. The Immobiline DryStrip kit facilitates the sample application and running of IPG IEF gels in the first-dimension of 2-DE. The strip tray consists of a thin glass plate with a polyester frame. The frame acts as an electrode holder and the metal bars affixed to the frame conduct voltage to the electrodes. The electrodes, which are made of polysulphone, are moveable to accommodate gel strips of varying inter-electrode distance and have a platinum wire that rests against the electrode strip. It is also fitted with a bar, also made of polysulphone, which supports the sample cups (styrene-acrylnitrile). These cups can be used to apply sample volumes up to 100 µL as an alternative to the in-gel rehydration technique of sample application described here.
6. It is essential that the power supply can deliver less than 1 mA at 3,500 V, as these conditions are achieved during IEF of IPG gels. Powerpacks from some manufacturers are designed to cut out if a low current condition at high voltage is detected. The EPS 3501 XL power supply (Amersham Pharmacia Biotech) meets this requirement.
7. Urea should be stored dry at 4°C to reduce the rate of breakdown of urea with the formation of cyanate ions, which can react with protein amino groups to form stable carbamylated derivatives of altered charge.
8. Lysis buffer should be prepared freshly. Small portions of lysis buffer can be stored at –80°C, but once thawed they should not be frozen again.
9. We generally use the zwitterionic detergent CHAPS as this can give improved sample solubilisation compared with nonionic detergents such as Triton X-100 and Nonidet NP-40. However, the more hydrophobic membrane proteins are poorly solubilised under these conditions and it may be preferable to use a more powerful chaotropic agent such as thiourea and/or alternative linear sulphobetaine detergents such as SB 3-10 or 3-12 *(**25**)*.
10. For analytical purposes (e.g., silver staining) between 60 and 80 µg total protein from complex mixtures such as whole cell and tissue lysates should be applied. It is possible to obtain successful chemical characterisation on at least the more abundant protein spots using such a loading, but it is preferable for micro-preparative purposes for the sample to contain between 400 µg and 1 mg total protein.
11. The total volume for rehydration must be adjusted depending on the separation length of the IPG strip used; 175 µL for 7 cm, 275 µL for 11 cm, 325 µL for 13 cm, and 600 µL for 24 cm IPG strips.

12. The temperature at which IEF with IPG is performed has been shown to exert a marked effect on spot positions and pattern quality of 2-D separations *(**26**)*. Temperature control is, therefore, essential in order to allow meaningful comparison of 2-DE patterns. Focusing at 20°C was found to result in superior 2-D separations with respect to sample entry, resolution, and background staining compared with separations carried out at 10°C or 15°C *(**26**)*.
13. Exposure of the gel strips to the air should be as brief as possible to prevent the formation of a thin layer of urea crystals on the gel surface.
14. As a guide, for IPG IEF gel strips with an 18 cm pH gradient separation distance we use 60,000 Vh for micro-preparative purposes.
15. The second SDS-PAGE dimension can also be carried out using a horizontal flatbed electrophoresis apparatus. This method is described in **ref.** *(**18**)*.
16. For the isolation of proteins for chemical characterisation, it is essential to minimize the risk of chemical modification of the proteins during the various steps of 2-DE. The polymerisation efficiency of polyacrylamide is rarely greater than 90%, with the inevitable risk for modification of amino acid residues by free acrylamide. This area is reviewed in detail by Patterson *(**27**)*. The amino acid most at risk of acrylamide adduction has been found to be cysteine, resulting in the formation of cysteinyl-S-β-propionamide. In addition, the partial oxidation of methionine to methionine sulphoxide, presumably owing to the presence of residual persulphate in the gel, has also been demonstrated. Several approaches have been used to prevent gel electrophoresis-induced modification of proteins, including the use of scavengers such as glutathione or sodium thiogycolate for SDS-PAGE or free cysteine for IPG IEF gels *(**27**)*. However, we have not found it necessary to adopt these procedures for the successful chemical characterisation of proteins purified by 2-DE. This may be a consequence of the deionization step (*see* **Subheading 2.**, **step 13**) which we routinely employ and/or to the quality of the acrylamide and Bis obtained from the supplier (we use Electran grade reagents from Merck, Poole, Dorset, UK).
17. Organic dyes such as Coomassie blue R-250 and G-250 are compatible with most chemical characterisation methods, including mass spectrometry, but are limited by their relative insensitivity *(**28**)*. Silver staining allows the detection of low nanogram amounts of protein. However, standard silver-staining protocols almost invariably use glutaraldehyde and formaldehyde, which alkylate α- and ϵ-amino groups of proteins, thereby interfering with their subsequent chemical characterisation. To overcome this problem, silver-staining protocols compatible with mass spectrometry in which glutaraldehyde is omitted have been developed *(**29**,**30**)*, but these suffer from a decrease in sensitivity of staining and a tendency to a higher background. This problem can be overcome using postelectrophoretic fluorescent staining techniques *(**28**)*. The best of these at present appears to be SYPRO Ruby, which has a sensitivity approaching that of standard silver staining and is fully compatible with protein characterization by mass spectrometry *(**31**)*.

References

1. Fleischmann, R. D., et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512
2. Venter, J. C. et al. (2001) The sequence of the human genome. *Science* **291**, 1304–1351.
3. International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. *Nature* **409**, 860–922.
4. Banks R., Dunn M. J., Hochstrasser D. F., et al. (2000) Proteomics: new perspectives, new biomedical opportunities. *Lancet* **356**, 1749–1756.
5. Merchant, M. and Weinberger, S. R. (2000) Recent advances in surface enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Electrophoresis* **21**, 1165–1177.
6. Nelson, R. W., Nedelkov, D., and Tubbs, K. A. (2000) Biosensor chip mass spectrometry: a chip-based approach. *Electrophoresis* **21**, 1155–1163.
7. Link, A. J., Eng, J., Schieltz, D. M., et al. (1999) Direct analysis of protein complexes using mass spectrometry. *Nature Biotechnol.* **17**, 676–682.
8. Gygi, S. P., Rist, B., Gerber, S. A., et al. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnol.* **17**, 994–999.
9. Rigaut, G., Shevchenko, A., Rutz, B., et al. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nature Biotechnol.* **17**, 1030–1032.
10. Uetz, P., Giot, L., Cagney, G., et al. (2000) A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. *Nature* **403**, 623–627.
11. Dunn, M. J. and Görg, A. (2001) Two-dimensional polyacrylamide gel electrophoresis for proteome analysis, in *Proteomics, From Protein Sequence to Function* (Pennington, S. R. and Dunn, M. J., eds.), BIOS Scientific Publishers, Oxford, pp. 43–63.
12. Patton, W. F. (2001) Detecting proteins in polyacrylamide gels and on electroblot membranes, in *Proteomics, From Protein Sequence to Function* (Pennington, S. R. and Dunn, M. J., eds.), BIOS Scientific Publishers, Oxford, pp. 65–86.
13. Dunn, M. J. (1992) The analysis of two-dimensional polyacrylamide gels for the construction of protein databases, in *Microcomputers in Biochemistry* (Bryce, C. F. A., ed.), IRL Press, Oxford, pp. 215–242.
14. Wilkins, M. R. and Gooley, A. (1997) Protein identification in proteome analysis, in *Proteome Research: New Frontiers in Functional Genomics* (Wilkins, M. R., Williams, K. L., Appel, R. D. and Hochstrasser, D. F., eds.), Springer-Verlag, Berlin, pp. 35–64.
15. Patterson, S. D., Aebersold, R., and Goodlett, D. R. (2001) Mass spectrometry-based methods for protein identification and phosphorylation site analysis, in *Proteomics, From Protein Sequence to Function* (Pennington, S. R. and Dunn, M. J., eds.), BIOS Scientific Publishers, Oxford, pp. 87–130.
16. O'Farrell, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021.

17. Klose, J. (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Humangenetik* **26**, 231–243.
18. Görg, A., Obermaier, C., Boguth, G., et al. (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **21**, 1037–1053.
19. Görg, A., Obermaier, C., Boguth, G., et a;. (1997) Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. *Electrophoresis* **18**, 328–37.
20. Hanash, S. M., Strahler, J. R., Neel, J. V., et al. (1991) Highly resolving two-dimensional gels for protein sequencing. *Proc. Natl. Acad. Sci. USA* **88**, 5709–5713.
21. Bjellqvist, B., Sanchez, J.-C., Pasquali, C., et al. (1993) Micropreparative two-dimensional electrophoresis allowing the separation of samples containing milligram amounts of proteins. *Electrophoresis* **14**, 1375–1378.
22. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
23. Wildgruber, R., Harder, A., Obermaier, C., et al. (2000) Towards higher resolution: 2D-Electrophoresis of Saccharomyces cerevisiae proteins using overlapping narrow IPG's. *Electrophoresis* **21**, 2610–2616.
24. Cordwell, S. J., Nouwens, A. S., Verrils, N. M., et al. (2000) Sub-proteomics based upon protein cellular location and relative solubilities in conjunction with composite two-dimensional gels. *Electrophoresis* **21**, 1094–1103.
25. Santoni, V., Molloy, M., and Rabilloud, T. (2000) Membrane proteins and proteomics: un amour impossible? *Electrophoresis* **21**, 1054–1070.
26. Görg, A., Postel, W., Friedrich, C., et al. (1991) Temperature-dependent spot positional variability in two-dimensional polypeptide gel patterns. *Electrophoresis* **12**, 653–658.
27. Patterson, S. D. (1994) From electrophoretically separated protein to identification: Strategies for sequence and mass analysis. *Anal. Biochem.* **221**, 1–15.
28. Patton, W. F. (2000) A thousand points of light: The application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics. *Electrophoresis* **21**, 1123–1144.
29. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 85–858.
30. Yan, J. X., Wait, R., Berkelman, T., et al. (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* **21**, 3666–3672
31. Yan, J. X., Harry, R. A., Spibey, C., and Dunn, M. J. (2000) Postelectrophoretic staining of proteins separated by two-dimensional gel electrophoresis using SYPRO dyes. *Electrophoresis* **21**, 3657–3665.

4

Electroblotting of Proteins from Polyacrylamide Gels for Chemical Characterization

Michael J. Dunn

1. Introduction

Since the first complete genome sequence, that of the bacterium *Hemophilus influenzae*, was published in 1995 *(**1**)*, a flurry of activity has seen the completion of the genomic sequences for more than 80 prokaryotic and eukaryotic organisms. Early in 2001 a major milestone was reached with the completion of the human genome sequence *(**2**,**3**)*. A major challenge in the post-genome era will be to elucidate the biological function of the large number of novel gene products that have been revealed by the genome-sequencing initiatives, to understand their role in health and disease, and to exploit this information to develop new therapeutic agents. The assignment of protein function will require detailed and direct analysis of the patterns of expression, interaction, localization, and structure of the proteins encoded by genomes; the area now known as "proteomics" *(**4**)*.

Techniques of polyacrylamide gel electrophoresis (PAGE) have an almost unrivaled capacity for the separation of complex protein mixtures. In particular, two-dimensional methods (2-DE) can routinely separate up to 2,000 proteins from whole cell and tissue homogenates, and using large format gels separations of up to 10,000 proteins have been described *(**5**,**6**)*. For this reason 2-DE remains the core technology of choice for protein separation in the majority of proteomics projects. Combined with the currently available panel of sensitive detection methods *(**7**)* and computer analysis tools *(**8**)*, this methodology provides a powerful approach to the investigation of differential protein expression. This has been complemented by the development over the last years of a battery of highly sensitive techniques of microchemical characterization, including N-terminal and internal protein microsequencing by automated

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

Edman sequencing, and amino acid compositional analysis *(9)*. More recently techniques based on the use of mass spectrometry for mass peptide profiling and partial amino acid sequencing have made this group of technologies the primary toolkit for protein identification and characterization in proteomics projects *(10)*.

A major obstacle to successful chemical characterization is efficient recovery of the separated proteins from the polyacrylamide gel as most procedures are not compatible with the presence of the gel matrix. The two major approaches used to overcome this problem for the recovery of intact proteins are electroelution and Western electroblotting. In the first method, protein zones are detected after PAGE by staining with Coomassie Brilliant Blue R-250. Protein-containing gel pieces are then excised and placed in an electroelution chamber where the proteins are transferred in an electric field from the gel into solution, and concentrated over a dialysis membrane with an appropriate molecular-weight cut-off. While this method can result in excellent protein recovery (>90%), it suffers from several disadvantages including:

1. The ability to handle only small numbers of samples at one time;
2. Contamination of the eluted protein with SDS, salts and other impurities which can interfere with subsequent chemical analysis;
3. Peptide chain cleavage during staining or elution; and
4. Chemical modification during staining or elution leading to N-terminal blockage *(11)*.

Although electroelution has largely been replaced by electroblotting, it is still occasionally successfully used, for example for protein mass analysis by matrix-assisted laser desorption mass spectrometry (MALDI-MS) *(12)*.

In Western blotting, proteins separated by 1-D or 2-D PAGE are blotted onto an appropriate membrane support, the total protein pattern visualized using a total protein stain, and the protein band or spot of interest excised. The protein, while still on the surface of the inert membrane support, can then be subjected to the appropriate microchemical characterization technique. The most popular method for the transfer of electrophoretically separated proteins to membranes is the application of an electric field perpendicular to the plane of the gel. This technique of electrophoretic transfer, first described by Towbin et al. *(13)*, is known as Western blotting. Two types of apparatus are in routine use for electroblotting. In the first approach (known as "tank" blotting), the sandwich assembly of gel and blotting membrane is placed vertically between two platinum-wire electrode arrays contained in a tank filled with blotting buffer. The disadvantages of this technique are that:

1. A large volume of blotting buffer must be used;
2. Efficient cooling must be provided if high current settings are employed to facilitate rapid transfer; and

3. The field strength applied (V/cm) is limited by the relatively large interelectrode distance.

In the second type of procedure (known as "semidry" blotting) the gel-blotting membrane assembly is sandwiched between two horizontal plate electrodes, typically made of graphite. The advantages of this method are that:

1. Relatively small volumes of transfer buffer are used;
2. Special cooling is not usually required although the apparatus can be run in a cold room if necessary; and
3. A relatively high field strength (V/cm) is applied due to the short interelectrode distance resulting in faster transfer times.

In the following sections both tank and semidry electroblotting methods for recovering proteins separated by 1-D or 2-D PAGE for subsequent chemical characterization will be described. In addition, a total protein staining procedure compatible with chemical characterization techniques is given. Electroblotting is ideal for the recovery of gel-separated proteins for automated Edman sequencing. It has also often been used (usually with trypsin) for subsequent peptide mass profiling by MALDI-MS. However, on-membrane digestion has now largely been superseded by methods of in-gel digestion as the latter process gives better overall sensitivity ***(14)***.

2. Materials

2.1. Electroblotting

Prepare all buffers from analytical grade reagents and dissolve in deionized water. The solutions should be stored at 4°C and are stable for up to 3 mo.

1. Blotting buffers are selected empirically to give the best transfer of the protein(s) under investigation (*see* **Note 1**). The following compositions are commonly used:
   a. For characterization of proteins with pIs between pH 4.0 and 7.0: Dissolve 6.06 g Tris base and 3.09 g boric acid and make up to 1 L (*see* **Note 2**). Adjust the solution to pH 8.5 with 10 *M* sodium hydroxide ***(15)***.
   b. For characterization of proteins with pIs between pH 6.0 and 10.0: Dissolve 2.21 g 3-(cyclohexyl-amino)-1-propanesulphonic acid (CAPS) and make up to 1 L (*see* **Note 2**). Adjust the solution to pH 11.0 with 10 *M* sodium hydroxide ***(16)***.
2. Filter paper: Whatman 3MM filter paper cut to the size of the gel to be blotted.
3. Transfer membrane: FluoroTrans (Pall) cut to the size of the gel to be blotted (*see* **Note 3**).
4. Electroblotting equipment: A number of commercial companies produce electroblotting apparatus and associated power supplies. For tank electroblotting we use the Hoefer TE 42 Transphor II unit (Amersham Pharmacia Biotech), while

for semidry electroblotting we use the Multiphor II NovaBlot apparatus (Amersham Pharmacia Biotech).
5. Rocking platform.
6. Plastic boxes for gel incubations.

2.2. Protein Staining

1. Destain: 450 mL methanol, 100 mL acetic acid made up to 1 L in deionized water.
2. Stain: 0.2 g Coomassie Brilliant blue R-250 made up to 100 mL in destain.

3. Method

3.1. Electroblotting

3.1.1. Semidry Blotting

1. Following separation of the proteins by gel electrophoresis, place the gel in equilibration buffer, and gently agitate for 30 min at room temperature (*see* **Note 4**).
2. Wet the lower (anode) plate of the electroblotting apparatus with deionized water.
3. Stack 6 sheets of filter paper wetted with blotting buffer on the anode plate and roll with a glass tube to remove any air bubbles.
4. Place the prewetted transfer membrane (*see* **Note 5**) on top of the filter papers and remove any air bubbles with the glass tube.
5. Place the equilibrated gel on top of the blotting membrane and ensure that no air bubbles are trapped.
6. Apply a further six sheets of wetted filter paper on top of the gel and roll with the glass tube.
7. Wet the upper (cathode) plate with deionized water and place on top of the blotting sandwich.
8. Connect the blotter to power supply and transfer at 0.8 mA/cm^2 of gel area (*see* **Note 6**) for 1 h at room temperature (*see* **Note 7**).

3.1.2. Tank Blotting

1. Following separation of the proteins by gel electrophoresis, place the gel in equilibration buffer, and gently agitate for 30 min at room temperature (*see* **Note 4**).
2. Place the anode side of the blotting cassette in a dish of blotting buffer.
3. Submerge a sponge pad taking care to displace any trapped air and place on top of the anodic side of the blotting cassette.
4. Place two pieces of filter paper onto the sponge pad and roll with a glass tube to ensure air bubbles are removed.
5. Place the prewetted transfer membrane (*see* **Note 5**) on top of the filter papers and remove any air bubbles with the glass tube.
6. Place the equilibrated gel on top of the blotting membrane and ensure that no air bubbles are trapped.

7. Place a sponge pad into the blotting buffer taking care to remove any trapped air bubbles and then place on top of the gel.
8. Place the cathodic side of the blotting cassette on top of the sponge and clip to the anode side of the cassette.
9. Remove the assembled cassette from the dish and place into the blotting tank filled with transfer buffer.
10. Connect to the power supply and transfer for 6 h (1.5-mm thick gels) at 500 mA at 10°C (*see* **Note 7**).

3.2. Protein Staining

1. Remove the blotting membrane from the sandwich assembly.
2. Place the membrane into a dish containing the Coomassie blue staining solution for 2 min and agitate gently on the rocking platform.
3. Place the membrane into destaining solution and agitate for 10–15 min (or until the background is pale).
4. Wash the membrane with deionized water and place on filter paper and allow to air dry.
5. Place the membrane into a clean plastic bag and seal until required for further analysis. The membrane can be stored in this state at room temperature for extended periods without any apparent adverse effects on subsequent chemical characterization.
6. An example of a membrane stained by this method is shown in **Fig. 1**.

4. Notes

1. The use of transfer buffers containing glycine or other amino acids must be avoided for proteins to be subjected to microchemical characterization.
2. Methanol (10–20%, v/v) is often added to transfer buffers as it removes SDS from protein-SDS complexes and increases the affinity of binding of proteins to blotting membranes. However, methanol acts as a fixative and reduces the efficiency of protein elution, so that extended transfer times must be used. This effect is worse for high molecular-weight proteins, so that methanol is best avoided if proteins greater than 100 kDa are to be transferred.
3. Nitrocellulose membranes are not compatible with the reagents and organic solvents used in automated Edman protein sequencing. A variety of alternative (e.g., glass fiber-based and polypropylene-based) membranes have been used for chemical characterization *(**17**)*, but PVDF-based membranes (FluoroTrans, Pall; ProBlott, Applied Biosystems; Immobilon-P and Immobilon-CD, Millipore; Westran, Schleicher and Schuell; Trans-Blot, Bio-Rad) are generally considered to be the best choice for this application *(**16**)*. Nitrocellulose can be used as a support in applications such as internal amino acid sequence analysis and peptide mass profiling, where the protein band or spot is subjected to proteolytic digestion prior to characterization of the released peptides.
4. Gels are equilibrated in blotting buffer to remove excess SDS and other reagents that might interfere with subsequent chemical analysis (e.g., glycine). This step

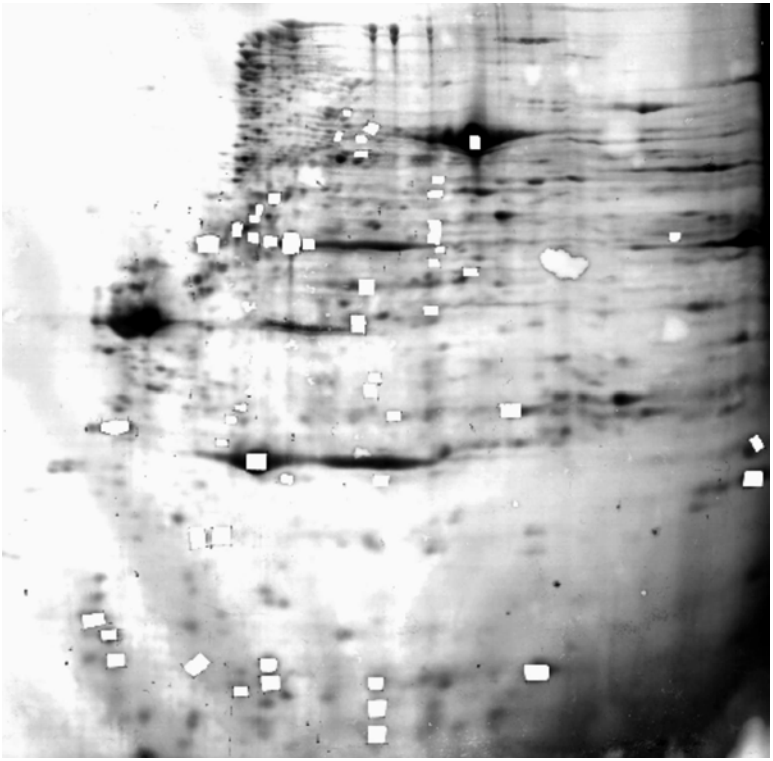


Fig. 1. PVDF (FluoroTrans) Western electroblot transfer of 300 μg human heart proteins separated by 2-DE and stained with Coomassie Brilliant blue R-250. The white areas indicate protein spots that have been excised for chemical characterization.

also minimizes swelling effects during protein transfer. Equilibration may result in diffusion of zones and reduced transfer efficiencies of high molecular weight proteins. It is important to optimize the equilibration time for the protein(s) of interest.

5. Nitrocellulose membranes can be wetted with blotting buffer, but PVDF-based membranes must first be wetted with methanol prior to wetting with the buffer.
6. The maximum mA/cm^2 of gel area quoted applies to the apparatus we have used. This should be established from the manual for the particular equipment available.
7. Blotting times need to be optimized for the particular proteins of interest and according to gel thickness. Larger proteins usually need a longer transfer time, whereas smaller proteins require less time. Proteins will also take longer to be transferred efficiently from thicker gels. The transfer time cannot be extended indefinitely (> 3 h) using the semidry technique as the small amount of buffer used will evaporate. If tank blotting is used, the transfer time can be extended almost indefinitely (> 24 h) providing that the temperature is controlled.

References

1. Fleischmann, R. D., et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512
2. Venter, J. C., et al. (2001) The sequence of the human genome. *Science* **291**, 1304–1351.
3. International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. *Nature* **409**, 860–922.
4. Banks R., Dunn M. J., Hochstrasser D. F., et al. (2000) Proteomics: new perspectives, new biomedical opportunities. *Lancet* **356**, 1749–1756.
5. Görg, A., Obermaier, C., Boguth, G., et al. (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **21**, 1037–1053.
6. Dunn, M. J. and Görg, A. (2001) Two-dimensional polyacrylamide gel electrophoresis for proteome analysis, in *Proteomics, From Protein Sequence to Function* (Pennington, S. R. and Dunn, M. J., eds.), BIOS Scientific Publishers, Oxford, pp. 43–63.
7. Patton, W. F. (2001) Detecting proteins in polyacrylamide gels and on electroblot membranes, in *Proteomics, From Protein Sequence to Function* (Pennington, S. R. and Dunn, M. J., eds.), BIOS Scientific Publishers, Oxford, pp. 65–86.
8. Dunn, M. J. (1992) The analysis of two-dimensional polyacrylamide gels for the construction of protein databases, in *Microcomputers in Biochemistry* (Bryce, C. F. A., ed.), IRL Press, Oxford, pp. 215–242.
9. Wilkins, M. R. and Gooley, A. (1997) Protein identification in proteome analysis, in *Proteome Research: New Frontiers in Functional Genomics* (Wilkins, M. R., Williams, K. L., Appel, R. D., and Hochstrasser, D. F., eds.), Springer-Verlag, Berlin, pp. 35–64.
10. Patterson, S. D., Aebersold, R., and Goodlett, D. R. (2001) Mass spectrometry-based methods for protein identification and phosphorylation site analysis, in *Proteomics, From Protein Sequence to Function* (Pennington, S. R. and Dunn, M. J., eds.), BIOS Scientific Publishers, Oxford, pp. 87–130.
11. Aebersold, R. (1991) High sensitivity sequence analysis of proteins separated by polyacrylamide gel electrophoresis, in *Advances in Electrophoresis, vol. 4* (Chrambach, A., Dunn, M. J., and Radola, B. J., eds.), VCH, Weinheim, pp. 81–168.
12. Patterson, S. D. (1994) From electrophoretically separated protein to identification: Strategies for sequence and mass analysis. *Anal. Biochem.* **221**, 1–15.
13. Towbin, H., Staehelin, T., and Gordon, G. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
14. Lahm, H. W. and Langen, H. (2000) Mass spectrometry: a tool for the identification of prtoeins separated by gels. *Electrophoresis* **21**, 2105–2114.
15. Baker, C. S, Dunn, M. J., and Yacoub, M. H. (1991) Evaluation of membranes used for electroblotting of proteins for direct automated microsequencing. *Electrophoresis* **12**, 342–348.

16. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**, 10035–10038.
17. Eckerskorn, C. (1994) Blotting membranes as the interface between electrophoresis and protein chemistry, in *Microcharacterization of Proteins* (Kellner, R., Lottspeich, F., and Meyer, H. E., eds.), VCH Verlagsgesellchaft, Weinheim, pp. 75–89.

5

Enzymatic Cleavage of Proteins

Bryan John Smith

1. Introduction

Endoproteinases catalyse hydrolysis of polypeptide chains, most usefully at specific sites within the polypeptide, as described in **Table 1**. The number and nature of peptides generated by a proteinase of good specificity is characteristic of a protein, since it reflects the protein's sequence. The term "peptide map" is applied to the chromatogram or pattern of peptides resolved by a method such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (*see* Chapter 8). Peptide mapping is widely used for quality control of recombinant proteins, where appearance of novel peptides indicates the presence of variant forms of protein (for example, *see* **refs. *1–4***). The mass spectroscopic equivalent of peptide mapping is called "mass mapping", whereby the masses of the products of proteolysis are characteristic of a given protein (*see* Chapters 17 and 18). Individual peptides may also be purified and subjected to various sequencing techniques as described elsewhere in this volume, the purpose being to identify a protein by its sequence, determine the partial sequence of a novel protein for cloning purposes, or identify sites of modification (for example, phosphorylation ***[5]***).

Exopeptidases (carboxy- and aminopeptidases) also digest polypeptide substrates, but at their termini rather than at internal sites. Exopeptidases may be used to study C-terminal and blocked N-terminal sequences, for instance pyroglutamate aminopeptidase may remove an N-terminal pyroglutamate (*see* Chapter 29) Chemical methods of proteolysis have also been developed (*see* Chapter 6). They can usefully complement enzymatic methods because they have different specificity, but they may be unsuited to some purposes in that the harsh conditions employed may destroy biological activity.

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

It is usually preferable for the polypeptide cleavage event to be as specific as possible; cleavage at a multiplicity of different sites and in a variety of yields can lead to a complex mixture of peptides. The specificities of some proteinases are broad, but others are quite specific. Various of these are commercially available in especially pure from, usually obtained by extra rounds of chromatography. The purpose of the extra purification is to maximize specificity and minimize alternative cleavage owing to traces of contaminating enzymes. These especially pure enzyme preparations are given names such as "sequencer grade," and are recommended for more consistent peptide mapping and preparations. The specificities and other properties of various commercially available "sequencer grade" endoproteinases are summarized in **Table 1**. Trypsin (EC 3.4.21.4) is one of the most widely used proteinases, and the use of this enzyme is described in this chapter as an example of the approach to digestion of polypeptides by proteases.

Proteolysis is commonly carried out in solution but in recent years methods have been developed to allow proteolysis of samples that are bound to or trapped within a solid support, most notably nitrocellulose or polyvinylidene difluoride (PVDF) such as used in blotting of proteins from polyacrylamide gels (*see* Chapter 4), or the polyacrylamide gel itself. The aim of these developments has been to interface with the technique of polyacrylamide electrophoresis, a common separation method that has very high-resolving powers. In particular, two-dimensional electrophoresis is the method of choice for the resolution and analysis of complex protein mixtures and is a frequent starting point for the identification of proteins by chemical sequencing, amino acid analysis or mass-spectrometry. Proteolysis of samples resolved in gels can be achieved by digestion of the proteins in solution after they are eluted from the gel, but the recovery of proteins can be problematic in that it may be at low yield and with contamination by nonprotein components from the polyacrylamide gel. Protein digestions are therefore generally performed either within the gel itself (in-gel digestion) or after transfer of the sample to a membrane such as PVDF or nitrocellulose (*see* Chapter 4) and the digest performed *in situ*. These two approaches are generally equally successful. In contrast to digests in solution, however, digestion of proteins in gels and on blots may suffer from hinderance of access of enzymes to the protein substrate and possibly by poor retrieval of some peptides, which lead to incomplete peptide maps compared with digests in solution.

Optimization of the efficiency of proteolysis and the recovery of peptides from gels and blots is therefore of paramount importance.

Methods for digestion of polypeptides in these various states are presented separately.

Table 1
Characteristics of "Sequencer-Grade" Endoproteases

Enzyme	E C No.	Enzyme Class	Source	Approx Mol. Weight	Operating pH	Preferred Cleavage Site[a]	Example Digestion Buffer[b]	Inhibitor, Effective Concentration[b]	Notes
Chymotrypsin	3.4.21.1	Serine	Bovine pancreas	25 kDa	8–9	Y-X; F-X; W-X; (L-X; M-X; A-X; D-X; E-X)	Tris-HCl, 100 m*M*; $CaCl_2$, 10 m*M*, pH 7.8	AEBSF, 0.4–4 m*M*	Sites in brackets cleaved less rapidly. X may be amide or ester group
Endo Arg C	3.4.22.8	Cysteine	Clostridium histolyticum	50 kDa	8.0	R-X	Tris-HCl, 90 m*M*; $CaCl_2$, 8.5 m*M*	TLCK, 100-135 μ*M* and DTT, 5 m*M*; pH 7.6	Reducing agent and Ca^{2+} for required activity (so oxidisin agents divalent metal ion chelators, e.g. EDTA, are alternative inhibitors) X may be amide or ester group. Alternative name: Clostripain.
Endo Asp N	–	Metallo	Pseudomonas fragi	27 kDa	6–8.5	X-D; X-C	Sodium phosphate, 50 m*M*; pH 8.0	EDTA, molar excess over divalent metal	
Endo Glu C	3.4.21.9	Serine	Staphylococcus aureus V8	27 kDa	7.8 4	E-X; D-X E-X	Ammonium carbonate; pH 7.8. Ammonium acetate; pH 4.0	3,4 dichloro- isocoumarin, 5–200 μ*M*	Alternative name: Protease V8
Endo Lys C	3.4.99.30	Serine	Lysobacter enzymogenes	30 kDa	7–9	K-X	Tris-HCl, 25 mM; pH 8.5	TLCK, 100–135 μ*M*	X may be amide or ester group. Apparent molecular weight increased to 33 KDa upon reducrion.
Trypsin	3.4.21.4	Serine	Bovine pancreas	23.5 kDa	8	K-X; R-X; Aminoethyl C-X	Tris. HCl, 100 m*M*; pH 8.5	AEBSF, 0.4–4 m*M*	X may be amide or ester group. Cleavage after Lys may be inhibited by succinylation or methylation of the Lys side chain.

[a] X = any amino acid. Susceptibility to proteolysis may be reduced or lost if the potentially cleavable bond is linked to P (e.g., K-P for chymotrypsin) or if between two like residues (e.g., E-E for Endo Glu C)
[b] Other conditions for reaction: enzyme:substrate (w/w):: 1:20 to 1:200, at 25–37ºC for 2–18 h, optimized empirically for substrate in question. DTT = dithiothreitol. Abbreviations: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride. EDTA, ethylenediaminetetra acetic acid. TLCK, L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone. *See* **Notes 7–9**, and **11**.

2. Materials

2.1. Proteolysis in Solution

1. Stock enzyme solution - Trypsin (EC.3.4.21.4) (*see* **Notes 1–3**): Available from various commercial suppliers. It is stable for periods of years as a dry solid kept at –20°C or –70°C. Make a stock solution of 1 mg/mL in 10 m*M* HCl (made in Milli-Q or HPLC-grade distilled water). Use fresh or divide into suitably sized aliquots and freeze to –70°C. A frozen stock solution may be thawed and refrozen several times without loss of the majority of the activity, but for consistent results thaw once only.
2. Reaction buffer: Ammonium bicarbonate (0.4 *M*) approximately pH 8.0 as prepared. For long-term storage, sterilize by filtration through a 0.2 mm filter and store refrigerated or frozen (*see* **Notes 4** and **5**)
3. Enzyme Inhibitor 4- (2-aminoethyl-benzenesulphonyl fluoride (AEBSF). 100 m*M* in water (Milli-Q or HPLC grade). Use fresh or store for 1 month at –20°C (*see* **Note 6**).

2.2. Proteolysis on Membranes

1. Destaining solution for stained proteins on membranes (see Note 10): dependent on the stain used, thus:
   a. For Coomassie brilliant blue R-stained PVDF: 70% (v/v) acetonitrile in water.
   b. For Amido Black-stained nitrocellulose or PVDF: 50% methanol, 40% water, 10% acetic acid (v/v/v).
   c. For Ponceau S-stained nitrocellulose or PVDF: 200 m*M* NaOH.
2. Digestion buffer-for trypsin: 10% methanol in ammonium carbonate (25 m*M*), pH 7.8 containing 1% (w/v) octyl ß glucoside. Make fresh or store for periods of days in the refrigerator to discourage microbial growth (*see* **Notes 11** and **12**).
3. Stock enzyme solution-for trypsin: dissolve the solid enzyme preparation in digestion buffer containing 10 % v/v methanol, to give a concentration of 1 mg/mL of enzyme. Stock solutions can be stored frozen in aliquots at –20°C, but should be thawed once only and then used immediately. Dilute to 0.1 mg/mL in digestion buffer prior to use (*see* **Note 13**).
4. Membrane extraction solution: 50% v/v formic acid in absolute ethanol. Prepare in advance and store at room temperature until used (*see* **Note 15**).

2.3. Proteolysis in Polyacrylamide Gel

1. Destain for Coomassie brilliant blue R-stained gel: 50% (v/v) acetonitrile in ammonium carbonate buffer (200 m*M*), pH 8.9.
2. Digestion buffer-for trypsin: 200 m*M* ammonium carbonate, pH 8.9, containing 0.02% (w/v) octyl β glucoside.
3. Stock enzyme solution - for trypsin: as in **Subheading 2.1.**, **step 1.**
4. Working enzyme solution: dilute the stock enzyme solution fourfold with digestion buffer to give an enzyme concentration of 250 µg/mL. Prepare immediately before use and discard excess solution after use (*see* **Note 13**).

5. Extraction solution: 60% (v/v) acetonitrile in 40% (v/v) trifluoroacetic acid (TFA) (0.1% v/v in water).

3. Methods

3.1. Proteolysis in Solution

1. Dissolve the sample protein in water (HPLC or Milli-Q grade) to a suitable concentration, such as 2 mg/mL. Add an equal volume of 0.4 *M* ammonium bicarbonate buffer i.e. final concentrations of 1mg/ml substrate, 0.2 *M* ammonium bicarbonate, pH 8.0. Add trypsin stock solution to a final enzyme : substrate ratio of 1:50 (w/w) i.e., to 1 mL of sample solution add 20 µL of 1 mg/mL trypsin solution. Mix thoroughly (*see* **Notes 1** to **5**).
2. Incubate the mixture at 37°C for 24 h.
3. Terminate proteolysis by addition of specific trypsin inhibitor, AEBSF, to a final concentration of 1 m*M*, i.e., add 1/100 volume of inhibitor solution. Store stopped reaction frozen (–20°C or –70°C) or immediately analyze or resolve by HPLC or other method (*see* **Note 6**).

3.2. Proteolysis on Membranes

1. Stained protein spots on PVDF membranes are excised and destained with 500 µL aliquots of the appropriate destaining solution (*see* **Subheading 2.2.**, **step 1**), refreshing the solution until all possible stain has been removed (*see* **Note 10**).
2. Dry the membrane pieces by laying them on filter paper, then cut each of them into 1–2 mm squares using a sharp scalpel. Transfer them to clean polypropylene microcentrifuge tubes.
3. Enzyme solutions are diluted to 0.1 µg/µL prior to use with the appropriate digestion buffer.
4. To each membrane piece add 1-4 µL of enzyme solution (diluted to 0.1 mg/mL in digestion buffer), the volume added depending on the size of the membrane piece. Note that PVDF is wetted by this solution without the need for prewetting in methanol. Allow the solution to absorb. Only add sufficient enzyme solution to wet the membrane. Centrifuge the tube briefly to deposit the membrane and digestion solution together in the bottom of the tube.
5. Cap tubes to restrict evaporation and incubate them in a water bath at 37°C for 3 h to overnight.
6. Extract peptides from each membrane piece by incubation with 50 µL of 50% formic acid/ethanol (v/v) for 1 h at 37°C. The extraction solution is then pipetted into a clean microcentrifuge tube and the membrane extracted with a further 50 µL of extraction solution for 1 h. Pool the two extraction aliquots and dry by centrifugal evaporation (*see* **Notes 14** and **15**).
7. Store the dried residue at 4°C until required for further analysis.

3.3. Proteolysis Within Polyacrylamide Gel

1. Excise protein spots of interest from the gel and then further destain each piece with two 500 µL aliquots of destain (50% acetonitrile in 200 m*M* ammonium

carbonate buffer, pH 8.9, *see* **Subheading 2.3.**, **step 1**) for 20–30 min at 30°C until colourless (*see* **Note 17**).

2. Place each gel slice on a clean sheet of sealing tissue (e.g., Parafilm) and air dry for 5–10 min until the gel has shrunk to less than half the original size (not to complete dryness), then cut into it small pieces (1–2 mm cubes) and place in a microcentrifuge tube (of small volume, to minimize the risk of gel drying by evaporation of liquid into the head space).
3. The slices are partially rehydrated with 5 µL of digestion buffer (ammonium carbonate, 200 m*M*, pH 8.9, containing 0.02% (w/v) octyl β glucoside, *see* **Subheading 2.3.**, **step 2**).
4. Rehydrate the gel further with 5 µL aliquots of digestion buffer containing trypsin (250 µg./mL, *see* **Subheading 2.3.**, **step 4**) until the gel has returned to its original size.
5. Seal and incubate the microcentrifuge tubes in a waterbath at 37°C overnight.
6. Extract peptides by addition of 250 µL extraction solution (*see* **Subheading 2.3.**, **step 5**). Incubate for 1 h at 37°C. Repeat the extraction and pool the extraction solutions. Dry by centrifugal evaporation. Store dried peptides at 4°C until required for analysis.

4. Notes

4.1. Digestion in Solution

1. Commercially available trypsin is prepared from bovine pancreas where it is synthesised as trypsinogen, the sequence of which is known (for example, **ref.** ***[6]***). Active trypsin is generated in vivo by removal of the amino terminal hexapeptide. Its molecular weight is approximately 23,500 Daltons. Trypsin is optimally active at about pH 8.0. Stock solutions are made in 10 m*M* HCl at pH 2 (or 50 m*M* acetic acid is an acceptable alternative) and at –70°C, both of these conditions deterring autolysis. The trypsin regains activity when the pH is raised to above 4.0. Neutral or acidic buffers may be used (despite not providing the optimal pH for trypsin activity) where it is necessary to minimize the risk of disulphide bond interchange (as when isolating peptides to identify the positions of disulphide bridge in a sample protein).

   Trypsin has a serine at its active site and so belongs to the serine protease family. AEBSF, or phenylmethylsulphonyl fluoride (which is more unstable in water, and more toxic than is AEBSF) inhibit the enzyme by covalently modifying active site serine. Macromolecular trypsin inhibitors such as soybean trypsin inhibitor are not recommended because, being proteins, they may interfere with subsequent analyses.

   Trypsin displays good specificity, catalyzing the hydrolysis of the peptide bond to the COOH side of the lysyl and arginyl residues. Hydrolysis is slower if an acidic residue occurs to either side of the basic residue, and still slower if the residue to the COOH side is prolyl. Polylysine sequences may not be cleaved at every lysine. Bonds to the C-terminal side of methylated lysines may not be cleaved at all.

Trypsin available from some sources has been treated with L-1-chloro-3-tosylamido-4-phenylbutan-2-one in order to inhibit any chymotrypsin, which may be contaminating the preparation. Different trypsin preparations may vary in activity, so use only one batch of protease if reproducibility is important (e.g., for peptide mapping or for GMP work). As with other proteases, if trypsin is used at high concentration (say, trypsin:substrate::1:50 (w/w), or less) rare and unexpected cleavages may become apparent. This may be the case in digestion of samples on blots or in gels.

For some years it has been recognized that trypsin and other proteases may catalyze formation (rather than breakage) of peptide bonds, but for a significant level of this to occur addition of organic solvent to the buffer is generally required. To a small degree this transpeptidation reaction may occur in aqueous buffers neutral or acid pH conditions and this may produce small amounts of artificial polypeptides, detectable in peptide maps. As an example, Canova-Davis et al. ***(7)*** have reported that during digestion of relaxin by trypsin at pH 7.2 two (normally noncontiguous) peptides became linked by a peptide bond to a 10% level.

2. During digestion, autolysis of the trypsin occurs, to produce a background of trypsin peptides that is particularly noticeable when the protease is used at high concentration (e.g., for digestion of blots or in gels). These have been described in the literature ***(8)*** but it is always best to identify these in each experiment by inclusion of a control reaction of trypsin without substrate. To identify trace contaminants in the substrate (or buffers) include a control of substrate without trypsin. Reductive methylation of lysines (to ε-N,N-dimethyllysine) in the trypsin renders these sites insensitive to autolytic cleavage ***(9)***. This modified trypsin is more stable and produces fewer interfering peptides. It is available commercially (for instance, from Promega).
3. Lysine is a common constituent of proteins and digestion with trypsin can generate a large number of peptides of small average size. This is a good point when peptide mapping, but some purposes, such as sequencing, may require longer peptides. The action of trypsin may be modified in an attempt to achieve this. It is done by modification of the side chains of lysyl or arginyl residues in the substrate, such that cleavage only occurs at unmodified residues. Perhaps the most common such method is succinylation of lysyl side chains, leading to tryptic cleavage at arginyl (and any remaining unmodified lysyl) residues (*see* Chapter 27, or **ref.** ***[10]***). Introduction of additional sites of cleavage by trypsin may be achieved by conversion of cysteinyl residues, to aminoethyl cysteinyl residues by reaction with ethyleneimine as described in Chapter 27.
4. The method described is the basic procedure, and various of the conditions described for the method of digestion of substrates with trypsin in solution may be altered. Thus, the ammonium bicarbonate buffer (which is volatile and allows removal of salt by drying under vacuum) may be replaced by another buffer at pH 8.0. Again, 50 m*M* Tris-HCl will provide similar results but beware of the marked effect of temperature on the dissociation constant of Tris, the pH at 37°C being about one pH unit lower that at 4°C. Check the buffer pH at the temperature to be

used for the digestion. Addition of salt to high concentration (e.g., 0.5 *M* NaCl) favours compact folding of a structured polypeptide chain. Tightly folded domains are generally more resistant to proteolytic attack than are unstructured regions (though not indefinitely resistant), so high-salt conditions may be used to prepare structural domains which may retain biological function.

For complete digestion (as may be required for peptide mapping) prolonged incubation may be required, with further addition of trypsin (to replace trypsin which has been inactivated by digestion). Thus, conditions may be as follows: 37°C for 24 h at pH 8.0, then addition of further trypsin (similar to the first addition) followed by a further 24h incubation. On the other hand, preferential cleavage of particularly sensitive bonds or generation of partial cleavage products may be achieved by using less trypsin (say on enzyme:substrate ratio of 1:200 w/w, or more), shorter incubation times, lower incubation temperatures and/or a pH adjusted away from the optimum of pH8. Partial digestion products may be useful for determination of the order of neighbouring peptides in the parent sequence.

5. The condition of the substrate is important. First, the substrate should be soluble, or as finely divided as possible, in the digestion buffer. If a sample is not readily soluble in water or ammonium bicarbonate solutions, suitable solvents can be used initially and then adjusted by dilution or titration of pH to allow for trypsin action. If the polypeptide remains insoluble, the precipitate should be kept in suspension by stirring. Thus, 8 *M* urea may be used to solubilize a protein or disrupt a tightly folded structure and then diluted to 2 *M* urea for digestion by trypsin. Trypsin will also function in 2 *M* guanidinium chloride, in the presence of sodium dodecylsulfate (SDS, e.g., 0.1% w/v), or in the presence of acetonitrile (up to about 50%, v/v). Therefore fractions from reverse-phase HPLC (acetonitrile gradients in water/trifluoroacetic acid, 0.1%, v/v) may be readily digested after adjustment of pH by addition of ammonium bicarbonate or other buffer, and dilution (if necessary to lower the acetonitrile concentration).

   Second, a native protein may be tightly folded, such as to markedly slow up or inhibit proteolytic attack. To remedy this, the substrate may be denatured and the structure opened out to allow for access of the proteinase. This may be done by boiling in neutral pH solution, or by use of such agents as urea, SDS, or organic solvent, as described above. Low concentrations of these agents (e.g., 5–10% acetonitrile, v/v) may give more rapid digestion than will a buffer without them, but high concentrations (e.g., 50% acetonitrile, v/v) will slow the digestion (also *see* **Note 1**, regarding the possibility of protease-catalyzed synthetic reactions in organic solvent).

   An additional, and very common, technique is reduction and alkylation, i.e., permanent disruption of disulfide bonds. This treatment opens out the protein structure to allow for ready digestion and to minimize complications in peptide separation that are caused by pairs (or larger combinations) of peptides remaining connected by S-S bonds. This treatment is carried out as described in Chapter 27. Alternatively, if a sample has already been digested, S-S bonds may be reduced by simple addition of small amounts of dithiothreitol (as solid or as an

aqueous solution) and incubation at room temperature (at pH 8.0) for 30 min or so. This treatment is followed immediately by HPLC to separate the various peptides. The amount of dithiothreitol required (i.e., slight molar excess over S-S bonds) may be calculated accurately if the cystine content of the protein concerned is known.

6. The digestion by trypsin may be stopped by addition of a serine protease inhibitor such as AEBSF as mentioned in **Note 1**. Alternatively, the solution's, pH may be adjusted far away from the optimal pH, e.g., lowered to pH 2.0 or so, by addition of acid (*see* **Note 1**). Keep the acidified solution cold, on ice, to minimize acid-catalyzed hydrolysis. Again, the reaction mixture may be immediately submitted to analysis (wherein trypsin is separated from substrate). Analysis and/or peptide preparation may be by reverse phase HPLC, capillary electrophoresis or polyacrylamide electrophoresis, or mass spectometry, as described in other chapters.
7. Other commercially available proteinases purified for sequencing purposes are summarized in **Table 1**. Use of these is essentially as described above for trypsin, except for use of buffers of appropriate pH and inclusion of divalent cations and reducing agents as required. Beware that complexing may occur between buffer salts and cations, thus affecting both cation concentration and buffering capacity for hydrogen ions. As an example calcium forms an insoluble phosphate in phosphate buffer.

   One of the most useful of these other proteinases is Endo proteinase Glu-C (Endo Glu C). It cuts to the COOH side of glutamyl residue. A lower frequency of cleavage to the COOH side of aspartyl residues may also occur at neutral pH, although at pH 4.0 this may not occur. Endoproteinase Glu-C functions in buffers containing 0.2% (w/v) SDS or 4 *M* urea. Its sequence is known ***(11)***. It has been noted by a number of investigators that Endo Glu C can cause aspartamide formation by condensation of the side-chain of aspartate residues leading to a loss of 18 Da. Formation of aspartamide does not interfere with chemical sequencing, but may cause problems in peptide mass-fingerprinting
8. Other readily available proteinases are of broader specificity and may be affected by surrounding sequences. Their action is therefore difficult to predict. In particular instances, however, their observed action may prove beneficial by cleaving at one or a few particularly sensitive sites when incubated in suboptimal conditions (e.g., short duration digestion or nondenatured substrate).

   Good examples of this come from work on preparation of $F(ab')_2$, antigen-binding fragments of immunoglobulin IgG, that are bivalent and lack the constant Fc region of the molecule. Incubation of nondenatured IgG molecules with a proteinase of broad specificity can lead to proteolytic cleavage at a few sites or a single site in good yield. Pepsin has been used for this purpose (e.g., pH 4.2–4.5, enzyme:substrate::1:33, w/w, 37°C ***[12]***). Different subclasses of mouse IgG were found to be digested at different rates, in the order IgG3 > IgG2a > IgG1. Different antibodies of the same subclass may also be degraded differently, some rapidly and without formation of $F(ab')_2$ ***(12)***. Papain has been used to prepare $F(ab')_2$ fragments from the IgG1 subclass, which is the subclass that is most

resistant to pepsin. The method described by Parham et al, *(13)* uses papain (which has been activated just before use by reaction with cysteine), at pH 5.5 (01.1 *M* acetate, 3 m*M* EDTA) 37°C, with an IgG concentration of about 10 mg/mL. The enzyme is added at time 0, and again later (e.g., at 9 h) to an enzyme:substate ratio of 1:20 (w/w). Digestion can be halted by addition of iodacetamide (30 m*M*) *(7)*. Rousseaux et al. *(14)* also described conditions for generating rat $F(ab')_2$, using papain (in the presence of 10 m*M* cysteine), pepsin or Endo Glu-C. Incubation of the IgG1 and 2a subclasses at pH 2.8 prior to digestion with pepsin improved the yields of $F(ab')_2$ fragments, presumably because the proteins thus denatured were effectively better substrates.

9. Two enzymes of broader specificities are worthy of further mention. The first is thermolysin, for its good thermostability that may prove useful when keeping awkward substrates in solution. Thermolysin remains active at 80°C or in 8 *M* urea. The second enzyme is pepsin, which acts at low pH. Disulfide bonds rearrange less frequently in acid than in alkaline conditions, so use of low pH buffers may not only help solubilize a substrate, but may also help preserve naturally disulfide bonded pairs of peptides. Endoproteinase Glu-C may also be used at low pH, having an optimum activity at pH 4.0.

4.2. Digestion on Blots

10. As a matter of course, destaining of blots removes what may be a source of interference in subsequent analyses. An alternative stain, sulforhodamine B, is compatible with mass spectrometry and need not be removed (*see* Chapter 18). If nitrocellulose membrane is used in place of PVDF, beware that it will dissolve in high concentrations of organic solvents and therefore care must be taken with staining and destaining steps (*see* **refs. *15–17*** for alternative formulations of stain and destain.
11. It is essential for the digestion of proteins on membranes to prevent adsorbtion of the enzyme to the membrane. This is achieved by using detergents, but the purity and stability of the detergents are important to prevent the formation or addition of reagent impurities, which will interfere with subsequent peptide analysis. For this reason detergents which are available in very high purity are necessary. Octyl β glucoside is one such detergent and it has little effect on subsequent analysis being both compatible with reversed-phase peptide fractionation and also with matrix-assisted laser desorption ionization mass-spectrometry (MALDI-MS). Octyl glucoside generates small peaks on reverse phase HPLC (monitored at 214 nm), but these are substantially less than those seen with Tween 20 or Triton X100, and are generally not a problem in analysis or peptide purification. Reduced Triton X-100 is also recommended owing to its low levels of UV absorbing contaminants compared with the unreduced detergent *(16)*.

    Any enyzme-compatible buffers can be used for membrane digestions (*see* **Table 1**), but they should be supplemented with detergents and methanol to aid efficient digestion and peptide recovery. Volatile buffers such as ammonium carbonate and ammonium acetate are particularly useful as they do not interfere

with subsequent biochemical procedures such as MALDI-MS. Buffers may also need to be supplemented with $CaCl_2$, dithiothreitol, or EDTA depending on the enzyme. The particular requirements of the enzyme in terms of pH and other co-factors is best determined by reference to the manufacturers technical bulletin which is normally supplied with the enzyme.

12. Access of the protease to substrate adsorbed to membrane is enhanced by the addition of detergents as mentioned in **Note 11**, which together with the addition of small quantities of organic solvents promotes wetting of the membrane. The compatibility of the membrane and enzyme with such solvents should however be ascertained before proceeding. The use of up to 50% (v/v) dimethyl sulfoxide (DMSO) in the digestion buffer has been reported to give much higher recoveries of hydrophobic peptides from digests on PVDF membranes. Generally, concentrations of up to 20% (v/v) methanol, n-propanol, or acetonitrile are tolerated by nitrocellulose and PVDF membranes, and are compatible with the proteases Lys-C, Glu C, Arg-C, and trypsin. However, the presence of solvents during proteolysis can cause transpeptidation (*see* **Note 1**), apparent in peptide maps and mass maps. A methanol concentration of 10% (v/v) can provide satisfactory results. If solvents are used they should be of HPLC-grade to prevent addition of contaminants that may interfere with subsequent analytical procedures. Likewise, water for all solutions should also be of HPLC grade or "polished" using a laboratory water-purification system (e.g., Milli-Q, Millipore).
13. A high concentration of protease is used for on-membrane digestions. This may encourage unusual cleavages and may also result in noticeable levels of autolysis products (especially if methylated trypsin is not used). These high concentrations of enzyme make high-purity enzymes (sequence-grade) essential for this type of analysis (*see* **Notes 1** and **4**).
14. Enzyme digestions of blotted samples are readily terminated by the addition of extraction solutions due to the high concentration of organic solvent and extreme pH. A double extraction is used to maximize the amount of peptides recovered and this is likely to be very important for hydrophobic peptides. Extraction of peptides from PVDF is an especial problem due to the high binding capacity and affinity of the membrane. Formic acid, which is a good solvent for peptides, can be used with ethanol and this gives good results with peptides digested on PVDF membranes and is also compatible with nitrocellulose.
15. After extraction, peptides can be dried down for subsequent analysis and centrifugal evaporation is the method of choice to reduce losses. However, with small quantities of peptides, drying completely can lead to irreversible binding of the peptides to the walls of tubes. In common with sample preparation for HPLC analysis, it may be advisable to only reduce the volume and remove organic solvents before further analysis. (*See also* Chapter 1.)

4.3. Digestion Within Gels

16. Successful digestion of small amounts of protein within polyacrylamide gels and their subsequent analysis is especially dependent on removal of residual SDS,

gel contaminants, and stain. This is most readily achieved by extracting the gel with 50% (v/v) acetonitrile *(18)*. Partial drying of the gel slices after destaining shrinks the gel and allows rapid entry of the enzyme solution into the gel as it rehydrates. Rosenfeld et al. have reported that complete drying of the slices leads to reduced recovery of peptides after digestion *(18)*. Hellman et al. *(19)*, on the other hand, have reported 50–85% yields of peptides after proteolysis in gels that have been completely dried. Jeno et al. *(20)* have described a further modification intended to suppress disulfide bond formation and so complexities in subsequent peptide maps. This is done by reduction (by DTT) and alkylation of Cys residues (by iodoacetamide) in the presence of 0.1% (w/v) SDS. The SDS is removed before HPLC analysis by addition of about 0.2 volume of 1 *M* guanidinium-HCl, followed by centrifugation.

17. Fixation or precipitation of protein in the gel during staining may reduce yields of extractable peptides. This can occur if the stain or destain is acidic or includes a fixation step such as with formaldehyde prior to some methods of silver staining. Sypro Ruby gel stain (Molecular Probes, Inc.) is a sensitive luminescent stain which has a pH of about 4.5 that is less likely to cause this problem. Equivalent Sypro Ruby blot stain is also available.

References

1. Jaquinod, M., Holtet, T. L., Etzerodt, M., Clemmensen, I., Thorgeson, H. C., and Roepstorff, P. (1999) Mass spectrometric characterisation of post-translational modificatin and genetic variation in human tetranectin. *Biol. Chem.* **380**, 1307–1314.
2. Gulati, D., Bongers, J., and Burman, S. (1999) RP-HPLC tryptic mapping of IgG1 proteins with post-column fluorescence derivatization. *J. Pharm. Biomed. Anal.* **21**, 887–893.
3. Samtora, L. C., Krull, I. S., and Grant, K. (1999) Characterization of recombinant human monoclonal tissue necrosis factor-alpha antibody using cation-exchange HPLC and capillary isoelectric focusing. *Anal. Biochem.* **275**, 98–108.
4. Bongers, J., Cummings, J. J., Ebert, M. B., Federici, M. M., Gledhill, L., Gulati, D., et al. (2000) Validation of a peptide mapping method for a therapeutic monoclonal antibody: what could we possibly learn about a method we have run 100 times? *J. Pharm. Biomed. Anal.* **21**, 1099–1128.
5. Xhou, W., Merrick, B. A., Khaledi, M. G., and Tomer, K. B. (2000) Detection and sequencing of phosphopeptides affinity bound to immobilized metal ion beads by matrix-assisted laser desorption/ionization mass spectrometry. *J. Am. Soc. Mass Spec.* **11**, 273–282.
6. Le Huerou, I., Wicker, C., Guilloteau, P., Toullec, R., and Puigserver, A (1990) Isolation and nucleotide sequence of cDNA clone for bovine pancreatic anionic trypsinogen. Structural identity within the trypsin family. *Eur. J. Biochem.* **193** 767–773.
7. Canova-Davis, E., Kessler, J., and Ling, V. T. (1991) Transpeptidation during the analytical proteolysis of proteins. *Anal. Biochem.* **196**, 39–45.

8. Vestling, M. M., Murphy, C. M., and Fenselau, C. (1990) Recognition of trypsin autolysis products by high-performance liquid chromatography and mass spectrmetry. *Anal. Chem.* **62**, 2391–2394.
9. Rice, R. H., Means, G. E., and Brown, W. D. (1977) Stabilization of bovine trypsin by reductive methylation. *Biochim. Biophys. Acta* **492**, 316–321.
10. Glazer, A. N., Delange, R. J., and Sigman, D. S. (1975) *Chemical Modification of Proteins*. Elsevier, North Holland, Amsterdam.
11. Drapeau, G. (1978) The primary structure of staphylococcal protease. *Can. J. Biochem.* **56**, 534–544.
12. Lamoyi, E. and Nisonoff, A. (1983) Preparation of $F(ab')_2$ fragments form mouse IgG of various subclasses. *J. Immunol. Meth.* **56**, 235–243.
13. Parham, P., Androlewicz, M. J. Brodsky, F. M., Holmes, N. J., and Ways, J. P. (1982) Monoclonal antibodies: Purification, fragmentation and application to structural and functional studies of class I MHC antigen. *J. Immunol. Meth.* **53**, 133–173.
14. Rousseaux, J., Rousseaux-Prevost, R., and Bazin, H. (1983) Optimal conditions for the preparation of Fab and $F(ab')_2$ fragments from monoclonal IgG of different rat IgG subclasses. *J. Immunol. Meth.* **64**. 141–146.
15. Sanchez, J.-C., Ravier, F., Pasquali, C., Frutiger, S., Paquet, N., Bjellqvist, B., et al. (1992) Improving the detection of proteins after transfer to polyvinylidene difluoride membranes. *Electrophoresis* **13**, 715–717.
16. Fernandez, J., DeMott, M., Atherton, D., and Mische, S. M. (1992) Internal protein sequence analysis: Enzymatic digestion of less than 10 mg of protein bound to polyvinylidene difluoride or nitrocellulose membranes. *Anal. Biochem.* **201**, 255–264.
17. Sutton, C. W., Pemberton, K. S., Cottrell, J. S., Corbett, J. M., Wheeler, C. H., Dunn, M. J., and Pappin, D. J. (1995) Identification of myocardial proteins from two-dimensional gels by peptide mass fingerprinting. *Electrophoresis* **16**, 308–316.
18. Rosenfeld., J., Capdevielle, J., Guillemot, J. C. and Ferrara, P. (1992) In-gel digestion of proteins for internal sequence analysis after one or two-dimensional gel electrophoresis. *Anal. Biochem.* **203**, 173–179.
19. Hellman, U, Wernstedt, C., Gonez, J and Heldin, C.-H. (1995) Improvement of an "in-gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem.* **224**, 451–455.
20. Jeno, P., Mini, T., Moes, S., Hintermann, E., and Horst, M. (1995) Internal sequences from proteins digested in polyacrylamide gels. *Anal. Biochem.* **224**, 75–82.

6

Chemical Cleavage of Polypeptides

Bryan John Smith

1. Introduction

Although proteins and peptides may be cleaved at various residues by use of endoproteolytic enzymes (*see* Chapter 5), they may be cleaved at still further sites by chemical methods. The most popular, best-yielding site for chemical cleavage is probably at the methionine residue. Cysteine is a significant residue, however, because it forms the disulfide bonds (by formation of cystine) which are so important in maintaining protein structure. Cleavage at cysteine may therefore be relevant to structural studies, and in any case can provide usefully large peptides because cysteine is a relatively uncommon amino acid. Likewise, cleavage of asparaginyl-glycyl bonds, and at tryptophanyl residues can provide large peptides. Further, since tryptophan is represented in the genetic code by a single codon, cleavage at that residue may be useful in cloning strategies in providing an unambiguous oligonucleotide sequence as part of a probe or primer. At the other extreme, aspartyl residues are relatively common, and cleavage of a protein there can generate a large number of small peptides (and partial cleavage products). Small proteins and peptides may be usefully cleaved at this point however, especially if other sites susceptible to other methods of cleavage are absent. Furthermore, partial hydrolysis can provide overlapping peptides, that can be analysed by mass spectrometric methods and used to order peptides in the sequence.

This chapter describes methods for chemical cleavage of Asn-Gly, Asp-X, Cys-X, Met-X, and Trp-X bonds, with the sample polypeptides being in solution. In recent years, however, methods have been adapted to suit samples on solid supports such as polyvinylidene difluoride (PVDF), as mentioned in the Notes section.

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Cleavage of Asn-Gly Bonds

1. Cleavage buffer: 2 *M* hydroxylamine-HCl, 2 *M* guanidine-HCl, 0.2 *M* K_2CO_3, pH 9.0. Use Analar grade reagents and HPLC grade water. Beware the mutagenic, toxic, and irritant properties of hydroxylamine. Wear protective clothing. Clear wet spillages with absorbent material or clear dry spillages with a shovel, and store material in containers prior to disposal.
2. Stopping solution: trifluoroacetic acid (TFA), 2% (v/v) in water. Both of HPLC-grade.

2.2. Cleavage of Asp-X Bond

1. Dilute hydrochloric acid (approx 0.013 *M*) pH 2 +/-0.04: dilute 220 µL of constant boiling (6 M) HCl to 100 mL with distilled water.
2. Pyrex glass hydrolysis tubes.
3. Equipment includes a blowtorch suitable for sealing the hydrolysis tubes, a vacuum line, and an oven for incubation of samples at 108°C.

2.3. Cleavage of Cys-X Bond

1. Modification buffer: 0.2 *M* tris acetate, pH 8.0, 6 *M* guanidine-HCl, 10 m*M* dithiothreitol (DTT). Use Analar grade reagents and HPLC grade water.
2. NTCB: 2-nitro-5-thiocyanobenzoate. Commercially available (Sigma) as yellowish powder. Contact with skin, eyes, etc., may cause short-term irritation. Long term effects are unknown, so handle with care (protective clothing). Sweep up spillages. Store at 0–5°C.
3. NaOH: sodium hydroxide solution, sufficiently concentrated to allow convenient alteration of reaction pH. For example 2 *M* in HPLC grade water.
4. Deblocking buffer: 50 m*M* Tris-HCl, pH 7.0.
5. Raney nickel-activated catalyst:
   Commercially available (e.g., from Sigma as 50% slurry in water, pH >9.0). Wash in deblocking buffer prior to use. A supply of N_2 gas is also required for use with the Raney nickel.

2.4. Cleavage of Met-X Bond

1. 0.4 *M* Ammonium bicarbonate solution in distilled water. Stable for weeks in refrigerated stoppered bottle.
2. 2-Mercaptoethanol. Stable for months in dark, stoppered, refrigerated bottle.
3. TFA, HPLC- or sequencing grade.
4. Cyanogen bromide. Stable for months in dry, dark, refrigerated storage. Warm to room temperature before opening. Use only white crystals, not yellow ones. Beware of the toxic nature of this reagent.
5. Sodium hypchlorite solution (domestic bleach).
6. Equipment includes a nitrogen supply, fume hood and suitably sized and capped tubes (e.g., Eppendorf microcentrifuge tubes).

2.5. Cleavage of Trp-X Bond

1. Oxidizing solution: mix together 30 vol glacial acetic acid, 15 vol 9 *M* HCl, and 4 vol dimethylsulfoxide. Use best-grade reagents. Though each of the constituents is stable separately, mix and use the oxidizing solution when fresh.
2. 15 *M* Ammonium hydroxide.
3. Cyanogen bromide solution in formic acid (60% v/v): make 6 mL formic acid (minimum assay 98%, Aristar grade) to 10 mL with distilled water. Add white crystalline cyanogen bromide to a concentration of 0.3 g/mL. Use when fresh. Store cyanogen bromide refrigerated in the dry and dark, where it is stable for months. Use only white crystals. Beware of the toxic nature of this reagent.
4. Sodium hypochlorite solution (domestic bleach).
5. Equipment includes a fume hood and suitably sized capped tubes (e.g., Eppendorf microcentrifuge tubes).

3. Methods

3.1. Cleavage of Asn-Gly Bonds (see Notes 1–7)

1. Dissolve the protein sample directly in the cleavage buffer, to give a concentration in the range 0.1–5 mg/mL. Alternatively, if the protein is in aqueous solution already, add 10 volumes of the cleavage buffer (i.e., sufficient buffer to maintain pH 9.0 and high concentration of guanidine-HCl and hydroxylamine). Use a stoppered container (Eppendorf tube or similar) with small headspace, so that the sample does not dry out during the following incubation.
2. Incubate the sample (in stoppered vial) at 45°C for 4 h.
3. To stop reaction, cool and acidify by addition of 3 volumes of stopping solution. Store frozen (–20°C) or analyze immediately.

3.2. Cleavage of Asp-X Bond (see Notes 8–13)

1. Dissolve the protein or peptide in the dilute acid to a concentration of 1–2 mg/mL in a hydrolysis tube.
2. Seal the hydrolysis tube under vacuum, i.e., with the hydrolysis (sample) tube connected to a vacuum line, using a suitably hot flame, draw out and finally seal the neck of the tube.
3. Incubate at 108°C for 2 h.
4. To terminate the reaction, cool and open the hydrolysis tube, dilute the sample with water, and lyophilize.

3.3. Cleavage of Cys-X Bond (see Notes 14–20)

1. Dissolve the polypeptide to a suitable concentration (e.g., 2 mg/mL) in the modification buffer (pH 8.0). To reduce disulfides in the DTT, incubate at 37°C for 1–2 h.
2. Add NTCB to 10-fold excess over sulphydryl groups in polypeptide and buffer. Incubate at 37°C for 20 min.
3. To cleave the modified polypeptide, adjust to pH 9.0 by addition of NaOH solution. Incubate at 37°C for 16 h or longer.

4. Dialyse against water. Alternatively, submit to gel filtration or reverse phase HPLC to separate salts and peptides. Lyophilize peptides.
5. If it is necessary to convert the newly formed iminothiazolidinyl N-terminal residue to an alanyl group, dissolve the sample to, say, 0.5 mg/mL in de-blocking buffer (pH 7.0) and add to Raney nickel (10-fold excess, w/w, over polypeptide) and incubate at 50°C for 7 h under an atmosphere of nitrogen. Cool and centrifuge briefly to pellet the Raney nickel. Store supernatant at –20°C, or further analyze as required.

3.4. Cleavage of Met-X Bond (see Notes 21–30)

1. Reduction:
   a. Dissolve the polypeptide in water to between 1 and 5 mg/mL, in a suitable tube. Add 1 vol of ammonium bicarbonate solution, and add 2-mercaptoethanol to between 1 and 5% (v/v).
   b. Blow nitrogen over the solution to displace oxygen, seal the tube, and incubate at room temperature for approx 18 h.
2. Cleavage:
   a. Dry down the sample under vacuum, warming if necessary to help drive off all of the bicarbonate. Any remaining ammonium bicarbonate will form a salt on subsequent reaction with acid.
   b. Redissolve the dried sample in TFA to 1–5 mg/mL. Add water to make the acid 50% (v/v) finally.
   c. Add excess white crystalline cyanogen bromide to the sample solution, to between two- and 100-fold molar excess over methionyl residues. Practically, this amounts to approximately equal weights of protein and cyanogen bromide. To very small amounts of protein, add one small crystal of reagent. Carry out this stage in the fume hood.
   d. Seal the tube and incubate at room temperature for 24 h.
   e. Terminate the reaction by drying down under vacuum. Store samples at –10°C or use immediately.
   f. Immediately after use, decontaminate equipment (spatulas, tubes and so on) that has contacted cyanogen bromide, by immersion in hypochlorite solution (bleach) until effervescence stops (a few minutes).

3.5. Cleavage of Trp-X Bond (see Notes 31–42)

1. Oxidation: dissolve the sample to approx 0.5 nmol/μL in oxidizing solution (e.g., 2–3 nmol in 4.9 μL oxidizing solution). Incubate at 4°C for 2 h.
2. Partial neutralization: to the cold sample, add 0.9 vol of ice cold NH_4OH (e.g., 4.4 μL of NH_4OH to 4.9 μL oxidized sample solution). Make this addition carefully so as to maintain a low temperature.
3. Cleavage: add 8 vol of cyanogen bromide solution. Incubate at 4°C for 30 h in the dark. Carry out this step in a fume hood.
4. To terminate the reaction, lyophilize the sample (all reagents are volatile).
5. Decontaminate equipment, such as spatulas, that have contacted cyanogen bromide, by immersion in bleach until the effervescence stops (a few minutes).

4. Notes

4.1. Asn-Gly Cleavage

1. The reaction involved in the cleavage of the Asn-Gly bond is illustrated in **Fig. 1** (with more detail provided by **refs.** ***1*** and ***2***). The reaction of hydroxylamine actually is with the cyclic imide which derives from the Asn-Gly pair. Asp-Gly cannot form this succinimide, so that bond is resistant to cleavage by hydroxylamine. Kwong and Harris ***(3)*** have reported cleavage at an Asp-Gly bond, via a presumed succinimide at that site. Bornstein and Balian ***(1)*** have reported an Asn-Gly cleavage yield of about 80% but yields are somewhat dependent on the sequence of the protein. Other reactions may occur upon treatment of polypeptide with hydroxylamine. Cleavage is to the C-terminal side of the succinimide. The peptide to the C-terminal side is available for N-terminal sequencing. Because Asp-Gly is relatively rare (about 0.25% of dipeptide sequences), quite large peptides may result from cleavage by hydroxylamine.

   The succinimide residue is involved in spontaneous asparagine deamidation and aspartate racemisation and isomerization, for it can hydrolyze in neutral or alkaline conditions to aspartyl-glycyl and isoaspartyl-glycyl (or α aspartyl-glycyl and β aspartyl-glycyl). The isomerization of Asp to iso-Asp can affect immunogenicity and function (for instance, *see* **ref.** ***[4]***). The succinimide is stable enough to be identified in proteins, the succinimidyl version being slightly more basic (by 1 net negative charge) than the aspartate version, which forms after incubation in neutral pH ***(3)***. Assays are available for quantification of iso-Asp (*see* **ref.** ***[5]*** and refs. therein). Both iso-Asp and succinimide are detected as a termination of peptide sequencing, for both are refractory to Edman chemistry. Cleavage by hydroxylamine may be used to map the positions of succinimides and presumed iso-Asp that may arise from them (e.g., **ref.** ***4***).
2. In addition to cleavage at Asn-Gly, there may be other, lower yielding cleavages. Bornstein and Balian ***(1)*** mention cleavage of Asn-Leu, Asn-Met, and Asn-Ala, while Hiller et al. ***(6)*** report cleavage of Asn-Gln, Asp-Lys, Gln-Pro, and Asn-Asp. Prolonged reaction times tend to generate more of such cleavages. Treatment with hydroxylamine may also generate hydroxamates of asparigine and glutamine, these modifications producing more acidic variants of the protein ***(7)***.
3. Inclusion of guanidine-HCl as a denaturant seems to be a factor in improving yields. Kwong and Harris ***(3)*** reported that omission of guanidine-HCl eliminated Asn-Gly cleavage while still allowing cleavage at Asp-Gly. However, the literature does have examples of the use of buffers lacking guanidine-HCl. **References** ***(6)*** and ***(7)*** exemplify the use of a Tris-HCl buffer of approximate pH 9.0, with **ref.** ***5*** including 1 m*M* EDTA and ethanol (10% v/v). Other examples ***(1,8,9)*** describe the use of more concentrated (6 *M*) guanidine-HCl.
4. As when making peptides by other chemical or enzymatic cleavage methods, it may be advisable, prior to the cleavage steps, to reduce disulfide bonds and alkylate cysteinyl residues (*see* Chapter 27). This denatures the substrate and prevents formation of inter-peptide disulfide bonds. Alkylation and subsequent cleavage by hydroxylamine on a few-μL scale is described in **ref.** ***(8)***.

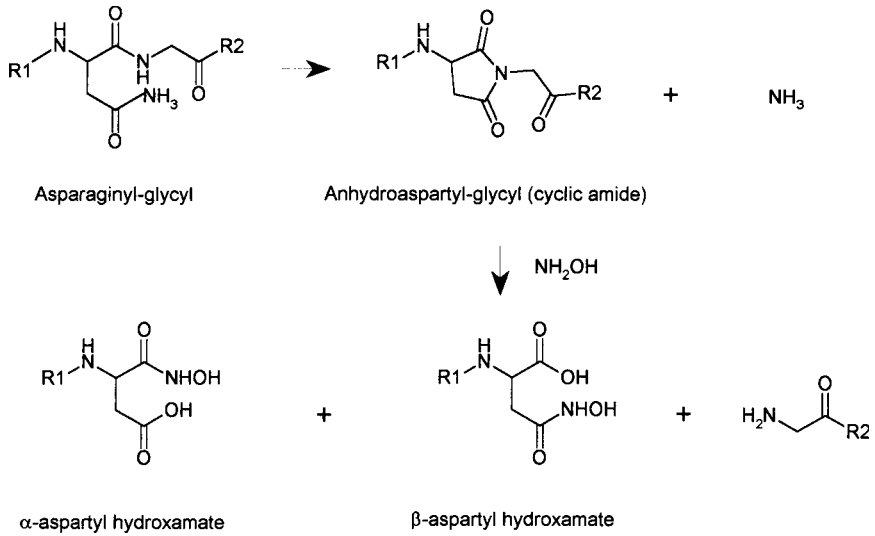


Fig. 1. Illustration of reactions leading to cleavage of Asn-Gly bonds by hydroxylamine.

5. After the cleavage reaction has been stopped by acidification, the sample may be loaded directly onto reverse-phase HPLC or gel filtration for analysis/peptide preparation. Alternatively, electrophoresis *(**6**)* will separate reactants and stop the reaction. Electrophoresis may be suitable for analysis of cleavage because large peptides are generally produced, but small peptides may be lost.
6. The hydroxylamine cleavage method has been adapted by Saris et al. *(**10**)* to cleave proteins in polyacrylamide gel pieces as follows:
   a. Wash gel piece(s) containing sample in 5% (v/v) methanol in order to remove SDS.
   b. Dry the gel pieces under vacuum.
   c. Submerge (and rehydrate) the gel pieces in cleavage solution, adding about 10–50 μL solution per 1 μL of gel piece. The cleavage solution is: 2 *M* hydroxylamine-HCl, 6 *M* guanidine-HCl. in 15 m*M* tris titrated to pH 9.3 by addition of 4.5 *M* lithium hydroxide solution. Preparation of the lithium hydroxide solution may generate insoluble carbonates, but these can be removed by filtration.
   d. Incubate at 45°C for 3 h.
   e. For analysis of cleavage, place the gel piece on the top of a second gel and undertake electrophoresis.

   Saris et al. *(**10**)* reported that peptides of 10,000 Da or less could be lost during washes of the gel piece, while about 10% of the sample remained bound to the treated gel piece. Recoveries were about 60% in the second (analytical) gel, and cleavage yield was about 25%. Recovery may be adversely affected by fixing of protein in the staining procedure (by the use of acidic stain or destain solutions, for example).

7. In approximately neutral pH conditions, reaction of protein with hydroxylamine may cause esterolysis, and so may be a useful method in studying post-translational modification of proteins. Thus, incubation in 1 *M* hydroxylamine, pH 7.0, 37°C for up to 4 h cleaved carboxylate ester-type ADP-ribose-protein bonds (on histones H2A and H2B) and arginine-ADP-ribose bonds (in histones H3 and H4) ***(11)***. Again, Weimbs and Stoffel ***(12)*** identified sites of fatty acid-acylated cysteine residues by reaction with 0.4 *M* hydroxylamine at pH 7.4, such that the fatty acids were released as hydroxamates. Omary and Trowbridge ***(13)*** adapted the method to release [^{3}H] palmitate from transferrin receptor in polyacrylamide gel pieces, soaking these for 2 h in 1 *M* hydroxylamine-HCl titrated to pH 6.6 by addition of sodium hydroxide.

4.2. Asp-X Cleavage

8. The bond most readily cleaved in dilute acid is the Asp-X bond, by the mechanism outlined in **Fig. 2A**. The bond X-Asp may also be cleaved, in lesser yields (*see* **Fig. 2B**). Thus, either of the peptides resulting from any one cleavage may keep the aspartyl residue at the point of cleavage, or neither might, if free aspartic acid is generated by a double cleavage event. Any of these peptides is suitable for sequencing.
9. The method described is that of Inglis ***(14)***. The amino acid sequence of the protein can affect the lability of the affected bond because the aspartic acid side chain can interact ionically with basic changes elsewhere in the molecule. Yields of cleavage are less than 100%, up to about 70% have been reported ***(14)***.

   The aspartyl-prolyl bond is particularly labile in acid. Landon ***(15)*** has suggested that cleavage of Asp-Pro bonds may be maximised by minimizing the effect of intramolecular interactions, this being achieved by use of denaturing agent, as follows:

   a. Dissolve the sample in guanidine.HCl (7 *M*) in acetic acid (10% v/v, adjusted to pH 2.5 by addition of pyridine).
   b. Incubate at 37°C for a prolonged period (e.g., 24 h).
   c. Terminate by lyophilization.

   Because of the influence of protein sequence, the results of incubation of polypeptide in dilute acid are somewhat unpredictable and best investigated empirically.
10. The conditions of low pH can be expected to cause a number of side reactions: cleavage at glutamyl residues; deamidation of (and possibly some subsequent cleavage at) glutaminyl and asparaginyl residues; partial destruction of tryptophan; cyclization of N-terminal glutaminyl residues to residues of pyrolidone carboxylic acid; α-β shift at aspartyl residues. The last two changes create a blockage to Edman degradation. The short reaction time of 2 h is intended to minimize these side reactions. A small degree of loss of formyl or acetyl groups from N-termini ***(14)*** is another possible side reaction but is not recognized as a significant problem, generally.
11. The method described has the benefit of simplicity. It is carried out in a single reaction vessel, with reagents being removed by lyophilization at the end of reac-

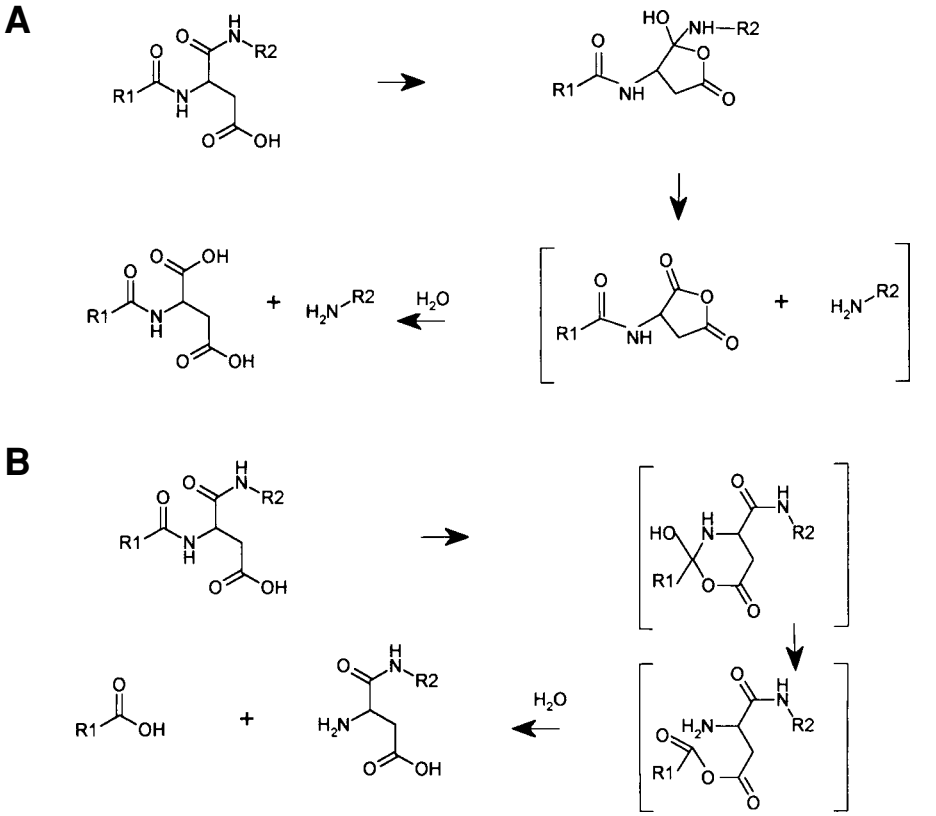


Fig. 2. Mechanisms of the cleavage of bonds (**A**) to the COOH side and (**B**) to the NH_2 side of aspartyl residues in dilute acid.

tion. Thus, sample handling and losses incurred during this are minimized. This makes it suitable for sub-nanomolar quantities of protein, though the method may be scaled up for larger amounts also.

12. A polypeptide substrate that is insoluble in cold dilute HCl may dissolve during the incubation at 108°C. Formic acid is a good protein denaturant and solvent and may be used instead of HCl as follows: Dissolve the sample in formic acid (minimum assay 98%, Aristar grade), then dilute 50-fold to pH 2.0; proceed as in method for HCl. Note, however, that incubation of protein in formic acid may result in formylation (detected as a 28 amu increase in mass *[**16**]*) and damage to tryptophan and tyrosine residues (altered spectral properties *[**17**]*).
13. Note that bonds involving aspartyl residues may also be cleaved by commercially available enzymes: endoproteinase Asp-N hydrolyses the bond to the N-terminal side of an aspartyl residue, but also of a cysteinyl residue; Glu-C cleaves the bond to the C-terminal side of glutamyl and aspartyl residues.

4.3. Cys-X Cleavage

14. The reactions involved in the method for Cys-X cleavage are illustrated in **Fig. 3**. The method described is basically that used by Swenson and Frederickson *(**18**)*, an adaptation of that of Jacobson et al. (*19*; also *see* **ref. 20**). The principle difference is that the earlier method *(**19,20**)* describes desalting (by gel filtration or dialysis) at the end of the modification step (**Subheading 3.3.**, **step 2**), followed by lyophilization and redissolution in a pH 9.0 buffer to achieve cleavage. Simple adjustment of pH as described in **Subheading 3.3.**, **step 3** has the advantages of speed and avoiding the danger of sample loss upon desalting.

    With conversion of the iminothiazolidinyl residue to an alanyl residue (in **Subheading 3.3.**, **step 5**), the peptide to the C-terminal side of the cleavage point is available for sequencing by Edman chemistry. If blockage of the N-terminal residue of the newly generated peptide to the C-terminal side of the cleavage point is not a problem (i.e. if sequencing is not required) **Subheading 3.3.**, **step 5** may be omitted.
15. Swenson and Frederickson *(**18**)* describe cleavage (**Subheading 3.3.**, **step 3**) at 37°C for 6 h, but report yields of 60–80%. Other references recommend longer incubations of 12 h or 16 h at 37°C to obtain better yields *(**19–21**)*.
16. Peyser et al. *(**21**)* have described a slightly modified procedure that may be more convenient for treating small samples. The procedure is as follows:
    a. Dissolve the sample to 1 mg/mL in a buffer of borate (20 m*M*) pH 8.0, urea (6 *M*).
    b. Add NTCB (0.1 *M* solution in 33% [v/v] dimethylformamide) at the rate of 40 μL of sample solution.
    c. Incubate at 25°C for 1 h.
    d. Adjust to pH 9.0 by addition of NaOH. Incubate at 55°C for 3 h. This brings about cleavage.
    e. Stop the reaction by addition of 2-mercaptoethanol to 80-fold excess over NCTB.
17. The conditions for reduction may be altered (**Subheading 3.3.**, **step 1**). Thus, if the sample contains no intramolecular or intermolecular disulfide bonds, the dithiothreitol (DTT) content of the modification buffer may be less, at 1 m*M*. Beware that nominally non-bonded cysteinyl residues may be involved in mixed disulfides with such molecules as glutathione or free cysteine. Reduction may be omitted altogether to allow reaction with native protein. Cys residues that remain protected in the native protein remain noncyanylated on reaction with 2-nitro-5-thiocyanobenzoate, and remain uncleaved upon alteration of pH to pH 9.0. Thus Cys residues that are buried within a native protein's structure or in a complex of proteins can be mapped within the proteins sequence, and regions involved in protein-protein interaction "footprinted" *(**22**)*.
18. Although Raney nickel is available commercially, Otiene *(**23**)* has reported that a more efficient catalyst may be obtained by the method he described, starting from Raney nickel-aluminium alloy. This is reacted with NaOH, washed, deionized and washed again (under H_2 gas).

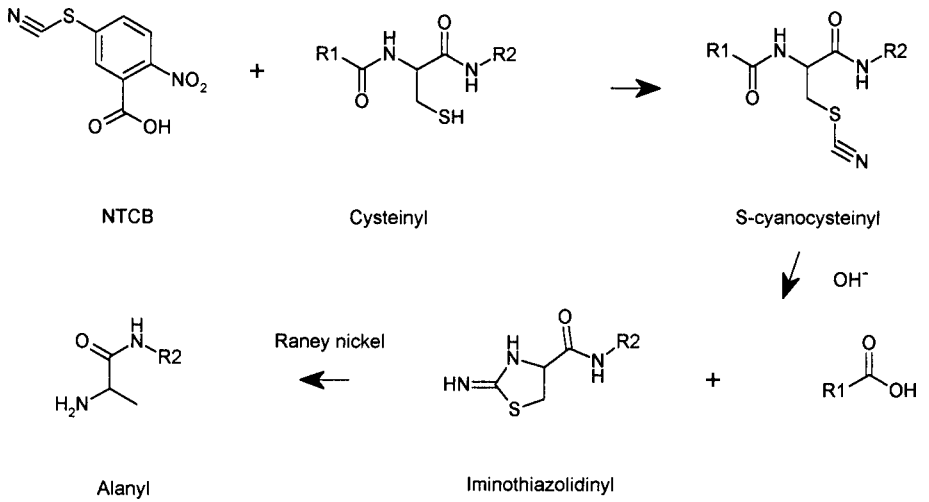


Fig. 3. Reactions in modification of, and cleavage at, cysteinyl residues by NTCB, and subsequent generation of alanyl N-terminal residue.

19. Treatment with Raney nickel (**Subheading 3.3., step 5**) causes desulfurization of residues. Thus, methioninyl residues are converted to 2-aminobutyryl residues. If the sample has not been reacted with NTCB, then remaining cysteinyl and cystinyl residues are converted to alanyl residues (without any cleavage). Otiene *(23)* has suggested that this modification might be used to study the dependence of protein function on Met and Cys content.
20. Endoproteinase Asp-N catalyses hydrolysis of bonds to the N-terminal side of either aspartyl or cysteinyl residues. Specificity for X-Cys can be generated by modification of aspartyl side chains *(24)*. Modification of cysteinyl residues to 2-aminoethylcysteinyl residues renders the Cys-X bond susceptible to cleavage by trypsin, and the X-Cys bond to cleavage by Lys-N, as discussed in **ref.** *(25)*.

4.4. Met-X Cleavage

21. The mechanism of the action of cyanogen bromide on methionine-containing peptides is shown in **Fig. 4**. For further details, see the review by Fontana and Gross *(26)*. The methioninyl residue is converted to homoseryl or homoseryl lactone. Peptides generated are suitable for peptide sequencing by Edman chemistry. Methionine sulfoxide does not take part in this reaction and the first step in the method is intended to convert any methionyl sulphoxide to methionyl residues, and so maximize cleavage efficiency. If the reduction is not carried out, the efficiency of cleavage may not be greatly diminished. If virtually complete cleavage is not necessary, partial cleavage products are desired (*see* **Note 26**), the sample is small and difficult to handle without loss, or speed is critical, the reduction step may be omitted.

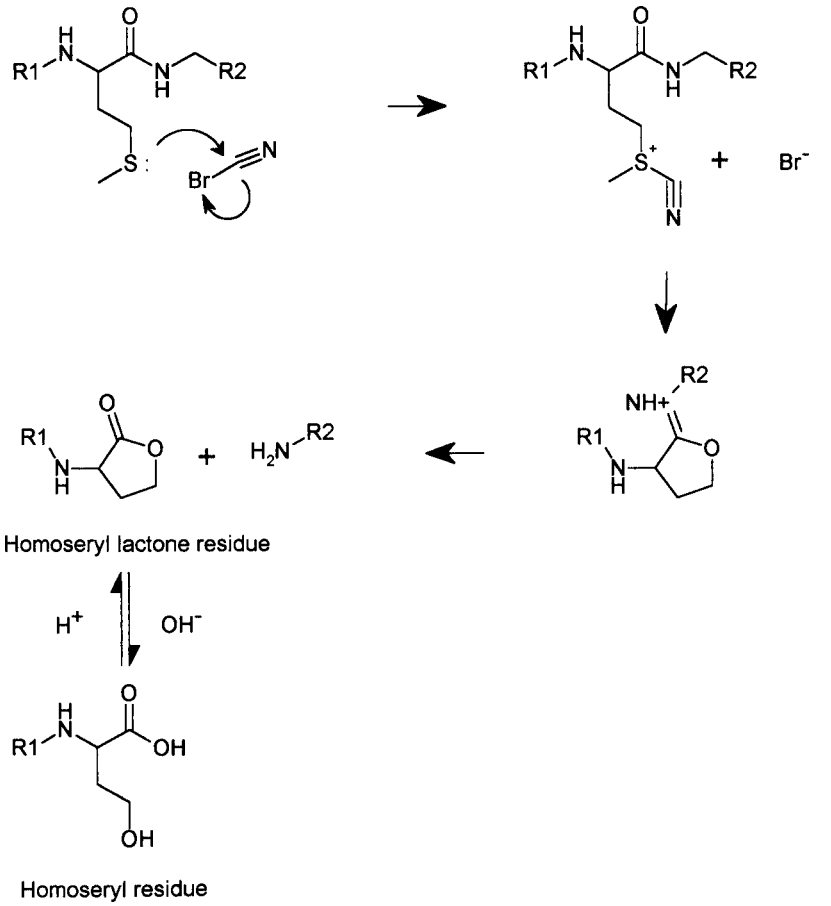


Fig. 4. Mechanism of cleavage of Met-X bonds by cyanogen bromide.

An acid environment is required to protonate basic groups and so prevent reaction there and maintain a high degree of specificity. Met-Ser and Met-Thr bonds may give significantly less than 100% yields of cleavage and simultaneous conversion to methionyl to homoseryl residues within the uncleaved polypeptide. This is because of the involvement of the β-hydroxyl groups of seryl and threonyl residues in alternative reactions, which do not result in cleavage *(26)*. Morrison et al. *(27)* however, have found that use of 70% (v/v) TFA gives a better yield of cleavage of a Met-Ser bond in apolipoprotein A1 than does use of 70% formic acid (*see* **Note 2**). Using model peptides, Kaiser and Metzka *(28)* have analyzed the cleavage reaction at Met-Ser and Met-Thr and concluded that cleavage that efficiency is improved by increasing the amount of water present, and for practical purposes 0.1 *M* HCl is a good acid to use, giving about 50%

cleavage of these difficult bonds. Remaining uncleaved molecules contained either homoserine or methionyl sulfoxide instead of the original methionyl. Cleavage efficiency improved with increasing strength of acid, but there was an accompanying risk of degradation in the stronger acids.

22. Acid conditions are required for the reaction to occur. 70% (v/v) formic acid (pH 1.0) was formerly commonly used because it is a good protein solvent and denaturant, and also volatile. However, it may damage tryptophan and tyrosine residues *(27)* and also cause formylation of Seryl and Threonyl side chains (showing up during analysis by mass spectroscopy as an increase of 28 amu per modification *[28,29]*). Use of other acids avoids this problem. Trifluoroacetic acid (TFA) (also volatile) may be used in concentrations in the range 50% to 100% (v/v). The pH of such solutions is approx pH 0.5 or less. The rate of cleavage in 50% TFA may be somewhat slower than in 70% formic acid, but similar reaction times of hours, up to 24 h will provide satisfactory results. Caprioli et al. *(30)* and Andrews et al. *(31)* have illustrated the use of 60% and 70% TFA (respectively), for cyanogen bromide cleavage of proteins. Acetic acid (50%–100% v/v) may be used as an alternative but reaction is somewhat slower than in TFA. Alternatively, 0.1 *M* HCl has been used *(28,29)*. To increase solubilization of proteins, urea, or guanidine-HCl may be added to the solution. Thus, in 0.1 *M* HCl, 7 *M* urea, for 12 h at ambient temperature, a Met-Ala bond was cleaved with 83% efficiency, and the more problematical Met-Ser and Met-Thr bonds with 56% and 38% efficiency (respectively) *(28)*.
23. Although the specificity of this reaction is excellent, some side reactions may occur. This is particularly so if colored (yellow or orange) cyanogen bromide crystals are used, when destruction of Tyr and Trp residues may occur.

    The acid conditions employed for the reaction may lead to small degrees of deamidation of glutamine and asparagine side chains (which occurs below pH 3.0) and cleavage of acid-labile bonds, e.g., Asp-Pro. A small amount of oxidation of cysteine to cysteic acid may occur, if these residues have not previously been reduced and modified (e.g.. carboxymethylated). Occasional cleavage of Trp-X bonds may be seen, but this does not occur with good efficiency, as it does when the reduction step of this technique is replaced by an oxidation step (*see* **Subheading 4.5.** for cleavage of Trp-X bonds). Rosa et al. *(32)* cleaved both Met-X and Trp-X bonds simultaneously by treatment of protein with 12 m*M* cyanogen bromide in 70% TFA solution, plus 240 μ*M* potassium bromide.
24. The protocol in **Subheading 4.5.** describes addition of solid cyanogen bromide to the acidic protein solution, to give a molar excess of cyanogen bromide over methionyl residues. This has the advantage that pure white crystals may be selected in favor of pale yellow ones showing signs of degradation (*see* **Note 23**). It does not allow accurate estimation of the quantity of reagent used, however. The work of Kaiser and Metzka *(28)* suggests that more than a 10-fold molar excess of cyanogen bromide over methionyl residues does not increase the extent of cleavage. If in doubt as to the concentration of methionyl residues, however, err on the side of higher cyanogen concentration.

If accurate quantification of cyanogen bromide is required, solid cyanogen bromide may be weighed out and dissolved to a given concentration by addition of the appropriate volume of 70% (v/v) TFA, and the appropriate volume of that solution added to the sample. The cyanogen bromide will start to degrade once in aqueous acid, so use when fresh. An alternative is to dissolve the cyanogen bromide in acetonitrile, in which it is more stable. Cyanogen bromide in acetonitrile solution is available commercially, for instance, at a concentration of 5 *M* (Aldrich). While such a solution may be seen to be degrading by its darkening color, this is not so obvious as it is with cyanogen bromide in solid form. For use, sufficient acetonitrile solution is added to the acidic protein solution to give the desired excess of cyanogen bromide over protein (e.g., 1/20 dilution of a 5 *M* cyanogen bromide solution to give a final 250 m*M* solution). The data of Kaiser and Metzka ***(28)*** indicate that high concentrations (70–100%) of acetonitrile can interfere with the cleavage reaction by decreasing the amount of water present, but below a concentration of 30% (in 0.1 *M* HCl) the effect is noticeable in causing a small decrease of Met-Ser and Met-Thr bond cleavage, but negligible for the Met-Ala bond.

25. The reagents used for Met-X cleavage are removed by lyophilization, unless salt has formed following failure to remove all of the ammonium bicarbonate. The products of cleavage may be fractionated by the various forms of electrophoresis and chromatography currently available. If analyzed by reverse-phase HPLC, the reaction mixture may be applied to the column directly without lyophilization. Since methionyl residues are among the less common residues, peptides resulting from cleavage at Met-X may be large and so in HPLC, use of wide-pore column materials may be advisable (e.g., 30-μ*M* pore size reverse-phase columns, using gradients of acetonitrile in 0.1% [v/v] TFA in water). Beware that some large peptides that are generated by this technique may prove to be insoluble (for instance if the solution is neutralized after the cleavage reaction) and so form aggregates and precipitates.
26. Incomplete cleavage that generates combinations of (otherwise) potentially cleaved peptides may be advantageous, for determination of the order of peptides within a protein sequence. Mass spectrometric methods are suitable for this type of analysis ***(29)***. Such partial cleavage may be achieved by reducing the duration of reaction, even to less than 1 h ***(29)***.
27. Methods have been described for cyanogen bromide treatment of low μg amounts of proteins in polyacrylamide gel ***(33)***, on PVDF ***(34)***, or on glass fiber, as used in automated protein sequencers ***(31)***. The method described for treating a protein in polyacrylamide gel ***(33)*** is as follows:
    a. Lyophilize the piece of gel containing the protein of interest.
    b. Expose the gel piece to vapor from a solution of cyanogen bromide in TFA, for 24 h at room temperature, in the dark. The vapor is generated from a solution of 20 mg cyanogen bromide per mL of 50% (v/v) TFA, by causing it to boil under partial vacuum. A sealed container is used for this incubation.
    c. Lyophilize the treated piece of gel.

d. Analyze cleavage by electrophoresis from the treated gel piece into a second gel.

28. For treatment of protein on PVDF *(34)* the method is as follows:
    a. Cut the protein band of interest from the PVDF, cutting closely around the band (since excess PVDF can reduce the final yield of peptide).
    b. Wet the dry PVDF piece with about 50 µL of cyanogen bromide solution in 50% (v/v) TFA (or 70% v/v TFA, or 70% v/v, formic acid - all of which can directly wet PVDF). Stone et al. *(34)* suggested application of cyanogen bromide at the rate of about 70 µg per 1 g of protein.
    c. Incubate in a sealed tube (to prevent drying out), at room temperature, 24 h, in the dark.
    d. Peptides generated may be extracted in the incubation solution itself, then successively in washes in 100 µL acetonitrile (40% v/v, 37°C, 3 h) and 100 µL TFA (0.05% [v/v] in 40% acetonitrile, 50°C). Pool extracts, dilute in water (to reduce acetonitrile concentration) and apply to reverse-phase HPLC, or dry down for analysis by PAGE.

    If the protein is run on PAGE prior to blotting onto PVDF, there is not a significant problem of methionine oxidation during electrophoresis; Stone et al. *(34)* reported approx 100% cleavage of myoglobin in these circumstances.

29. Protein may be treated with cyanogen bromide after having been subjected to Edman sequencing chemistry in an automated sequencer. This is useful for circumventing N-terminal blockage or for testing the alternatives of blockage or no sample in the event of failing to obtain any sequence. The method is similar to that described in **Note 28**, applicable either if the sample has been applied to a glass-fiber disk or to a piece of PVDF in the sequencer reaction cartridge. The method is as follows:
    a. Remove the glass fiber or PVDF from the sequencer, or leave in place in the reaction cartridge.
    b. Saturate the glass-fiber or PVDF piece with a fresh solution of cyanogen bromide in 50% (v/v) TFA (or 70% v/v formic acid). Make the cyanogen bromide solution to 100 mg/mL in the acid.
    c. Wrap the reaction cartridge, or loose glass fiber or PVDF in a small capped tube, in sealing film to prevent drying out. Incubate at room temperature in the dark, 24 h.
    d. Dry the sample under vacuum. Replace in the sequencer and start sequencing again. Yields tend to be poorer than the standard method described above for protein solutions; they may be down to 50% or less, and other, non-Met-X bonds may be cleaved at still lower yields. If the sample contains more than one methioninyl residue, more than one new N-terminus is generated. This may be simplified by subsequent reaction with orthophthalaldehyde, which blocks all N-termini except those bearing a prolyl residue *(35)*. For this approach to work, prior knowledge is required of the location of prolyl residues in the sequence, so that the orthophthalaldehyde reaction may be conducted at the correct cycle.

In order to test rapidly for the presence of any sample, a piece of CNBr-soaked PVDF may be treated at 65°C, for 1 h, followed by drying and sequencing. Yields, again, can be 30–50% with some (unpredictable) preference in Met-X bond cleavage, with somewhat greater levels of non-Met-X bond cleavage.

30. As described in **Note 21**, the peptide to the N-terminal side of the point of cleavage, has at its C-terminus a homoserine or homoserine lactone residue. The lactone derivative of methionine can be coupled selectively and in good yield *(**36**)* to solid supports of the amino type, e.g., 3-amino propyl glass. This is a useful technique for sequencing peptides on solid supports. The peptide from the C-terminus of the cleaved protein will, of course, not end in homoserine lactone (unless the C-terminal residue was methionine!) and so cannot be so readily coupled. Similarly, the C-terminal peptide carboxyl can react (if not amidated) with acidic methanol, to become a methyl ester (with a corresponding mass increment of 14 amu). Homoserine lactone, present as the C-terminal residue on other peptides in a cyanogen bromide digest, will react with acidic methanol and show a mass increase of 32 amu. With account made for side chain carboxyl residues, this is a means to identify C-terminal peptides by mass spectroscopy *(**37**)*.

4.5. Trp-X Cleavage

31. The method described for Trp-X bond cleavage is that of Huang et al. *(**38**)*. Although full details of the mechanism of this reaction are not clear, it is apparent that tryptophanyl residues are converted to oxindolylalanyl residues in the oxidation step, and the bond to the C-terminal side at each of these is readily cleaved in excellent yield (approaching 100% in **ref.** *[**39**]*) by the subsequent cyanogen bromide treatment. The result is seemingly unaffected by the nature of the residues surrounding the cleavage site.

    During the oxidation step, methionyl residues become protected by conversion to sulfoxides, bonds at these residues not being cleaved by subsequent cyanogen bromide treatment. Cysteinyl residues will also suffer oxidation if they have not been reduced and alkylated beforehand (*see* Chapter 27). Rosa et al. *(**32**)* cleaved both Trp-X and Met-X bonds simultaneously by omission of the oxidation step and inclusion of 240 μ*M* potassium iodide in the reaction of protein with 12 m*M* cyanogen bromide.

    The peptide to the C-terminal side of the cleavage point has a free N-terminus and so is suitable for sequencing.

32. Methioninyl sulfoxide residues in the peptides produced may be converted back to the methioninyl residues by the action (in aqueous solution) of thiols (e.g., DTT, as described in **ref.** *[**39**]*, or as described in **Subheading 3.3.**, **step 1**, or **Subheading 3.4.**, **step 1**).

33. The acid conditions used for oxidation and cleavage reactions seem to cause little deamidation *(**38**)*, but one side reaction that can occur is hydrolysis of acid-labile bonds. The use of low temperature minimises this problem. If a greater degree of such acid hydrolysis is not unacceptable, speedier, and warmer alternatives to the reaction conditions described earlier can be used as follows:

a. Oxidation at room temperature for 30 min, but cool to 4°C before neutralization.
b. Cleavage at room temperature for 12–15 h.

34. As alternatives to the volatile base NH_4OH, other bases may be used (e.g., the nonvolatile potassium hydroxide or tris base).
35. As mentioned in **Note 22**, it has been found that use of 70% (v/v) formic acid can cause formylation of the polypeptide (seen as a 28 amu increase in molecular mass *[29]*) and damage to tryptophan and tyrosine (evidenced by spectral changes *[27]*). As an alternative to 70% formic acid, 5 *M* acetic acid may be used. Possibly, as in the use of cyanogen bromide in cleaving Met-X bonds 50% or 70% (v/v) TFA may prove an acceptable alternative *(32)*.
36. Samples of protein that have been eluted from sodium dodecylsulfate (SDS) gels may be treated as described, but for good yields of cleavage, Huang et al. *(32)* recommend that the sample solutions are acidified to pH 1.5 before lyophilization in preparation for dissolution in the oxidizing solution. Any SDS present may help to solubilize the substrate and, in small amounts at least, does not interfere with the reaction. However, nonionic detergents that are phenolic or contain unsaturated hydrocarbon chains (e.g., Triton, Nonidet P-40), and reducing agents are to be avoided.
37. The method is suitable for large-scale protein cleavage; this requires simple scaling up. Huang et al. *(38)* made two points, however:
    a. The neutralization reaction generates heat. Since this might lead to protein or peptide aggregation, cooling is important at this stage. Ensure that the reagents are cold and are mixed together slowly and with cooling. A transient precipitate may be seen at this stage. If the precipitate is insoluble, addition of SDS may solubilize it (but will not interfere with the subsequent treatment).
    b. The neutralization reaction generates gases. Allow for this by choosing a reaction vessel with reasonably large headspace.
38. At the end of the reaction, all reagents may be removed by lyophilization and the peptide mixture analyzed, for instance by polyacrylamide gel electrophoresis or by reverse-phase HPLC. Peptides generated may tend to be large, ranging up to a size in the order of 10,000 Da or more. Some of these large peptides may not be soluble, for instance if the solution is neutralized following the cleavage reaction, and consequently they aggregate and precipitate.
39. Note that all reactions are done in one reaction vial, eliminating transfer of sample between vessels, and so minimizing peptide losses that can occur in such exercises.
40. Various alternative methods for cleavage of the Trp-X bond have been described in the literature. The method that employs N-chlorosuccinimide is possibly the most specific, but shows only about 50% cleavage yield *(40)*. BNPS-skatole is a popular Trp-X-cleaving reagent whose reaction and products have been studied in some detail (for instance, *see* **refs.** *[41]* and *[42]*).
41. Methods have been described for Trp-X bond cleavage in small amounts (μg or less) of protein on solid supports or in polyacrylamide gel. These use N-chlorosuccinimide or BNPS-skatole (3-bromo-3-methyl-2-(2'-nitrophenylsulphenyl)-indolenine). For cleavage of protein in gel *(43)*:

a. Soak the gel piece for 30 min in a small volume of the solution: N-chlorosuccinimide, 0.015 *M* in urea (0.5 g/mL in 50% v/v acetic acid).
b. Wash the gel piece and electrophorese peptides from the treated gel into a second analytical gel.

42. Proteins bound to glass fiber (as used in protein sequencers) or to PVDF may be cleaved at Trp-X bond(s) by the method described in **ref.** ***(44)***:
    a. The glass-fiber disk, or PVDF, is wetted with a solution of BNPS-skatole (1 μg/mL in 70% v/v acetic acid).
    b. Incubate in a sealed container to prevent drying out, at 47°C, 1 h in the dark.
    c. Dry under vacuum. Replace in the sequencer and start sequencing.

References

1. Bornstein, P. and Balian, G. (1977) Cleavage at Asn-Gly bonds with hydroxylamine. *Methods Enzymol.* **47**, 132–145.
2. Blodgett, J. K., Londin, G. M., and Collins, K. D. (1985) Specific cleavage of peptides containing an aspartic acid (beta-hydroxamic) residue. *J. Am. Chem. Soc.* **107**, 4305–4313.
3. Kwong, M. Y., and Harris, R. J. (1994) Identification of succinimide sites in proteins by N-terminal sequence analysis after alkaline hydroxylamine cleavage. *Protein Sci.* **3**, 147–149.
4. Cacia, J., Keck, R., Presta, L. G., and Frenz, J. (1996) Isomerization of an aspartic acid residue in the complementarity-determining regions of a recombinant antibody to human IgE: identification and effect on binding affinity. *Biochemistry* **35**, 1897–1903.
5. Schurter, B. T. and Aswad, D. A. (2000) Analysis of isoaspartate in peptides and proteins without the use of radioisotopes. *Anal. Biochem.* **282**, 227–231.
6. Hiller, Y., Bayer, E. A., and Wilchek, M. (1991) Studies on the biotin-binding site of avidin. Minimised fragments that bind biotin. *Biochem. J.* **278**, 573–585.
7. Canova-Davis, E., Eng, M., Mukka, V., Reifsnyder, D. H., Olson, C. V., and Ling, V. T. (1992) Chemical heterogeneity as a result of hydroxylamine cleavage of a fusion protein of human insulin-like growth factor I. *Biochem. J.* **278**, 207–213.
8. Niles, E. G. and Christen, L. (1993) Identification of the vaccinia virus mRNA guanyltransferase active site lysine. *J. Biol. Chem.* **268**, 24986–24989.
9. Arselin, G., Gandar, J. G., Guérin, B., and Velours, J. (1991) Isolation and complete amino acid sequence of the mitochondrial ATP synthase ϵ-subunit of the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266**, 723–727.
10. Saris, C. J. M., van Eenbergen, J., Jenks, B. G., and Bloemers, H. P. J. (1983) Hydroxylamine cleavage of proteins in polyacrylamide gels. *Anal. Biochem.* **132**, 54–67.
11. Golderer, G. and Gröbner, P. (1991) ADP-ribosylation of core histones and their acetylated subspecies. *Biochem. J.* **277**, 607–610.
12. Wiembs, T. and Stoffel, W. (1992) Proteolipid protein (PLP) of CNS myelin: Positions of free, disulfide-bonded and fatty acid thioester-linked cysteine residues and implications for the membrane topology of PLP. *Biochemistry* **31**, 12289–12296.

13. Omary, M. B. and Trowbridge I. S. (1981) Covalent binding of fatty acid to the transferrin receptor in cultured human cells. *J. Biol. Chem.* **256**, 4715–4718.
14. Inglis, A. S. (1983) Cleavage at aspartic acid. *Methods Enzymol.* **91**, 324–332.
15. Landon, M. (1977) Cleavage at aspartyl-prolyl bonds. *Methods Enzymol.* **47**, 132–145.
16. Beavis, R. C. and Chait, B. T. (1990) Rapid, sensitive analysis of protein mixtures by mass spectrometry. *Proc. Natl. Acad. Sci. USA* **87**, 6873–6877.
17. Morrison, J. R., Fiolge, N. H., and Grego, B. (1990) Studies on the formation, separation and characterisation of cyanogen bromide fragments of human A1 apolipoprotein. *Anal. Biochem.* **186**, 145–152.
18. Swenson, C. A. and Frederickson, R. S. (1992) Interaction of troponin C and troponin C fragments with troponin I and the troponin I inhibitory peptide. *Biochemistry* **31**, 3420–2427.
19. Jacobson, G. R., Schaffer, M. H., Stark, G., and Vanaman, T. C. (1973) Specific chemical cleavage in high yield at the amino peptide bonds of cysteine and cystine residues. *J. Biol. Chem.* **248**, 6583–6591.
20. Stark, G. R. (1977) Cleavage at cysteine after cyanylation. *Methods Enzymol.* **47**, 129–132.
21. Peyser, Y. M., Muhlrod, A., and Werber, M. M. (1990) Tryptophan-130 is the most reactive tryptophan residue in rabbit skeletal myosin subfragment-1. *FEBS Lett.* **259**, 346–348.
22. Tu, B. P. and Wang, J. C. (1999) Protein footprinting at cysteines: probing ATP-modulated contacts in cysteine-substitution mutants of yeast DNA topoisomerase II. *Proc. Natl. Acad. Sci. USA* **96**, 4862–4867.
23. Otiene, S. (1978) Generation of a free α-amino group by Raney nickel after 2-nitrothiocyanobenzoic acid cleavage at cysteine residues: applications to automated sequencing. *Biochemistry* **17**, 5468–5474.
24. Wilson, K. J., Fischer, S., and Yuau, P. M. (1989) Specific enzymatic cleavage at cystine/cysteine residues. The use of Asp-N endoproteinase, in *Methods in Protein Sequence Analysis* (Wittman-Liebold, B., ed.), Springer-Verlag, Berlin, 310–314.
25. Aitken, A. (1994) Analysis of cysteine residues and disulfide bonds, in *Methods in Molecular Biology, vol. 32: Basic Protein and Peptide Protocols* (Walker, J. M., ed.), Humana Press, Inc., Totowa, NJ, pp. 351–360.
26. Fontana, A. and Gross, E. (1986) Fragmentation of polypeptides by chemical methods, in *Practical Protein Chemistry: A Handbook* (Darbre, A., ed.), Wiley, Chichester, UK, pp. 67–120.
27. Morrison, J. R., Fidge, N. H., and Grego, B. (1990) Studies on the formation, separation, and characterisation of cyanogen bromide fragments of human A1 apolipoprotein. *Anal. Biochem.* **186**, 145–152.
28. Kaiser, R. and Metzka, L. (1999) Enhancement of cyanogen bromide cleavage yields for methionyl-serine and methionyl-threonine peptide bonds. *Anal. Biochem.* **266**, 1–8.
29. Beavis, R. C. and Chait, B. T. (1990) Rapid, sensitive analysis of protein mixtures by mass spectrometry. *Proc. Natl. Acad. Sci. USA* **87**, 6873–6877.

30. Caprioli, R. M., Whaley, B., Mock, K. K., and Cottrell, J. S. (1991) Sequence-ordered peptide mapping by time-course analysis of protease digests using laser description mass spectrometry in *Techniques in Protein Chemistry II* (Angeletti, R. M., ed.), Academic Press Inc., San Diego, CA, pp. 497–510.
31. Andrews, P. C., Allen, M. M., Vestal, M. L., and Nelson, R. W. (1992) Large scale protein mapping using infrequent cleavage reagents, LD TOF MS, and ES MS, in *Techniques in Protein Chemistry II* (Angeletti, R. M., ed.), Academic Press Inc., San Diego, CA, pp. 515–523.
32. Rosa, J. C., de Oliveira, P. S. L., Garrat, R., Beltramini, L., Roque-Barreira, M.-C., and Greene, L. J. (1999) KM+, a mannose-binding lectin from Artocarpus integrifolia: amino acid sequence, predicted tertiary structure, carbohydrate recognition, and analysis of the beta-prism fold. *Protein Sci.* **8**, 13–24.
33. Wang, M. B., Boulter, D., and Gatehouse, J. A. (1994) Characterisation and sequencing of cDNA clone encoding the phlorem protein pp2 of Cu curbita pepo. *Plant Mol. Biol.* **24**, 159–170.
34. Stone, K. L., McNulty, D. E., LoPresti, M. L., Crawford, J. M., DeAngelis, R., and Williams, K. R. (1992) Elution and internal amino acid sequencing of PVDF blotted proteins, in *Techniques in Protein Chemistry III* (Angeletti, R. M., ed.), Academic Press Inc., San Diego, CA, pp. 23–34.
35. Wadsworth, C. L., Knowth, M. W., Burrus, L. W., Olivi, B. B., and Niece R. L. (1992) Reusing PVDF electroblotted protein samples after N-terminal sequencing to obtain unique internal amino acid sequence, in *Techniques in Protein Chemistry III* (Angeletti, R. M., ed.), Academic Press Inc., San Diego, CA, pp. 61–68.
36. Horn, M. and Laursen, R. A. (1973) Solid-phase Edman degradation. Attachment of carboxyl-terminal homogenine peptides to an insoluble resin. *FEBS Lett.* **36**, 285–288.
37. Murphy, C. M. and Fenselau, C. (1995) Recognition of the carboxy-terminal peptide in cyanogen bromide digests of proteins. *Anal. Chem.* **67**, 1644–1645.
38. Huang, H. V., Bond, M. W., Hunkapillar, M. W., and Hood, L. E. (1983) Cleavage at tryptophanyl residues with dimethyl sulfoxide-hydrochloric acid and cyanogen bromide. *Methods Enzymol* **91**, 318–324.
39. Tseng, A., Buchta, R., Goodman, A. E., Loughman, M., Cairns, D., Seilhammer, J. et al. (1991) A strategy for obtaining active mammalian enzyme from a fusion protein expressed in bacteria using phospholipase A2 as a model. *Protein Expr. Purific.* **2**, 127–135.
40. Lischwe, M. A. and Sung, M. T. (1977) Use of N-chlorosuccinimide/urea for the selective cleavage of tryptophanyl peptide bonds in proteins. *J. Biol. Chem.* **252**, 4976–4980.
41. Vestling, M. M., Kelly, M. A., and Fenselau, C. (1994) Optimization by mass spectrometry of a tryptophan-specific protein cleavage reaction. *Rapid Commun. Mass Spectrom.* **8**, 786–790.
42. Rahali, V. and Gueguen, J. (1999) Chemical cleavage of bovine β-lactoglobulin by BNPS-skatole for preparative purposes: comparative study of hydrolytic procedures and peptide characterization. *J. Protein Chem.* **18**, 1–12.

43. Lischwe, M. A. and Ochs, D. (1982) A new method for partial peptide mapping using N-chlorosuccinimide/urea and peptide silver staining in sodium dodecyl sulphate-polyacrylamide gels. *Anal. Biochem.* **127**, 453–457.
44. Crimmins, D. L., McCourt, D. W., Thoma, R. S., Scott, M. G., Macke, K., and Schwartz, B. D. (1990) In situ cleavage of proteins immobilised to glass-fibre and polyvinylidene difluoride membranes, cleavage at tryptophan residues with 2-(2'-nitropheylsulfenyl)-3-methyl-3'-bromoindolenine to obtain internal amino acid sequence. *Anal. Biochem.* **187**, 27–38.

7

Enzymatic Preparation and Isolation of Glycopeptides

David J. Harvey

1. Introduction

The preparation and isolation of glycopeptides is the first step in the site-specific characterization of oligosaccharides attached to proteins. As discussed in Chapter 30, if the glycoprotein contains only one glycosylation site, the glycan profile can be obtained directly by mass spectrometry *(**1**)* providing that the mass of the glycoprotein is below about 30 kDa and the number of glycoforms is not large *(**2**)*. For larger glycoproteins and those containing multiple glycosylation sites, it is necessary to cleave the glycoprotein into smaller peptide fragments, either enzymatically or chemically (*see* Chapters 5 and 6, respectively), with the aim of isolating each glycosylation site into one glycopeptide. For some glycoproteins, it may be necessary to use a combination of cleavage methods in order to develop an approach for separating all of the glycosylation sites. The derived glycopeptides can then be isolated by high-performance liquid chromatography (HPLC) or lectin chromatography. The affinity of glycopeptides for different lectins provides preliminary information on the carbohydrates attached to the peptide. A number of strategies for serial lectin chromatography have been developed that result in isolation and characterization of individual glycopeptides *(**3**,**4**)*.

The proteases usually employed for digestion of glycoproteins, trypsin (which cleaves on the carboxy-terminal side of the basic amino acids lysine and arginine) and endoproteinase lys-C (which cleaves on the carboxy-terminal side of lysine), are available commercially to a very high standard and have been well-characterized. Other enzymes are discussed in Chapter 5. Both trypsin and Lys-C produce very low levels of autolytic products, which enables high ratios of sample protein to protease (1:10 to 1:40) to be used over long periods of time without significant loss of proteolytic activity. As both pro-

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

teases are active in 2 *M* urea, 8 *M* urea can be used to unfold target proteins and allow better access to the cleavage sites. Only a small dilution is then required for the proteases to be active while maintaining the protein at a high concentration (> 0.5 mg/mL) for digestion.

HPLC is the standard method for resolution of peptide and glycopeptide mixtures and is frequently used to provide specific profiles of digested proteins, for example in quality control of recombinant products. Other than a convenient method for separation and isolation of (glyco)peptides, HPLC does not provide any structural information on glycosylation without further investigation (*see* Chapter 30). Other methods for glycoprotein and glycopeptide fractionation are given in **ref.** ***(5)***

As lectins are specific for particular sugars or discrete oligosaccharide structures, their ability to bind glycopeptides provides a means of characterizing the glycans present, in addition to providing a means of purifying them. Lectins immobilized on Agarose-based gels can be purchased from a number of commercial sources (*see* **Table 1**). However, the vast majority of lectins are available as highly purified preparations, which can readily be immobilised on CNBr-activated Sepharose or *N*-hydroxysuccinimide ester-activated Agarose ***(6)***.

2. Materials

2.1. Trypsin and Endoproteinase Lys-C Digestion

1. Resuspension buffer: 8 *M* urea, 100 m*M* ammonium bicarbonate pH 7.8.
2. Reducing agent: 50 m*M* dithiothreitol (DTT) in distilled water (*see* **Note 1**).
3. Cysteine modifying reagent: 100 m*M* iodoacetamide in distilled water. Store in the dark or cover with aluminium foil.
4. Proteases (Boehringer Mannheim Ltd., Lewes, UK or Promega Ltd., Madison, WI):
   a. Sequence-grade, modified trypsin.
   b. Sequence-grade endoproteinase lys-C.

   Prepare each at 1 mg/mL in 2 m*M* hydrochloric acid. These solutions can be stored for up to 3 mo at –20°C.

2.2. Isolation of the Glycopeptide

2.2.1. Reversed-Phase HPLC

1. Suitable HPLC system capable of flow rates of 100 µL/min with a 200 µL sample loop, a C-18 or C-8 reversed-phase column (e.g., Beckman Ultrasphere 150 × 4.6 (id) mm C-18 from Alltech (Carnforth, Lancashire, UK), and with the detector set to a wavelength of 225 nm.
2. Eluant A: 0.1% trifluoroacetic acid (TFA) in water (500 mL) (*see* **Note 2**).
3. Eluant B: Acetonitrile containing sufficient TFA to give an absorbance at a wavelength of 225 nm equivalent to that of eluant A (approx 0.085% v/v) (500 mL) (*see* **Note 2**).
4. Fraction collector.

2.2.2. Lectin Chromatography

1. Sepharose- or Agarose-immobilized lectin (**Table 1**) (1–5 mL) packed into a PolyPrep (2 mL bed volume, 0.8 × 4.0 cm) or EconoPac (1.0–20 mL bed volume, 1 × 12 cm) disposable column (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).
2. Equilibration buffer: 20 mM phosphate buffer, pH 7.2, 150 m*M* sodium chloride (with 1 m*M* magnesium chloride and 1 m*M* calcium chloride for appropriate lectins; *see* **Table 1**) and 0.02% (w/v) sodium azide.
3. Elution buffer(s): equilibration buffer containing 100–500 m*M* of the appropriate sugar (*see* **Table 1**).

3. Methods

3.1. Trypsin and Endoproteinase Lys-C Digestion (see Note 3)

1. Solubilize the lyophilized protein (120 μg) in 50 μL of resuspension buffer to give a protein concentration of 2.4 mg/mL.
2. Add 5 μL of reducing agent (50 m*M* DTT).
3. Incubate at 50°C for 15 min.
4. Cool to room temperature and add 5 μL of 100 m*M* iodoacetamide. (All further steps should be performed in a foil-covered tube to exclude light.)
5. Incubate at room temperature for 15 min.
6. Add 140 μL of water.
7. Add 5 μL of trypsin or endoproteinase lys-C to give a protein:proteinase ratio of 24:1 (w/w).
8. Incubate at room temperature for 24 h.
9. Stop reaction by cooling to –20°C.

3.2. Isolation of the Glycopeptides

3.2.1. Reversed-Phase HPLC

1. Regenerate the C-8 column in 95% eluant B for 30 min at a flow rate of 100 μL/min.
2. Equilibrate the column in 5% eluant B for 30 min at a flow rate of 100 μL/min.
3. Diluted the digested protein mixture with eluant A to give a total volume of 200 μL.
4. Inject 200 μL of digested protein onto the column and elute using the following gradient:

Time (Min)	Eluant B (%)
0	5
25	5
95	50
97	95
102	95
105	5
120	5

Table 1
Lectins

Taxonomic name	Common name	Abbreviation	Specificity	Cation	Elution Sugar	Supplier[a]
Arachis hypogea	Peanut	PNA	Galβ1-3GalNAc	Ca^{2+}	Galactose	C, S
Artocarpus integrifolia	Jackfruit		Core Galβ1-3GalNAc Terminal αGalactose	Ca^{2+}	α-Methyl-D-galactoside	C
Canavalia ensiformis	Jack bean (Con-A)	Con A	α-Mannose, α-Glucose	Ca^{2+}	α-Methyl-D-mannoside	C, P, S
Datura stramonium		DSL	GlcNAcβ1-GlcNAc, LacNAc	Ca^{2+}	Chitobiose, Chitotriose	C
Dolichos biflorus	Horse gram	DBA	Terminal α-GalNAc	Ca^{2+}	*N*-Acetylgalactosamine	C, S
Glycine soja	Soybean	SBA	α- or β-GalNAc	–	*N*-Acetylgalactosamine	C, S
Griffonia simplicifolica			α-Galactose	Ca^{2+}	Raffinose, Galactose	C
Helix pomatia	Edible snail	HPA	O-Linked GalNAc		*N*-Acetylgalactosamine	S
Lens culinaris integrifolia	Lentil	LcH	Fucosylated antennary complex α-Mannose	Ca^{2+}	α-Methyl-D-mannoside plus α-Methyl-D-glucoside	C, P, S
Limax flavus	Slug	LFA	NeuAc, NeuGc	–	*N*-acetyl-neuraminic acid	C
Lotus tetragonolobus	Asapargus		Terminal α-fucose	Ca^{2+}	L-Fucose	C
Lysopersicon esculentum	Tomato		GlcNAcβ1-4GlcNAc oligomers	Ca^{2+}	Chitobiose and chitotriose	C
Maackia amurensis		MAL I	NeuNAcα2-3Galα1-4GlcNAc	Ca^{2+}	Lactose	C
Phaseolus vulgaris	Red kidney bean	PHA L4	Tri- and tetra-antennary complex with N-acetyl-lactosamine branches		*N*-Acetylgalactosamine	C, S

Table 1
Lectins *(continued)*

Taxonomic name	Common name	Abbreviation	Specificity	Cation	Elution Sugar	Supplier[a]
Phaseolus vulgaris	Red kidney bean	PHA E4	Bisected biantennary complex	Ca^{2+}	*N*-Acetylgalactosamine	C, S
Phytolacca americana	Pokeweed	PWM	GlcNAcβ oligomers complex		Chitobiose	S
Pisum Sativum	Garden pea	PSA	Bi- and tri-antennary with α1-6 fucose	Ca^{2+} Mg^{2+}	α-Methyl-D-mannoside α-Methyl-D-glucoside	C, S
Ricinus communis	Castor bean	RCA1, RCA60	Bi- and tri-antennary complex		Lactose	S
Sambucus nigra	Elderberry	SNA	NeuNAcα2-6Gal NewNAcα2-6GalNAc	–	N-Acetylgalactosamine, Lactose	C
Solanum tuberosum	Potato (STA)	STA	GlcNAcβ oligomers	Ca^{2+}	Chitobiose, Chitotetraose	C, S
Tetragonolobus purpureas	Winged pea	–	L-Fucose		L-Fucose	S
Triticum vulgaris	Wheatgerm	WGA	GlcNAcβ1⌧ 4GlcNAc (*N*,*N*-Diacetylchitobiose), NeuNAc	–	*N*-Acetylgalactosamine	C, P, S
Tritrichomonas mobilensis		TML	NeuNAc, NeuNGc	–	NeuNAc	C
Ulex europaeus	Gorse	UEA1 UEA2	Fucα1-2Galβ1-4GlcNAc	Ca^{2+}	L-Fucose	C, S
Wisteria floribunda	Wisteria		Terminal galNAcβ1-4-	Ca^{2+}	*N*-Acetylgalactosamine	C
Vicia villosa	Hairy vetch	VVA	GalNAcα1-O-serine	Ca^{2+}	*N*-Acetylgalactosamine	C

[a]C = Calbiochem, CN BioSciences, Nottingham, UK, S = Sigma-Aldrich, Poole, Dorset, UK, P = Pharmacia, Milton Keynes, UK.

5. Collect 2 min fractions (200 μL) fractions from time 0 to time = 100 min.
6. Lyophilize fractions and store at –20°C until required for analysis.

3.2.2. Glycopeptide Purification by Immobilized Lectin Chromatography

1. Equilibrate the column with five column volumes of equilibration buffer (including 1 m*M* divalent metal ions as appropriate; *see* **Table 1**).
2. Dilute the digested protein with equilibration buffer to a final volume of 1 mL and apply to the column.
3. Wash the column with five column volumes of equilibration buffer and collect the flow; this contains nonbound material.
4. Elute bound glycopeptides with five column volumes of elution buffer (*see* **Note 4**) and collect 1 mL fractions.
5. Store fractions at –20°C until required for analysis.
6. Measure the absorbance of the fractions spectrophotometrically at a wavelength of 225 nm or use methods outlined in Chapter 30.
7. Regenerate the column with 10 column volumes of equilibration buffer.

4. Notes

1. Distilled water is preferable to standard ion-free water, found in many laboratories, as the latter sources frequently contain residual organic material such as polyethylene glycol. This material can subsequently interfere with analyses performed by mass spectrometry.
2. Solutions should be sparged with helium for 10 min.
3. Protease digestion: Some flexibility is possible in the quantities and concentrations of the components used for digestion. The initial concentration on resuspension with 8 *M* urea, 100 m*M* ammonium bicarbonate, pH 7.8, in **step 1** should be between 2 and 10 mg/mL. Add a volume of 50 m*M* DTT that is between 1/10 and 1/25 the volume of suspended protein. DTT is present in at least 10-fold molar excess of the number of cysteine residues present in the protein to ensure complete reduction. Add an equal amount of iodoacetamide to ensure complete carboxamidomethylation of the reduced cysteine. The mixture is then diluted fourfold to reduce the urea concentration. Both trypsin and endoproteinase lys-C retain activity in 2 *M* urea. The final protein:proteinase ratio should optimally be between 20 to 40:1 (w/w).
4. Lectin chromatography: Fractionation of bound glycopeptides can be achieved by using a series of elution steps employing 0.1 *M* increments in specific sugar concentration up 0.5 *M*. Use five column volumes to elute bound material, collecting 1-mL fractions at each stage. Because of the selectivity of lectins for specific sugars, some glycopeptides will not bind and will appear in the void volume; others will elute in low sugar and others in high sugar concentrations.

References

1. Mock, K. K., Davy, M., and Cottrell, J. S. (1991) The analysis of underivatised oligosaccharides by matrix-assisted laser desorption mass spectrometry. Biochem. *Biophys. Res. Commun.* **177**, 644–651.
2. Tsarbopoulos, A., Bahr, U., Pramanik, B. N., and Karas, M. (1997) Glycoprotein analysis by delayed extraction and post-source decay MALDI-TOF-MS. *Int. J. Mass Spectrom. Ion Processes* **169/170**, 251–261.
3. Osawa, T. and Tsuji, T. (1987) Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Ann. Rev. Biochem.* **56**, 21–42.
4. Robertson, E. R. and Kennedy, J. F. (1996) Glycoproteins: a consideration of the potential problems and their solutions with respect to purification and characterisation. *Bioseparation* **6**, 1–15.
5. Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G., and Strecker, G. (1986) Glycoproteins, in *Carbohydrate Analysis: A Practical Approach* (Chaplin, M. F. and Kennedy, J. F., eds.), IRL Press, Oxford, pp. 143–204.
6. Sutton, C. W. (1989) Lectin affinity chromatography, in Protein Purification Protocols: A Practical Approach (Harris, E. L. V. and Angal, S., eds.), Oxford University Press, Oxford, pp. 268–282.

8

Analytical and Micropreparative Capillary Electrophoresis of Peptides

Alan J. Smith

1. Introduction

Capillary electrophoresis (CE) was developed as a high sensitivity, high resolution, quantitative, electrophoretic separation technique. Since its commercial introduction in 1987, it has proved to be a versatile analytical tool for the separation of both small molecules, e.g., inorganic cations and anions, and drugs; and large molecules, e.g., peptides, proteins, carbohydrates, and nucleic acids.

In the case of proteins and peptides CE has found utility both as an analytical technique and as a micropreparative separation technique. CE methods have been developed for monitoring the enzymatic digestion of proteins, for purity checks on both natural and synthetic peptides, for screening protein and peptide fractions from chromatographic separations, and for the micropreparative isolation of peptides from complex digestion mixtures.

The separation of peptides by CE is based on both size and charge considerations *(**1**)*. It can be viewed as an orthogonal separation technique to reverse-phase high-performance liquid chromatography (RP-HPLC) which separates on the basis of hydrophobicity and to a lesser extent on size. A comparison between the two separation methods is shown in **Fig. 1**. The same tryptic digest of β lactoglobulin was separated by CE in **Fig. 1A** and RPHPLC in **Fig. 1B**. The high-sensitivity and high-resolution capabilities of CE are based on performing electrophoretic separations in long glass capillaries under conditions that keep diffusion to a minimum. Standard analytical capillaries have internal diameters of 50–75 micron and lengths of 20–50 cm. These glass capillaries are fragile and in order to improve their tensile strength of they are coated with a thin plastic (polyimide) film. The small internal volume of the capillaries (low

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

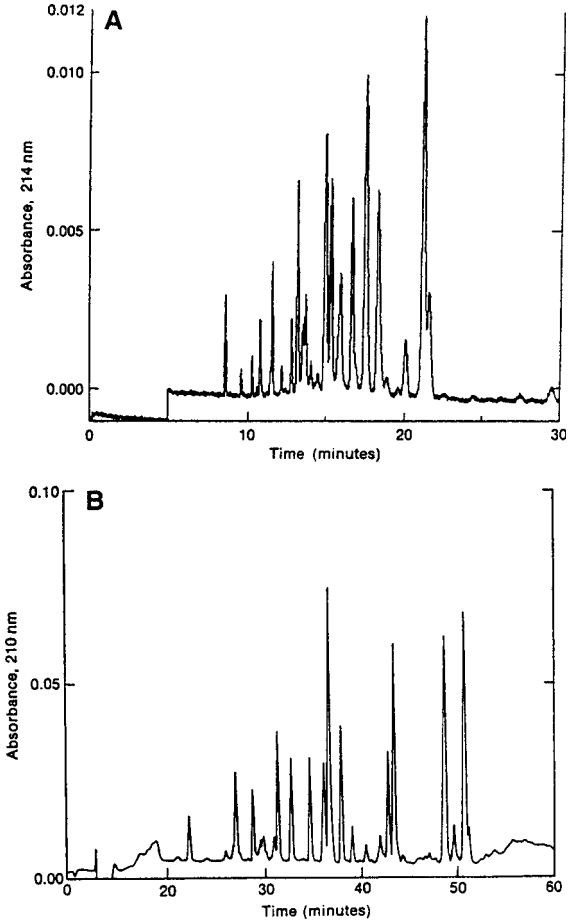


Fig. 1. **(A)** Analytical CE of β-lactoglobulin digest. **(B)** Narrowbore RP-HPLC anaytical separation of β-lactoglobulin digest.

microlitre) require very high voltages (10–25kV) to achieve optimal electrophoretic separations, and for this reason they are incorporated into dedicated CE instruments. In its simplest form, (**Fig. 2**) a CE instrument consists of two electrode buffers joined by a glass capillary, and a UV detector. A third reservoir containing the sample is also required. The sample is loaded on to the capillary from the sample reservoir either electrokinetically or mechanically (pressure or vacuum). The cathode end of the capillary is then removed from the sample reservoir, returned to the cathodic buffer reservoir, and electrophoresis is commenced.

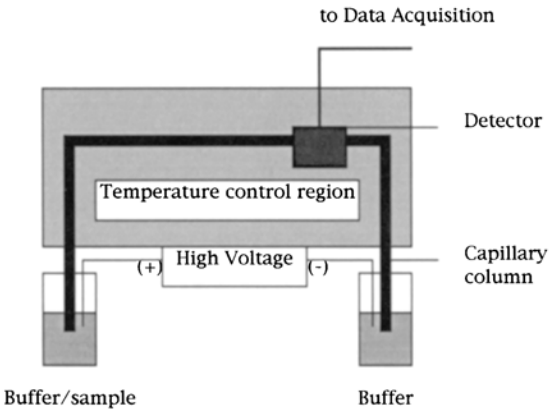


Fig. 2. Schematic of a CE instrument.

Once electrophoresis is started, an electroendosmotic flow (EOF) is set up within the capillary. The EOF is produced by the migration of cations and their associated water of hydration from the cathode towards the anode. Thus not only positively charge, but neutral, and negatively charged peptides are swept through the capillary to the anode. A small region of the polyimide coating is removed from the glass capillary and is inserted into the detector light path. Thus the capillary itself becomes the flow cell. The UV absorbing peptides are recorded and quantitated as they pass through the detector. The glass capillary can have a surface of native silica (open tube) or, it can be derivatized (coated) and can contain either electrolyte alone or, electrolyte and a suitable separation matrix.

One complication with the separation of peptides in open-tube capillaries is the potential for their irreversible adsorption to the silica wall. At low pH *(**2**)*, or high pH in the presence of a modifier *(**3**)*, this has not proved to be a significant problem. However, it is a serious problem with proteins. Recent work *(**4**)* from the Righetti group has shown that both peptides and proteins can be effectively separated by using uncoated capillaries and amphoteric, isoelectric buffers at low pH. The running buffer also contains hydroxyethylcellulose as a dynamic coating for the silica surface. This processes effectively eliminates the nonspecific adherence of peptides and proteins to the capillary walls. In the case of proteins, a denaturant such as 5 *M* urea or reduced Triton X-100 is added in order to keep them in their unfolded state. The use of amphoteric buffers such as aspartic acid at their isoelectric point means that higher electric fields and shorter capillary lengths can be used. In combination, these conditions can reduce the analytical separation times of protease digestion mixtures

to less than 15 min. The definition as to what constitutes a protein and what constitutes a peptide becomes important when selecting an optimal separation protocol. In the context of CE, peptides should be viewed as containing less than 50 amino acids.

A second important aspect of CE is the definition of sensitivity. Because only nanoliter sample volumes are loaded on to the capillary for analytical separations, the absolute sensitivity is very high when compared to HPLC. However, in a practical sense, the sample must be at an approximate concentration of one microgram per microliter and a volume of at least 10 microliters even though only a very small percentage of the sample is used in the separation. For example, a 10-s injection would transfer approx 50 nl to the capillary, which would be equivalent to approx 50 ng of protein. At times these concentration ranges may not be readily available. It is possible to mitigate these difficulties to a certain extent by the preconcentration of dilute samples in the capillary by pressure loading a water slug prior to electrokinetic injection of the sample *(**5**)*. More recently isotacophoretic injection methods have worked well for dilute samples *(**6**)*.

Although CE was originally developed as an analytical technique, considerable interest has developed in utilizing the high-resolution capabilities as a preparative technique. The subsequent section will describe protocols that can be employed when micropreparative peptide separations are required.

Two major approaches have been developed for the successful micropreparative separation of peptides that have been generated from a protease digestion of a target protein. One method utilizes a single electrophoretic separation on a capillary of much larger diameter than those normally used for analytical separations *(**7**)*. The other utilizes multiple separations on a single analytical capillary *(**8**)*. Fractions with the same electrophoretic mobility are pooled in order to obtain sufficient material for further characterization.

In both cases, the amount of digest fractionated by micropreparative CE is in the 5–50 pmole range. In contrast, it is at these load levels that losses owing to adsorption become a significant problem for narrowbore (2.1 mm ID) RPHPLC separations. In this sense, the two techniques can be regarded as complimentary to each other.

2. Materials

*2.1. Preconditioning Underivatized Capillaries (*see ***Note 1)***

1. Commercial CE instrument.
2. Sodium hydroxide: 100 m*M* in distilled water.
3. Sodium phosphate: 250 m*M* in distilled water pH 2.3.
4. Underivatized capillary: 75 micron × 50 cm.

2.2. Monitoring Protease Digestions

1. Commercial CE instrument equipped with a temperature controlled sample table.
2. Tris: 250 m*M*, pH 7.8, containing 8 *M* urea (*see* **Note 2**)
3. Protease: 1 μg/μL in distilled water (*see* **Note 3**).
4. Sodium phosphate: 50 m*M* in distilled water, pH 2.3.
5. Sodium phosphate: 250 m*M* in distilled water, pH 2.3.
6. Sodium hydroxide: 100 m*M* in distilled water (*see* **Note 4**).
7. Dithiothreitol (DTT): 50 m*M* in distilled water (*see* **Note 5**).
8. Preconditioned silica capillary.

2.3. Screening Fractions from Preparative Reverse Phase HPLC

2.3.1. Standard Protocol

1. HPLC fraction in actonitrile/TFA (*see* **Note 6**).
2. Vacuum centrifuge.
3. Ethylene glycol: 100% (*see* **Note 7**).
4. Sodium phosphate: 50 m*M* in distilled water, pH 2.3.
5. Preconditioned silica capillary.

2.3.2. Alternate Protocol (see **Note 5**)

1. HPLC fraction in actonitrile/TFA (*see* **Note 6**).
2. Vacuum centrifuge.
3. Ethylene glycol: 100% (*see* **Note 7**).
4. Sodium tetraborate: 100 m*M* with boric acid, pH 9.0.
5. Sodium dodecylsulphate (SDS): 0.2% in water.
6. Preconditioned silica capillary.

2.4. Micropreparative Separation

2.4.1. Single Separation Protocol

1. Commercial CE instrument equipped with active cooling (*see* **Note 8**).
2. Sodium phosphate: 50 m*M* in distilled water, pH 2.3.
3. Sodium phosphate: 250 m*M* in distilled water, pH 2.3.
4. Sodium hydroxide: 100 m*M* in distilled water.
5. Underivatized capillary: 50 micron × 57 cm. (Polymicro Inc.) (*see* **Note 9**).
6. Microvials for collection: 25 μL volume.
7. Ethylene glycol: 100% solution (*see* **Note 7**).
8. Protease digestion mixture: approx 1 μg/μL of substrate in Tris-urea buffer (*see* **Subheading 3.2.**).

2.4.2. Multiple Separations Protocol

1. Commercial CE instrument with forced air (passive) cooling.
2. Reagents as in **Subheading 2.4.1.**
3. Underivatized capillary: 75 micron × 57 cm (Polymicro Inc.).
4. Standard 100 μL vials.

3. Methods

3.1. Preconditioning Underivatized Capillaries

1. Place capillary in instrument.
2. Flush capillary with 10 column volumes of 100 m*M* sodium hydroxide at 0.5 psi.
3. Flush with 10 column volumes water.
4. Flush with 4 column volumes 250 m*M* sodium phosphate.
5. Store in same buffer until use.
6. When switching to a new buffer a 4 h equilibration is advised and **steps 2** and **3** eliminated.

3.2. Monitoring Protease Digestions

1. Dissolve 20 μg of the protein in 20 μL of Tris-urea buffer.
2. Add 5 μL of 50 m*M* DTT solution and incubate at 50°C for 15 min to reduce disulphide bonds in the protein.
3. Add 75 μL distilled water to dilute the tris buffer to 50 m*M* and the urea to 2 *M*.
4. Add protease 1/10 enzyme to protein ratio (w/w).
5. Load onto CE sample table that has been temperature equilibrated to 37°C.
6. Operate instrument in accordance with manufacturer's instructions.
7. Separate the peptide mixture using the following conditions:
   a. Electrolyte: 50 m*M* sodium phosphate pH 2.3.
   b. Sample table temperature: 37°C.
   c. Run Temp: 25°C.
   d. Voltage: 25kV.
   e. Sample injection: 10 s at 0.5 psi.
   f. Injection interval: 4 h.
   g. Detection: 200 nm.
   h. Run time: 37 min.
8. An example of a separation protocol for a Beckman P/ACE 5000 instrument with a 37-min run time from injection to injection is as follows:
   a. Screening of peptides (digests, HPLC fractions, and synthetic peptides).
   b. Sample vial = 11, Injection = 10 s, Voltage = 25kV, Separation = 30 min, 50 m*M* phosphate, pH 2.3.
   c. Vial contents:

      Position #7. Waste vial, water level just to contact capillary effluent.
      Position #9. Electrolyte: 50 m*M* phosphate, pH 2.3.
      Position #11. Sample.
      Position #29. Electrolyte 50 m*M* phosphate, pH 2.3.
      Position #32. 0.1 *N* NaOH for regeneration.
      Position #33. Water for rinse.
      Position #34. 0.25 *M* phosphate, pH 2.3.
9. The electropherograms are examined to determine the length of time required to produce a stable profile (*see* **Note 10**).

3.3. Screening HPLC Fractions for Peptide Purity

3.3.1. Standard Protocol

1. Concentrate the HPLC fractions in an evacuated centrifuge to approximately 20 μL in order to remove excess acetonitrile, but do not dry.
2. Add 5 μL of 100% ethylene glycol to reduce evaporation on the sample table and minimize adsorption to the walls of the tube.
3. Run same conditions as in **Subheading 3.2.** except that the sample table temperature is 25°C and only single injections are made.
4. Fractions that contain single or major components are suitable candidates for protein sequencing.

3.3.2. Alternate Protocol

1. Sample preparation is as in **Subheading 3.3.1.**, **steps 1** and **2**.
2. Prepare electrolyte by mixing equal volumes of sodium tetraborate and SDS solutions.
3. Separate the peptide mixture using the following conditions:
   a. Electrolyte: 50 m*M* sodium borate/0.1% SDS pH 9.0.
   b. Sample table temp: 25°C.
   c. Run temp: 25°C.
   d. Voltage: 30 kV.
   e. Sample injection: 15 s at 0.5 psi.
   f. Detection: 200 nm.
   g. Run time: 25 min.

3.4. Micropreparative Separation

3.4.1. Single Separation Protocol (see ***Note 11***)

1. Place 10 μL of a 50 m*M* sodium phosphate/ethylene glycol (80:20 v/v) mixture in the microvials.
2. Program the Beckman Instruments P/ACE 5000 instrument to collect 3-min fractions.
3. All other operating conditions are as in **Subheading 3.2.** except that the operating voltage is 7.5 kV and the sample table temperature is 25°C.
4. Sample injection: 15 s at 0.5 psi.
5. Collect fractions over a 90-min separation period (*see* **Note 12**).

3.4.2. Multiple Separations Protocol (see ***Note 13***)

1. The fraction collection tubes are filled with 10 μL of 50 m*M* sodium phosphate buffer to serve as the analyte electrodes.
2. Separation conditions and sample concentrations are as in **Subheading 3.2.** with the sample table temperature lowered to 25°C.
3. Fractions are collected every minute across the separation (*see* **Note 14**).

4. The CE instrument is programmed to perform 10 consecutive separations automatically.
5. The length of each separation is 25 min, which includes capillary regeneration.

4. Notes

1. Preconditioning of the capillary refers to the generation of ionized silanol (SiO^-) groups on the inner wall of the capillary. This is essential for the generation of EOF.

   Preconditioning underivatized capillaries is essential when they are new or when it is necessary to change the separation buffer. Do NOT precondition coated capillaries as this will remove the coating and ruin the capillary. Buffers can be changed in these coated capillaries by pre-equilibration.
2. The proteins are dissolved in 8 *M* urea to facilitate unfolding and dissolution. This solution is diluted to 2 *M* urea with water prior to adding the protease. Most proteases are fully active in 2 *M* urea solutions.
3. Store the protease at –20°C.
4. The sodium hydroxide solution is used for regenerating the surface of the capillary before reequilibrating with the 250 m*M* and 50 m*M* phosphate buffers.
5. It is essential to reduce all the potential disulphide bonds in the protein prior to digestion and separation in order to obtain an accurate representation of the constituent peptides.
6. Fractions of approx 100 μL are normally collected from a narrowbore RP-HPLC separation of a protease digest that has been developed with an acetonitrile/water 0.5% (v/v) TFA gradient.
7. Obtained as a viscous solution from the supplier.
8. The large diameter capillary single-separation method requires that the CE instrument possesses refrigerated (active) cooling. The use of large (150–200 micron ID) capillaries produces significant quantities of Joule heating which can distort the electrophoretic separations unless they are adequately temperature controlled. Some CE instruments utilize forced ambient air (passive) cooling and this is inadequate to achieve effective temperature control when using these large diameter capillaries.
9. Much larger sample volumes can be loaded onto these capillaries (100–500 nl) and peptides can be recovered at the 5–50 pmole level from a single separation. These recoveries are adequate to allow direct protein sequencing of the fractions *(7)*. The upper sample volume limit (500 nl) is dictated by the total volume of the capillary (approx 10 μL). A 15-s injection time would load approx 250 nl (0.5 μg protein digest) onto the capillary.
10. Overdigestion will result in the autodigestion of the protease, which will produce unwanted peptides.
11. The method suffers from the disadvantage that fractions have to be collected "blind" since it is not possible to obtain a continuous chromatographic profile during the run. This is due to the interruption in current that occurs when the end of the capillary is moved from one fraction collection tube to the next.

12. Fractions can be screened with either analytical CE or MALDI mass spectrometry prior to sequencing.
13. The multiple collection approach combines identical peaks from 10 consecutive analytical separations in order to obtain sufficient material for further characterization. Fractions are collected at 1-min time intervals across each separation. A single set of fraction collection tubes are used such that like-fractions are pooled in the same tube. The success of the method requires that the absolute electrophoretic migration times of the individual peaks must not vary by more than 0.1 min over the series of separations. This electrophoretic stability is not easily achieved with some commercial instruments.
14. As with the other micropreparative protocol, it is advisable to screen the fractions prior to selection for protein sequence analysis.

References

1. McCormick, R. M. (1994) Capillary zone electrophoresis of peptides, in *Handbook of Capillary Electrophoresis* (Landers, J. P., ed.), CRC, Boca Raton, FL, 287–324.
2. Strickland, M. and Strickland, N. (1990) Free solution capillary electrophoresis using phosphate buffer and acidic pH. *Am. Lab.* **22**, 60–65.
3. Chen, F.A., Kelly, L., Palmieri, R., Biehler, R., and Schwartz, H. (1992) Use of high ionic strength buffers for the separation of proteins and peptides with CE. *J. Liq. Chrom.* **15**, 1143–1150.
5. Burgi, D. S. and Chien, R. L. (1992) On-column sample concentration using field-amplification in CZE. *Anal. Chem.* **64**, 849–855A.
7. Kenny, J. W., Ohms, J. I., and Smith, A. J. (1993) Micropreparative capillary electrophoresis (MPCE) and micropreparative HPLC of protein digests, in *Techniques in Protein Chemistry IV* (Hogue Angeletti, R.A., ed.), Academic, San Diego, CA, pp. 363–370.
6. Bergman, T. and Jornvall, H. (1992) Capillary electrophoresis for preparation of peptides and direct determination of amino acids, in *Techniques in Protein Chemistry III* (Hogue Angeletti, R. A., ed.), Academic, San Deigo, CA, pp. 129–134.
7. Righetti, P. G., Gelfi, C., Perego, M., Stoyanov, A. V., and Bossi, A. (1997) Capillary zone electrophoresis of oligonucleotides and peptides in isoelectric buffers: theory and Methodology. *Electrophoresis* **18**, 2143–2153
8. Palmer, J., Burgi, D. S., Munro, N. J., and Landers, J. P. (2001) Electrokinetic injection for stacking neutral analytes in capillary and microchip electrophoresis. *Anal. Chem.* **73**, 725–731.

9

High-Performance Liquid Chromatography On-Line Derivative Spectroscopy for the Characterization of Peptides with Aromatic Amino Acid Residues*

Christoph W. Turck

1. Introduction

High-performance liquid chromatography (HPLC) is one of the most common separation techniques in today's protein chemistry. With the development of diode-array UV detectors for HPLC instruments on-line derivative spectroscopy has become possible and provides an extremely powerful tool for the analysis of peptides and proteins *(2)*. The method of on-line spectral analysis has been particularly useful in the analysis of peptides containing aromatic residues *(1,2)*. The formation of second derivatives of the absorption maxima leads to further increases in the resolution between spectral differences and allows one to distinguish between different aromatic residues in a peptide. Based on these findings we have utilized HPLC on-line derivative spectroscopy for the analysis of an important post-translational modification of peptides and proteins *(1)*.

Tyrosine phosphorylation has been shown to be a key step in the regulation of several cellular events *(3,4)* including signal-transduction mechanisms of stimulated growth factor receptors *(5,6)*. Traditional methods for direct mapping of phosphorylated tyrosines use biosynthetic radiolabeling procedures with [^{32}P] orthophosphate and subsequent isolation and cleavage of the protein of interest followed by peptide map analysis *(7)*. If the sequence of the studied protein is known, synthetic peptides can be prepared and compared to cleavage fragments carrying the phosphotyrosine residue. The mapping of protein phos-

*This article is a modified reprint of an article published in Peptide Research *(1)*.

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

phorylation sites is also possible with mass spectrometry *(8)*. However, owing to the low stoichiometries of protein phosphorylation and the unfavorable behavior of phosphopeptides during mass spectrometry analysis, this method is still not routine *(8)*. Also protein sequence analysis of phosphotyrosine containing peptides has been possible by several methods *(9)*.

We have developed a method for the detection of phosphotyrosine residues in peptides based on reversed-phase HPLC (RP-HPLC) on-line spectral analysis. It was found that tyrosine containing peptides show a hypsochromic shift of the aromatic absorbance maximum when the tyrosine is phosphorylated *(1)*. Subsequent second-order derivative spectra likewise reveal a hypsochromic shift of the corresponding minima of the phosphotyrosine residues compared to the unmodified tyrosine. This method allows mapping of tyrosine phosphorylation sites in proteins after cleavage into smaller peptides and separation and on-line spectral analysis of the latter by RP-HPLC. It furthermore provides a useful way for the characterization of synthetic phosphotyrosine containing peptides. The difference in absorption between phosphotyrosine and unmodified tyrosine can be exploited to determine phosphotyrosine residues in microgram amounts of polypeptides during their elution from reversed-phase columns with the help of an on-line scanning diode array detector and subsequent analysis of the second-order derivative spectra that exhibit characteristic minima at peaks and shoulders of the zero-order spectra *(2)*. This method can be used for the determination of tyrosine phosphorylation sites after isolation and cleavage of the protein of interest followed by on-line spectral analysis of the cleavage products during separation and subsequent Edman degradation or mass spectrometry analysis of phosphotyrosine containing peptides.

2. Materials

1. Analytical RP-HPLC is carried out with 1–5 μg of each amino acid or peptide in 25 μL 50% acetic acid using a Model 1090 Hewlett Packard instrument (Palo Alto, CA) equipped with a HP 1040 diode-array detector and HP 79996A data acquisition software (Hewlett Packard).
2. A narrow bore C_{18}-column from Vydac (2.1 mm × 25 cm) (Hesperia, CA) is used in 0.1% trifluoroacetic acid (TFA) with a gradient of acetonitrile from 0–90% in 60 min at a flow rate of 0.5 mL/min.
3. Effluents are monitored with a diode-array detector (flow cell: 6 mm pathlength, 8 μL volume). Peptide zero- and second-order derivative spectra are obtained between 230 and 300 nm and subsequently stored in a spectra library (HP 79996A).

3. Methods

1. Spectra of peptides collected in the above manner are analyzed for the presence of tryptophan, phenylalanine, tyrosine or phosphotyrosine by overlaying them with spectra of reference amino acids and peptides.

Fig. 1A and **1B** show the zero-and second-order derivative spectra of 5 μg of free tyrosine and phosphotyrosine, respectively. A hypsochromic shift of the aromatic ring absorption maximum of tyrosine can be observed when the phenolic hydroxyl group is modified with a phosphate moiety. Likewise in the second-order derivative spectra a hypsochromic shift of the corresponding two minima can be detected (281–272 nm and 273–264 nm, respectively).

2. In **Fig. 1C** and **1D** zero- and second-order derivative spectra for the other two natural aromatic amino acids, namely phenylalanine and tryptophan, are shown. The characteristic minima in the second-order derivative spectra of phenylalanine at 257 nm and 264 nm and for tryptophan at 268 nm and 278 nm are in accord with published data *(2)* and are used for subsequent comparison in the spectral analysis of the model peptides. A complete list of second-order derivative spectra minima of all four aromatic amino acids is shown in **Table 1**.
3. Results from on-line derivative spectroscopy of synthetic peptides (5 μg) with phosphorylated and unphosphorylated tyrosine residues are shown in **Table 2**. **Fig. 2A** and **2B** demonstrate that the hypsochromic shift of the absorption maximum between 250 and 300 nm is also observed for the tyrosine phosphorylated peptide. The method of real time spectral analysis hence can distinguish very clearly between phophotyrosine and unmodified tyrosine-containing peptides when no other aromatic residues are in the peptide sequence.

   Although additional minima are introduced into second-order derivative spectra when phenylalanine is present in the peptide sequence (**Fig. 2C** and **2D**) the hypsochromic shift of phosphotyrosine can still be detected (phosphotyrosine minima at 268 and 274 nm, phenylalanine minima at 261 and 268 nm).

   Less pronounced is the spectral difference between phosphotyrosine and tyrosine containing peptides when the sequence contains an additional tryptophan residue. **Fig. 3A** and **3B** show that the two peptide spectra are more similar than the ones without tryptophan but even in this case slight hypsochromic shifts of the second-order derivative minimum from 272 to 270 nm are observed. The tryptophan minimum at 290 nm is also present. A similar picture is obtained when all three amino acids, i.e., either phosphotyrosine or tyrosine and phenylalanine and tryptophan, are in the peptide sequence (minimum shift from 272–270 nm, **Fig. 3C** and **3D**). The results of a peptide containing a phosphotyrosine and an unmodified tyrosine residue (spectra not shown) are listed in the last row of **Table 2**. Its spectrum represents a combination of the spectra of the two peptides containing either only phosphotyrosine or only tyrosine (**Table 2**, rows 1 and 2). The second-order derivative minima at 261 nm and 282 nm are derived from the phosphotyrosine and tyrosine residues, respectively, whereas the third minimum at 273 nm represents two overlapping minima.
4. The spectral data of all the peptides listed in **Table 2** may be stored in a spectra library and can be used for the analysis of peptides derived from digests of proteins whose tyrosine phosphorylation sites are to be mapped.

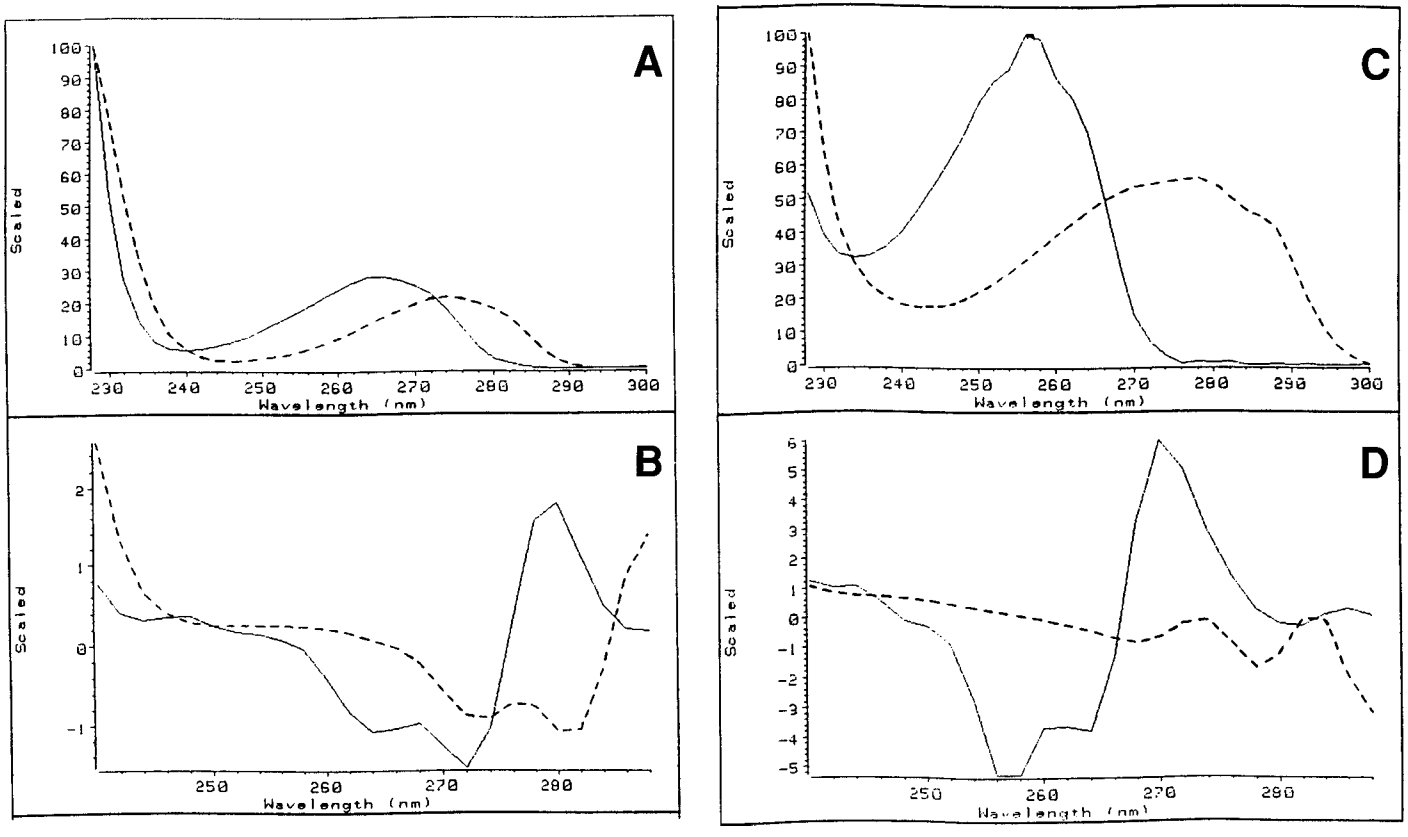


Fig. 1. Zero- (**A**,**C**) and second-order (**B**,**D**) derivative spectra of phosphotyrosine, tyrosine, phenylalanine and tryptophan. (**A**,**B**): phosphotyrosine (—) and tyrosine (- -) (**C**,**D**): phenylalanine (—) and tryptophan (- -).

Table 1
RP-HPLC On-Line Spectral Analysis of Free Aromatic Amino Acids

Amino acid		Second-order derivative spectra minima/nm
	*	
Phosphotyrosine	Y	264, 272
Tyrosine	Y	273, 281
Phenylalanine	F	257, 264
Tryptophan	W	268, 278, 290

Table 2
RP-HPLC On-Line Spectral Analysis of Synthetic Model Peptides

Peptide sequence	Aromatic amino acids	Second-order derivative spectra Minima/nm
*	*	
YVPML	Y	264,270
YVPML	Y	273,282
*	*	
YVPFL	Y,F	261,268,274
YVPFL	Y,F	258,264,274,282
*	*	
YVPWL	Y,W	262,270,280,290
YVPWL	Y,W	270,272,280,290
*	*	
YVFWL	Y,F,W	270,272,280,290
YVFWL	Y,F,W	272,280,290
*	*	
YVPYL	Y,Y	261,273,282

*: Phosphotyrosine.

4. Notes

1. A method has been developed that can detect the presence of phosphate groups on tyrosine residues with the help of on-line RP-HPLC spectral analysis, a method often used for purity check of peptides before sequence analysis *(**2**)*. We have demonstrated that owing to the hypsochromic shift of the aromatic UV-absorption maximum between 250 and 300 nm, characteristic second-order derivative minima can be detected in tyrosine-phosphorylated vs -unphosphorylated peptides.
2. Although other factors, such as sequence context, pH, concentration of the organic solvent during elution *(**10**)*, cause shifts in peptide absorption maxima, previous studies have shown that these differences are only in the range of 1–2 nm as compared to the corresponding free amino acids and thus are within the limit of resolution of the diode-array detector *(**11**)*.

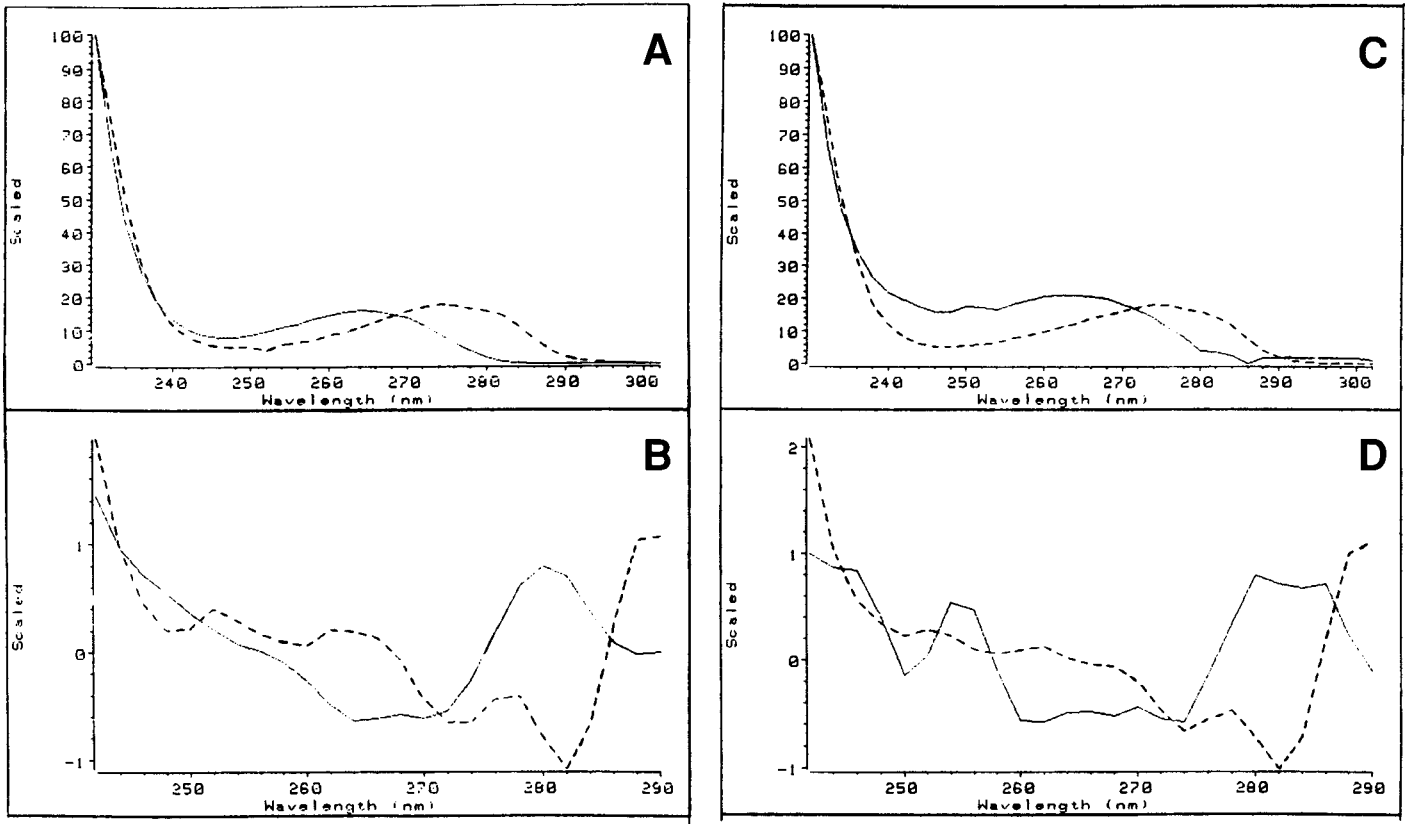


Fig. 2. Zero- (**A**,**C**) and second-order (**B**,**D**) derivative spectra of peptides $\overset{*}{Y}$VPML, $\overset{*}{Y}$VPML, YVPFL and YVPFL. (**A**,**B**): $\overset{*}{Y}$VPML (—) and YVPML (- -) (**C**,**D**): YVPFL (—) and YVPFL (- -).

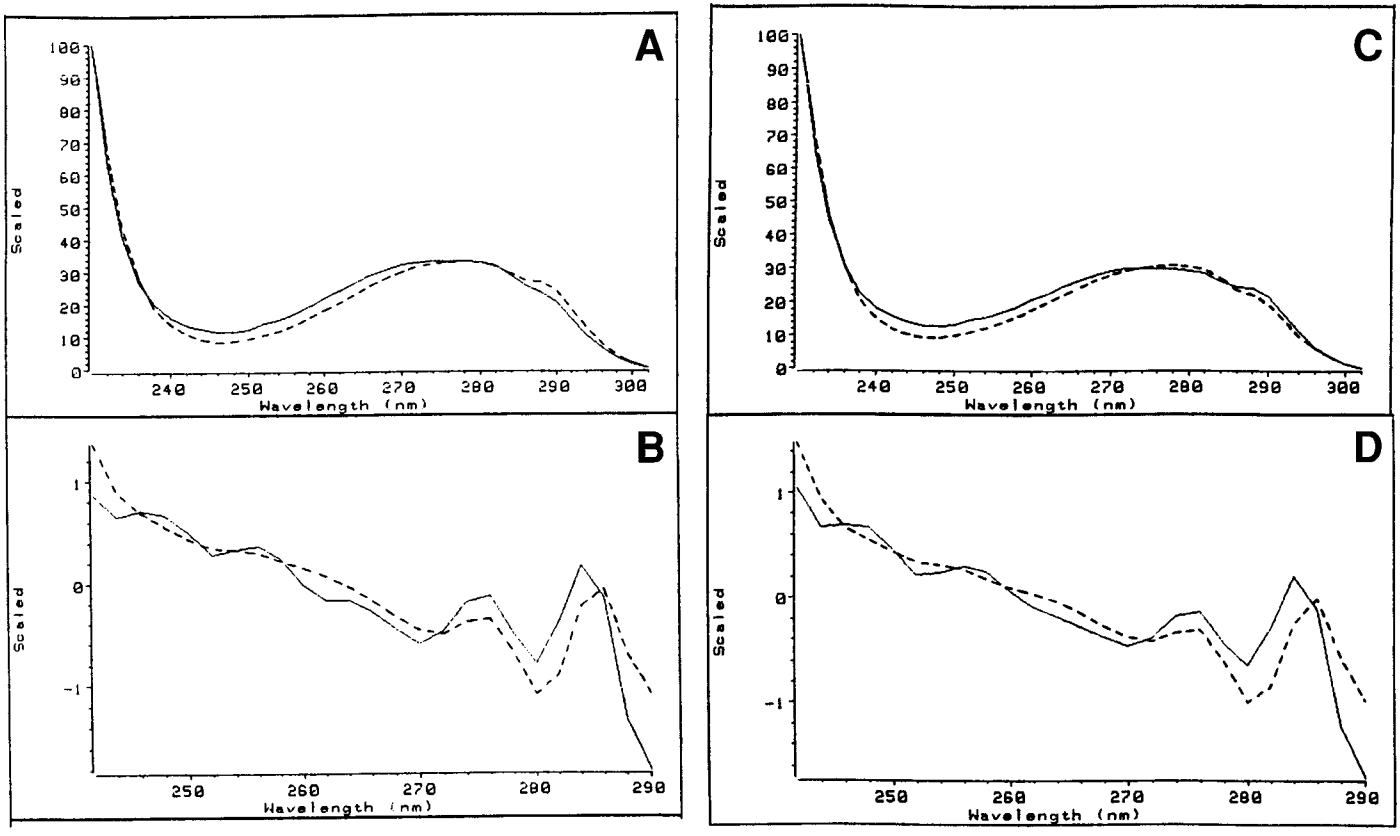


Fig. 3. Zero- (**A**,**C**) and second-order (**B**,**D**) derivative spectra of peptides *YVPWL, *YVPWL, YVFWL and *YVFWL. (**A**,**B**): *YVPWL (—) and YVPWL (- -) * (**C**,**D**): YVFWL (—) and YVFWL (- -).

3. With the exception of tryptophan-containing peptides where the hypsochromic shift of the absorbance maximum can be within the error of detection, tyrosine phosphorylation sites of microgram amounts of peptides can be detected with this method.
4. The established spectra library using the data from the peptides listed in **Table 2** can be expanded and should be useful in the analysis of peptide mixtures derived from tyrosine-phosphorylated natural as well as recombinant proteins during RP-HPLC separation.
5. The presented method for the identification of phosphotyrosine residues in peptides provides a simple and nondestructive way for the mapping of phosphorylation sites in proteins avoiding prior radiolabeling.
6. An additional application is the characterization of synthetic peptides containing phosphotyrosine. The synthesis of these compounds is associated with a high risk of phosphate ester hydrolysis in the assembled peptide chain during cleavage and deprotection steps leading to unphosphorylated peptide *(**11**)*. The on-line spectral analysis during RP-HPLC allows for a relatively simple way of checking for the presence of the phosphate group on tyrosine.
7. It is expected that other methods that use derivative spectroscopy to determine quantitatively the number of tyrosine and tryptophan residues in proteins *(**12**,**13**)* can be adapted for HPLC on-line analysis.

Acknowledgments

This work was supported by the Howard Hughes Medical Institute.

References

1. Turck, C. W. (1992) Identification of phosphotyrosine residues in peptides by high performance liquid chromatography on-line derivative spectroscopy. *Peptide Res.* **5**, 156–160.
2. Grego, B., Nice, E. C., and Simpson, R. J. (1986) Use of scanning diode array detector with reversed-phase microbore columns for the real-time spectral analysis of aromatic amino acids in peptides and proteins at the submicrogram level. *J. Chromatogr.* **352**, 359–368.
3. Cohen, P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **296**, 613–616.
4. Krebs, E. G. and Beavo, J. A. (1979) Phosphorylation-dephosphorylation of enzymes. *Ann. Rev. Biochem.* **48**, 923–939.
5. Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203–212.
6. Hunter, T. (2000) Signaling-2000 and beyond. *Cell* **100**, 113–127.
7. Martensen, T. M. (1984) Chemical properties, isolation, and analysis of O-phosphates in proteins. *Methods Enzymol.* **107**, 3–23.
8. Neubauer, G. and Mann, M. (1999) Mapping of phosphorylation sites of gel-isolated proteins by nanoelectrospray tandem mass spectrometry: potentials and limitations. *Anal. Biochem.* **71**, 235–242.

9. Meyer, H. E., Hoffmann-Posorske, E., Korte, H., Donella-Deana, A., Brunati, A.-M., Pinna, L. A., et al. (1990) Sequence analysis of phosphotyrosine-containing peptides. Determination of PTH-phosphotyrosine by capillary electrophoresis. *Chromatogr.* **30**, 691–695.
10. Donovan, J. W. (1969) Ultraviolet Absorption, in *Physical Principles and Techniques of Protein Chemistry, Part A* (Leach, S. J., ed.), Academic Press, New York, NY, pp. 101–105.
11. Valerio, R. M., Perich, J. W., Kitas, E. A., Alewood, P. F., and Johns, R. B. (1989) Synthesis of O-phosphotyrosine-containing peptides. II Solution-phase synthesis of Asn-Glu-PTyr-Thr-Ala through methyl phosphate protection. *Aust. J. Chem.* **42**, 1519–1525.
12. Bray, M. R., Carriere, A. D., and Clarke, A. J. (1994) Quantitation of tryptophan and tyrosine residues in proteins by fourth-derivative spectroscopy. *Anal. Chem.* **221**, 278–284.
13. Mach, H. and Middaugh, C. R. (1994) Simultaneous monitoring of the environment of tryptophan, tyrosine, and phenylalanine residues in proteins by near-ultraviolet second-derivative spectroscopy. *Anal. Biochem.* **222**, 323–331.

10

Hydrolysis of Samples for Amino Acid Analysis

Ian Davidson

1. Introduction

There is no single hydrolysis method that will effectively cleave all proteins to single amino acids completely and quantitatively. This is owing to the varying stability of the peptide bonds between the different amino acids and the amino acid side chains, which are themselves susceptible to the reagents and conditions used to cleave the peptide bonds (*see* **Table 1**). The classical hydrolysis conditions, to which all other methods are compared, is liquid-phase hydrolysis in which the protein or peptide sample is heated in 6 *M* hydrochloric acid under vacuum at 110°C for 18–24 h ***(1)***. The various methods of hydrolysis described here are summarized in **Table 2**.

2. Materials

1. Rotary evaporator, e.g., Savant "Speed Vac" equipped with a supply of high-purity nitrogen to the atmosphere inlet.
2. Rotary Vacuum pump, capable of evacuating the system to 50mTorr or better, equipped with a cold trap and high-purity nitrogen bleed valve. The cold trap should be kept at a temperature of –60°C or below. This can be achieved by cooling propan-2-ol in a Dewar Flask with dry ice or, e.g., Neslab (Waalresberg, Netherlands) Immersion Cryocool Type CC60 or by filling the Dewar flask with liquid nitrogen.
3. Argon gas supply, equipped with a needle valve to enable slow bubbling of gas.
4. Laboratory Oven with a variable temperature control up to 200°C
5. Vapor-phase hydrolysis tubes (Borosilicate glass 50 × 6 mm) (Sigma-Aldrich, Dorset, UK, Part no. Z144509). Before using the 50 × 6mm sample tubes they should first be cleaned thoroughly by inverting them in a borosilicate glass beaker, pyrolysing at 500°C for approx 4 h. Allow to cool, rinse with ultra high-quality water, dry, and store covered. Use these cleaned tubes only once.

From: *Methods in Molecular Biology, vol. 211: Protein Sequencing Protocols, 2nd ed.*
Edited by: B. J. Smith © Humana Press Inc., Totowa, NJ

Table 1
Stability of Amino Acid Residues and Peptide Bonds During Hydrolysis on 6 M Hydrochloric Acid at 110°C

Residue/bond	Stability/modification	Consequence	Remedy
Serine; Threonine	1. Side-chain hydroxyl group modified by dehydration, which is increased with increased hydrolysis time and temperature. 2. Ester formation with, e.g., glutamic acid can occur at the drying stage ***(3)***.	Serine and Threonine generated in low yield.	1. Hydrolyze protein samples for different times between 6–72 h. Calculate the yields, extrapolate results to time = 0 to compensate for losses ***(1,10)***. 2. Dry the hydrolysates rapidly in a rotary evaporator.
Tyrosine	The phenolic group (-C6H4OH) side chain is modified by traces of hypochlorite/chlorine radicals present in the acid ***(3)***.	Tyrosine is generated in low yield.	Incorporate phenol in acid to compete for hypochlorite/ chlorine radicals ***(1,4,5)***.
Methionine	The thioether (-CH2-S-CH3) side chain is oxidized to the sulphoxide or sulphone ***(3)***.	Methionine, usually a less common residue anyway is converted to smaller peaks, more difficult to quantify on amino acid analyzers.	Add reducing agent (e.g., dodecanthiol or thioglycolic acid) to the acid/phenol mixture ***(5,6)***.
Cystine; Cysteine	The free sulfhydryl (-SH) and diusulphide (-S-S) side chain groups are oxidized ***(3)***.	Cystine and Cysteine recovered in low non-quantifiable yields.	Chemically modify prior to hydrolysis ***(7)*** (Chapter 27).

Table 1 *(continued)*
Stability of Amino Acid Residues and Peptide Bonds During Hydrolysis on 6 M Hydrochloric Acid at 110°C

Residue/bond	Stability/modification	Consequence	Remedy
Trytophan	The Indole group side chain is destroyed by oxidation under acid conditions ***(3)***.	Trytophan is not quantifiable under these conditions.	Add reducing agents (e.g., dodecanthiol or thioglycolic acid) to the acid mixture ***(5,6,10)*** or hydrolyze under alkaline conditions ***(3,8,9)***.
Aspargine; Glutamine	Asn and Gln are deaminated to form the respective acids ***(3)***.	Mixtures of Asp/Asn amd Glu/Gln are normally assigned as Asx and Glx respectively, in quantification data.	
Bonds between hydrophobic amino acids (e.g., Val-Val or any combination of Ala, Ile, Leu, Val).	Bonds are realtively stable ***(3)***.	Hydrolyze in poor yield. May be seen as dipeptides or similar, on amino acid analyzer or not at all.	Hydrolyze for longer time or elevated temperature, e.g., 165°C ***(1,5)***.
Phosphorylation	Phosphorylated amino acids are labile.	Destroyed under these extreme conditions.	Reduce time for hydrolysis to 1–4 h ***(13)***.
Glycosylation	Amino acid-sugar interactions produces complex secondary reaction products ***(3)***.	Complex reaction products are difficult to interpret even if they are seen on an analyzer.	Deglycosylate before hydrolysis as described in Chapter 30.

Table 2
Advantages and Disadvantges of Various Hydrolysis Techniques

Method of hydrolysis	Advantages	Disadvantages
Vapor phase under argon (165°C for 45 min) (*see* **Subheading 3.1.**).	High sensitivity. Relatively fast hydrolysis times. Samples can be processed in batches.	Owing to the high pressures the reaction vial and seals require regular inspection. Danger of exploding vials and eacaping hot acid can occur with defective vials and seals.
Vapor phase under vacuum (110°C for 18 h) (*see* **Subheading3.2.**).	Conditions not as extreme as in **Subheading 3.1.** Samples can be processed in batches.	Long duration time for hydrolysis. Most analyzers have relatively short derivatization and analysis times. The seals of the vial require regular inspection as above.
Liquid phase (110°C for 18 h) (*see* **Subheading 3.3.**)	Conditions not as extreme as in **Subheading 3.1.**	Long duration time for hydrolysis. Samples are processed individually over a number of manipulations, which is very time consuming.
Microwave irradiation (8 min) (*see* **Subheading 3.4.**).	Rapid hydrolysis times. Samples can be processed in batches.	More extreme conditions than 3.1.; thereefore more dangers from exploding vials. Potential contamination from the reusable, expensive tubes.
PVDF blots (*see* **Subheading 3.5.**).	Some useful composition data from samples that may not have been pure prior to SDS gel electrophoresis.	Low recoveries. Samples are difficult to remove successfully from the blot.
Liquid phase under alkaline conditions (*see* **Subheadings 3.6.** and **3.7.**).	Thrytophan is preserved throughout.	Nonvolatile reagents. Relatively high salt content. pH for derivatization difficult to control with such small volumes.

6. Liquid-phase hydrolysis tubes are made by cutting a piece of borosilicate glass tubing, 6 mm ID × 8 mm OD, 150–200 mm in length. The shortened tubing can be cleaned by immersing in 50% Nitric acid in water overnight, rinsing with distilled water, and finally with ultra high-quality water. Dry the tubes in an oven. Seal one end with a glass blowing torch, equipped with a gas and oxygen flame, to form a tube.

7. Preparation of acid hydrolysis mixture (add reducing agent as and when necessary).
   a. Hydrochloric acid (constant boiling) 6 *M* (Applied Biosystems, Foster City, CA, Part No. 400939 or Pierce, Rockland, IL, Part No.24309) (1 mL ampoules).
   b. Phenol (Sigma-Aldrich, Part No. 328111). (Store small amounts in a clean vial sealed under argon)
   c. Reducing agent, e.g., Dodecanethiol (Sigma-Aldrich, Part No. 62592) or Thioglycolic acid (Sigma-Aldrich, Part No. 88650).
   d. Gently heat the vial containing a little phenol, stored as above (**Note 7b**), on a hotplate set at 80°C in a fume cupboard to liquefy the phenol. Add 5 µL of the liquefied phenol before it cools, to 500 µL hydrochloric acid and mix thoroughly. Add 5 µL of the reducing agent and mix thoroughly. Allow the stock phenol to cool before purging with argon and storing.
   e. Alternative hydrolysis reagents for samples containing Tryptophan: 3 *M* mercaptoethanesulphonic acid (Pierce, Part no. 25555) or 4 *M* methanesulphonic acid (containing 0.2% 3-(2-Aminoethyl) indole) (Pierce, Part no. 25600).
   f. Alkaline hydrolysis reagent for samples containing tryptophan. 4.2 *M* sodium hydroxide (Sigma-Aldrich, Part no. 06213).
8. Reaction vial and valve (*see* **Fig. 1**) (e.g., Waters, Milford, MA, Part no. 07363). The Waters reaction vial is specified for vapour phase hydrolysis conditions at 110°C under vacuum. For hydrolysis conditions requiring elevated temperatures then a Reaction Vial (120 mm × 25 mm OD) consisting of a teflon lined Mininert valve (Pierce, Part no. 10150) fitted to a heavy walled, glass 25 mL universal container is required. A bulb is blown at 50 mm from the base of the universal container (a good glassblower will help with this) so that the tops of the sample tubes do not come into contact with the walls of the reaction vial. Any condensed hydrolysis mixture will therefore not run down the vial wall into the sample tube.
9. Microwave hydrolysis tubes, 100 ×10 mm borosilicate tubes with side arm and Teflon screw-on valve (Pierce, Part no. 29560). These tubes are reusable but should be cleaned thoroughly as described in **Subheading 2.6.**
10. Microwave Oven 650 Watt Full Power (Minimum requirement).
11. Norleucine as an internal standard is prepared by dissolving 32.75 mg of L-Norleucine (Pierce, Cat. no. 36323) in 100 mL of 0.1 *M* hydrochloric acid. Aliquots of approx 200 µL of this stock solution are frozen, almost indefinitely, at –20°C until required. A daily working solution is prepared by dissolving 20 µL of the stock solution up to 1 mL with water and vortexing thoroughly. A 5 µL aliquot of this solution contains 250 pmol Norleucine (*see* **Note 1**).
12. Digestion block (Pierce, Part no. 18970).
13. There are several different types of tips and packing material available and the choice is dependent on the molecular weight and type of peptide or protein of interest. (Supro tips, Nest Group, Southborough, MA, Part no. SSPUVC08F or Zip tips from Millipore, Bedford, MA, Part no. ZTC18S960. Alternatively, these tips can be prepared and adapted as required ***(2)***. Constrict the end of a gel-loader tip (Eppendorf-Netherel-Hinz Gmbh, Hamburg, Germany, Part no. 0030

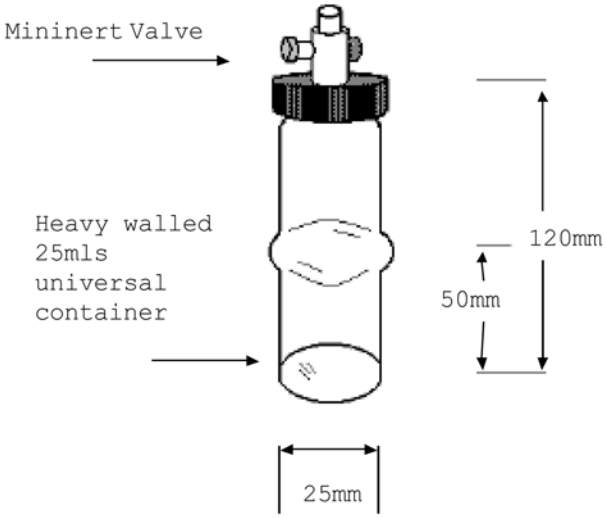


Fig. 1. Hydrolysis reaction vial.

001.222) by gently squeezing and pulling the end of the tip with a spatula so that liquid can still pass through the constricted tip but a few millimeters of a slurry of POROS R1 10 (Applied Biosystems, Framingham, MA, Part no. 1-1118-02) does not. The slurry is a mixture of the POROS material and 30% propan-2-ol in water. Use a 1-mL syringe to act as pump to push the POROS through but not out of the gel loader tip.

3. Methods

3.1. Manual Vapor-Phase Hydrolysis Under Argon

1. Pipet the sample aliquot, including any internal standard (*see* **Notes 1–5**) to a maximum volume of 200 μL, into the vapor-phase hydrolysis sample tube (50 × 6 mm borosilicate tube) as described in **Subheading 2.5.**, marked with a diamond-tipped pen as the ink from felt-tipped pens will run. Normally 50 ng to 1 μg is required depending on the derivatization method employed (*see* Chapters 11–15). Centrifuge so that the entire sample collects at the bottom of the tube and not on the sides.
2. Dry the sample. The samples should be dried in a rotary evaporator ensuring that there are no loose particles left in the bottom of the tube. Should loose particles be present after drying then add a little water and redry.
3. Add 500 μL of hydrolysis mixture to the reaction vessel (*see* **Fig. 1**).
4. Slowly purge the hydrolysis acid mixture in the reaction vessel and the mininert valve with argon for approx 2 min to displace atmospheric oxygen.

5. Add the sample tubes and purge with argon again. Purge each tube in turn taking care to avoid cross-contamination and displacing material from the tube. A gentle continuous flow from a 50 µL micropipet is a useful guide.
6. Screw the mininert valve securely onto the reaction vessel ensuring the valve is in the closed position.
7. Heat the reaction vessel to 165°C for 45 min (*see* **Note 6**).
8. **CAUTION!! The contents are under pressure.** Open the reaction vessel mininert valve very carefully in a fume cupboard. Use thermal gloves and face shield. Release the pressure very gradually to avoid the liquid boiling up into the sample tubes.
9. Remove the tubes from the reaction vessel while still hot. Wipe the outside of the tube with a clean tissue and dry off the sample tubes in a rotary evaporator without centrifugation to prevent any condensed acid vapor being drawn onto the sample at the bottom of the sample tube.
10. Store the samples dry at –20°C until required for derivatization.

3.2. Manual Vapor-Phase Hydrolysis Under Vacuum

1. The same as for **Subheading 3.1., steps 1–3**.
2. Add the tubes to the reaction vial.
3. Screw the mininert valve securely onto the reaction vial ensuring the valve is in the closed position.
4. Attach the reaction vessel to the vacuum pump and open the valve slowly to avoid "bumping."
5. Evacuate for a few minutes with gentle agitation.
6. Close the valve.
7. Heat the reaction vessel to 110°C for 18 h (*see* **Note 6**).
8. Attach the reaction vessel to a nitrogen supply set at approx 1–5 psi and slowly open the mininert valve, taking care to avoid the samples being displaced from the tubes.
9. Remove the tubes from the reaction vial while still hot. Wipe the outside of the tube with a clean tissue and dry off the sample tubes in the rotary evaporator without centrifugation to prevent any condensed acid vapor being drawn onto the sample at the bottom of the sample tube.
10. Store the samples dry at –20°C until required for derivatization.

3.3. Manual Liquid-Phase Hydrolysis Under Vacuum

1. Pipet the sample aliquot including any internal standard, into a liquid phase (*see* **Notes 1–5**) hydrolysis borosilicate glass tube (200 mm × 6 mm ID) as described in **Subheading 2.6.** marked with a diamond tipped pen on the bottom one-third of the tube (the top two-thirds will be discarded later). Normally 50 ng to 1 µg is required depending on the derivatization method employed (*see* Chapters 11–15). Centrifuge so that the entire sample collects at the bottom of the tube and not on the sides.
2. Dry the samples in a desiccator under vacuum, slowly to avoid "bumping."

3. Add 200 µL of hydrolysis mixture to the hydrolysis tube.
4. Vortex thoroughly.
5. Centrifuge the hydrolysis tubes again so that all of the sample and hydrolysis mixture is on the bottom of the tube.
6. Cool the samples to –20°C or below to avoid the sample "bumping" when it is attached to the vacuum line. This can be achieved easily by dipping the tube into the –60°C cold trap of the vacuum pump for a few minutes.
7. Evacuate for a few minutes with gentle agitation.
8. Seal the tube while still under vacuum, approx 30–50 mm from the top, with a glass-blowing torch, equipped with a gas and oxygen flame.
9. Heat the reaction vessel to 110°C for 18 h (*see* **Note 6**).
10. Cool the tubes in ice. Centrifuge so that the samples are at the bottom of the tubes and not on the walls of the tubes.
11. Cut the tube open with a sharp glass knife approx 30–50 mm from the top by scoring around the tube, placing both thumbs either side of and close to the score and pulling both hands together against the thumbs.
12. Dry off the excess acid in a desiccator or rotary evaporator.
13. Store the samples dry at –20°C until required for derivatization.

3.4. Manual Liquid-Phase Hydrolysis by Microwave Irradiation

1. Pipet the sample aliquot (*see* **Notes 1–5**) as described in **Subheading 3.3.**, **step 1** into the specifically designed vacuum hydrolysis tube described in **Subheading 2.**, **item 9**.
2. Proceed as described **Subheading 3.3.**, **steps 2–7**.
3. Seal the tube by screwing down the Teflon plunger.
4. Place the tubes in a Microwave Oven on full power (650W) for 8 min. **Extreme Caution!! High pressures of up to 140 psig. The tubes have been reported to explode at this point.** ***(11)***
5. Cool the hydrolysate tubes in ice, centrifuge to collect the entire sample on the bottom of the tube and not on the tube walls.
6. Pipet the hydrolyzed samples into a clean microcentrifuge tube.
7. Dry off the excess acid in a rotary evaporator.
8. Store the samples dry at –20°C until required for derivatization.

Note: A commercial Microwave Digestion System Type CEM-MDS-81D is available from CEM Corporation (Mathews, NC) ***(10,11)***.

3.5. Manual Hydrolysis of Samples Blotted onto Polyvinylidene Difluoride (PVDF) Membrane (see Note 6)

1. Using any of the described methods from **Subheadings 3.1.–3.3.**, cut the band from the membrane (with a scalpel) containing the sample and place the PVDF membrane into the bottom of a hydrolysis tube and proceed as normal taking care that the membrane is not dislodged during evacuation or when opening the tubes.
2. Proceed as described in **Subheadings 3.1.–3.3.**
3. Dry off the excess acid under vacuum in a rotary evaporator.

4. Extract the hydrolyzed amino acids in the sample from the hydrolyzed PVDF membrane by adding 100 μL of 70% 0.1 *M* hydrochloric acid in methanol (v/v).
5. Vortex thoroughly, by attaching the sample tubes to the mixer with a piece of parafilm, or similar, for 5 min.
6. Transfer the liquid to a clean microcentrifuge tube.
7. Repeat **steps 5** and **6**.
8. Dry the hydrolysed sample in a rotary evaporator.
9. Store the sample dry at –20°C until required for derivatization.

3.6. Alternative Reagents for Hydrolysis of Samples Where Tryptophan is to be Preserved (see Note 8)

1. Proceed as described in **Subheading 3.3.**, **steps 1–8**, substituting 30 μL 3 *M* mercaptoethanesulphonic acid (or 4 *M* methanesulphonic acid) for the acid hydrolysis mixture.
2. Heat the tube at 110°C for 22 h.
3. Cool the tube in ice and centrifuge so that the entire sample is collected at the bottom of the tube and not on the tube walls.
4. Neutralize with 50 μL of 1.38 *M* sodium hydroxide solution and mix thoroughly.
5. Store the samples at –20°C until required for derivatization.

3.7. Manual Alkaline Hydrolysis of Samples Where Tryptophan is to be Preserved (see Note 9)

1. Proceed as described in **Subheading 3.3.**, **steps 1–7** substituting the 200 × 60 mm Borosilicate glass tube with the specifically designed vacuum hydrolysis tube as described in **Subheading 2.9.** Note the tubes will not be cut at a later stage.
2. Add 200 mL of 4.2 *M* sodium hydroxide solution and vortex.
3. Seal the tube by screwing down the Teflon plunger.
4. Heat the hydrolysis tube to 110°C for 18 h (*see* **Note 5**) in the digestion block (*see* **Subheading 2.**, **item 12**).
5. Proceed as described in **Subheading 3.4.**, **steps 5–6**.
6. Neutralize to the correct pH for derivatization with approx 60 μL concentrated hydrochloric acid.
7. Alternatively store the hydrolyzed samples buffered at pH 4.25 at –20°C until required for derivatization.

4. Notes

1. The choice of internal standard depends on the following:
   a. Stability during hydrolysis.
   b. The derivatization procedure employed. The yield should be linear with concentration.
   c. The ability to be easily separated from other amino acids.
   d. Not occurring naturally.
   e. Commercially available and inexpensive.

2. Samples should ideally be dissolved in volatile solvent (i.e., no buffer salts present). Removal of buffer salts prior to hydrolysis may be required. This can be achieved by desalting either by HPLC as described in Chapter 1 or with the use of Supro tips or Zip Tips (see **Subheading 2.1.**, **step 3**). It should be noted that that the commercially available tips are for high sensitivity work and it may be necessary to apply several desalting cycles per sample to achieve sufficient material for amino acid analysis.
   To equilibrate the tip:
   a. Add 10 μL of a solution of Acetonitrile/0.1% Trifluoroacetic acid in water (9:1), Acetonitrile HPLC-grade, Trifluoroacetic acid (Sigma-Aldrich) to the zip tip. Attach the 1-mL disposable syringe to the above tip to act as a pump and gently expel the solution to waste. Remove the syringe. Repeat twice.
   b. Add 10 μL of 0.1% TFA in water to the Zip Tip. Attach the 1-mL disposable syringe to the above tip and gently expel the solution to waste. Remove the syringe. Repeat twice.
   c. Add the sample through the tip, the volume will depend on the concentration of the sample and the derivatization method for the analysis but a few picomoles should be sufficient. With some analyzers it may be necessary to repeat the procedure several times to obtain sufficiently pure material. Remove the syringe.
   d. Remove the buffer salts from the protein or peptide sample by adding 10 μL of 0.1% TFA in water and expelling slowly to waste using the syringe. Repeat 2 or 3 times depending on the concentration of buffer salts expected.
   e. Finally elute the purified peptide or protein from the column packing material with 10 μL of a solution of Acetonitrile/0.1% TFA in water (9:1 v/v) and collect the purified material.
   f. The tips are cleaned and stored as in **step a** and may be used several times but this will depend on the level of impurities in the samples.
3. Powder-free gloves should always be worn when handling samples and associated glassware to avoid contamination. Powdered gloves can give rise to contamination from proteins, peptides, and amino acids adsorbing to the cornstarch in the powder. The particles of cornstarch can also accumulate in and eventually block narrow-bore tubing found in most modern analyzers.
4. The use of dedicated glassware is also recommended. The glassware should be cleaned, where possible, with 50% nitric acid, rinsed thoroughly with high-quality water and stored covered. Washing the glassware in a 3% solution of EDTA (Ethylenediaminetetraacetic acid, tetrasodium salt, Sigma-Aldrich, Part no. E26290) every couple of months will clean off and prevent metal ions, which can leach out from the glass, building up on the surfaces.
5. Reagents for all methods should be of the highest quality available. Water should be of ultra-high quality. (HPLC-grade or better).
6. A hydrolysis time-course experimental study is highly recommended to optimize laboratory conditions best suited to particular requirements. In a (1993) survey of

amino acid analysis test sites ***(10)***, the times of hydrolysis averaged 111 ± 2.7°C for 22 ± 2.4 h or 160 ± 15°C for 1.4 ± 0.4 h.

7. PVDF blots are best analysed by manual liquid phase hydrolysis which requires excess acid but the recoveries are low, in the region of 25–30% with the total loss of methionine. Some useful composition information can be produced with care.
8. As with alkaline hydrolysis (*see* **Note 9**) the reagents are nonvolatile and have relatively high amounts of salts present. The hydrolyzed amino acids can be purified or determined by Ion Exchange Chromatography or derivatization followed by Ion exchange Chromatography for analysis.
9. Alkaline hydrolysis is dependent on the derivatization method to be used. The hydrolysis reagents are nonvolatile and have relatively large amounts of salts present, which is unsuitable for phenylisothiocyanate (PITC) derivatization for example.

Acknowledgment

My thanks to Lynne McKay for the illustration (**Fig. 1**).

References

1. Moore, S. and Stein, W. H. (1963) Chromatographic determination of amino acids by the use of automatic recording equipment. *Methods Enzymol.* **6**, 819–831.
2. Wilm, M., Shevchenko, A., Houthaeve, T., Brit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometer. *Nature* **379**, 466–469.
3. Hunt, S. (1985) Degradation of amino acids accompanying *in vitro* protein hydrolysis, in *Chemistry and Biochemistry of the Amino Acids* (Barrett, G. C., ed.) Chapman and Hall, London, pp. 376–398.
4. Bidlingmeyer, B. A., Tarvin, T. L., and Cohen, S. A. (1986) Amino acid analysis of submicrogram hydrolysate samples, in *Methods in Protein Sequence Analysis* (Walsh K., ed.) Humana Press, Totowa, NJ, pp. 229–244.
5. Dupont, D., Keim, P., Chui, A., Bozzini, M. L., and Wilson, K. J. (1988) Gas-phase hydrolysis for PTC-amino acids. *Appl. Biosys. User Bull.* **2**, 1–10.
6. Matsubara, H. and Sasaki, R. M. (1969) High recovery of tryptophan from acid hydrolysis of proteins. *Biochem. Biophys. Res. Commun.* **35**, 175–181.
7. Carne A.F. Chemical Modifications of Proteins, Methods in Molecular Biology, *Basic Protein and Peptide Protocols*, vol. 32 (Walker, J. M., ed.) Humana Press, Totowa, NJ, pp. 311–320.
8. Hugli, T. E. and Moore, S. (1972) Determination of the tryptophan content of proteins by ion exchange chromatography of alkaline hydrolysis. *J. Biol. Chem.* **247(9)**, 2828–2834.
9. Simpson, R. J., Neuberger, M. R., and Lui, T.-Y. (1976) Complete amino acid analysis of proteins from a single hydrolysate. *J. Biol. Chem.* **251**, 1936–1940.
10. Strydom, D. J., Anderson, T. T., Apostal, I., Fox, W. J., Paxton, R. J., and Crabb, J. W. (1993) Cysteine and tryptophan amino acid analysis of ABRF92-

AAA, in *Techniques in Protein Chemistry IV* (Angeletti, R.H., ed.) Academic Press, San Diego, CA, pp. 279–288.
11. Chiou, S. H. and Wang, K.-T. (1990) A rapid and novel means of protein hydrolysis by microwave irradiation using Teflon-Pyrex tubes, in *Current Research in Protein Chemistry, vol. 3* (Villafranca, J. J., ed.) Academic Press, San Diego, CA, pp 3–10.
12. Gilman, L. B. and Woodward, C. (1990) An evaluation of microwave heating for the hydrolysis of proteins, in *Current Research in Protein Chemistry, vol. 3* (Villafranca, J. J., ed.) Academic Press, San Diego, CA, pp. 23–26.
13. Capony, J.-P. and Demaille J. G. (1983) A rapid microdetermination of phosphoserine, phosphothreonine and phosphotyrosine in proteins by automatic cation exchange on a conventional amino acid analyser. *Anal. Biochem.* **128**, pp. 206–212.