THE EFFECT OF TRANSGLUTAMINASE (TG) ON

DOUGH AND BREAD CONTAINING WHEAT-

SOYBEAN TEMPE FLOUR

By

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Abstract

Consumer's need for good quality and healthy products are also applicable to wheat baked products. Variation of gluten protein quality and the limiting amino acid lysine from the nutritional point of view are two challenges of wheat flour. Gluten protein can be modified by transglutaminase (TG) and improvement of desirable product properties can be designed with the right treatment dose. Tempe, a fermented soy/grain, provides healthy components such as isoflavones, bioactive peptides, vitamins and high lysine content. This study was aimed to measure the effect of TG on gluten, dough and baking properties. A set of six commercial wheat flours with protein content (11.0 ± 0.5) was treated with TG (0; 0.1; 0.2; 0.4; 0.8; 1.6 % w/w flour basis) based on randomized complete block design with 3 replications. Results indicated that wet gluten decreased while gluten index and elastic recovery increased with increasing TG levels. Addition of mixing and resting dough prior to the gluten extraction positively increased wet gluten and gluten elastic recovery. At level of 0-0.4%, TG positively increased farinograph development time and stability, decreased amount of CO_2 release during dough fermentation, increased the coefficient of gas retention and produced finer bread crumb structure. However, at 0.8% TG, excessive formation of covalent bonds dramatically decreased dough rheological properties, Hm and bread volume and increased crumb texture hardness.

The study was also aimed to prepare tempe wheat-soy flour and to analyze the effect of TG and tempe flour on rheological and fermentation properties of composite flour. A desirable tempe cake and lysine content closest to WHO standard lysine requirement for adults was obtained with wheat:soy at 1:1 ratio (w/w). Substitution tempe flour at 0, 10, 20, 30 and 40% in wheat flour treated with TG (0, 0.05 and 0.1%) significantly influenced most of the mixing and fermentation properties of composite flour. It increased development time, stability and breakdown time and decreased CO_2 lost during fermentation. Tempe flour up to 20% did not change gaseous release. This study suggests that composite flours using up to or less than 20% tempe can be used in bread formulation using 0.1% TG.

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LIST OF ABBREVIATION

AA	=	Amino acids
AACCI	=	AACC international
BLAST	=	Basic Local Alignment Search Tool
BU	=	Brabender unit
FAO	=	Food and agriculture organization
Gluten CORE	=	Gluten compression-relaxation
h	=	The height of the dough at the end of the test
H_f	=	Final height at 60 s test
H_i	=	Initial height
H_m	=	Minimum height reached through compression test
Hm	=	Maximum height of the dough
(Hm-h)/Hm	=	Lowering the dough development percentage after 3 h
H'm	=	Maximum height of the gaseous release curve
HMW-GS	=	High molecular weight glutenin subunits
ICC	=	International association for cereal science and technology
LMW-GS	=	Low molecular weight glutenin subunits
M-TG	=	Microbial transglutaminase
NCBI	=	National Center for Biotechnology Information
Rep	=	Repetitive domain
Sig	=	Signal peptide
T1	=	The time required for reaching the maximum height
T2	=	Time at which Hm decreases 12%
T'1	=	Time spent to reach H'm
TG	=	Transglutaminase
Tx	=	Time when the dough begins to release CO_2
WHO	=	World health organization
USDA	=	United State department of agriculture

CHAPTER I

INTRODUCTION

Statement of Problem

Wheat flour is the major ingredient for producing various leavened yeast products like bread and pastry. Bread baking quality, according to Caballero et al (2007), is mainly determined by the quality and quantity of gluten protein. However, the quality of wheat flours and characteristics of gluten protein can differ among wheat flours due to differences of growing environment, genetic background and post-harvest conditions (Joye et al 2009). Improvers such as the enzyme TG could be added to aid the cross-linking of protein and improve the baking quality of wheat flour.

TG has the ability to introduce covalent cross-links between glutamine and lysine residues of protein to form high molecular weight polymer (Tseng and Lai, 2002; DeJong and Kopelman, 2002; Ahn et al 2005). In the presence of TG, the gluten network becomes more developed (Autio et al 2005) and it is expected that higher polymerization will influence viscoelastic properties and the ability to hold CO_2 gas produced by yeast during fermentation. However, excessive protein cross-linking by increasing TG levels too much will have detrimental effects on the bread baking quality (Renzetti et al, 2010).

Efficiency of TG in improving flour/ bread quality is dependent on the concentration of TG (Moore et al 2006) and protein sources (Marcoa and Rosell 2008).

The effect of different TG levels on bread systems has been studied. The recommended level of TG addition varies from low dose (0.05% TG) for improving the crumb of wheat:soy (85:15 w/w) bread (Roccia et al 2012), 5000 ppm (0.5% TG) for improving bread and croissant dough (Gerrard et al 2001), 1% TG for improving visco-elastic oat dough (Huang et al 2010) and (gluten-free) rice flour dough quality (Marcoa and Rosell 2008) and 2.5%TG for improving wheat bread fortified with 20% barley flour (Basman et al 2003). Roccia et al (2012) reported that 0.3% TG caused adverse effects on wheat:soy (85:15 w/w) bread volume and crumb firmness. It seems that different systems need a specific range of TG levels to obtain a desired functionality. The objective of the study was to evaluate the effects of increasing levels of TG in properties of wheat flours with similar protein content but with different quality.

Another objective of this study was to incorporate tempe flour into bread formulation. This would potentially offer consumers a choice to a line of breads containing tempe. According to Delcour et al (2012), bread is an excellent carrier of health-promoting components because bread is a staple food and has been a steady contribution to the human diet.

Tempe is an Indonesian traditional fermented food mainly made from soybean using inoculums *Rhizopus* spp. (Astuti et al 2000). Tempe has been recognized as a good food. It contains bioactive peptides, isoflavones and tocopherols considered potent antioxidants (Hoppe et al 1997; Hernandez-Ledesma et al 2011), and supports human brain and general health (Hogervorst et al 2011). Tempe also has high molecular weight

(HMW) fraction /'arabinose' compounds that have anti-bacterial effect (Kiers et al 2007; Roubos-van den Hil et al, 2010). The United States was the number 1 and number 3 world's largest soy and wheat producer with production of 82,054,800 and 61,775,240 T, respectively in 2012 (FAOSTAT, 2014). Therefore, it is of interest to combine wheat grain with soybean and adopt the tempe fermentation method to prepare tempe flour. The flour could have the compounds found in tempe, deliver broad utilization of wheat and soy as new food ingredients, and increase the consumption of these crops.

Fortification of soy flour into bread or baked product formulations delivers benefits. It decreases carbohydrate and energy (calories) (Mohamed et al 2006) increases minerals, fiber (Serrem et al 2011), protein and lysine content (Shogren et al 2003; Serrem et al 2011). The bread making process modifies the structure of isoflavones (glucosides to aglycones) but does not change total isoflavone content. Therefore, baked products still contain isoflavones that are believed to have antioxidant effects and other health benefits (Shao et al 2009).

On the other hand, adding soy flour has negative effects on both gluten network formation and dough properties leading to the decrease of final bread quality (Roccia et al 2012) such as decrease of bread loaf volume and increase bread weight (Islam et al 2007). Too much soy also causes 'beany' and bitter flavor (Shogren et al 2003). Regarding limitations of soy flour, so far a 10% substitution is recommended to make bread (Islam et al 2007); 35% soy fortification in spaghetti formulation without any adverse effect on flavor and texture (Shogren et al 2006); and a 50% fortification to sorghum biscuit formulation (Serrem et al 2011). However, there are no studies in the

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literature on the effect of incorporating increasing levels of soy in the form of tempe flour into a bread formulation.

Several treatments such as soaking, boiling, fermentation, drying and milling are applied during tempe flour preparation. Heat treatments modify the conformation of the protein which disrupt the secondary structure resulting in an unfolded structure and denatured protein (Nordqvist et al 2012; Jin et al 2009), a decrease of free SH due to crosslinking between gliadin and glutenin through disulphide bonds, and leading to formation of aggregates of wheat gluten protein (Wang et al 2009). Therefore, all these processes may lead to decreasing baking quality if tempe flour is substituted into a wheat bread formulation. Application of TG is expected to alleviate this problem. According to Ahn et al (2005), TG increases functional properties of weak wheat flour even when it is blended with barley (40%) or soy flour (20%).

According to DeJong and Koppelman (2002) cross-linking reaction will occur only if TG is exposed to readily available glutamine and lysine in the protein substrate. Availability of glutamine and lysine may not be a big problem since during fermentation protein is partially hydrolyzed resulting in smaller molecules (Wang et al 2009) and causes more available lysine (Handoyo et al 2006) and glutamine. Thus we hypothesize that is possible to utilize TG to improve bread quality of wheat flour substituted with tempe flour.

Purpose of Study

The main objective of this study was to analyze the effect of TG on the rheological, fermentation and baking properties of wheat flour and of substituted wheat flour with tempe flour.

The specific aims of this study were

1. To analyze the effect of increasing level of TG on the rheological, gluten, fermentation and baking characteristics of wheat flours

2. To analyze the effect of substitution tempe flour and the addition of TG on the rheological and fermentation characteristics of composite wheat-tempe flour.

Hypothesis

Null hypotheses of this study as listed below were tested.

1. There was no significant effect on the rheological (mixing farinogram), gluten, fermentation, and baking properties between control flour and flours treated with TG.

2. There was no significant effect on rheological and fermentation characteristics between wheat flour control and the treatments with tempe flour and TG.

If the null hypotheses are rejected, then the effect of TG and fortified tempe flour to the rheological, gluten, fermentation and baking properties will be explained by possible structural changes that occur in the gluten and dough due to TG treatment, and possible changes of protein during tempe fermentation.

Assumptions

It was assumed that the effect of TG will depend on the gluten quantity and quality. When TG was added to the wheat flour, it caused inter- and intra-molecular protein cross-link interactions by introducing covalent bonds leading to a stronger protein network. Characteristics of wheat and soy protein were altered during tempe fermentation, and caused decrease of baking quality of wheat flour substituted with tempe flour. TG was able to cross-link glutamine and lysine from different sources of protein (from soy and wheat), so that it could improve rheological and fermentation properties of composite wheat-tempe flour.

CHAPTER II

REVIEW OF LITERATURE

Wheat Flour

Wheat is considered one of the top three world cereals crops with 670 million tons total global production in 2012 (FAOSTAT 2014). The most world cultivated type of wheat is hexaploid bread wheat (about 95%) and most of the 5% remaining are tetraploid durum wheat, with small portion belonging to specific wheat type such as einkorn, emmer, spelt and kamut (Shewry et al 2009). One of hexaploid wheat is common wheat (*Triticum aestivum*). This type of wheat is often distinguished on the basis of seed coat color (red and white), endosperm texture (hard and soft), dough strength (strong and weak) and sowing season (winter and spring) (Gooding 2009). According to Carson and Edwards (2009), Hard Red Winter (HRW) is the largest wheat concentration produced in the United States. HRW is composed of medium to hard endosperm, red seed-coated, and is a fall-sown wheat variety. With protein content ranges from 10.5 to 14.0% (12% mb), HRW is mostly used for pan bread production, some lower-protein crusty bread and all-purpose flour.

Wheat flour is composed of about 80% starch (Sasaki et al 2008, Song and Zheng 2007) or 60-70% in whole grain flour and 65-75% in white flour (Shewry 2009).

It also contains 8-18% protein (Selinheimo et al 2006) and minor components such as lipid, ash, enzymes and non-starch polysaccharides (for example arabinoxylan) (Courtin and Delcour 2002).

These compounds help to develop baking product performances differently where the most important contributor is gluten following by starch and soluble fraction (Graßberger et al 2003). Starch contributes to the rheological properties of dough (Petrofsky and Hoseney 1995; Sasaki et al 2008), acts as dough inert filler, and determines retrogradation and stalling process of bread products (Courtin and Delcour 2002). Lipid and water soluble protein may be involved in the visco-elastic network formed by protein-polysaccharides interactions in wheat flour dough (Addo et al 2001). Whereas, arabinoxylan have the capability to cross-link and form gel under oxidizing condition, stabilize protein film and retard thermal disruption (Courtin and Delcour 2002).

Wheat Protein

According to Tilley and Chen (2012) protein content is one of the most important properties of wheat, because it determines price of bread wheat (HRW, HRS and HW) and properties such as water absorption, dough mixing time, gluten strength and finish product quality. Better quality bread products are usually resulted from higher protein wheat flour.

Wheat protein is comprised of 10-20% soluble (enzymes and enzyme inhibitors) and insoluble macromolecules non-prolamin protein (membrane protein, cell wall protein and nucleoprotein) (Hargreaves et al 1995) and 80% of glutens proteins (Shewry et al

2009). The components of gluten can also be broken down into the categories of 85% protein, 3.5-6.8% lipid, 0.5-0.9% minerals and 7.0-16.0% carbohydrate. In addition, about 80-90% of protein gluten are gliadin and glutenin (Petrofsky and Hoseney 1995; Song and Zheng 2007). The amount of glutenin subunits (and ratio between gliadin to glutenin) in wheat flour are contributing factors for maximum resistance of dough and gluten and gluten index (Wieser and Kieffer 2001).

Traditionally (functional) wheat gluten proteins are grouped as monomeric gliadins (ω-, α- and γ-type) and polymeric HMW-GS and LMW-GS (Shewry et al 1986). According Shewry et al (2009), gluten protein is composed about 30% gliadins, 32.5% LMW-GS and 16.6% HMW-GS. Gliadins consist of 11.2% ω-gliadin, 52.6% α-gliadin and 36.2% γ-gliadin.

The proportion of HMW-GS may be only about 1% of the dry weight of the mature endosperm (Payne et al 1987), but these subunits contribute highly to bread baking quality (Shewry et al 1992). Payne et al (1987) explain that these subunits are responsible for the elasticity of gluten where insufficient elasticity will produce a poor dough strength. Shewry et al (1992) depict a structural model of HMW glutenin subunits. HMW glutenin subunits consist of 1) (at the left part) a N-terminal domain; a globular conformation with α- helix cysteine residues that able to form cross-links and branches, 2) repetitive sequence in the middle forms loose spiral, β turn that may determine elastic properties, and 3) (at the right part) C-terminal domain, a globular with α helix with single cysteine residue forming a cross-link.

HMW-GS are coded by genes that occur on chromosome 1A (1 and 2* subunits), 1B (17+18, 7+8, 7+9, 7 and 6+8 subunits) and 1D (5+10, 2+12, 3+12, 4+12). Variation

of bread baking quality is more significantly contributed by subunits coded by chromosome 1A and 1D than chromosome 1B (Payne et al 1987). Related to bread quality, Shewry et al (2003) review that subunit 5+10 is associated to the highest quality, whereas subunits 2+12, 3+12 and 5+12 are associated to poor quality. Presence of 1 and 2* subunits is superior to the null allele. Among other alleles in chromosome B, 17+18 is considered of superior quality.

When wheat flour is mixed with water, gluten protein is hydrated and transformed into a cohesive and visco-elastic gluten protein network (Caballero et al 2007). Gliadin contributes to extensibility and viscosity, while glutenin determines rigid and elastic properties of gluten (Autio et al 2005). The visco-elastic properties of gluten from various wheat sources are different (Petrofsky and Hoseney 1995) depending on the glutenin sub-fraction and ratio gliadin/glutenin (Khatkar et al 1995) and are affected by intermolecular interactions (Song and Zheng 2007).

The functional properties of protein gluten are important in determining dough mixing properties, gas holding during proofing and baking, and overall baking performance (Autio et al 2005). The functionality of gluten, according to Goesaert et al (2005), are strongly influenced by molecular weight of glutenin, the occurrence of covalent and non-covalent bonds between glutenin molecules and interactions between glutenin and other flour constituents. The visco-elastic property of gluten could be increased by cross-link reactions (Jerez et al 2005).

Transglutaminase (TG)

TG is an oxidative enzyme (Caballero et al 2005; Autio et al 2005; Dunnewind et al 2002) with wide application in the food industry. According to Gerrard (2002), cross-linking is a chemical reaction that brings major consequences to protein functionality either in native or denatured state (Gerrard 2002). TG (protein glutamine γ-glutamyl-transferase, EC 2.3.2.13) catalyzes the formation cross-links between the γ-carboxyamide group of peptide-bound glutamine residues and various primary amines. According to Motoki and Seguro (1998) ε-amino of lysine can act as primary amine and contribute to the formation of inter- and intra-molecular cross-link with glutamine. The formation of glutamyl-lysine covalent bonds does not reduce the nutrition quality in foods (Motoki and Seguro 1998). Glutamyl-lysine bonds can be produced during an extreme heating process (Motoki and Seguro, 1998), but this is mostly found in foods that naturally contains high levels of enzyme TG such as meat and fish muscle (Gerrard 2002).

TG can be found in many organisms such as mammals, plants, fish and microbes. Today, microbial (*Streptomyces mobaraensis*) is the most important source of TG (DeJong and Koppelman 2002; Dube et al 2007). Compared to other sources, microbial TG has the most stable activity, effective in wide range temperature and pH and has been approved as GRAS (generally recognized as safe) and the most desirable source for many applications such as meat, poultry and crab imitation products (Ohtsuka et al 2001). Microbial TG also unlikely to have potential allergenity since no IgE (immunoglobulin E) mediated allergy to any bacteria has been reported. After 5 min treated with pepsin, microbial TG is fully degraded (Pedersen et al 2004).

According to DeJong and Koppelman (2002) TG can participate in three main reactions: cross-linking, acyl-transfer and deamination (Figure 1). Ohtsuka et al (2001) compare the deamination activity of three different sources of TG, guinea pig liver (GTG), fish red sea bream liver (FTG) and microorganism (MTG). They observe that microbial TG has less than 1/7 deamination activity from both GTG and FTG. A review of Dube et al (2006) indicates that hydrolytic deamination is much slower than formation of cross-links in the presence of accessible glutamine and lysine residues. Cross-linking reaction occurs before the acyl transfer reaction and deamination (de Góes-Favoni and Bueno 2014). In other words, the dominant activity of microbial TG is cross-link (DeJong and Koppelman 2002; Bauer et al 2003).

```
I                               I              I
Gln  -  C  -  NH2  +  NH2  -  Lys  ⟶  Gln  -  C  -  NH-Lys    +  NH3
I       II                      I         I       II
        O                                         O
Cross-linking reaction
I                                              I
Gln  -  C  -  NH2  +  RNH2        ⟶  Gln  -  C  -  NHR       +  NH3
I       II                               I       II
        O                                        O
Acyl-transfer reaction
I                                              I
Gln  -  C  -  NH2  +  H2O         ⟶  Gln  -  C  -  OH        +  NH3
I       II                               I       II
        O                                        O
Deamidation reaction
```
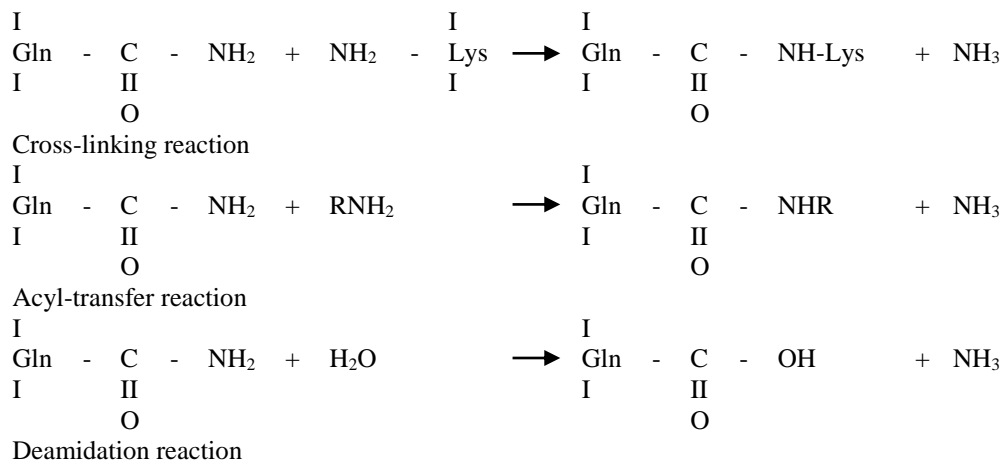
Figure 1. Three important reactions catalyzed by TG (DeJong and Koppelman 2002).

Factors that Influence the Activity/ Rate Reaction of TG

a. Temperature, pH and Co-factor

According to the information provided by the manufacturer Ajinomoto, TG, in general, actives at wide range of temperatures from 32 to 150°F (optimum at 122-131°F); and inactivates at higher temperatures (during cooking process). It has

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optimum pH of 6-7 but its activity still can be detected at pH 4-9. According to

Yokoyama et al (2004) microbial TG has optimum pH 5-8, and temperature 55°C, and

loses activity very quickly at 70°C.

A review of de Góes-Favoni and Bueno (2014) indicates that TG derived from

guinea pig liver requires Ca^{2+} to be activated, but activity of microbial TG is totally

independent to the presence of ion calcium. Microbial TG is strongly inhibited by ion

metal such as Cu^{2+}, Zn^{2+}, Pb^{2+} due to the ability these metals to bind the thiol group of

cysteine residue in enzyme's active site.

b. Accessible Glutamine and Lysine

TG introduces inter- and intra-molecular covalent cross-link by forming

isopeptide bonds between glutamine and lysine residues of protein, and high molecular

weight polymer are formed due to those cross-links (Tseng and Lai, 2002; Jong and

Kopelman, 2002; Ahn et al, 2005; Autio et al, 2005). Cross-linking reaction will occur

only if TG is exposed to readily available glutamine and lysine in the protein substrate

(Jong and Koppelman 2002). Lysine acts as acyl acceptors, and glutamine acts as acyl

donor (Yokoyama et al, 2004).

c. Macromolecular Structure of Protein Substrate

According to Dickinson (1997), good substrates for TG are substrates with

glutamine in flexible regions of polypeptide chain (for example casein) or in the region

of reverse turns. In contrast, globular proteins such as ovalbumin and ß-lactalbumin

that in their native state are stabilized by disulfide bonds, cannot be attacked by TG.

Therefore, several pre-treatments such as enzymatic, acid/chemical hydrolysis, and

heating for disrupting intermolecular -S-S- bonds, adsorption at the oil-water interface

and modification to be molten globule state are needed to facilitate TG crosslinks on these substrates. Level of reactivity of commercial TG (Activa, produced by Ajinomoto) to certain substrates can be seen in Table 1.

Table 1. Level reactivity of TG to food protein substrates

Source	Protein	Reactivity level
Milk	▪ Casein ▪ Na-Caseinate ▪ α-Lactalbumin ▪ β-Lactoglobulin	▪ very well ▪ very well ▪ depending on conditions ▪ depending on conditions
Egg	▪ Egg White Protein (ovalbumin) ▪ Egg Yolk Protein	▪ depending on conditions ▪ well
Meat	▪ Myoglobin ▪ Collagen ▪ Gelatin ▪ Myosin (myofibrillar) ▪ Actin (myofibrillar)	▪ depending on conditions ▪ well ▪ very well ▪ very well ▪ Fair to poor reaction
Soybean	▪ 11S globulin ▪ 7S globulin	▪ very well ▪ very well
Wheat	▪ Gliadin ▪ Glutenin	▪ well ▪ well

Source: fantes.com/manuals/ajinomoto-activa.pdf

d. Origin Sources of TG

According to Dickinson (1997) and Nielson (1995) TG can be derived from different sources; mammalian systems (including human tissue, blood, guinea pig liver, etc.), fish, plant and microbial. TG from different sources shows different reactivity toward glutamine residue; for example ß-casein is a good substrate for factor XIII, but poor substrate for liver-TG.

Extracellular microbial-TG from *Streptoverticillium mobaraense* is a simple monomeric protein with 331 amino acids residues; this enzyme does not require calcium ion to catalyze substrates. M-TG is widely used and is feasible to be

commercially applied in food processing. On the other hand, endogenous TG such as mammalian TG requires ion calcium to be activated and is impractical for use in food industry due to high cost of extraction and purification (de Góes-Favoni and Bueno 2014).

e. Presence of Salt (NaCl), Phosphate, $CaCl_2$ and $MgCl_2$

Salt, phosphate and TG have a synergic effect on the binding property of restructured meat/poultry (Kilic 2003)/fish. Salt helps to unfold the protein, so that it is more accessible to be cross-linked by TG. Calcium activates TG to cross-link protein and makes surimi gel set at a low temperature (Nielson, 1995).

f. Presence of Different Sources Protein

The activity of TG is more effective with the presence of additional protein sources such as gelatin, sodium caseinate, hydrolyzed wheat protein and lactose. TG effectively improves binding properties of restructured meat when gelatin is available, improves texture and water absorption in chicken/turkey kabob with availability of sodium caseinate (Kilic, 2003), improves firmness, 'mouth feel' and shelf life of noodle with hydrolyzed wheat protein (Babiker et al 1996), improves 'gel setting' of yoghurt with lactose (Rajakari et al 2007).

TG Application in Baking Industry

Since being first introduced in 1996, enzymes have been widely used as improvers in bread making to substitute the role of chemical oxidants (Poutanen 1997; Autio et al 2005). Enzymes could properly improve the handling properties of dough, develop a better crumb structure, prolong shelf life of bread, and increase bread volume

(Poutanen 1997). Since enzymes are also generally considered as safe, they may be a good alternative ingredient to chemical bread improvers (Joye et al 2009; Steffolani et al 2010). Application of TG, according to Gerrard et al (1998) is promising because it reduces the required work input and improves dough water absorption, where both effects lower processing costs of commercial baking.

TG introduces inter- and intra-molecular covalent cross-link by forming isopeptide bonds between glutamine and lysine residue of protein, and high molecular weight polymer is formed due to those cross-links (Tseng and Lai 2002; DeJong and Koppelman 2002; Ahn et al 2005; Autio et al 2005). Occurrence of protein polymerization is proved by the lowering of free amino acids (Huang et al 2010) and thiol groups (Ahn et al 2005; Marcoa and Rosell 2008). The reduction of bands in areas between 67 to 330 kDa, and the appearance of a new big band on the top of gel (larger molecular weight) are detected by SDS page after treatments of TG (Tseng and Lai 2002). New cross-link interactions cause the formation of large protein aggregates, and this is associated with lowering extensibility and increasing resistance of dough (Steffolani et al 2010). According to Autio et al (2005), glutenin fraction has lysine content higher than gliadin; this suggests that glutenin is cross-linked easier by TG than gliadin.

TG improves formation of protein network in bread making (Autio et al 2005), even in gluten-free bread (Marcoa and Rosell 2008). TG also increases the water holding capacity, water absorption, viscoelastic and thermal properties, and fat absorption (Ahn et al 2005; Huang et al 2010). TG restores viscoelastic property and thermal stability of the protein of wheat flour damaged by insects (Caballero et al 2005). TG makes the dough

stronger, increases both the mixing stability and maximum resistance (R max) (Roccia et al 2012), and also lowers extensibility (Tseng and Lai 2002; Basman et al 2003; Autio et al 2005). TG also recovers stickiness and less elastic dough caused by ferulic acid or over-mixed (Koh and Ng 2008). TG broadens application of gluten-free flour and increases protein content (nutritional improvement) of product (Marcoa and Rosell 2008). TG decreases allergic potential of roasted peanut flour containing 2.5-5% casein due to the covalent modification masking IgE peanut protein binding epitopes (Clare et al 2008).

The efficiency of TG on improving dough and bread is dependent on the enzyme levels (Moore et al 2006), reaction time (Gerrard et al 1998; Koh and Ng 2008) and protein sources (Marcoa and Rosell 2008).The recommended TG dose varies from low level (0.05% TG) for improving the crumb of wheat:soy (85:15 w/w) bread (Roccia et al 2012), 5000 ppm (0.5% TG) for improving bread and croissant dough (Gerrard et al 2001), 1% TG for improving visco-elastic oat dough (Huang et al 2010) and (gluten-free) rice flour dough quality (Marcoa and Rosell 2008) and 2.5%TG for improving wheat bread fortified with 20% barley flour (Basman et al 2003).

Incorporating other flours into wheat flour-based foods formulation becomes easier with the help of TG as a processing aid. Basman et al (2003) substituted 20% barley flour into wheat flour bread without any deterioration in loaf volume by applying 0.25% TG. Further, Basman et al (2006) also applied TG with level of 0.2% and 0.4% to improve quality of 15% and 30% bran supplemented spaghetti. The spaghetti had a lower total organic matter (TOM) score and higher sensory score for firmness, stickiness and bulkiness compared to spaghetti produced without TG.

TG may not be appropriate to apply at higher dose and into a strong wheat cultivar. In higher doses, TG has adverse effects on bread volume and crumb firmness (Roccia et al 2012), retards growth of air bubbles during fermentation and creates uneven protein network formation in which protein-rich areas are located at around air bubbles (Autio et al 2005). When applied in a strong wheat cultivar, TG may cause excessive crosslink leading to form over-strong dough and give slight decrease in loaf volume and increase in bread firmness (Basman et al 2003).

Wheat Grain and Soybean

Wheat and Soybean are two out of the eight main components considered the most allergenic foods. The immunodominant allergen in soy is P34 and it has a complex structure, therefore, Wilson et al (2005) suggest applying a coupling heat treatment with another treatment to effectively change its structure and decrease allergenicity. Heat treatment and fermentation as part of the tempe process could decrease the level of allergenicity in buckwheat (Handoyo et al 2006). Heat treatment will denature the protein and reduce the anti-nutritional properties of soybean. During fermentation, the hydrolysis of protein produces smaller peptides that may not be recognized by antibodies leading to less allergenicity (Wilson et al 2005).

Wheat grain contains bran, germ and endosperm, components that are considered good sources of essential amino acids (9 out of 10 essential amino acids, minus lysine), minerals (iron, zinc and selenium), vitamins (folate, tocol), and beneficial phytochemicals (lignin, phenolic acids) and dietary fiber (arabinoxylans, glucans) components to the

human diet (Shewry 2009; Slavin 2010). Consumption of whole grain could reduce body fat mass and may have cardio-protective effects (Kristensen et al 2012).

Legumes, including soy bean, are an excellence source of protein, dietary fiber, micronutrients and phytochemicals. Soybean has isoflavones, active compounds that are believed to help the prevention the risk of several chronic diseases (Messina 1999). Four common structures of isoflavones are aglycones (daidzein, genistein, and glycitein), β-glycosides (daidzin, genistin and glycitin), conjugates of 6"-*O*-malonyl-β-glycosides and conjugates of 6"-*O*-acetyl-β-glicosides (Villares et al 2011). Isoflavones can be decreased or be transformed in the new conjugates during food processing which can cause changes in texture and bioavailability (Villares et al 2011). Isoflavones degrade during thermal treatment and reduce during storage (Chien et al 2013); however, the loss rate is relatively low at ambient temperatures (Eisen et al 2003). Total isoflavones decreases and the composition of isoflavones changes during the fermentation process. However, aglycones content increases and double after 24 hours of fermentation (Nakajima et al 2005).

Fermentation

Fermentation has been widely applied in food processing. Fermentation reduces anti-nutritional factors (phytate, tannins, saponins, and oxalate and hydrogen cyanide (HCN)), increases minerals bioavailability, and protein and carbohydrate digestibility (Reddy and Pierson 1994). By tempe fermentation, pytate content of whole grain barley and oat could be decreased up to 97 percent. While, the amount of important minerals, Fe and Zn, are well preserved (Eklund-Jonsson et al 2006). Fermentation of bran by yeast

prior to incorporation into bread making will overcome the taste problems of whole grain bread, such as the harsh and strong taste of bran. Thus, bran-fortified bread with higher loaf volume and softer bread crumb will be produced (Delcour et al 2012).

Tempe

Tempe is an Indonesian traditional fermented food which is mainly made with soybeans or other legumes and seeds through fungi fermentation (Astuti et al 2000). Nout and Kiers (2005) illustrate tempe as a major fermented soybean food that is well recognized because of its attractive flavor, texture and superior digestibility. Currently, besides prepared traditionally in small scale, tempe also has been commercially produced at industrial scale with improved controls of starter and fermentation conditions (Nout and Kiers, 2005). The main processes to make tempe involves soaking, boiling/steaming, inoculating, and fermentation at room temperature (Astuti et al 2000).

Germination of fungal sporangiospores starts after several hours of beans inoculations, followed by the growing of mycelium (Nout and Kiers, 2005). The hyphae of fungus can penetrate about 2 mm deep (at 40 h fermentation) into soybean cotyledon, diffuse extracellular enzymes and degrade solid substrates (Varzakas 1998). Soybeans are bound together by a dense cottony mycelia and form a compact cake after the fermentation is completed (Hachmeister and Fung 1993).

Filamentous fungi is utilized in food processing due to its ability to secrete a wide variety of enzymes and produce secondary metabolites such as peptide, fatty acids, other organic acids, vitamins and flavor compounds (Archer et al 2008). *Rhizopus oligosporus* is an important fungus that contributes in tempe making (Muzzarelli et al 2012). *Rhizopus*

spp can produce enzymes such as protease (Heskamp and Barz 1998), lipase (Flood and Kondo 2003), glucoamylase (Yu and Hang 1991), intracellular acid and sodium phytases with broad affinity for various phosphorylated compounds (Azeke et al 2011), extracellular tripeptidyl peptidase, and a metalo and serine protease (Lin et al 2007).

Physical and chemical characteristics of raw materials are modified during tempe fermentation. Enzymes hydrolyze substrates and support development of a desirable taste, texture, flavor, acidity and aroma of final product (Hachmeister and Fung 1993). Digestibility and shelf life of cereal such as sorghum, wheat and rice are also improved during fermentation (Nout 2009). Modification of chemical compounds during tempe fermentation is mainly contributed by mold activity which is mostly completed after 46 h incubation. Generally, during fermentation dry matter diminishes, fatty acids declines sharply due to its role as main source of energy for mold, free amino acids increase while nitrogen content remains stable (Bisping et al 1993; Sparringa and Owens 1999). The pH rises gradually due to ammonia production, from 4.6 at 0 h up to 6.6 at mature tempe (46 h) and 7.1 at aging stage (72 h) of fermentation (Sparringa and Owens 1999). Oligosaccharides content reduces toward very low level after 25 h fermentation of black bean with *R. oligosporus* (Rodríguez-Bürger et al 1998). Sucrose, raffinose and stachiose are completely removed from cowpea after 15 h of *R. oligosporus* fermentation (Prinyawiwatkul et al 1996). Significant conversion of conjugated glucosides isoflavones to aglycones is observed during fermentation of whole soy bean flour at 30°C for 48 h where fermented flour contains mainly aglycones (75.51%) compared to 6.98% in raw soy flour (da Silva et al 2011). Fermentation increases essential amino acids, in vitro and true protein digestibility, protein efficiency ratio and corrected protein digestibility of

chickpeas (Angulo-Bejarano et al 2008). Improvement of essential amino acid profile occurs during fungal-fermentation of teff seeds (Yigzaw et al 2004).

Bioactive peptides, containing 2-50 amino acids residues, are liberated from protein during tempe fermentation (Hernandez-Ledesma et al 2011). A wide range of benefits (such as antimicrobial, angiotensin-converting-enzyme (ACE)-inhibitory, antioxidant, cholesterol-lowering agent, increase absorption/bioavailability of minerals, and opioid activity) has been attributed as the effects of bioactive peptides (Hartmann and Meisel 2007).

Formation of vitamins also occurs during tempe fermentation. The vitamins are B_{12} (cyanocobalamin) (Bisping et al 1993), β-carotene (produced at 34-48 h of fermentation), ergosterol (produced about 750 µg/g at 34 h even up to 1610 µg/g at 96 h of fermentation) , and γ-tocopherol (192.4 to 231.8 µg/g) (Denter et al 1998).

Tempe contains γ-amino butyric acid (GABA) (Aoki et al 2003) and saponin in average 1.93 µmol/g wet weight (Murphy et al 2008). Further cooking of the tempe would not alter distribution of saponin forms (Murphy et al 2008).

Safety is an important aspect of food consumption. Tempe is relatively safe, because it has been consumed by various age groups for a long period of time without delivering adverse effects (Astuti et al 2000). *R. oligosporus* does not produce any potentially poisonous metabolites (Jennessen et al 2008).

Confirmation of the health claim that soy protein may reduce the risk of coronary heart disease by the FDA in 1999 may help to increase the popularity of soy foods, including tempe. Tempe has potential benefits as an antioxidant, antibacterial and it also supports the human brain and health. Antioxidant capacity, such as inhibition against

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superoxide anion radical, lower amount of ferric-reducing and scavenging of diphenyl picryl hydrazil (DPPH) radical of soy germ, increases during fermentation (Hubert et al 2008). The highest antioxidant activity is observed from fermentation tempe using *R. oligosporus* for 10 days (Chang et al 2009).

The antioxidant property of tempe oil results from a synergistic effect of tocopherols and liberated amino acids by activity of *R. oligosporus* during fermentation (Hoppe et al 1997). Antioxidant capacity of tempe are mostly (2/3) contributed by peptides derived from microbe-hydrolysis of protein during fermentation, and only 1/3 by isoflavones (Sheih et al 2000).

Tempe extract has active compounds (molecular weight >30 kDA and contains arabinose of soy bean pectin cell wall released during fermentation) that can protect against the food borne pathogen *Bacillus cereus* (Roubos-van den Hil et al 2010). It also has high molecular weight fraction that can protect against diarrhea, avoid fluid and electrolyte loss from small intestine by inhibiting attachment/adhesion of enterotoxigenic *Escericia coli* (ETEC) to the brush border membrane of small intestine (Kiers et al 2000). The anti-adhesion effect of tempe against *E. coli* is even greater after digested in the human gastrointestinal system (Mo et al 2012).

Isoflavones found in fermented soybean can act as antidiabetic by improving glucose control and reducing insulin resistance, leading to delay of the progression of type 2 diabetes (Kwon et al 2010). Tempe intake has been associated with supporting a better verbal memory among older rural Indonesians due to its estrogenic (isoflavones) compounds and folates content (Hogervorst et al 2011). Tempe has been included as one

of the non-dairy probiotic products because lactic acid bacteria (LAB) (*L. plantarum*) is involved in tempe fermentation (Rivera-Espinoza and Gallardo-Navarro 2010).

Though tempe has many benefits, it also has beany flavor and bitter after taste. The bitter taste is contributed by isoflavones (Drewnowski and Gomez-Carneros 2000) with genistein as main bitter contributor (Roland et al 2011). Peptides of Asp-Ala-Leu-Pro-Glu (NALPE) have a very bitter taste equal to the taste of 4.0 mM quinine-HCl solution (Kim et al 2008). Metabolites of microbial, enzymatic degradation, heat treatment, and lipid oxidation may contribute to the off taste of food products (Galindo-Cuspinera 2011). Nevertheless salt, sugar and herb/spices have been used to help mask the off tastes. There are strategies to mask bitter flavor suggested by Galindo-Cuspinera (2011) including mixture suppression and encapsulation.

Tempe Flour

Wang et al (1968) prepared wheat-soy tempe by slightly cracking both wheat and soy, washing and boiling 12 min for wheat and 25 min for soy. The cooked materials then were cooled at room temperature, inoculated with suspension of *R. oligosporus* NRRL 2710 spores, incubated for 24 h at 31°C. Tempe cake then was steamed for 5 min to inactive the mold.

The way to dry tempe flour from fresh tempe cake varies among researchers, Wang et al (1968) steamed the fresh tempe for 5 min to inactive the mold prior freeze dried and ground it to produce tempe flour. Angulo-Bejarano et al (2008) and Cuevas-Rodríguez et al (2006) used the same method and they dried fresh chickpea and corn tempe in oven at 52°C for 12 h, milled it in cyclone sample mill and sieved the flour to pass through an 80-US mesh screen.

There is an agreement in the increased nutritional value of tempe compared to the unfermented material. Wang et al (1968) found that a mixture between soy and wheat at ratio 1:1 produced a good pattern of amino acids. Additional values including an increase of PER value (comparable to casein) and lysine were also observed. Chickpea and corn tempe flours had a higher in vitro and true protein digestibility, protein efficiency ratio, corrected protein digestibility, and resistant starch content (Angulo-Bejarano et al 2008; Cuevas-Rodriguez et al 2006).

Tempe flour has a higher particle size index, gelatinization temperature and dispersability (Angulo-Bejarano et al 2008; Cuevas-Rodriguez et al 2006). Considering these physical and nutritional benefits, Cuevas-Rodriguez et al (2006) suggested that fermented flour may be used in fortification of widely consumed cereal-based food products (tortillas, bread, cookies, atoles).

Analysis Wheat Flour Quality

Mixing Properties (Farinograph)

The farinograph is one of the most common equipment used for measuring dough rheological properties by both plant breeders and scientific research (Konopka et al 2004). There are five parameters produced from analysis rheological properties of wheat flour using Farinograph; water absorption, development time, stability, tolerance index and breakdown time.

Water absorption is the amount of water added to the flour to produce a curve with maximum consistence centered on the 500 BU line (AACCI 2011). Optimum water level is needed for developing cohesive and viscoelastic dough as well as for forming

optimum gluten strength (Zaidel et al 2010). Water absorption has been related to bread quality parameters such as weight, volume and form ratio (Liu et al 2010). Flour with higher water absorption has the potential to produce a more moistness (Puhr and D'Appolonia 1992), softer and longer shelf life baking products.

Development time (also termed as peak time, mixing time) is defined as the time interval between the first addition of water to the point when dough reaches maximum consistency/ minimum mobility (Shuey 1984). During development time, protein is hydrated leading to the formation of continuous matrix and coherent viscoelastic mass of dough (Macritchie 1975). Strong flour usually requires a longer mixing time than a weak one (Zaidel et al 2010).

Stability is the interval time between arrival time (curve reaches 500 BU) and departure time (curve leaves 500 BU), and this parameter indicates tolerance to mixing of the flour (Shuey 1984) and is related to overall quality of the protein (Dowell et al 2008).

Mixing tolerance index is the differences of BU value between peak time point and 5 minutes after peak. Flour with good tolerance to mixing will have a low tolerance index and vice versa (Shuey 1984).

Time to breakdown is the time counted when mixing begins to the point which consistency decreases 30 BU from the peak (Shuey 1984). It measures the ability of dough to retain its structure during mixing process. According to Macritchie (1975) breakdown stage of dough is marked by progressively losses of elasticity and further, dough becomes undesirably too sticky to handle.

Gluten Quality Indices

Wet gluten is defined in AACC International method 38-12.02 as a visco-elastic gliadin-glutenin protein material obtained from washing out the starch from wheat flour dough. This parameter is important to estimate the quantity of protein as well as the quality of flour. Amount of wet gluten usually increases with increasing flour protein content (Kulkarni et al 1987). However, the amount of wet gluten does not automatically reflect either gluten/protein quality (Curic et al 2001) or explain differences in flour and in dough properties (Wieser and Kieffer 2001) and all variation in baking quality (Chapman et al 2012).

Gluten index is defined as the amount of wet gluten (in weight percentage) that remains on the sieve after centrifugation with the larger gluten strands/aggregates remaining on the sieve (Mis 2000; Dowell et al 2008). This parameter is associated to gluten strength, reflecting inadequate, sufficient, average or excellent gluten quality (ICC Standard no 158, 1995). During centrifugation the more elastic (stronger) part of gluten remains on the top part of sieve, while the more extensible (the weaker) passes through the sieve (Tilley and Chen, 2012). It is generally assumed that quality of gluten increase with an increase of gluten index scores (Mis 2000). Gluten index is a good variable to predict bread loaf volume (Dowell et al 2008). Wieser and Kiefer (2001) found that gluten index was strongly correlated to glutenin and type of glutenin subunit was negatively correlated to ratio of gliadin to glutenin, but was no influenced by amount of gliadin.

Gluten recovery (%) is obtained from new bi-axial compression instrument (Gluten CORE) to evaluate gluten elastic recovery and is well adapted for dough and

other viscoelastic materials. The degree of recovery illustrates the ratio of the overall distance recovered over the distance compressed. It has a high (0.86) correlation with gluten strength from tensile test (Chapman et al 2012).

Fermentation Properties

The fermentation characteristics are measured in F3 Rheofermentometer. This analysis produced two curves: dough development curve and gaseous curve recorded during 3 h fermentation test. The dough development curve provides information about maximum height can be reached by piston (Hm), the height of dough at the end of test (h), the required time for reaching the maximum height (T1), and the lowering of the dough development percentage after 3 h time frame compared to T1 (Hm-h)/Hm (Ktenioudaki et al 2011; Tripette and Renaud 2004). Flour with average dough development and very good tolerance (has no T2, where T2 is the time at which Hm decreases 12%) can be defined as good quality flour (Tripette and Renaud 2004).

Hm indirectly represents gas production during fermentation and gas holding capacity of the dough. A high Hm indicates a good gas production and a sustaining macrostructure of the proofed dough piece (Huang et al 2008). (Hm-h)/Hm is the lowering of development percentage and it shows the best time for working the dough (Tripette and Renaud 2004). A small percentage of (Hm-h)/Hm indicates that the flour has good fermentation tolerance because the dough has a well maintained height during fermentation. Time of maximum rise (T1) is the time in hour required to reach the dough maximum height. T1 closely relates to the activity of yeast to produce gas (Tripette and Renaud 2004).

Gaseous release curve provides seven fermentation characteristics: H'm, T'1, Tx, total gas volume, CO_2 lost, CO_2 retention and retention coefficient. The gaseous release curve is resulted from measuring pressure that is developed in the tank during fermentation and two cycles (direct and indirect) that measure air and CO_2 (Ktenioudaki et al 2011).

The total volume of gas is a cumulative volume of gas retention and CO_2 lost. Tx is the time when the dough begins to release CO_2 (Tripette and Renaud 2004). During fermentation, some of CO_2 gas escapes from the dough system and is recorded by the F3 Rheofermentometer. The CO_2 retention (ml) is a parameter that measures the volume of CO_2 kept inside the dough until the end of the test (Tripette and Renaud 2004).

Retention coefficient is calculated by dividing the volume of retention by the total gas release. Amount of retention coefficient close to 100 means the flour consists of a good quality and usually is extracted from the healthy grain (Tripette and Renaud 2004).

Baking Properties

According to Tilley and Chen (2012) two of the most important characteristic of the bread are loaf volume and texture. It is preferable for a white pan bread to have high loaf volume accompanied with good crumb grain and texture. Most American consumers prefer fresh white-pan bread with soft, resilient and short crumb texture.

AACC International has approved three test methods to determine potential bread-making of flour. One of them is the standard optimized straight-dough bread-making method (AACCI 10-10.03, 2011). External characteristic of bread such as loaf volume can be measured by rapeseed displacement method (AACCI method 10-05.01, 2011). Crumb grain is usually tested by the cell size, cell shape and cell wall thickness

(Tilley and Chen 2012), AACCI provided the guidelines for scoring the bread, approved method 10.12.01 (AACCI, 2011).

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CHAPTER III

COMPOSITION OF LYSINE, GLUTAMINE AND CYSTEINE OF SELECTED GLUTEN PROTEINS AND THEIR POSSIBLE BONDING AS AFFECTED BY TRANSGLUTAMINASE (TG)

ABSTRACT

It has been widely reported that transglutaminase (TG) catalyzes formation of isopeptidic bonds between glutamine and lysine residues, and gluten proteins are good substrates for TG reaction. None of the reports illustrates in detail how gluten proteins are modified by TG. This study was aimed to evaluate the potential of TG to modify gluten proteins using the content and position of lysine, glutamine and cysteine residues as tools and to propose a model based on insight from increasing covalent crosslinks of gluten via TG. The data were obtained from the sequences of the complete coding regions of high molecular weight-glutenin subunits (HMW-GS), low molecular weight-glutenin subunits (LMW-GS), α/β-, γ-, and ω-gliadins in the National Center for Biotechnology Information (NCBI) website. All gluten proteins possessed lysine and glutamine with variation in number and position. Glutamine was abundantly (about 30% of total amino acids) available in all of gluten proteins, but the number of lysine was limited. HMW-GS contained 4 to 10 lysine residues and it was the highest number of lysine among other gluten proteins where LMW-GS had 1 to 2, α/β-gliadin has 0 to 2, γ-gliadin had 1 to 3

and ω-gliadin had 1 to 3 lysine residues. This finding suggests that HMW-GS have 4 to 10 chances to form inter- and intra-molecular glutamyl-lysine cross-links and create a large protein polymer. We propose the formation of closed loop strands which can help to understand an increase in covalent crosslinks of gluten via TG.

Key words: gluten structure, TG, lysine, glutamine, cysteine, isopeptidic covalent bonds.

INTRODUCTION

TG is an enzyme with activity to catalyze inter- and intra-molecular glutamyl-lysine cross-link between glutamine residue at the γ-carboxyamide group of peptide and primary amine of lysine residue (Motoki and Seguro 1998). In order to illustrate the effect of TG in modifying the structure of gluten, it is important to evaluate the amino acids sequence of each of gluten component, mainly the possession and position of lysine and glutamine contents. Identification of the number and position of the lysine and glutamine amino acids will help to predict the possibility of inter- and intra-molecular glutamyl-lysine interaction to take place and proposed accessibility of TG to the substrates. In addition, the presence of cysteine is also valuable to identify, because TG could indirectly cause the formation of secondary disulfide interaction due to close proximity between thiol groups. According to Gujral and Rosell (2004) proximity among amino acids, including sulphur containing amino acids, may become closer since polymeric protein become more compact due to creation of glutamyl-lysine bonds. Thus, the formation of disulfide bonds by oxidation is favored.

Gluten proteins have commonly been grouped into HMW-GS, LMW-GS, alpha/beta (α/β)-gliadin, gamma (γ)-gliadin and omega (ω)-gliadin. Lysine, glutamine and cysteine in N-terminal, repetitive motifs and C-termini was determined. According to Shewry et al (2000), repetitive motifs may form an unusual spiral super secondary structure based on β- or γ-reverse turns, whereas, structures of N- and C-terminal domains more similar to globular proteins, containing α-helix and irregular structure. N- and C-termini are also rich in cysteine residues which provide sites for the formation of inter-chain disulfide bonds. This paper was aimed to evaluate the potential of TG to modify gluten proteins using lysine, glutamine and cysteine residues as tools and to propose a model based on insights from increasing the covalent bonds of gluten via TG.

METHODOLOGY

Multiple sequence alignment was used to compare gluten proteins with emphasis on HMW-GS, LMW-GS, and α/β-, γ- and ω-gliadin from *Triticum aestivum* wheat. A number of accessions with full amino acid sequence of gluten proteins (in database of the NCBI (www.ncbi.nlm.nih.gov/) until October 2013) were selected. Multiple alignments were determined using BLAST program Multiple Alignment tool (available in NCBI website). The content and location of lysine, glutamine and cysteine in N-terminal, repetitive motifs and C-termini were tabulated and compared.

A 10g sample of wheat flour with protein content 11.0±0.5% was treated with TG (Activa TI with activity 100U/g from Ajinomoto, Fort Lee, NJ) at levels of 0, 0.1, 0.2, 0.4, 0.8 and 1.6% (w/w). Gluten was extracted by washing wheat flour with 2% sodium chloride (NaCl) solution using Glutomatic 2202 (Perten AB, Segeltorp, Sweden).

RESULTS AND DISCUSSION

HMW-GS

A total of 27 accessions with 648 to 1,025 amino acids were selected from hundreds of HMW-GS available in the NCBI database. Multiple alignments and a summary of the amino acids of interest of HMW-GS are reported in Figure and Table 1.

Table 1. Amino acids, molecular weight, and lysine and cysteine residues of HMW-GS

Subunit	Accession	∑ AA	MW (kDa)	Number of lysine residue				Number of cysteine residue			
				Nter	Rep	Cter	total	Nter	Rep	Cter	total
Ax1	CAA43331	830	104639	2	1	2	5	3	0	1	4
Ax2*	ABG68031	815	103027	2	2	2	6	3	0	1	4
Ax2*	AAB02788	815	103027	2	2	2	6	3	0	1	4
Bx7	ABY59654	795	99430	1	1	2	4	3	0	1	4
Dx1	ABF14401	839	104246	3	2	2	7	3	0	1	4
Dx2	DAA06557	835	103854	3	1	2	6	3	0	1	4
Dx2.1	AAR98780	836	103905	3	2	2	7	3	0	1	4
Dx2.2	AFP58009	971	120350	3	1	2	6	3	0	1	4
Dx2.2*	CAI72574	1024	127057	3	0	2	5	3	0	1	4
2.6	BAG12019	1025	127195	3	0	2	5	3	0	1	4
Dx5'	ADH04662	833	103760	3	0	2	5	3	0	1	4
Dx5	ABG68042	848	105438	3	1	2	6	3	1	1	5
Dx5	CAA31395	848	105438	3	1	2	6	3	1	1	5
Dx5	DAA06555	848	104856	3	1	2	6	3	1	1	5
Dx5	BAH37041	848	105482	3	1	2	6	3	1	1	5
Dx5	P10388	848	105438	3	1	2	6	3	1	1	5
GS	AAN78346	971	120398	3	1	2	6	3	1	1	5
GS	ABK54365	815	103012	2	2	2	6	3	0	1	4
GS	AEO19857	866	109112	2	1	2	5	3	0	1	4
GS	ABF82252	824	104183	2	1	2	5	3	0	1	4
GS	ABX89297	839	104322	3	2	2	7	3	0	1	4
GS	AEO45112	971	120349	3	1	2	6	3	0	1	4
GS	ADF32930	827	102693	3	1	2	6	3	0	1	4
GS	P08489	838	104140	3	1	2	6	3	0	1	4
GSx	CAC40686	811	100781	3	5	2	10	3	0	1	4
Dy10	P10387	648	81196	2	4	1	7	5	1	1	7
Dy12	DAA06556	658	82428	2	5	1	8	5	1	1	7

∑AA = Total number amino acids; MW = molecular weight; Nter = N-terminal; Rep = Repetitive domain; Cter = C-terminal. Source: https://www.ncbi.nlm.nih.gov/

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The molecular weight (MW) of HMW-GS ranged from about 81,196 to 127,753 kDa for the selected set. These subunits had four to ten lysine residues, where the lowest number of lysine was reported from Bx7 subunit and the highest from GSx. Number of cysteine ranged from 4 to 7, with the highest one reported from Dy10 and Dy12.

Subunit Dx5 with 848 amino acids had six lysine residues, three in N-terminal, one in repetitive domain and two in C-terminal (Table 1). The number of glutamine was abundant where fifteen residues were in N-terminal, 279 residues in repetitive domain and five in C-terminal. Dx5 subunit also had five cysteine residues that spread into three in N-terminal and one in repetitive domain and C-terminal.

Dy10 subunit possessed seven lysine residues, seven cysteine residues and 206 glutamine residues. Glutamine spread mostly in the repetitive domain (182 residues), where 18 residues were in N-terminal and 6 residues in C-terminal. HMW-GS has been reported as a good substrate for TG since it has more lysine residues than LMW-GS (Autio et al 2005). These lysine residues can create both intra- and inter-molecular covalent links with glutamine residues. Similar to lysine, the cysteine of HMW-GS in N- and C-terminal forms inter- and intra-molecular disulfide bonds. Whereas, repeated sequences of amino acids in repetitive domain may promote formation of hydrogen bonds (Shewry et al 2002) that stabilize the protein structure.

Figure 1. Multiple alignment of selected amino acids sequences of HMW-GS. Accession 1) CAA43331, 2) ABG68031, 3) AAB02788, 4) ABY59654, 5) ABF14401, 6) DAA06557, 7) AAR98780, 8) AFP58009, 9) CAI72574, 10) BAG12019, 11) ADH04662, 12) ABG68042, 13) CAA31395, 14) DAA06555, 15) BAH37041, 16) P10388, 17) AAN78346, 18) ABK54365, 19) AEO19857, 20) ABF82252, 21) ABX89297, 22) AEO45112, 23) ADF32930, 24) P08489, 25) CAC40686, 26) P10387, 27) DAA06556, Sig = Signal peptide; Nter = N-terminal; Rep = Repetitive domain (that has been secluded from the figure); Cter = C-terminal. K = lysine, C = cysteine, Q = glutamine. Source: https://www.ncbi.nlm.nih.gov/

LMW-GS

Thirty two accessions of LMW-GS with 301 to 390 amino acids sequences

(MW about 39437 to 51986 kDa) were selected. This group of proteins was poor in

lysine residues with either one and two residues located in C-terminal and/or contain one

additional lysine located in the N-terminal.

Table 2. Number of amino acids, molecular weight, and lysine and cysteine residues for
LMW-GS

Accession	∑ AA	MW (kDa)	Number of lysine residue						Number of cysteine residue					
			Nter	rep	Cter I	Cter II	Cter III	Total	Nter	rep	Cter I	Cter II	Cter III	Total
ACA63852	365	48284	0	0	1	0	0	1	0	1	5	1	1	8
ACA63856	370	48704	0	0	1	0	0	1	0	1	5	1	1	8
ACA63857	369	48558	0	0	1	0	0	1	0	1	5	1	1	8
ACA63865	392	51532	0	0	1	0	0	1	0	1	5	1	1	8
ACA63867	392	51628	0	0	1	0	0	1	0	1	5	1	1	8
ACA63873	369	48585	0	0	1	0	0	1	0	1	5	1	1	8
ACA63874	369	48585	0	0	1	0	0	1	0	1	5	1	1	8
AFX69667	350	46049	1	0	1	0	0	2	0	1	5	0	1	7
AGE13922	390	51966	0	0	1	0	0	1	0	0	6	1	1	8
AGE13923	390	51986	0	0	1	0	0	1	0	0	6	1	1	8
AFI81529	350	46049	1	0	1	0	0	2	0	1	5	0	1	7
AFI81530	350	46105	1	0	1	0	0	2	0	1	5	0	1	7
AFI81531	365	48200	1	0	1	0	0	2	0	1	5	0	1	7
AFI81533	376	49987	0	0	1	0	0	1	0	0	6	1	1	8
AFI81539	351	46195	1	0	1	0	0	2	0	1	5	0	1	7
AFI81540	350	46101	1	0	1	0	0	2	0	1	5	0	1	7
AFI81541	365	48200	1	0	1	0	0	2	0	1	5	0	1	7
AFI81542	354	46340	1	0	1	0	0	2	0	1	5	1	1	8
AFI81547	350	46049	1	0	1	0	0	2	0	1	5	0	1	7
AFI81548	350	46105	1	0	1	0	0	2	0	1	5	0	1	7
AFI81550	365	48200	1	0	1	0	0	2	0	1	5	0	1	7
AFI81551	354	46340	1	0	1	0	0	2	0	1	5	1	1	8
AFI81552	376	49961	0	0	1	0	0	1	0	0	6	1	1	8
AAS66083	388	51630	0	0	1	0	0	1	0	0	6	1	1	8
AEI00657	350	46115	1	0	1	0	0	2	0	1	5	0	1	7
AAP44991	346	45483	1	0	1	0	0	2	0	1	5	0	1	7
ACX46517	351	46211	1	0	1	0	0	2	0	1	5	0	1	7
ACK44491	343	45156	0	0	1	0	1	2	0	1	5	1	1	8
ADH51279	301	39437	0	0	1	0	0	1	1	0	5	0	1	7
AAS66084	304	40017	0	0	1	0	0	1	1	0	5	1	1	8
AAS66085	340	44502	1	0	1	0	0	2	0	1	5	1	1	8
AFU48612	369	48725	0	0	1	0	0	1	0	1	5	1	1	8

∑AA = Total number amino acids; MW = molecular weight; Nter = N-terminal; Rep =
Repetitive domain; Cter = C-terminal. Source: https://www.ncbi.nlm.nih.gov/

Accession	Sig	Nter	Rep	Cter 1
	20	13	70-186	75

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ACA63852  MKTFLIFALLAVAATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIVL
ACA63856  MKTFLIFALLAIVATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
ACA63857  MKTFLIFALLAIVATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
ACA63865  MKTFLIFALLAVAATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIVYSIIL
ACA63867  MKTFLIFALLAVAATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQRSCHVMQQQCCQQLPQIPQQSRYEAIRAIVYSIIL
ACA63873  MKTFLIFALLAIVATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
ACA63874  MKTFLIFALLAIVATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
AFX69667  MKTFLVFALLAIAATSAIAQMETSRVPGLEKPW-----------IPVVQPSVLQQLNPCKVFLQQQCSHVAMSQ-RLARSQMWQQSSCHVMQQQCCQQLPQIPEQSRSEAIRAIVYSIIL
AGE13922  MKTFLVFALLALAAARAVAQIS---------------------IPAIHPSVLQQLNPCKVFLQQQCIPVAMQRC-LARSQMLQSICHVTQQQCCQQLRQIPEQSRHESIRAIVSIIIL
AGE13923  MKTFLVFALLALAAARAVAQIS---------------------IPAIHPSVLQQLNPCKVFLQQQCIPVAMQRC-LARSQMLQSICHVMQQQCCQQLRQIPEQSRHESIRAIVSIIIL
AFI81529  MKTFLIFALLAIAATSAIAQMETSRVPGLEKPW-----------IPVVQPSVLQQLNPCKVFLQQQCSHVAMSQ-RLARSQMWQQSSCHVMQQQCCQQLPQIPEQSRSEAIRAIVYSIIL
AFI81530  MKTFLIFALLAVAATSAIAQMETSHIPSLEKPL-----------IPSVQPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLWQSSCHVMQQQCCRQLPQIPEQSRYDAIRAIIYSIVL
AFI81531  MKTFLIFALLAVAATSAIAQMETSHIPGLEKPS-----------IPYVQPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLWQSSCHVMQQQCCQQLPRIPEQSRYDAIRAIIYSIVL
AFI81533  MKTFLVFALLALAAASAVAQIS---------------------IPVIHPSVLQQLNPCKVFLQQQCIPVAMQRC-LARSQMLQSICHVMQQQCCQQLRQIPEQSRHESIRAIIYSIIL
AFI81539  MKTFLIFALLAIAATSAIAQMETSRVPGLEKPW-----------IPVVQPSVLQQLNPCKVFLQQQCSHVAMSQ-RLARSQMWQQSSCHVMQQQCCQQLPQIPEQSRSEAIRAIVYSIIL
AFI81540  MKTFLIFALLAVAATSAIAQMETSHIPSLEKPL-----------IPSVQPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLWQSSCHVMQQQCCRQLPQIPEQSRYDAIRAIIYSIVL
AFI81541  MKTFLIFALLAVAATSAIAQMETSHIPGLEKPS-----------IPYVQPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLWQSSCHVMQQQCCQQLPRIPEQSRYDAIRAIIYSIVL
AFI81542  MKTFLIFALLAVAATSAIAQIENSHIPGLEKPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
AFI81547  MKTFLIFALLAIAATSAIAQMETSRVPGLEKPW-----------IPVVQPSVLQQLNPCKVFLQQQCSHVAMSQ-RLARSQMWQQSSCHVMQQQCCQQLPQIPEQSRSEAIRAIVYSIIL
AFI81548  MKTFLIFALLAVAATSAIAQMETSHIPSLEKPL-----------IPSVQPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLWQSSCHVMQQQCCRQLPQIPEQSRYDAIRAIIYSIVL
AFI81550  MKTFLIFALLAVAATSAIAQMETSHIPGLEKPS-----------IPYVQPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLWQSSCHVMQQQCCQQLPRIPEQSRYDAIRAIIYSIVL
AFI81551  MKTFLIFALLAVAATSAIAQIENSHIPGLEKPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
AFI81552  MKTFLVFALLALAAASAVAQIS---------------------IPVIHPSVLQQLNPCKVFLQQQCIPVAMQRC-LARSQMLQQSICHVMQQQCCQQLRQIPEQSRHESIRAIIYSIIL
AAS66083  MKTFLVFALLALAAASAVAQIS---------------------IPVIHPSVLQQLNPCKVFLQQQCIPVAMQRC-LARSQMLQSICHVMQRQCCQQLRQIPEQSRHESIRAIIYSIIL
AEI00657  MKTFLIFALLAVAATSAIAQMETSHIPSLEKPL-----------IPSVQPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLWQSSCHVMQQQCCRQLPQIPEQSRYDAIRAIIYSIVL
AAP44991  MKTFLVFALLAIAATSAIAQMETSRVPGLEKPW-----------IPVVQPSVLQQLNPCKVFLQQQCSPVAMPQ-HLARSQMWQQSCNVMQQQCCQQLPRIPEQSRYEAIRAIIFSIIL
ACX46517  MKTFLVFALLAIAATSAIAQMETSRVPGLEKPW-----------IPVVQPSVLQQLNPCKVYLQQQCSHVAMSQ-RLARSQMWQQSSCHVMQQQCCQQLPQIPEQSRSEAIRAIVYSIIL
ACK44491  MKTFLIFALLAVAATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIVL
ADH51279  MKTFLVFALIAVVVATSAIAQMETSCISGLERPW-----------IPIVQPSVLQQLNPCKVFLQQQCSPVAMPQ-RLARSQMWQQSSCHVMQQQCCQQLQQIPEQSRYEAIRAIIYSIIL
AAS66084  MKTFLIFALLAVAATSAIAQMETRCIPGLERPW-----------IPVVQPSVLQQLNPCKLFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
AAS66085  MKTFLIFALLAVAATSAIAQIENSHIPGLEKPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
AFU48612  MKTFLIFALLAIVATSAIAQMENSHIPGLERPS-----------IPFVHPSILQQLNPCKVFLQQQCSPVAMPQ-SLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIIL
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Accession	Cter II			Cter III
				54

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ACA63852  QEQQQVRGSIQT   QQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPMMCRVNVPLYRTTTSVPFGVGTGVGAY
ACA63856  QEQQQVQGSIQT   PQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTSVPFGVGTGVGSY
ACA63857  QEQQQVQGSIQT   PQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVSLYRTTTRVPFGVGTGVGSY
ACA63865  QEQQQVQGSIQT   QQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCNVNVSLYRTTTRVPFGVGTGVGGY
ACA63867  QEQQQVQGSIQT   QQQQPQQLGQCVSQPQQQL    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCNVNVPLYRTTTRVPFGVGTGVGGY
ACA63873  QEQQQVQGSIQT   PQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTNVPFGVGTGVGSY
ACA63874  QEQQQVQGSIQT   PQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTNVPFGVGTGVGSY
AFX69667  QEQQ--QGFVQP   QQQQPQQSGQGVSQHQQQS[14]QQ1QQLGQQPQQQQIPQGIFLQPHQISQLEVMTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGAY
AGE13922  QQQQQQQQQQ-[ 7]QQQQPQQLGQCVSQPQQQL    QQ--QLGQQPQQQQLAHGTFLQPHQIAQLEVMTSIALHNLPMMCSVNVPLYETTTSVPLGIGIGVGVY
AGE13923  QQQQQQQQQQ-[ 7]QQQQPQQLGQCVSQPQQQL    QQ--QLGQQPQQQQLAHGTFLQPHQIAQLEVMTSIALHNLPMMCSVNVPLYETTTSVPLGIGIGVGVY
AFI81529  QEQQ--QGFVQP   QQQQPQQSGQGVSQHQQQS[14]QQ1QQLGQQPQQQQIPQGIFLQPHQISQLEVMTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGAY
AFI81530  QEQQHGQGLNQP   QQQQPQQSVQGVSQPQQQ[11]QQ--QQLGQWPQQQQVPQGTLLQPHQIAQLEVMTSIALRTLPMMCSVNVPVYGTTTIVPFGVGTRVGAY
AFI81531  QEQQHGQGFNQP   QQQQPQQSVQGVSQPQQQ[11]QQ--QQLGQWPQQQQVPQGTLLQPHQIAQLELMTSIALRTLPMMCSVNVPVYGTTTSVPFGVGTQVGAY
AFI81533  QQQQQQQQQQQ[ 7]QQQQPQQLGQCVSQPQQQL    QQ--QLGQQPQQQQLAHGTFLQPHQIAQLEVMTSIALRTLPTMCSVNVPLYETTTSVPLGIGIGVGVY
AFI81539  QEQQ--QGFVQP   QQQQPQQSGQGVSQHQQQS[14]QQ1QQLGQQPQQQQIPQGIFLQPHQISQLEVMTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGGY
AFI81540  QEQQHGQGLNQP   QQQQPQQSVQGVSQPQQQ[11]QQ--QQLGQWPQQQQVPQGTLLQPHQIAQLEVMTSIALRTLPTMCSVNVPVYGTTTIVPFGVGTRVGAY
AFI81541  QEQQHGQGFNQP   QQQQPQQSVQGVSQPQQQ[11]QQ--QLGQWPQQQQVPQGTLLQPHQIAQLELMTSIALRTLPMMCSVNVPVYGTTTSVPFGVGTQVGAY
AFI81542  QEQQQVQGSIQS   QQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTSVPFGVGAGVGAY
AFI81547  QEQQ--QGFVQP   QQQQPQQSGQGVSQHQQQS[14]QQ1QQLGQQPQQQQIPQGIFLQPHQISQLEVMTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGGY
AFI81548  QEQQHGQGLNQP   QQQQPQQSVQGVSQPQQQ[11]QQ--QQLGQWPQQQQVPQGTLLQPHQIAQLEVMTSIALRTLPTMCSVNVPVYGTTTIVPFGVGTRVGAY
AFI81550  QEQQHGQGFNQP   QQQQPQQSVQGVSQPQQQ[11]QQ--QQLGQWPQQQQVPQGTLLQPHQIAQLELMTSIALRTLPMMCSVNVPVYGTTTSVPFGVGTQVGAY
AFI81551  QEQQQVQGSIQS   QQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTSVPFGVGAGVGAY
AFI81552  QQQQQQQQQQQQ[ 7]QQQQPQQLGQCVSQPQQQL    QQ--QLGQQPQQQQLAHGTFLQPHQIAQLEVMTSIALRTLPTMCSVNVPLYETTTSVPLGVGIGVGVY
AAS66083  QQQQQQQQQQQQ[10]QQQPPQQLGQCVSQPQQQL    QQ--QLGQQPQQQQLAHGTFLQPHQIAQLEVMTSIALRTLPRMCSVNVPLYETTTSVPLGVGIGVGVY
AEI00657  QEQQHGQGLNQP   QQQQPQQSVQGVTQPQQQQ[11]QQ--QQLGQWPQQQQVPQGTLLQPHQIAQLEVMTSIALRTLPTMCSVNVPVYGTTTIVPFGVGTRVGAY
AAP44991  QEQQ--QGFVQP   QQQQPQQSVQGVYPQQQS[12]QQ--QLGQQPQQQQVQKGTFLQPHQIARLEVMTSIALRTLPTMCSVNVPLYSSITSAPLGVGTGVGAY
ACX46517  QEQQ--QGFVQP   QQQQPQQSGQGVSQHQQQS[14]QQ1QQLGQQPQQQQIPQGHQIPQHQISQLEVMTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGAY
ACK44491  QEQQQVRGSIQT   QQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPMMCRVNVPLYRTTTSVPFGVGTGVGAY
ADH51279  QEQQ--QGFVQA   QQQQPQQSGQGVSQSQQQS[12]QQ--QLGQQPQQQQVQKGTFLQPHQIAQLEVMTSIALRILPTMCSVNVPLYRTTTSVPFDVGTGVGAY
AAS66084  QEQQQVQGSIQS   QQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRILPTMCSVNVPLYRTTTSVPFDVGTGVGAY
AAS66085  QEQQQVQGSIQS   QQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRILPTMCSVNVPLYRTTTSVPFDVGTGVGAY
AFU48612  QEQQQVQGSIQT   PQQQPQQLGQCVSQPQQQS    QQ--QLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTNVPFGVGTGVGSY
```

Figure 2. Multiple alignment of selected amino acids sequences of 32 accessions of LMW-GS. Sig = Signal peptide; Nter = N-terminal; Rep = Repetitive domain (that has been secluded from the figure); Cter = C-terminal. K = lysine, C = cysteine, Q = glutamine. Source: https://www.ncbi.nlm.nih.gov/

Thus the contribution of LMW-GS to forming glutamyl-lysine crosslinks via TG

is lower compared to HMW-GS. However, LMW-GS may still potentially contribute in

formation of protein polymerization by two reasons. First, the content of LMW-GS is higher than HMW-GS in wheat protein, with the ratio LMW/HMW about 1.4 to 25.4 (Cinco-Moroyoqui and MacRitchie 2008). Second, LMW-GS are able to form inter-chain bonds with HMW-GS.

Glutamine residues were abundantly available (in average 34% of total amino acids). For example, LMW-GS with accession AAS66085 had total 111 glutamine residues (33% from 340 total amino acids) located in the repetitive domain (64 residues), C-terminal I (17 residues), C-terminal II (26 residues), C-terminal III (4 residues).

LMW-GS had either seven or eight cysteine residues distributed in repetitive domain (0 to 1 residue), C-terminal I (5 to 6 residues), C-terminal II (0 to 1 residue), and C-terminal III (1 residue). According to Shewry et al (2009), the position of cysteine within the sequence allows them either to form inter- or intra-molecular disulfide bonds. According to D'Ovidio and Masci (2004) the 1[st] (Cys-43, located either in N-terminal or repetitive domain) and 7[th] (Cys-295, located in C-terminal II) cysteine residues are most likely to participate in the formation of inter-molecular disulfide bonds.

α- and ß-Gliadin

Glutamine residues were well represented in α/β-gliadin (average 34% of total amino acids). For example, accession BAM08462 has 32% glutamine (93 out of 287) from the total amino acids. The glutamine residues were present in the repetitive domain (58 residues), C-terminal I (13), C-terminal II (21), C-terminal 3 (1) and none was available in N-terminal.

	Sig.	N ter	Rep.	Cter. I
	20	5	110-139	59

```
AAZ94421  MKTFLILALPAIVATTATTAVRVPV-----------------------LIPCMDV-VLQQHNIVHGRSQVLQQSTYQLLRELCCQHLWQIPEQSQCQAIHNVVHAIIL
AAZ94420  MKTFLILALLAIVATTATIAVRVPV-----------------------LIPCMDV-VLQQHNIAQGRSQVLQQSTYQLLQELCCQHLWQIPEQSQCQAIHNVVHAIIL
1307187B  MKTFLILALLAIVATTATTAVRVPV-----------------------LIPCRDV-VLQQHNIAHARSQVLQQSTYQPLQQLCCQQLWQIPEQSRCQAIHNVVHAIIL
P04727    MKTFLIL---ALVATTATTAVRVPV-----------------------LIPCRDV-VLQQHNIAHASSQVLQQSTYQLLQQLCCQQLLQIPEQSRCQAIHNVVHAIIM
P04726    MKTFLILALLAIVATTATTAVRVPV-----------------------LIPCRDV-VLQQHNIAHARSQVLQQSTYQPLQQLCCQQLWQIPEQSRCQAIHNVVHAIIL
AAA34279  MKTFLIL---ALVATTATTAVRVPV-----------------------LIPCRDV-VLQQHNIAHASSQVLQQSTYQPLQQLCCQQLLQIPEQSRCQAIHNVVHAIIM
AAA34277  MKTFLILALLAIVATTATTAVRVPV-----------------------LIPCRDV-VLQQHNIAHARSQVLQQSTYQPLQQLCCQQLWQIPEQSRCQAIHNVVHAIIL
BAM08464  MKTFLILALLAIVATTATSAVRVPV-----------------------LIPCRDV-VLQQHNIAHASSQVLQQSSYQQLQQLCCQQLFQTPEQSRCQAIHNVVHAIIL
BAM08451  MKTFLIISLLAIVATTATTAVRVPV-----------------------LIPCRDViVLQQHNIAHESSQVLQQSSYQVLQQLCCQQLRLIPEQSRCQAIHNVVHAIIL
BAM08463  MKTFLILALLAIVATTATTAVRVPV-----------------------LIPCMDV-VLQQHNIAQGRSQVLQQSTYQLLQELCCQHLWQTPEQSQCQAIHNVVHAIIL
BAM08462  MKTFLILALPAIVATTATTAVRVPV-----------------------LIPCMDV-VLQQHNIAHGRSQVLQQSTYQLLQELCCQHLWQIPEQSQCQAIQNVVHAIIL
BAM08461  MKTFLILSLLAIVATTATTAVRFPV-----------------------LIPCRDV-VLQQHNIAHASSQVLQQSSYQLLQQLCCQRLWQIPEQSRCQAIHNVVHAIIL
BAM08459  MKTFLILALLAIVATTTTTAVRVPV-----------------------LIPCRDV-VLQQPNIAHASSQVSQQS-YQLLQQLCCQQLWQTPEQSRCQAIHNVIHAIIL
BAM08456  MKTFLILALLAIMATTTTTAVRVPV-----------------------LIPCRDV-VLQQPNIAHASSQVSQQS-YQLLQQLCCQQLWQTPEQSRCQAIHNVIHAIIL
BAM08454  MKTFLILALLAIVATTTTTAVRVPV-----------------------LIPCRDV-VLQQPNIAHASSKVSQQS-YQLLQQLCCQQLWQTPEQSRCQAIHNVIHAIIL
BAM08452  MKTFLILALLAIVATTTTTAVRVPV-----------------------LIPCRDV-VLQQPNIAHASSKVSQQS-YQLLQQLCCLQLWQTPEQSRCQAIHNVIHAIIL
BAM08450  MKTFLIISLLAIVATTATTAVRVPV-----------------------LIPCRDViVLQQHNIAHESSQVLQQSSYQVLQQLCCQQLRQIPEQSRCQAINNVVHAIIL
BAM08458  MKTFLILALLAIVATTTTTAVRVPV-----------------------LIPCRDV-VLQQPNIAHASSQVSQQS-YQLLQQLCCQQLWQTPEQSRCQAIHNVIHAIIL
BAM08455  MKTFLILALLAIVATTTTTAVRVPV-----------------------LIPCRDV-VLQQPNIAHASSQVSQQS-YQLLQQLCCQQLWQTPEQSRCQAIHNVIHAIIL
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C terminal II and III

	Cter. II		Cter. III
	49-85		35-37

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AAZ94421  HQQ   Q---KQQQQ--    PSSQVSFQQPLQQYPLGQGSFRPSQQNPQAQGSVQPQQLPQFEEIRNLALQTLPAMCNVYIPPYC--TIAPFGIFGTN
AAZ94420  HQQ   HhhHQQQQQQQ[ 3]PLSQVSFQQPQQQYPSGQGFFQPSQQNPQAQGSVQPQQLPQFEAIRNLALQTLPAMCNVYIPPYC--TIAPFGIFGTN
1307187B  HQ-   Q----QQQQ--[ 1]PSSQVSLQQPQQQYPSGQGFFQPSQQNPQAQGSVQPQQLPQFEEIRNLALQTLPRMCNVYIPPYCSTTIAPFGIFGTN
P04727    HQQ[17]QQQQQQQQ--    PSSQVSFQQPQQQYPSSQGSFQPSQQNPQAQGSVQPQQLPQFAEIRNLALQTLPAMCNVYIPPHCSTTIAPFGIFGTN
P04726    HQ-   Q----QRQQ--[ 1]PSSQVSFQQPQQQYPSGQGFFQPSQQNPQAQGSVQPQQLPQFEEIRNLALQTLPRMCNVYIPPYCSTTIAPFGIFGTN
AAA34279  HQQ[17]QQQQQQQQ--    PSSQVSFQQPQQQYPSSQGSFQPSQQNPQAQGSVQPQQLPQFAEIRNLALQTLPAMCNVYIPPHCSTTIAPFGIFGTN
AAA34277  HQ-   Q----QRQQ--[ 1]PSSQVSLQQPQQQYPSGQGFFQPSQQNPQAQGSVQPQQLPQFEEIRNLALQTLPRMCNVYIPPYCSTTIAPFGIFGTN
BAM08464  HHH   Q----QQQ---[ 1]PSSQVSFQQPQEQYPSGQGSFQPSQQNPQAQGSVQPQQLPQFQEIRNLALQTLPAMCNVYIPPYCSTTIAPFGIFGTN
BAM08451  HQQ   Q----QQQQQQ[ 7]PSSQVSYQQPQQQYPSGQGSFQPSQQNPQAQGFVQPQHLPQLEEISNLALQTLPAMCNVYIPPYCSTTIAPFGIFGTN
BAM08463  HQQ   Q---KQQQQQQ[ 3]PSSQVSFQQPQQQYPLGQGSFRPSQQNPQAQGSVQPQQLPQFEEIRNLALQTLPAICNVYIPPYC--TIAPFGIFGTN
BAM08462  HQQ   Q---KQQQQ--    PSSQVSFQQPLQQYPLGQGSFRPSQQNPQDQGSVQPQQLPQFEEIRNLARQTLPAMCNVYIPPYC--TIAPFGIFGTN
BAM08461  QQQ[17]QQQQQQQQQQ[14]PSSQVSYQQPQQQYPSGQGSFQPSQQNPQAQASVQPQQLPQFEEIRNLARQTLPAMCNVYIPPYCSTTIAPSGIFGTN
BAM08459  HHQ[17]QQQQQQQQQQQ    PSSQVSYQQPQQQYPSGQGSFQPSQQNPQAQGFVQPQQLPQFEEIRNLALQTLPAMCNVYIPPYCSTTIAPFGIMSTN
BAM08456  HQQ[ 7]---QQQQQQQ    PSSQVSYQQPQQQYPSGQGFFQPSQQNPQAQGFVQPQQLPQFQFQEIRNLALQTLPAMCNVYIPPYCSTTIAPFGIMSTN
BAM08454  H-Q[ 7]---QQQQQQQ    PSSQVSYQQPQQQYPSGQGFFQPSQQNPQAQGFVQPQQLPQFEEIRNLALQTLPAMCNVYIPPYCSTTIAPFGIMSTN
BAM08452  HHQ[ 7]---QQQQQQQ    PSSQVSYQQPQQQYPSGQGFFQPSQQNPQAQGFVQPQQLPQFEEIRNLALQTLPAMCNVYIPPYCSTTIAPFGIMSTN
BAM08450  HQQ   Q----QQQ---[ 4]QSSQVSYQQPQQQYPSGQGSFQPSQQNPQAQGFVQPQHLPQLEEISNLALQTLPAMCNVYIPPYCSTTIAPFGIFGTN
BAM08458  HQQ[ 8]---QQQQQQQ    PSSQVSYQQPQQQYPSGQGFFQPSQQNPQAQGFVQPQQLPQFEEIRNLALQTLPAMCNVYIPPYCSTTIAPFGIMSTN
BAM08455  HQQ[ 8]---QQQQQQQ[ 5]PSSQVSYQQPQQQYPSGQGFFQPSQQNPQAQGFVQPQQLPQFEEIRNLALQTLPAMCNVYIPPYCSTTIAPFGIMSTN
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Figure 3. Multiple alignment of selected amino acids sequences of 19 accessions of α/β-gliadin. Sig = Signal peptide; Nter = N-terminal; Rep = Repetitive domain (that has been secluded from the figure); Cter = C-terminal. K = lysine, C = cysteine, Q = glutamine. Source: https://www.ncbi.nlm.nih.gov/

Lysine residues in α/ß-gliadin were limited (0-2 residues) and most of accessions

(12 out of 19 or 63%) had no lysine residues (Table 3, Figure 3). Lysine was located

either in the repetitive domain, C-terminal I or C-terminal II. The location of lysine in the

center compared to the terminal domains may cause difficulty for TG accessing and

forming crosslink from α/ß-gliadin. Such lack of accessibility of the amino acids

substrate has been suggested to prevent the enzymatic cross-link to take place (Heck et al

2013). Therefore, α- and β-gliadin and LMW-GS will have limited to no contribution in the formation of glutamyl-lysine bonds (Mujoo and Ng 2003).

Table 3. Number of amino acids, molecular weight, and lysine and cysteine residues of nineteen α-/ß-gliadin accessions

Accession	∑ AA	MW (kDa)	Number of lysine residues						Number of cysteine residues					
			N ter	rep	Cter I	Cter II	Cter III	total	N ter	rep	Cter I	Cter II	Cter III	total
AAZ94421	283	37606	0	0	0	1	0	1	0	0	4	0	2	6
AAZ94420	290	38350	0	0	0	0	0	0	0	0	4	0	2	6
1307187B	296	39240	0	0	0	0	0	0	0	0	4	0	2	6
P04727	313	41692	0	2	0	0	0	2	0	0	4	0	2	6
P04726	296	39212	0	0	0	0	0	0	0	0	4	0	2	6
AAA34279	313	41692	0	2	0	0	0	2	0	0	4	0	2	6
AAA34277	296	39212	0	0	0	0	0	0	0	0	4	0	2	6
BAM08464	282	37277	0	0	0	0	0	0	0	0	4	0	2	6
BAM08451	318	42196	0	0	0	0	0	0	0	0	4	0	2	6
BAM08463	297	39515	0	0	0	1	0	1	0	0	4	0	2	6
BAM08462	287	38188	0	1	0	1	0	2	0	0	4	0	2	6
BAM08461	331	44293	0	0	0	0	0	0	0	0	4	0	2	6
BAM08459	302	40234	0	0	0	0	0	0	0	0	4	0	2	6
BAM08456	290	38555	0	0	0	0	0	0	0	0	4	0	2	6
BAM08454	291	38670	0	0	1	0	0	1	0	0	4	0	2	6
BAM08452	292	38810	0	0	1	0	0	1	0	0	4	0	2	6
BAM08450	294	38737	0	0	0	0	0	0	0	0	4	0	2	6
BAM08458	296	39385	0	0	0	0	0	0	0	0	4	0	2	6
BAM08455	296	39400	0	0	0	0	0	0	0	0	4	0	2	6

∑AA = Total number amino acids; MW = molecular weight; Nter = N terminal; Rep = Repetitive domain; Cter = C terminal. Source: https://www.ncbi.nlm.nih.gov/

The 19 selected accessions of α/β-gliadin had six cysteine residues that spread into four in C-terminal I and two residues in C-terminal III. However, according to Müller and Wieser (1995), these six cysteine residues only contribute in formation of intra-molecular disulfide crosslinks.

γ-Gliadin

Total lysine of γ-gliadin varied from one to three residues per molecule, at least one of them was located in C-terminal I, and some accessions had additional lysine

residue in C-terminal III. There is no reports in the literature addressing the participation

of lysine of γ-gliadin in inter- and intra-molecular glutamyl-lysine interactions.

Table 4. Total amino acids, molecular weight, and lysine and cysteine residues of 22 accessions of γ-gliadin

Accession	∑ AA	MW (kDa)	Number of lysine residue						Number of cysteine residue					
			Nter	rep	Cter I	Cter II	Cter III	total	Nter	rep	Cter I	Cter II	Cter III	total
AAQ63858	311	41216	0	0	2	0	0	2	0	0	6	0	2	8
ACX37113	308	40513	0	0	2	0	0	2	0	0	6	0	2	8
ACX37112	314	41580	0	0	2	0	1	3	0	1	6	0	2	9
ACW82492	302	39677	0	0	1	0	1	2	0	1	6	0	2	9
1507333A	302	39675	0	0	1	0	1	2	0	1	6	0	2	9
AFC75727	326	42892	0	0	2	0	0	2	0	0	6	0	2	8
AEA52219	327	42955	0	0	2	0	0	2	0	0	6	0	2	8
AAA34289	327	42945	0	0	2	0	0	2	0	0	5	0	2	7
AGJ50341	327	42982	0	0	2	0	0	2	0	0	6	0	2	8
AGJ50340	339	44899	0	0	2	0	1	3	0	0	6	0	2	8
AFX69687	327	43026	0	0	2	0	0	2	0	0	6	0	2	8
AFX69685	302	39769	0	0	1	0	1	2	0	1	6	0	2	9
AED99848	311	41189	0	0	2	0	0	2	0	0	5	0	2	7
ACJ03479	302	39974	0	0	1	0	0	1	0	1	6	0	2	9
ACJ03465	310	41009	0	0	2	0	1	3	0	1	6	0	2	9
ACJ03464	327	43029	0	0	2	0	0	2	0	0	6	0	2	8
AGO17726	327	43048	0	0	2	0	0	2	0	0	6	0	2	8
AGO17724	328	43309	0	0	2	0	1	3	0	0	6	0	2	8
AGO17690	327	42970	0	0	2	0	0	2	0	0	6	0	2	8
AAF42989	308	40682	0	0	2	0	0	2	0	0	6	0	2	8
AFX69690	302	39711	0	0	1	0	1	2	0	1	6	0	2	9
ACJ03483	302	39869	0	0	1	0	0	1	0	1	6	0	2	9

∑AA = Total number amino acids; MW = molecular weight; Nter = N terminal; Rep = Repetitive domain; Cter = C terminal. Source: https://www.ncbi.nlm.nih.gov/

Glutamine residues in γ-gliadin were in average 33% of the total amino acids. For example, γ-gliadin from accession ACX37112 had 100 glutamine residues (32% from total 314 amino acids) distributed in the N-terminal (3 residues), repetitive domain (61 residues), C-terminal I (10 residues), C-terminal II (25 residues) and C-terminal III (1 residue).

Accession	Sig.	Nter.	Rep.	Cter.
	19	12	119-150	63
AAQ63858	----------------MNIQVDPSSQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
ACX37113	MKTLLIQTILVMAITIATANMQVDPSGQVQW		--------------------LNPCKNFLLQQCKPVSLVSSLWSMILPRSDCQVMRQQCCQQLAQIPQQLQCAAIHSIVHSIIM
ACX37112	MKTLLILTILAMATTIATANMQVDPSGQVQW		--------------------MNPCKNYLLQQCNPVSLVSSLVSMILPRSDCKVMRQQCCQQLAQIPQQLQCAAIHGIVHSIIM
ACW82492	MKTLLILTILAMATTIATANMQVDPSGQVQW		--------------------MNPCKNFLLQQCNHVSLVSSLSIILPRSDCQVMQQQCCQQLAQIPQQLQCAAIHSVAHSIIM
1507333A	MKTLLILTILAMATTIATANMQVDPSGQVQW		--------------------MNPCKNFLLQQCNHVSLVSSLVSMILPRSDCQVMRQQCCQQLAQIPQQLQCAAIHSVHSIIM
AFC75727	MKTLLILTILAMAITIGTANIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHS-II
AEA52219	MKTLLILTILAMAITIGTANIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AAA34289	MKTLLILTILAMAITIGTANIQVDPSGQVQW		--------------------LNPCKNILLQQSKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AGJ50341	MKTLLILTILAMAITIGTANIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AGJ50340	MKTLLILTIIAVALTTTTANIQVDPSGQVQW		--------------------MNPCKNYLLQQCNPVSLVSSLVSMILPRSDCKVMRQQCCQQLAQIPQQLQCAAIHGIVHSIIM
AFX69687	MKTLFILTILAMAITIGTANIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AFX69685	MKTLFILTILAMATTIATANMQVDPSGQVQW		--------------------MNPCKNYLLQQCNPVSLVSSLVSMILPRNDCQVMQQQCCQQLAQIPQQLQCAAIHSVAHSIIM
AED99848	------------MNIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLSIILPQSDCQVMRQQCYCQQLAQIPQQLQCAAIHSVVHSIIM
ACJ03479	MKTLLIVTILAMATTIATANMQVDPGYQVQW		--------------------MNPCKNFLLQQCNPVSLVSSLMILPRSDCQVMQQQCCQQLAQIPRQLQCAAIHSVVHSIVM
ACJ03465	MKTLFILTILAMATTIATANMQVDPSGQVQW		--------------------VNPCKNFLLQQCKPVSLVSSLWSMIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHTVIHSIIM
ACJ03464	MKTLLILTILVMAVTIGTANMQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AGO17726	MKTLLILTILAMAITIGTANIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWLQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AGO17724	MKTLFILTIIAVALTTTTANIQVDPSGQVQW		--------------------MNPCKNYLLQQCNPVSLVSSLVSMILPRSDCQVMRQQCCQQLAQIPQQLQCAAIHGIVHSIIM
AGO17690	MKTLLILTIFAAALTIATANIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AAF42989	----------------NIQVDPSGQVQW		--------------------LNPCKNILLQQCKPASLVSSLWSIIWPQSDCQVMRQQCCQQLAQIPQQLQCAAIHSVVHSIIM
AFX69690	MKTLFILTILAMATTIATANMQVDPSGQVQW		--------------------MNPCKNFLLQQCNHVSLVSSLVSIILPRSDCQVMQQQCCQQLAQIPQQLQCAAIHSVAHSIIM
ACJ03483	MKTLLIVTILAMATTIATANMQVDPGYQVQW		-----------------MNPCKNFLLQQCNPVSLVSSLISMILPRSDCQVMQQQCCQQLAQVPQQLQCAAIHSVVHSIIM

C ter II and III

Accession	Cter II		Cter III	
	30-42		41-43	
AAQ63858	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQyr	
ACX37113	-Q QEQQEQRQ GVQILVPLSQQQQVGQGTLVQGQGIIQPQQPAQLEVIRSLVLQTLATMCNVYV		PPYCSTIRAPFASIVAGIGGQ--	
ACX37112	QQ[8]QQQQQQQQ GIQIMRPLFQ-------LVQGQGIIQPQQPAQLEVIRSLVLGTLPTMCNVFV		PPECSTTKAPFASIVADIGGQ--	
ACW82492	-- --QQEQQQ GVPILRPLFQ-------LAQGLGIIQPQQPAQLEGIRSLVLKTLPTMCNVYV		PPDCSTINVPYANIDAGIGGQ--	
1507333A	-- --QQEQQQ GVPILRPLFQ-------LAQGLGIIQPQQPAQLEGIRSLVLKTLPTMCNVYV		PPDCSTINVPYANIDAGIGGQ--	
AFC75727	MR QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPRQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AEA52219	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCSVYV		PPECSIMRAPFASIVAGIGGQ--	
AAA34289	QQ QQQQQQQQ GIDIFLPLSQHEQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AGJ50341	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AGJ50340	QQ[2]QQQQQQQQ[6]GIQIMRPLFQ-------LVQGQGIIQPQQPAQLEVIRSLVLGTLPTMCNVFV		PPECSTTKAPFASIVADIGGQ--	
AFX69687	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AFX69685	-- --QQEQQQ GVPILRPLFQ-------LAQGLGIIQPQQPAQLEGIRSLVLKTLPTMCNVYV		PPDCSTINVPYANIDAGIGGQ--	
AED99848	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQyr	
ACJ03479	-- --QQEQQQ GIQILRPLFQ------LIQGQGIIQPQQPAQYEVIRSLVLRTLPNMCNVYV		RPDCSTINAPFASIVAGISGQ--	
ACJ03465	QQ ----EQQQ GMHILLPLYQQQQVGQGTLVQGQGIIQPQQPAQLEAIRSLVLQTLPTMCNVYV		PPECSIIKAPFSSVVAGIGGQ--	
ACJ03464	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AGO17726	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AGO17724	QQ[2]QQQQQQQQ GIQIMRPLFQ-------LVQGQGIIQPQQPAQLEVIRSLVLGTLPTMCNVFV		PPECSTTKAPFASIVADIGGQ--	
AGO17690	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AAF42989	QQ QQQQQQQQ GMHIFLPLSQQQQVGQGSLVQGQGIIQPQQPAQLEAIRSLVLQTLPSMCNVYV		PPECSIMRAPFASIVAGIGGQ--	
AFX69690	-- --QQEQQQ GVPILRPLFQ-------LAQGLGIIQPQQPAQLEGIRSLVLKTLPTMCNVYV		PPDCSTINVPYANIDAGIGGQ--	
ACJ03483	-- --QQEQRQ GVQIRRPLFQ-------LVQGQGIIQPQQPAQLEVIRSLVLRTLPTMCNVYV		SPDCSTINAPFASIVVGIGGQ--	

Figure 4. Multiple alignment of selected amino acids sequences of 22 accessions of γ-gliadin. Sig = Signal peptide; Nter = N-terminal; Rep = Repetitive domain (that has been secluded from the figure); Cter = C-terminal. K = lysine, C = cysteine, Q = glutamine. Source: https://www.ncbi.nlm.nih.gov/

Cysteine residues per γ-gliadin molecule ranged from seven to nine; two residues were found in the C-terminal III, five or six in C-terminal I and one additional cysteine found in the repetitive domain. According to Muller and Wieser (1997), γ-gliadin usually contains eight cysteine residues which only take part in intra-molecular disulfide interaction. According to Kasarda (1989) availability of a single unpaired cysteine residue may act as either chain terminator or participate in inter-molecular disulfide interaction.

ω-Gliadin

The lysine residues of ω-gliadin ranged from one to three and their positions were either in N-terminal or in the repetitive domain. Like γ-gliadin, so far there has been no reports on whether these lysine residues are forming isopeptide bonds with glutamine facilitated by TG or not. Glutamine residues of ω-gliadin were about 39% of the total amino acids sequence and were mostly (98%) located in the repetitive domain; only one or two were in the N-terminal and the other was in the C-terminal. Cysteine residues in ω-gliadin were very limited, either 0 or 1, and was located in the repetitive domain (Table 5, Fig. 5). According Shewry et al (2009) some ω-gliadin with a single cysteine may be able to incorporate into the large glutenin polymer.

Table 5. Total amino acids, molecular weight, and lysine and cysteine residues of eleven ω-gliadin accessions

Accession	∑AA	MW (Da)	Lysine				Cysteine			
			Nter	rep	Cter	total	Nter	rep	Cter	total
AAT01617	354	44764	0	2	0	2	0	1	0	1
ACN62214	321	43101	0	2	0	2	0	0	0	0
ACN62213	354	47646	0	1	0	1	0	0	0	0
ACN62212	381	51152	0	1	0	1	0	0	0	0
ACN62211	345	46047	0	3	0	3	0	0	0	0
AGO17774	377	50602	1	0	0	1	0	1	0	1
AGO17773	321	43116	1	0	0	1	0	0	0	0
AGO17772	266	35598	1	0	0	1	0	0	0	0
AGO17771	270	36105	1	0	0	1	0	0	0	0
BAN29067	366	49267	1	0	0	1	0	0	0	0
AAG17702	280	37539	1	0	0	1	0	0	0	0

∑AA = Total number amino acids; MW = molecular weight; Nter = N terminal; Rep = Repetitive domain; Cter = C terminal. Source: https://www.ncbi.nlm.nih.gov/

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Accession   |        Sig.        |   Nter.   |            Rep.            |    Cter.
            |         19         |    11     |          224-258           |     1-12
AAT01617    | MKTFIIFVLLSMPMSIVIAARHLNPSDQEL------------------------------------------S
ACN62214    | -----------------ARQLNPSEQEL------------------------------PFVVVE------
ACN62213    | -----------------ARELNPSEQEL------------------------------PFVVVE------
ACN62212    | -----------------ARELNPSEQEL------------------------------PFVSSGTSIGGQ
ACN62211    | -----------------ARQLNPSEQEL------------------------------PFVSSGTGIGGQ
AGO17774    | MKTFLIFVLLAMAMNIATAARQLNPSNKEL----------------------------PYGSSLTSIGGQ
AGO17773    | MKTFLIFVLLAMAMKIATAARELNPSNKEL----------------------------PYGSSLTSIGGQ
AGO17772    | MKTFLIFVLLAMAMKIATAARELNPSNKEL----------------------------PSGSSLTSIGGQ
AGO17771    | MKTFLIFVLLAMAMNIATAARQLNPSNKEL----------------------------PYGSSLTSIGGQ
BAN29067    | -----------------ARELNPSNKEL----------------------------PYGSSLTSIGGQ
AAG17702    | MKTFLIFVLLAMAMKIATAARELNPSNKEL----------------------------PYGSSLTSIGGQ
```

Figure 5. Multiple alignment of selected amino acids sequences of 11 accessions of ω-gliadin. Sig = Signal peptide; Nter = N-terminal; Rep = Repetitive domain (that has been secluded from the figure); Cter = C-terminal. K = lysine, C = cysteine, Q = glutamine. Source: https://www.ncbi.nlm.nih.gov/

Formation of Glutamyl-Lysine Bonds Catalyzed by TG

A preliminary evaluation of glutamyl-lysine cross-links could be done by comparing the location of lysine and glutamine residues in the gluten structure. A summary of the number and position of lysine and cysteine residue of selected gluten components is illustrated in Figure 6 and Table 6.
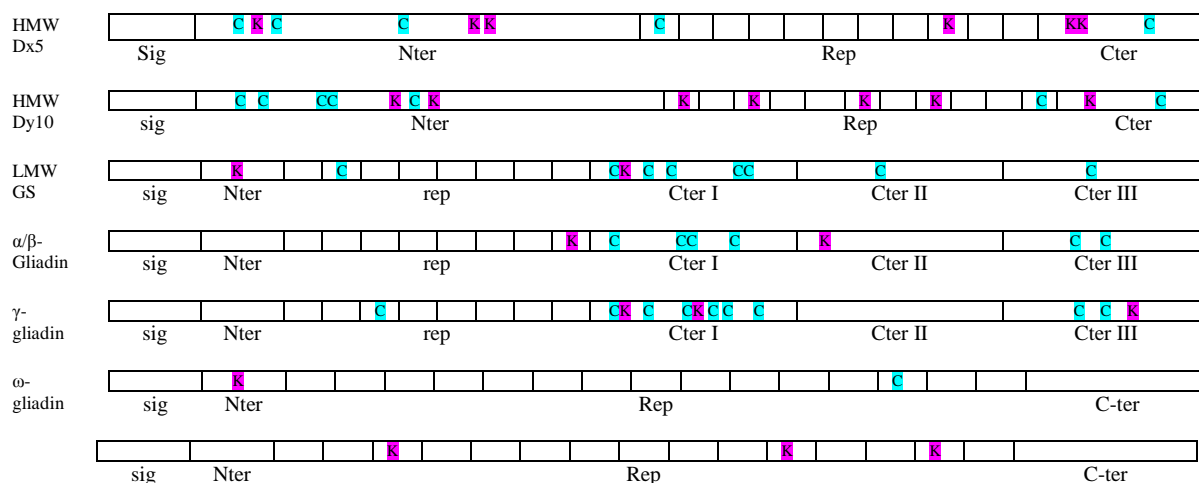
Figure 6. Summary of lysine and cysteine residues location of selected wheat gluten proteins. K is lysine; C is cysteine; sig is signal peptide; Nter is N-terminal; rep is repetitive domain; Cter is C-terminal.

Table 6. Comparison of lysine, glutamine and cysteine residues of selected gluten proteins.

Gluten protein	Accession	Number residues		
		Lysine	Glutamine	Cysteine
HMW-GS Dx5	DAA06555.1	6	299	5
HMW-GS Dy10	P10387	7	182	7
LMW-GS	AAS66085	2	111	8
α/β-gliadin	BAM08462	2	93	6
γ-gliadin	ACX37112	3	100	9
ω-gliadin	AG017774.1	1	146	1
	ACN62211.1	3	134	0

Source: https://www.ncbi.nlm.nih.gov/

Both glutenin and gliadin proteins are substrates for TG (Larré et al 2000; Autio et al 2005). However, HMW-GS are the most affected by TG activity (Larré et al 2000; Bauer et al 2003) since it contains the highest (up to ten) lysine residues compared with the rest of the gluten proteins.

There are two different suggestions on whether gliadins participate or not in formation of isopeptidic bonds. Rosell et al (2003) observed that the presence of all gliadin types, as detected by free-zone capillary electrophoresis, decreased after treated with TG. Bauer et al (2003) analyzed peptides sequences of a thermolytic digest of the insoluble residue and observed that HMW-GS and α-gliadins were the predominant gluten proteins involved in the formation isopeptidic cross-link by TG. In contrast, Gerrard et al (2001) reported that by SDS analysis there were no crosslink of gliadins created by TG. By immunoblotting assays Mujoo and Ng (2003) also observed that LMW-GS and some of gliadin types were not participating in the glutamyl-lysine bond formation. Nevertheless, according to DeJong and Koppelman (2002), as long as there is accessibility to lysine and glutamine residues, TG may catalyze formation of covalent

bonds. Since all of gluten components contain lysine (Table 2 and Figure 6) their accessibility to TG will be a key factor to determine the formation of glutamyl-lysine bonds.

A simplified illustration of the modification of gluten structure by TG is described in Figure 7. TG introduces covalent cross-links between glutamine and lysine residues of proteins leading to the formation of high molecular weight polymer (Tseng and Lai 2002; DeJong and Koppelman 2002; Ahn et al 2005). The formation of large polymers will improve viscoelastic properties of gluten (Larré et al 2000); even when a small number of interactions occur, it could significantly affect gluten visco-elastic properties. For example, when only one inter-chain interaction takes place between two HMW-GS (for instance two Dx5 and each has a MW of about 104,856 kDa), it would produce polymer with a MW of 209,712 kDa. If two inter-molecular isopeptidic bonds are created among three HMW glutenins subunits, the polymer will have a molecular weight about 314,568 kDa and so forth. Further, if the isopeptidic bond takes place between two polymers of gluten proteins, it would produce a large MW polymeric gluten protein. Polymerization of gluten will also affect the improvement of gluten strength and elasticity (Huang et al 2008). Sroan et al (2009) suggested that polymeric proteins with molecular weight larger than 250,000 Da confer the strength to the entangled gluten protein network.
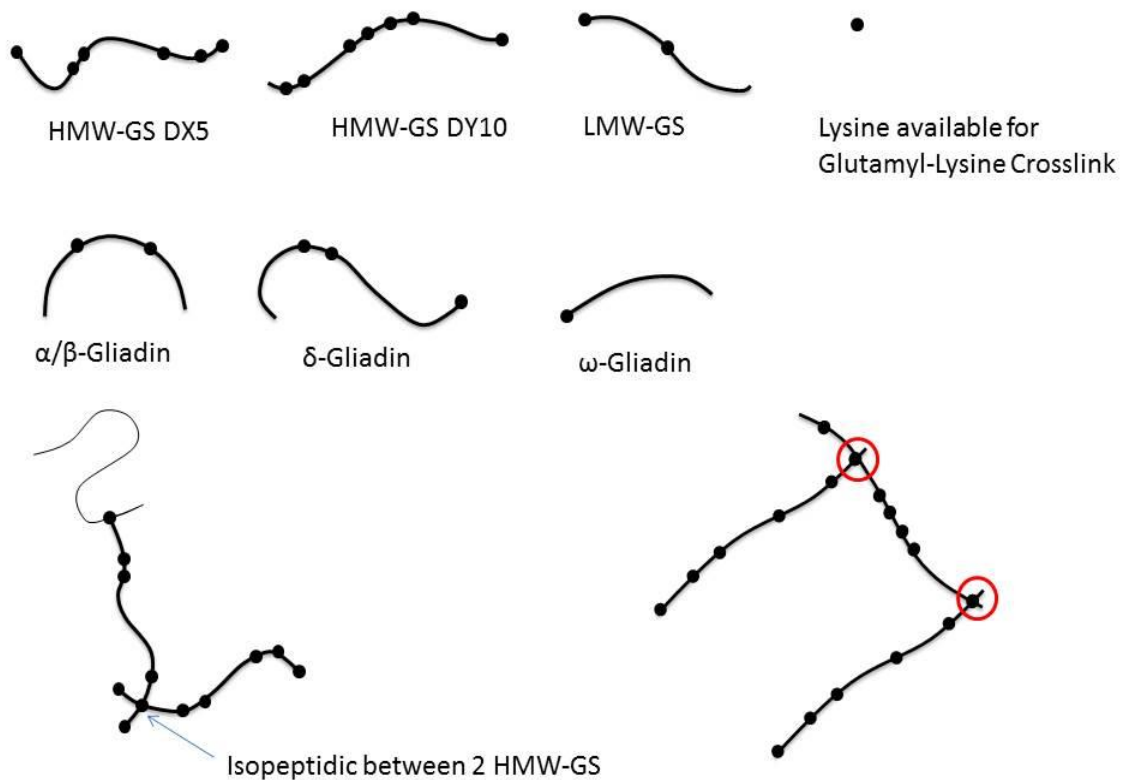
Figure 7. A proposed simplified model of polymerization of gluten as affected by TG, assuming no limitation of accessibility by protein conformations.

Activity of TG in creating isopeptidic covalent bonds is dose and time dependent. The activity will not stop until the substrate (glutamine/lysine) is no longer available or until the protein network limits accessibility and mobility of TG (DeJong and Koppelman 2002). TG creates both inter- and intra-molecular covalent bonds. Combination of inter- and intra-molecular interactions might produce a 'closed loop' in which the lysine in N-terminal may crosslink with glutamine in the C-terminal (Fig. 8). The more crosslinks are formed in a system, the smaller the closed loop will be.
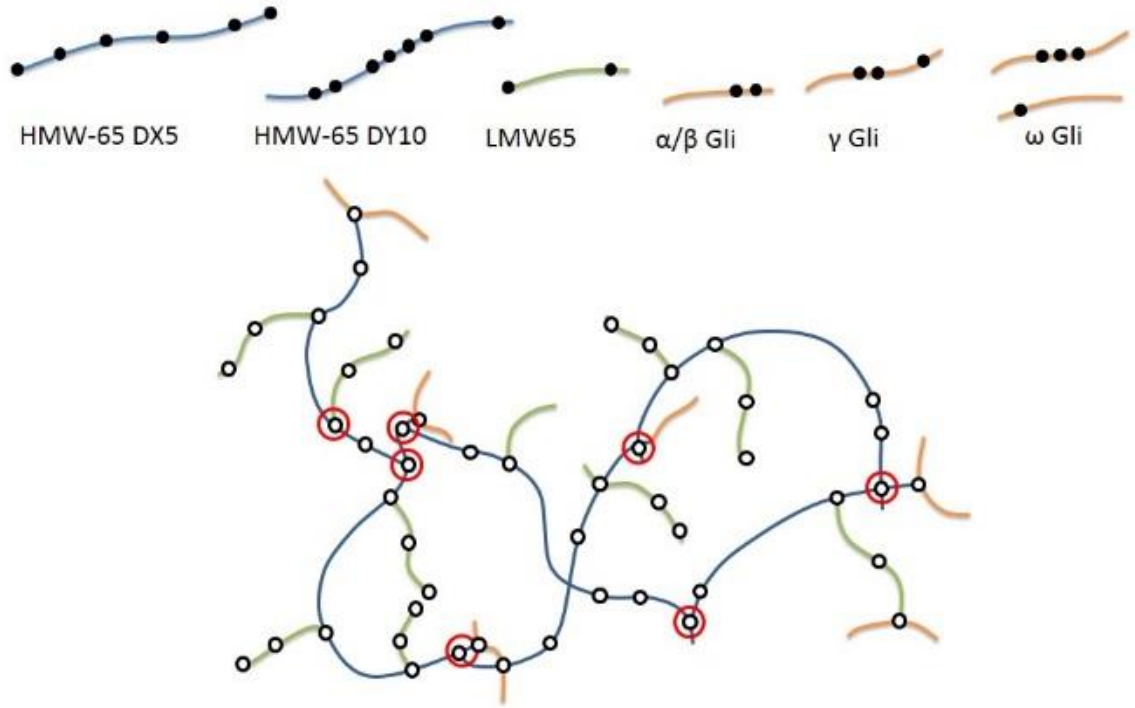
Figure 8. A predicted simplified model of formation of closed loop due to the formation of inter- and intra-chain isopeptide bonds.

Figure 9 has a predicted model when other gluten components; for example, γ-gliadin with three lysine residues might join and form branches with the polymer. The more gluten proteins participate in creating branches; the pulling of strands might occur and increase the proximity among amino acids in protein. This will lead to the formation of disulfide bonds as a result of oxidation between of close thiol group of gluten proteins (Gujral and Rosell 2004; Bonet et al 2005).
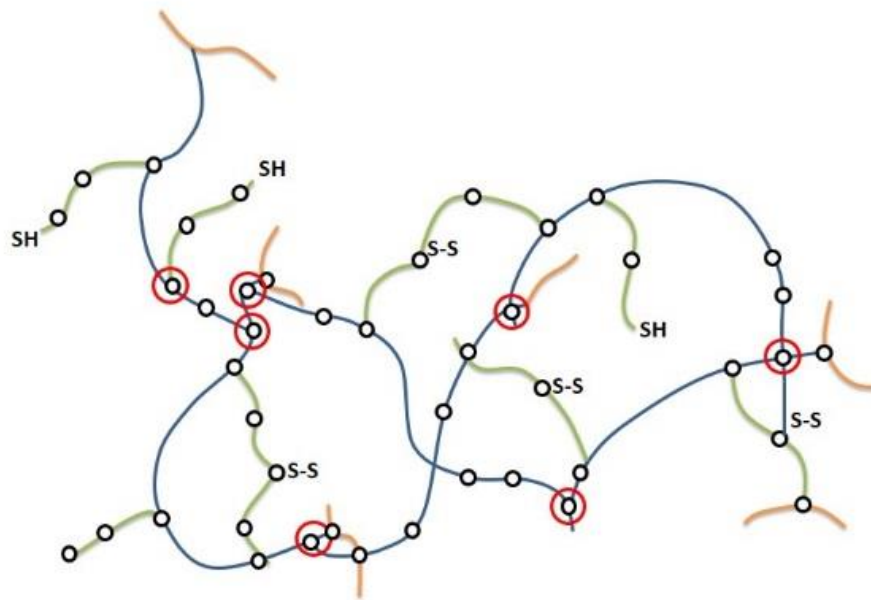
Figure 9. A model to illustrate the formation of disulfide bonds0 due to increase of proximity between amino acids of gluten proteins polymers.

The hypothesis models (Fig. 8 and 9) can be related to the physical appearance of gluten treated with different levels of TG displayed in Figure 10. The formation isopeptide interactions between glutamine and lysine of HMW glutenin subunits are expected to increase with increasing number of TG. But, then the agglomeration changes and the structure of gluten seem quite rough after treated with TG 0.8%. At this level, TG forms covalent bonds both glutamyl-lysine and disulfide that causes a different agglomeration with clumps of protein and loss of cohesiveness. It is speculated that the loss of cohesiveness has been also contributed by prevention of hydrogen bonds of a slightly over crosslinked gluten matrix. According to Belton (2005) hydrogen bonds have important role in stabilizing interaction between glutenin and giadins.
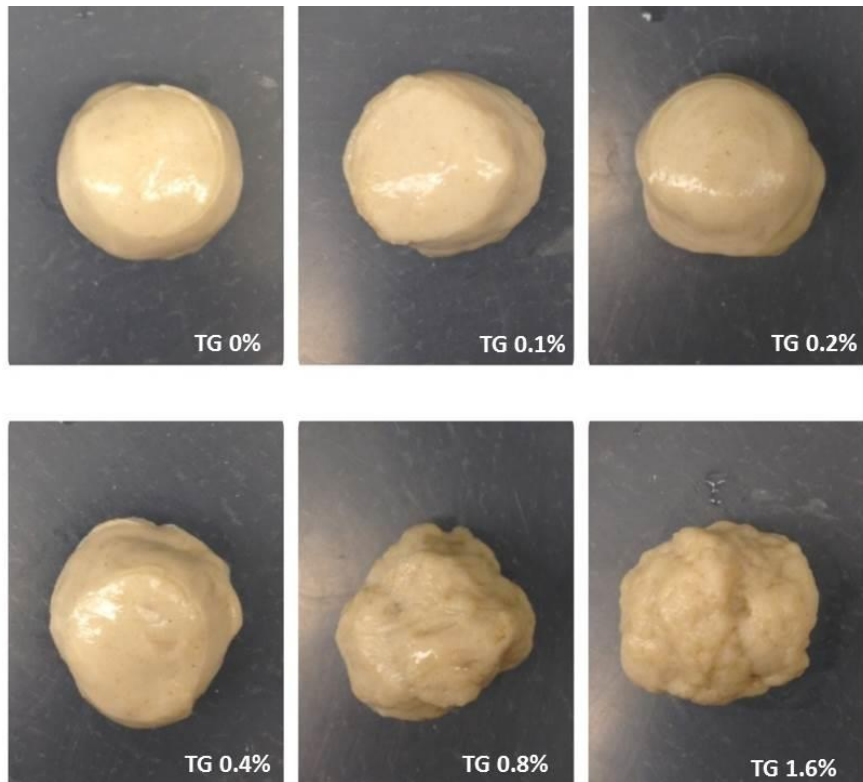
Figure 10. Pictures of gluten extracted from commercial wheat flour treated with TG at 0 to 1.6% levels.

CONCLUSION

Among gluten protein fraction, HMW-GS subunits possess the highest (4-10) lysine residues, and in theory the highest probability to participate in the formation glutamyl-lysine covalent bonds catalyzed by TG. Formation of these covalent bonds, both inter- and intra-molecular, increases the molecular size of gluten protein polymer along with increase proximity of chains by disulfide bonds. We propose the formation of closed loop strands which can help to understand an increase in covalent crosslinks of gluten via TG.

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CHAPTER IV

**MODIFICATION OF GLUTEN PROPERTIES AS INFLUENCED BY
TRANSGLUTAMINASE AND EXTRACTION METHODS**

ABSTRACT

Gluten quality is one of the most important determinants of wheat flour end use. This study was aimed to evaluate gluten properties as influenced by TG and to compare two gluten preparation methods. A set of six commercial wheat flours with protein content $11\pm0.5\%$ was treated with TG (0, 0.1, 0.2, 0.4, 0.8 and 1.6% w/w) using a Randomized Complete Block Design with 3 replicates. Gluten properties were determined including wet gluten, gluten index and gluten recovery. Two preparation methods for gluten were also compared: 1) standard gluten separation using a Glutomatic, and 2) dough mixed at optimum water absorption in a Farinograph, resting period 10 min, and followed by standard Glutomatic procedure. Data were analyzed using ANOVA and protected pair wise t-tests. TG modified gluten structure and quality where wet gluten decreased while gluten index and gluten elastic recovery increased with increasing TG levels. Compared to the standard method, modified method of gluten extraction produced significantly higher wet gluten and gluten recovery. The modified method is a good alternative for measuring the effect of time dependent additives, like TG, on gluten properties and thus avoid underestimating the full potential of TG.

Key words: gluten, TG, standard and modified methods.

INTRODUCTION

The specific processing functionality of wheat flour to form cohesive doughs which then can be baked into bread or formed into pasta and noodles are largely derived from gluten proteins (Shewry et al 2000). In the baking industry, functional properties of gluten proteins are important to determine dough mixing properties, hold gas during proofing and baking, and establish baking performances (Autio et al 2005). The functionality of gluten, according to Goesaert et al (2005), are strongly influenced by molecular weight of glutenin, the occurrence of covalent and non-covalent bonds between glutenin molecules and interactions between glutenin and other flour constituents. The visco-elastic property of gluten could be increased by cross-link reaction (Jerez et al 2005).

TG has the ability to introduce covalent cross-links between glutamine and lysine residues of proteins leading to the formation of high molecular weight polymer (Tseng and Lai 2002; DeJong and Koppelman 2002; Ahn et al 2005). New cross-link interactions cause the formation of large protein aggregate, the lowering extensibility and the increasing of resistance of dough (Steffolani et al 2010). Formation of polymers leads to improvement of viscoelastic properties of gluten (Larré et al 2000). Polymerization of gluten will also effect on the improvement of gluten strength and elasticity (Huang et al 2008). In the presence of TG, gluten network becomes more developed (Autio et al 2005), however, increasing of TG levels and excessive gluten proteins polymerization will bring detrimental effects on the bread baking quality (Renzetti et al 2010).

Several analyses have been commonly used for measuring gluten properties, such as wet gluten, gluten index, and in this work a new instrument "gluten CORE" was used

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to determine gluten elastic recovery (%).Wet gluten is defined in AACC International method 38-12.02 as a viscoelastic gliadin-glutenin protein material obtained from washing out the starch and other water soluble from wheat flour dough. This parameter is important to estimate the quantity of protein as well as the quality of flour. Amount of wet gluten usually increases with increasing flour protein content (Kulkarni et al 1987). However, the amount of wet gluten does not automatically reflect either gluten/protein quality (Curic et al 2001) or explain differences in flour and in dough properties (Wieser and Kieffer 2001) all variations in baking quality (Chapman et al 2012).

Gluten index is defined as the amount of wet gluten (in weight percentage) that remains on the sieve after centrifugation with the larger gluten strands/aggregates remaining on the sieve (Mis 2000; Dowell et al 2008). This parameter is associated to gluten strength, reflecting inadequate, sufficient, average or excellent gluten quality (ICC Standard no 158, 1995). It is generally assumed that quality of gluten increased with an increase of gluten index scores (Mis 2000). Gluten index is a good variable to predict bread loaf volume (Dowell et al 2008).

Gluten recovery (%) is obtained from a new bi-axial compression instrument (Gluten CORE) to evaluate gluten elastic recovery and is well adapted for dough and other viscoelastic materials. The degree of recovery illustrates the ratio of the overall distance recovered over the distance compressed. It has a high (0.86) correlation with gluten strength from tensile test (Chapman et al 2012).

The ability of TG to crosslink glutamine and lysine of proteins is dependent on factors such as dose, source of enzyme, compatibility between substrates and enzyme and time reaction (Shewry et al 1992; Dickinson 1997; Gerrard et al 1998; Koh and Ng

2008). This study was aimed to analyze the effects of levels of TG treatment on the gluten properties.

According Robertson and Cao (1998) the success of gluten extraction process depends on the ability to maintain / strengthen protein-protein interaction and weaken protein-starch interaction. Factors such as level of hydration, mixing and resting period will contribute to the protein interaction. Therefore, this study also is aimed to compare two gluten extraction methods, standard and modified, where the modified method provides a longer time for TG to interact with gluten proteins due to additional 4 min mixing time and 10 min resting dough prior to standard gluten extraction.

The objectives of this study were to evaluate the effect of TG levels on gluten properties and to compare two isolation methods of gluten consisting in different times, mixing type and resting steps.

MATERIALS AND METHODS

Materials

A set of six blends of commercial wheat flours had protein content of 11.0±0.5%. Microbial TG, Activa TI with activity 100 U/g, was obtained from Ajinomoto (Fort Lee, NJ) and sodium chloride was from Fisher Scientific (Fair Lawn, NJ).

Gluten Analysis Standard Method

Wet gluten (%) and gluten index were measured following approved method 38-12.02 (AACCI 2011). Gluten was extracted by washing wheat flour with 2% sodium chloride (NaCl) solution using Glutomatic 2202 (Perten Instruments AB, Segeltorp,

Sweden). Wheat flour sample weighing 10 g (TG was added to the base flour at levels of 0, 0.1, 0.2, 0.4, 0.8 and 1.6% (w/w)) was placed into the washing chamber that was equipped with an 88 μm polyester screen. A 4.8 ml of wash solution (2% sodium chloride solution) was added and gently spread over the top surface of sample before the washing chamber was attached to the Glutomatic. The dough sample was mixed for 60 sec and then washed with a wash liquid rate of 50-56 ml/min for 5 min to obtain gluten. Wet gluten was calculated:

$$\text{Wet gluten \% (14\%mb)} = \frac{\text{total wet gluten (g) X 860}}{100 - \%\text{sample moisture}}$$

Wet gluten was isolated from wheat flour using a Glutomatic that was placed in a separate gluten index cassette Ø 22 mm and centrifuged at 6000±5 rpm for 1 min to force gluten passing through the sieve. Gluten that passed through the sieve was removed using a spatula and weighed to the nearest 0.01g. The gluten on top of the sieve was added on the balance to obtain total wet gluten. Gluten index was obtained by dividing wet gluten that remained on sieve (g) with total wet gluten (g) and then multiplied by 100.

Gluten recovery (%) was determined using Gluten Core Analyzer (Perten Instruments AB, Segeltorp, Sweden) according to the procedure of Chapman et al (2012). Wet gluten was gently rounded and placed into a cylindrical shaper tube with a closely fitted plunger and centrifuged at 6000±5 rpm for 1 min (using centrifuge 2015, Perten Instruments AB, Segeltrop, Sweden). The shaped gluten samples were rested for 1-2 min before loading to Gluten Core Analyzer. The instrument was calibrated to a height of 8 mm and force of 104.7 g. Other conditions of the test were set as follows: sampling interval 100 ms, velocity start 20 mm/s, target force start 0.5 N, hold time start 0 s, target force compression 8 N, compression time 5 s, minimum distance 1 mm, target force

recovery 0.2 N, and recovery time 55 s. At the end of the test, a report was provided

including gluten initial height (H_i) in mm, minimum height reached through compression

test (H_m) in mm, final height at 60 s test (H_f) in mm, and Recovery in %. Gluten elastic

recovery % was calculated as follows:

$$\text{Gluten elastic recovery (\%)} = \frac{Hf-Hm}{Hi-Hm} \text{ x } 100$$

The gluten quality indices analysis were performed in triplicate.

Gluten Analysis Modified Method

The method for measuring wet gluten and gluten recovery (%) was modified with

additional dough preparation and resting before tested with standard procedures. A wheat

flour sample weighing 10 g was mixed at optimum water absorption (about 6-6.5 ml) in a

Farinograph (C.W. Brabender Instruments, Hackensack, NJ) until reached its maximum

development (about 4 min). The dough then was wrapped with plastic and rested for 10

min. The sample was placed in the Glutomatic and analyzed following method 38-12.02

(AACCI 2011). The test of wet gluten and elastic recovery were performed in triplicate.

Statistical Analysis

The study was conducted in a Randomized Complete Block Design. The flours

were treated with TG in 6 different levels (0, 0.1, 0.2, 0.4, 0.8 and 1.6%w/w), with 3

independent replications. Analysis of Variance (ANOVA) procedure with 0.05

significance level were performed using SAS version 9.2 (SAS Institute, Cary, NC).

Mean separation of significantly different data were compared using protected pair wise

t-tests. Comparisons were made between gluten quality indices obtained from standard and from modified methods at each TG level.

RESULTS AND DISCUSSION

TG significantly influenced ($p<0.05$) gluten quality indices; wet gluten, gluten index and gluten elastic recovery. The effect of TG and comparison of two methods used for extracting gluten to the gluten properties are displayed in Table 1 and Figure 1.

Table 1. Effect of TG on the gluten properties using standard and modified methods of gluten extraction [a].

TG	Wet gluten (%)		Elastic recovery (%)		Gluten index
(%)	Standard	Modified	Standard	Modified	
0	28.9±0.22 a	31.9±0.19 a	71.3±0.88 d	58.6±1.12 e	94.2±0.85 c
0.1	28.8±0.21 ab	32.1±0.19 a	71.4±1.01 d	59.8±1.19 e	93.6±1.09 c
0.2	28.6±0.24 bc	31.8±0.21 a	72.2±0.80 cd	63.7±1.36 d	94.9±0.63 bc
0.4	28.7±0.22 abc	31.7±0.21 a	73.9±0.75 bc	68.5±1.14 c	95.8±0.60 b
0.8	28.5±0.20 c	31.1±0.22 b	74.5±0.75 b	76.0±0.92 b	97.2±0.56 a
1.6	28.2±0.22 d	30.3±0.18 c	78.8±0.61 a	82.0±0.90 a	98.5±0.28 a
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] Means ± standard error from triplicate analysis. Means within the same column with the same letter are not significantly different at p= 0.05.
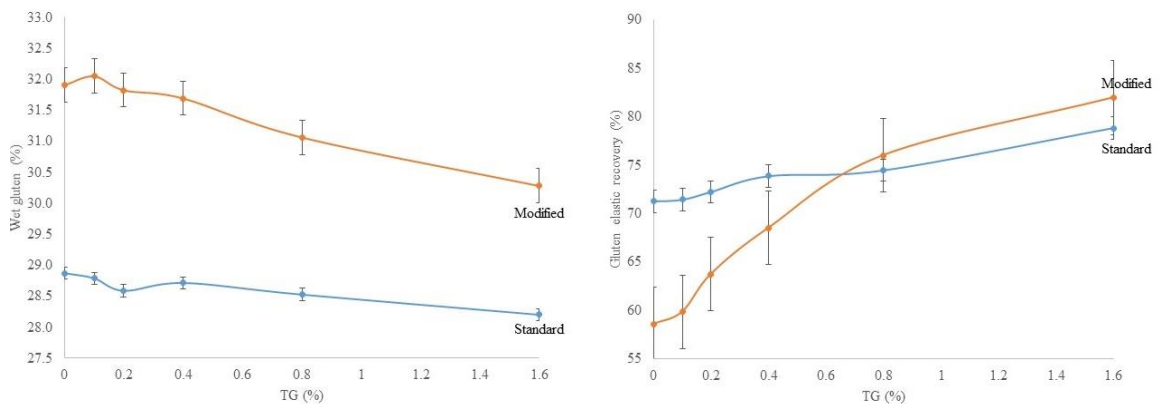
Figure 1. Graphic comparison of wet gluten and elastic recovery obtained by standard and modified method. The data are means from triplicate analysis and the bars indicate standard errors.

Wet Gluten

Wet gluten values of TG treatments analyzed by the standard and modified methods are presented in Table 1. The amount of wet gluten extracted from the standard method ranged from 28.2 to 28.9% and had a trend to increase (30.3-32.1%) when the gluten extraction method was modified. TG significantly influenced ($P < 0.05$) wet gluten obtained from both standard and modified procedure of extraction. In both methods a decrease of wet gluten with increasing TG levels was observed. Wet gluten obtained from flour treated with TG 0 to 0.4% were statistically similar, with the lowest wet gluten observed with levels 1.6% TG.

This observation can be related to gluten hydration shell that leads to water absorption. TG has the ability to create covalent bonds (glutamyl-lysine bond) and secondary disulfide bonds (intra- and inter-molecular) of protein gluten and the crosslinks increase with increasing level of TG. A higher amount of cross-links causes tighter structure protein with lower surface area. Kontogiorgos (2011) suggested the structure of hydrated gluten network model where within an atomic scale, gluten proteins interact with each other with various bonds (hydrogen, -S-S-, ionic) to form gluten sheets. Gluten sheets, in nano scale, are proposed to be arranged side by side so as to form nanoporous ultrastructure with the nanoporous areas filled with confined water that strongly interact with gluten matrix. If this hypothesis is accepted then one may postulate that increasing covalent bonds/crosslink due to increasing amount of TG causes the proposed ultrastructure of protein to be tighter. With a reduced diameter of nanoporous ultrastructure, the capacity to entrap water is reduced. The decreasing water absorption of wheat flour due to increasing level of TG treatment was previously reported by Barrett et

al (2002). Similar observation was also reported by Huang et al (2010) who found a decrease of water absorption of oat flour with increasing levels of TG.

Wet gluten obtained from the modified method was 7-11% higher and significantly different (P<0.0001) than those observed from the standard method (Table 1). An increase of wet gluten of modified method can be related to total mixing time and the resting dough step prior to gluten extraction. In the modified method, dough was mixed in a Farinograph until it reached optimum development time (about 4 min) and rested for 10 min before wet gluten was separated by standard procedure. Compared to the standard method that provides only 1 min mixing, dough from the modified procedure undergoes longer total mixing time (4 min in Farinograph and 1 min in Glutomatic) that encourages agglomeration of gluten and dough development. Mixing promotes the dough development by many physicochemical modifications, including evenly distribution of dough ingredients, hydration and swelling of flour components, development of gluten network through breakage and formation of covalent (-S-S-) or non-covalent (hydrogen, hydrophobic) bonds (Kaddour and Cuq 2011). During the early stage of mixing, protein fibril gluten is hydrated, glutenin polymers are folded with chains in a random orientation; continued mixing promotes more gluten proteins to become hydrated and glutenin subunits to align, at this stage dough is optimally developed (Zaidel et al 2010). In developed dough, gluten film is homogenously formed and regularly distributed around the starch granules. The more developed dough produces the higher gluten yield during washing process (Frederix et al 2004; Snehil Dua et al 2009).

During resting, dough has a chance to relax from the stress due to mixing, water is redistributed, hydration of flour components continues, interchange of sulfhydryl-

disulfide reactions take place (Dong and Hoseney 1995), new bonds are formed (Van Der Borght et al 2005), protein network is formed through aggregation of protein (Renzetti et al 2008), and aggregation of gluten protein forms more dense protein bodies (Kieffer et al 1998). Gluten is able to take up water about 1.9 x (Ohm and Chung 1999) or 1-3x (Bloksma and Bushuk 1988) its weight. Resting of dough encourages the reformation of unextractable glutenin polymer and causes the gluten network to become more elastic. A more elastic gluten network can retain starch from leaching out during washing (Auger et al 2009). Additional time from both mixing and resting gives more chance for TG to modify gluten. As a result, gluten has more time to strongly interact with other flour components (for example starch, non-starch polysaccharides) and absorbs more water. Therefore, the 30 to 32% increase in wet gluten obtained from the modified extraction method.

Gluten Index

Gluten index observed from this study ranged from 93.6 to 98.5. According to Perten (1990) and Curic et al (2001) flours for the production of bakery products have gluten index values from 60 to 90 and flours with gluten index between 75 and 90 resulted in test baking with loaves of largest volume and best sensory properties. Flours with gluten index exceeding 95 are too strong and those with the index value less than 40/60 are too weak for bread production. Therefore, the result of this study indicated that wheat flour treated with TG up to level 0.2% produced gluten in the range of strong category, whereas treatment TG 0.4% and more resulted in over-strong gluten.

Gluten index was significantly influenced by addition of TG. The general trend indicated that TG increased the gluten index. These results agree with those reported by Bonet et al (2006). In this study, increasing of gluten index relates to the ability TG to modify and strengthen the structure of gluten. TG produces inter- and intra-molecular covalent bonds between residue amino glutamine and lysine (DeJong and Koppelman 2002) and the molecular weight of gluten increased (Köksel et al 2001). The amount of protein polymerization is determined by amount of enzyme (TG) (Rosell et al 2003). Besides creating glutamyl-lysine bonds as the main product of TG, this enzyme also has been reported to encourage formation of disulfide bonds. This has been explained by a closer proximity among amino acids of protein, then leading to formation of secondary disulfide bonds (Gujral and Rosell 2004). Formation of covalent bonds and polymerization due to TG activity is assumed to contribute to stronger gluten structure. Therefore, it leads to a reduced amount of gluten which passes through the sieve. The amount of gluten that passes through the sieve is what determines gluten index value. So, when a longer polymeric gluten structure is formed, a lower amount of gluten passes through the sieve and a higher gluten index is observed.

When the level of TG was increased to 0.8%, gluten index was higher than 95 indicating it has become over-strong. Curic et al (2001) suggested that gluten is too strong when it has gluten index higher than 95.

Gluten Elastic Recovery (%)

Figure 2 illustrates an example of the gluten compression recovery of commercial wheat flour treated with different levels of TG. Gluten elastic recovery obtained from our

study ranged from 71.2 to 78.8% for the standard method and from 58.6 to 82.0% for the

modified one. Statistical analysis suggested that treatment wheat flour sample with TG

significantly modified gluten recovery (P<0.0001) (Table 1). Both standard and modified

methods share the same general trend of rising gluten recovery with increasing levels of

TG. The gluten recovery increased 9.6 and 39.9% for standard and modified methods,

respectively, compared to the control. Since gluten recovery highly correlated with gluten

strength (Chapman et al 2012), the assumption that as gluten recovery increases gluten

strength does as well is justified. The ability of TG to increase dough/gluten strength is

related to the creation of new peptide/covalent bonds between glutamine and lysine, and

the formation of disulfide bond as secondary product.
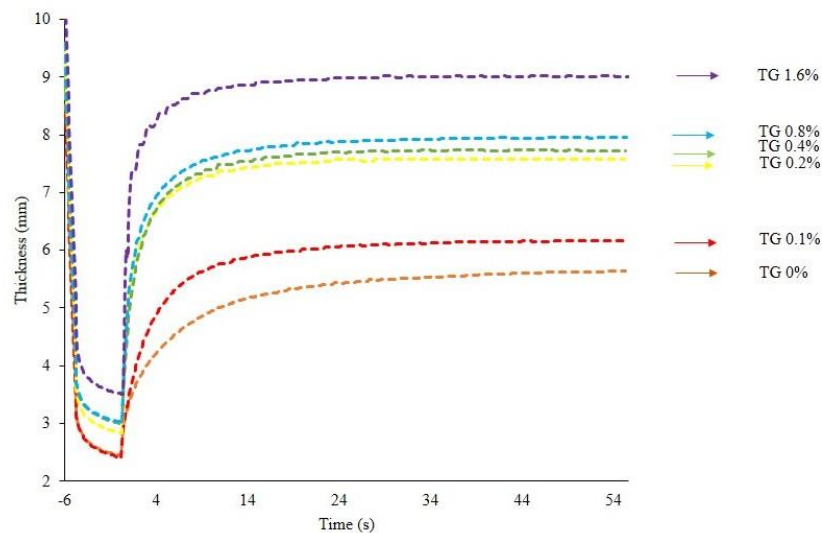
Figure 2. Example of compression recovery curves of a commercial wheat flour treated
with different levels of TG.

Gluten contains two primary proteins: gliadin and glutenin. Among gluten

components, glutenin dictates the elastic properties of gluten. Glutenin is a good substrate

for TG (Larré et al, 2000). Compared to LMW-GS, the HMW-GS are better substrates

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for TG because they have more lysine content (Autio et al 2005). Lysine in HMW-GS is considered to be 6 to 7 residues/mol protein, whereas LMW-GS has only 1-2 lysine residues/mol protein (see chapter III of this dissertation). Since the effect of TG is dose dependent, the formation isopeptide interactions between glutamine and lysine of HMW-GS increase with increasing number of TG which increases protein polymerization contributing to the increasing of elastic/strength of gluten.

Interactions between glutamine and lysine created by TG are different from disulfide or hydrogen bonds that occur during mixing wheat and water dough (Steffolani et al 2008). The isopeptidic interactions have higher energy (100 kcal/mol (418.4 kJ/mol)) than disulfide bonds (50 kcal/mol (209 kJ/mol)) (Macritchie 1975), hydrophobic forces (5-10 kJ/mol), hydrogen bonds (10-40 kJ/mol) and electrostatic interactions (25-80 kJ/mol) (Dickinson 1997). Therefore, increasing the number of glutamyl-lysine and disulfide interaction due to increasing the level of TG will strengthen gluten structure.

Compared to the standard method, the modified method with TG up to 0.4% produced smaller (7-18%) gluten elastic recovery (Table 1). However, when TG level increased to 0.8%, gluten extracted from modified method showed higher (2-4%) elastic recovery than gluten extracted from standard method. This result occurs because the level of gluten protein polymerization due to TG activity, as reported by Larré et al (1998), is dose and time dependent. The modified method provides additional time; about 4 min of dough mixing, to reach optimum development and 10 min of dough resting. The combination of high dose (≥0.8%) and additional 14 min of reaction time provided by the modified method facilitates TG to react and create more covalent bonds resulting in more cross-linked gluten.

CONCLUSION

All of the measured gluten properties such as wet gluten, gluten index and gluten recovery were significantly modified by TG treatment. Both the standard and modified methods shared the same pattern of gluten properties where gluten index and gluten elastic recovery increased and wet gluten decreased with increasing levels of TG. When TG levels was increased from 0 to 1.6%, wet gluten decreased 2.5% (standard method) and 5.3% (modified method) and gluten index increased 4.6%. The largest impact of TG levels was on elastic recovery of gluten where the 1 min mixing yielded an increase in recovery ranging from 3.6 to 10.5% and the modified method consisting in extended mixing and resting yielded an increase range from 8.7 to 39.9%.

Compared to the standard method, modified method of gluten extraction significantly produced higher wet gluten (7 to 11%) and higher increasing of gluten recovery (10.5% of standard method vs 39.9% of modified method). This results suggest that the modified method is a good alternative for measuring the effect of time dependent additives, like TG, on gluten properties and thus avoid underestimating the full potential of TG.

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CHAPTER V

EVALUATION OF WHEAT DOUGH RHEOLOGICAL AND FERMENTATION PROPERTIES AS AFFECTED BY TRANSGLUTAMINASE AND THEIR CORRELATION TO BAKING QUALITY

ABSTRACT

This study was aimed to determine the effect of transglutaminase (TG) on dough rheological and fermentation properties and to determine correlations with the bread loaf volumes obtained. A set of six commercial wheat flours with protein content of $11\pm0.5\%$ was treated with TG (0, 0.1, 0.2, 0.4, 0.8 and 1.6% w/w) following a Randomized Complete Block Design with 3 replicates. Dough mixing properties (water absorption, development time, stability, tolerance index, breakdown time), fermentation (dough development and gaseous volume released), and baking properties were determined. Data were analyzed using ANOVA and protected pair wise t-tests to determine the effect of TG. Pearson correlation was conducted to test the correlation among the wheat dough properties with bread loaf volume prepared from wheat flour treated with 0-0.2% TG. Treatment of wheat flour with TG at levels of 0.1 to 0.4% positively increased development time and stability, however a dramatic decrease of these properties took place when levels of TG were increased to 0.8% and up. TG positively impacted the fermentation characters of dough by decreasing the amount of CO_2 released during dough fermentation, and increased the coefficient of gas retention.

However, the maximum height of dough decreased with increasing level of TG. Bread loaf volume was not correlated to dough mixing properties. Two of the fermentation properties, maximum dough height (r = 0.84) and dough height at the end of the test (r= 0.83), were highly correlated to bread loaf volume of samples treated with TG.

Key words: correlation, dough rheological and fermentation properties, bread loaf volume, transglutaminase

INTRODUCTION

Mixing is an important step in preparing dough to have the strength and structure needed during proofing and baking. During mixing, proteins are hydrated and glutenins align due to the imposition of shear and stretching force. Gluten networks are more developed and become stronger by formation crosslink bonds (Zaidel et al 2010). A bread improver with the right treatment dose is usually applied to adjust dough with desirable strength, elasticity and tolerance. Enzymes have been widely used as improvers in bread making because they are generally considered as safe (Joye et al 2009; Steffolani et al 2010) and properly improve handling properties of dough (Poutanen 1997).

Transglutaminase (TG) (protein glutamine γ-glutamyl-transferase, EC 2.3.2.13) is a cross link enzyme (DeJong and Koppelman 2002; Dube et al 2007) that introduces inter- and intra- molecular covalent cross-links by forming isopeptide bonds between glutamine and lysine residues of protein. A high molecular weight polymer is formed due to those cross-links (Tseng and Lai 2002; DeJong and Koppelman 2002; Ahn et al 2005; Autio et al 2005). Application of TG, according to Gerrard et al (1998) is promising since

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it reduces the required work input and improves dough water absorption, where both affect to lower processing costs of commercial baking. In the presence of TG, the formation of protein networks during bread making is improved (Autio et al 2005). TG makes the dough stronger, more consistent, increases mixing stability, maximum resistance (R max) (Roccia et al 2012), and lower extensibility (Tseng and Lai 2002; Basman et al 2003; Autio et al 2005).

The efficiency of TG on improving dough and bread is dependent on factors such as enzyme levels (Moore et al 2006) and time reaction (Gerrard et al 1998; Koh and Ng 2008). Increasing of TG levels and excessive protein polymerization will bring detrimental effects on the bread baking quality (Renzetti et al 2010). So far, the recommendation of TG doses added in bread formulation varies from low level (0.05%) for improving the crumb of wheat:soy (85:15) bread (Roccia et al 2012), to 2.5% TG for improving wheat bread fortified with 20% barley flour (Basman et al 2003). It seems that TG treatment causes different effects on different bread systems.

Baking performance of the wheat flour can be predicted with rheological and fermentation properties. Estimation of baking quality is important for breeding programs to select best lines among thousands new lines with limited sample. For milling/baking industries, it is useful to adjust their process rapidly and produce high consistent-quality product (Dowell et al 2008). One aspect of bread quality that is commonly measured is the bread loaf volume. The objective of this study was to evaluate dough rheological, fermentation and baking properties as influenced by TG, and to correlate dough rheological and fermentation properties of wheat flour treated with TG and bread loaf volume.

MATERIALS AND METHODS

Six blends of commercial wheat flour with protein content 11±0.5% were prepared (see appendix 1) from flours purchased from Shawnee Milling Co. (Shawnee, Ok). Other materials used were microbial TG Activa TI with activity 100U/g (Ajinomoto, Fort Lee, NJ), instant active dry yeast (Lesaffre Yeast Corporation, Milwaukee, WI) and sodium chloride (Fisher Scientific, Fair Lawn, NJ).

Rheological Measurements

Mixing properties of wheat flour treated with TG (0, 0.1, 0.2, 0.4, 0.8 and 1.6%) were measured using Farinograph-E (C.W. Brabender Instruments, Hackensack, NJ) following AACC approved method 54-21.02 (AACCI 2011).

Fermentation Characteristics

Fermentation properties of flours were tested using a F3 Rheofermentometer (Chopin, Villeneuve la Garenne, France). Dough was prepared in a Chopin Alveo-Consistograph kneader. Flour (250 g), dry yeast (3 g), and TG (0, 0.1, and 0.2% w/w) were added to the kneader. Prior to mixing, sodium chloride (5 g) was dissolved in deionized water. The amount of water required was dependent on the moisture content of flour as suggested in the reference Table in the Chopin protocol. Mixing was done for a total of 8 minutes. The dough (315 g) was placed in the rheofermentometer bowl and fermentation properties were evaluated during 3 h under constant temperature of 28.5ºC.

Baking Tests

Preliminary baking test was done with doughs which had TG at levels of 0, 0.1, 0.2, 0.4, 0.8 and 1.6% w/w of wheat flour. However, at the fermentation stage where dough experienced processing treatments such as punching, sheeting and molding, structure of dough with 0.4, 0.8 and 1.6% TG became lumpy and difficult to manage (Fig. 3). Therefore, baking tests were performed duplicate for doughs with reduced TG levels: 0, 0.1 and 0.2% w/w of wheat flour.

Baking tests were performed according to the optimized straight-dough bread baking method 10-10.03 (AACCI 2011). In brief, a 100 g wheat flour, water, 6 g sucrose, 1.5 g salt, 1.3 g dry yeast, 3 g shortening, 50 ppm ascorbic acid and 0.1 g malt flour were mixed using a 100-g mixer Swanson-Working pin type (National Mfg. Co. TMCO Inc, Lincoln, NE) (head speed 100-125 rpm, about 5 min) to obtain a homogenous developed-dough. Dough was then rounded and placed inside a proofer for a total 3 h 55 min. Dough was subjected to two punching and one molding step during proofing time before it was ready to bake in a reel type oven for 30 min. Bread was cooled for 2 h at room temperature before the loaf volume was measured using rapeseed displacement method 10-05.01 (AACCI 2011) in a loaf Volumeter.

Bread Firmness

Bread firmness was measured after resting the bread 24 h at room temperature in a sealed plastic bag. Bread firmness was determined using a Texture Analyzer TA-XT2 (TA.XTPlus, Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK), where 2 slices of bread (12.5 mm thick each) were compressed

25% (6.25 mm) with a TA4 probe with diameter of 3.8 cm (1.5 inch) at speed 1 mm/sec (Basman et al 2003).

Statistical Analysis

Study was conducted following arrangement in a Randomized Complete Block design. The six flours coded A, B, C, D, E and F were treated with TG (0, 0.1, 0.2, 0.4, 0.8 and 1.6% w/w), and the rheological and fermentation properties were analyzed with 3 replications. Analysis of Variance (ANOVA) procedure with 0.05 significance level, Means separation protected pair wise t-tests, and Pearson correlation were performed using SAS version 9.2 (SAS Institute, Cary, NC). Pearson correlation was conducted to test the correlation among the dough properties with bread loaf volume prepared from wheat flour treated with 0-0.2% TG.

RESULTS AND DISCUSSION

The Effect of TG on the Mixing Properties of Wheat Flour

An example of the effect of increasing levels of TG to the flour mixing properties is illustrated in Figure 1. Flour treated with 0% TG (without TG, Fig. 1) displayed good mixing properties in which stability was higher than 10 min and tolerance index was less than 30 BU. Statistical analyses indicated that TG (at levels 0 to 1.6%) significantly influenced the development time (P<0.0001), stability (P<0.0001), tolerance index (P<0.0001) and breakdown (P<0.0001), but it did not change water absorption (P=0.78) of wheat flours (Table 1).

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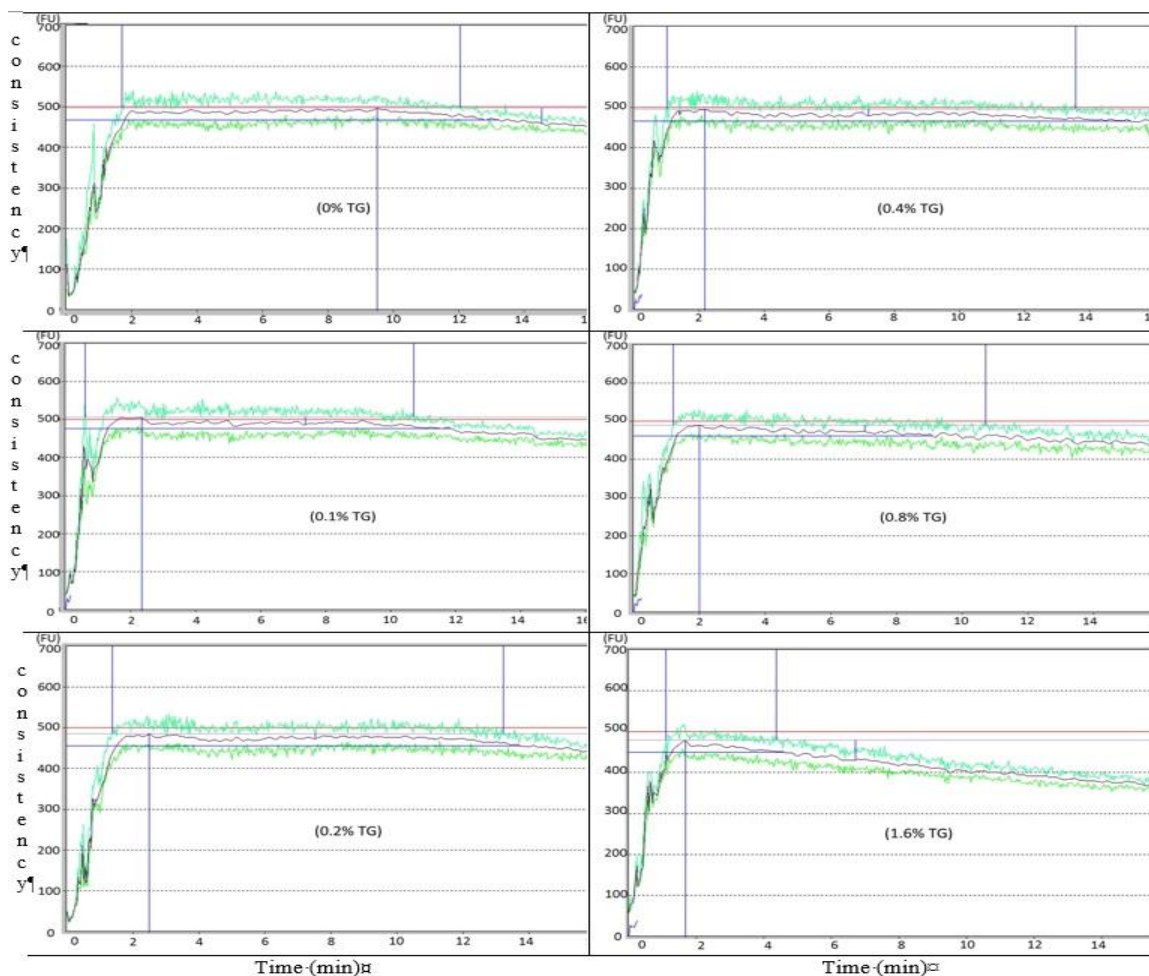
Figure 1. Example of farinograms of a commercial wheat flour as influenced by TG.

Table 1. Mixing properties of commercial wheat flours treated with TG[a].

TG (%)	Water absorption (14% mb[b]) (%)	Development time (min)	Stability (min)	Tolerance Index (BU)	Breakdown time (min)
0	54.9±0.11 a	4.9±1.02 bc	10.9±0.78 ab	24±2.55 b	11.7±1.06 a
0.1	54.9±0.13 a	7.0±1.25 a	11.1±0.81 ab	25±3.35 b	12.3±1.29 a
0.2	54.8±0.11 a	5.9±1.26 ab	11.9±1.08 ab	22±2.91 b	12.4±1.41 a
0.4	54.7±0.10 a	5.2±1.06 ab	12.5±1.22 a	22±3.05 b	11.3±1.45 a
0.8	54.8±0.11 a	2.9±0.63 cd	10.0±1.29 b	27±2.79 b	8.9±1.37 b
1.6	54.7±0.13 a	2.3±0.21 d	5.2±0.90 c	45±4.39 a	5.3±0.42 c
p-value	0.78	<0.0001	<0.0001	<0.0001	<0.0001

[a] Means ± standard error from triplicate analysis. Means within the same column with the same letter are not significantly different at p= 0.05. [b] mb = moisture basis

Water Absorption (14% moisture basis)

Water absorption ranged from 54.7 to 54.9% (Table 1), and was not significantly influenced (p=0.78) by levels of TG added. This observation may be related to the time of reaction. Dough hydration is a fast process (about 1-2 min) and it might be short for TG to react and modify gluten protein/dough structure. Gerrard et al (1998) and Koh and Ng (2008) suggested that the effect of TG in wetted flour is time dependent; more covalent bonds may result with longer time for TG effect.

These observations do not agree with literature reports. Wheat flour treated with increasing level of TG showed either a decrease (Basman et al 2002) or an increase (Gerrard et al 1998) of water absorption. Gerrard et al (1998) postulated that increasing water absorption is caused by the ability of TG to modify protein structure through crosslink and to cause deamination of glutamine residue. When amine in the medium is not available, water can act as acyl acceptors. Deamination will cause ionized hydrophilic protein side chains (Yokoyama et al 2004) and increased solubility of gliadin (Chobert et al 1996). However, this explanation seems weak because Ohtsuka et al (2001) reported that microbial TG only has 1/7th of the deamination activity compared to other TG types. This means crosslink is the predominant reaction catalyzed by TG. Presence of more crosslinks in a dough system can restrict the amount of water trapped inside ultrastructure of the protein pore (Kontogiorgos 2011).

Thus, it is possible that the diverse effects of TG on the flour water absorption could be related to a number of factors. Those factors, according to Berton et al (2002), are quantity and quality of gluten, flour extraction rate, damaged starch and fiber (mostly pentosans) content.

Development Time

At dose of 0.1 to 0.4%, TG increased the length of polymer and strengthens gluten protein by the formation of covalent bonds. Increase of the presence of covalent bonds in a dough system restricts the movement of water to hydrate flour components needed to form a homogenous gluten film; as a result development time is increased. However, when the level of TG was increased to 0.8% and up, it forms too many covalent bonds. Therefore, in a short time it restricts the mobility of flour components, and it may form the consistency of 'dough already developed' even though the flour components may not be evenly hydrated and mixed. Bauer et al (2003) suggested that excessive crosslink as a result of higher TG concentration, leads to loss of elasticity and structural damage of gluten network. Larré et al (1998) postulated that at certain point when covalent bonds are excessive in the network, it hinders the enzyme to diffuse and find any more substrates.

Similar observation was reported by Basman et al (2002). The dough development time of wheat flour increased when treated with TG at levels up to 0.25% and then it decreased at higher levels of TG. Water hydrates protein and other flour components during mixing, and then mechanical energy induces changes of protein structure through formation of covalent and non-covalent bonds, and a continuous viscoelastic gluten network is formed (Kaddour and Cuq 2011). When a homogenous gluten film is evenly distributed around starch granule, the dough is developed.

Stability

Stability of flours did not change at TG levels of 0 to 0.4%, but it sharply

decreased at 1.6% TG. It was expected that at low levels, TG was able to connect protein

gluten by glutamyl-lysine bridges (DeJong and Koppelman 2002) and these cross-links

contribute in increasing dough stability. Macritchie (1975) stated that any kind of agents

with ability to form crosslinks between protein chains through strong covalent bonds will

increase the mixing stability of dough. Formation of a higher molecular weight polymer

may contribute to improvement of viscoelastic gluten (Larré et al 2000).

At levels higher than 0.4%, TG decreased stability of flour. This observation may

be due to the ability of TG to create secondary disulfide cross-link beside the primary

glutamyl-lysine bridge. Macritchie (1975) explained that formation of disulfide

crosslinking greatly accelerated breakdown of flour. Another possible explanation is that

an excessive strength of dough due to excessive crosslinks will increase work input and

bond breakage (Shewry et al 2003).

Tolerance Index

Tolerance index of flour ranged from 22 to 45, and it was significantly influenced

($p<0.05$) by TG treatment (Table 1). Tolerance index of control was not different to that

treated with TG up to level of 0.8%. An increasing number of crosslinks created by TG

after 0.8% caused dough to become too stiff and behaved more like solid rather than

viscoelastic dough probably due to increased crosslinks by TG (Bauer et al 2003).

Shewry et al (2003) suggested that mechanical extension can extend mobile phase of

gluten protein (β-turn) but further extension strains and pulls the train regions (regions

where two or more molecules interact by the formation of intermolecular β-sheets) apart and causes breakage of cross-links with further mixing. Therefore, more mixing applied to stiffer dough will cause increasing of cross-links breakage and weaken the dough.

Breakdown Time

Breakdown time obtained from this study ranged from 5.3-12.4 min and it was influenced ($p<0.05$) by TG treatment. The results indicate that the dough can preserve its structure well if TG is added at level up to 0.4%, but at the higher level, TG causes dough breakdown faster. There are two mechanisms of dough breakdown suggested by Meredith and Bushuk (1962); the rupturing of covalent and other bonds due to pure physical force and breaking of structural cross-linkages by chemicals actions such as reduction, oxidation and hydrolysis. TG at high level causes the formation of brittle protein network and leads to loss of its structure (Kuraishi et al 2001). Similar effects of TG could be achieved either from low dose in longer time or high dose in short time (Gerrard et al 1998).

The Effect of TG on the Fermentation Characteristics of Wheat Flour

Fermentation test produces two curves, dough development and gaseous release. Figure 2 and 3 illustrate an example of the dough development and gaseous release curves of commercial wheat flour treated with 6 different levels of TG. Wheat flour with 0% TG showed an average dough development and had only slightly reduced height at the end of fermentation. According to Tripette and Renaud (2004), flour with average dough development and very good tolerance (has no T2, where T2 is the time at which

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Hm decreases 12%) can be defined as good quality flour. Therefore, the wheat flour used in this study was in the category of good quality for bread making.
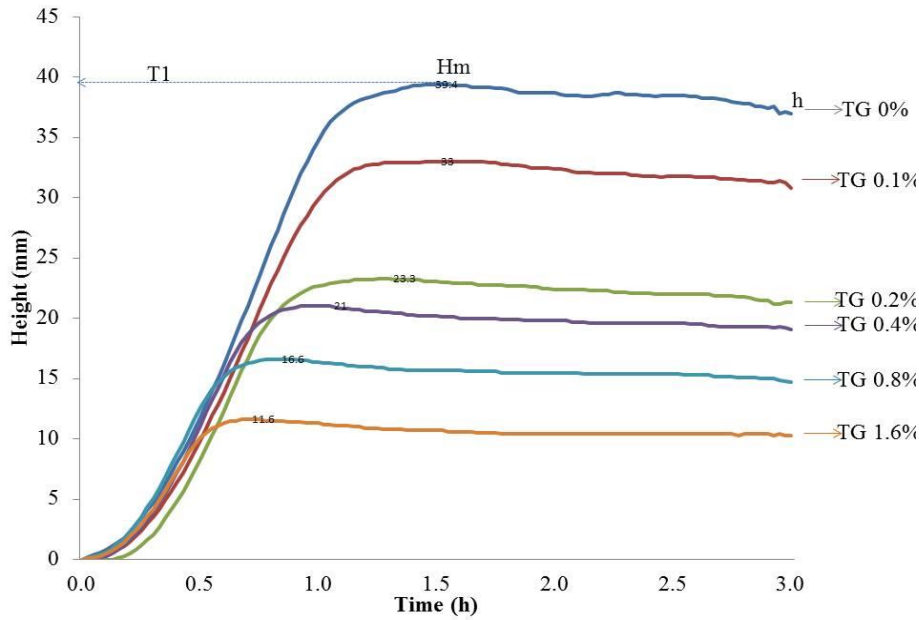
Figure 2. Example of dough development curves of commercial wheat flours treated with different levels of TG (0-1.6%).
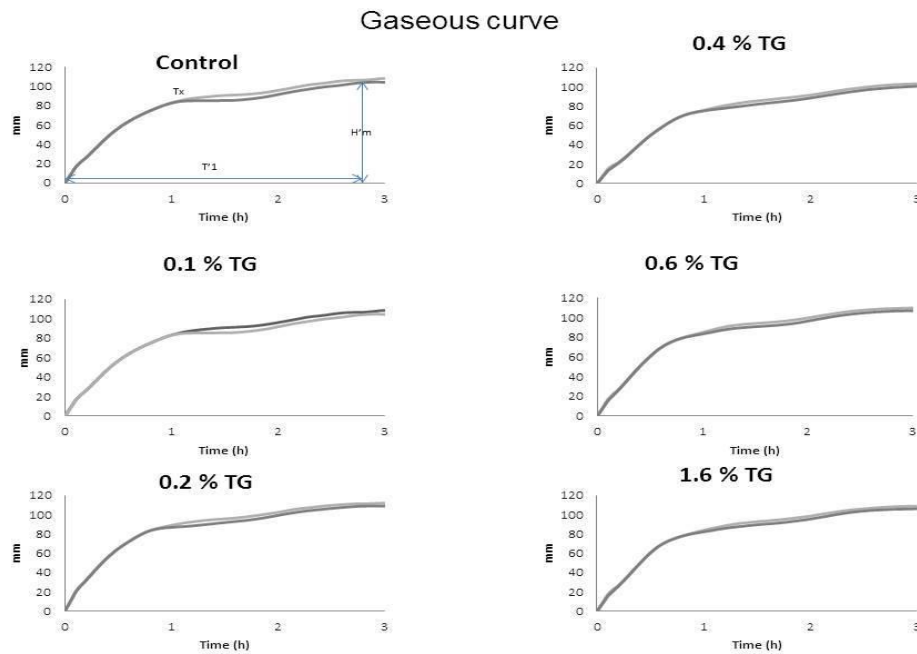
Figure 3. Example of gaseous release curves of commercial wheat flours treated with different levels of TG (0-1.6%).

Table 2. Fermentation parameters of commercial wheat flours treated by different levels of TG[a].

TG (%)	Dough development parameters				Gaseous release parameters		
	Hm (mm)	h (mm)	(Hm-h)/Hm (%)	T1 (min)	Tx (min)	CO_2 lost (ml)	Coef. Retention (%)
0	54.8±2.18 a	54.2±2.26 a	1.3±0.35 b	164.42±6.29 a	64.58±1.21 a	73±1.44 a	96.8±0.06 d
0.1	42.4±1.17 b	41.6±1.29 b	1.9±0.56 ab	149.86±8.96 a	62.67±1.62 a	68±1.32 b	97.0±0.08 c
0.2	32.4±0.73 c	31.7±0.87 c	2.3±0.82 ab	150.32±8.86 a	56.92±1.05 b	68±1.26 b	97.1±0.06 c
0.4	23.0±0.58 d	22.5±0.57 d	2.2±0.69 ab	131.50±11.42 b	50.79±2.52 c	63±1.42 c	97.2±0.07 b
0.8	17.6±0.34 e	17.0±0.34 e	3.2±1.05 a	113.08±12.88 c	44.25±0.75 c	62±1.25 c	97.4±0.06 a
1.6	13.5±0.31 f	13.1±0.34 f	3.1±0.72 a	93.66±13.16 d	45.00±2.60 c	61±1.61 c	97.4±0.09 ab
p=	<0.0001	<0.0001	0.03	<0.0001	<0.0001	<0.0001	<0.0001

[a] Means ± standard error from triplicate analysis. Means within the same column with the same letter are not significantly different at p= 0.05.

Dough Maximum Height (Hm)

The Hm of wheat dough treated with TG ranged from 13.5 to 54.8 mm, and declined with increasing level of TG (Table 2). Hm represents the expansion of dough piece during fermentation/proofing, and the decline of Hm after treatment with TG could be explained by the change of dough rheological properties. Formation of covalent glutamyl-lysine and secondary -S-S- bonds increases protein strength that it may hinder the expansion of gluten-starch matrix required to increase loaf volume (Sroan et al 2009). Strengthening the dough makes expansion more difficult (Gerrard et al 2000).

Second explanation of decreasing Hm with increase of TG is related to entrainment of initial bubbles of dough during mixing. It has been reported that creation of aerated/porous structure of dough is initiated during mixing to provide the nucleation sites for proofing (Chiotellis and Campbell 2003). TG influences the structure of dough due to formation of protein-protein and protein-starch interactions that occur during mixing process. The glutamyl-lysine bond, as primary product of TG, is a covalent bond non-disulfide. This is a strong bond that is not going to be weakened through interchange like disulfide to sulfhydryl in further mixing. Macritchie (1975) suggested that covalent

bond non disulfide is stronger than disulfide bonds, which peptide covalent bonds have energies close to 100 kcal/mole compared to 50 kcal/mole of disulfide bond. The creation of covalent bonds apparently became higher with increasing levels of TG. Presence of excessive covalent bonds will increase gluten strength and it restricts entrapping air bubbles inside the dough.

Expansion of the dough also relates to the effect of TG on the mobility of CO_2 into bubbles during proofing. Creation of protein-starch interaction due to TG activity can produce strong/stiff dough. Dough with 0.5% TG, as observed by Huang et al (2008) using Scanning Electron Microscopy, showed more starch granules embedded in or attached to the gluten network than those without TG. Hence when levels of TG are increased, it will strengthen protein-starch interaction and produce denser dough. It may also cause fewer initial air bubbles inside the dough that leads to producing a smaller Hm because according to Ktenioudaki et al (2011) during fermentation only the size of cell gas changes, as the amount of gas cells remains constant.

The height volume of dough during fermentation is determined by the growth of air bubbles. Factors, such as surface tension and rheology of the dough (Mills et al 2003), activity of yeast producing CO_2 gas and rate of CO_2 mass transfer into bubbles (Campbell et al 2001), gas cell's stability, ability of dough system to expand and retain the gas (Ktenioudaki et al 2011), define the growth of air bubbles and expansion of dough during fermentation. Our results indicated that TG did not affect the production of CO_2 gas by the yeast since the height maximum of gaseous curve, total gas volume and the amount of CO_2 retained were not significantly different (data not shown). However, TG may impair

rate of mass transfer of CO_2 into bubbles and decrease the ability dough system to expand.

Mass transfer of CO_2 into gas bubbles is related to viscoelasticity of gluten protein. Viscoelasticity is the key component of the dough to effectively hold CO_2 gas (Kim et al 2008). He and Hoseney (1992) suggested that the viscous characteristics is required for allowing gas expand to balance the pressure, whereas elastic properties provide strength to prevent overexpansion/collapse of dough. TG can improve viscoelastic properties of gluten protein through covalent bonds, but it is dose dependent. Basman et al (2003) stated that in high doses, TG can create excessive cross-link and over-strong dough. TG has also been reported by Huang et al (2008) to be able to increase interaction between starch and protein. It is expected that at higher levels of TG the protein-starch interaction is stronger. Both covalent bonds and protein starch interaction may decrease flow properties (viscous characters) of dough and inhibit CO_2 to diffuse into cells gas.

Height of Dough at the End of Test (h)

Like Hm, h shared similar common trend indicating that increasing level of TG decreased h values. The explanation for decreasing h value of wheat dough treated with TG is the same as the explanation for parameter maximum height (Hm). The height of all the dough produced from flours treated with TG only dropped slightly (1-3%) by the end of the test (Fig. 2). These results indicate that TG is able to maintain tolerance of dough during fermentation.

Lowering of Development Percentage ((Hm-h)/Hm)

Lowering of development percentage of wheat flours treated with TG ranges from 1.3 to 3.2 %. These small percentages of (Hm-h)/Hm indicate that the flour has good fermentation tolerance because the dough has a well maintained height during fermentation. Statistical analysis indicated that the values of (Hm-h)/Hm were significantly influenced by levels of TG (P=0.03). Increasing levels of TG increased the percentage of lowering development. However, it was observed that addition of TG, even in the highest dose (1.6%), did not interrupt the ability of the dough to maintain its height since the highest value of lowering development percentage of this study was only 3.2%.

Time of Maximum Rise (T1)

T1 was significantly influenced by TG (P<0.0001). Dough from wheat flour treated with higher levels of TG ($\geq$ 0.4%) showed earlier dough development compared to dough with low levels of TG (0-0.2%). Achievement of dough development usually occurs coincident with accelerating CO_2 production (Haros et al 2002). In our study, the acceleration of CO_2 production does not appear to be the reason of early dough development since data of gaseous release, such as H'm, T'1 and total gas volume, were not significantly influenced by TG (data not displayed). Therefore, the most likely explanation is the strengthening of protein/dough structure. Covalent bonds, both glutamyl-lysine and disulfide, produced by TG cause strong protein fibers. Autio et al (2005) suggested that strong protein fibers cannot extend as much as protein network in the control dough. TG, as reported by Autio et al (2005), is able to promote formation of thicker protein fibers and improve interaction of the fibers leading to formation of more

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developed gluten network. Dough with TG is less extensible compared to control, and it retards the growth of air bubbles during fermentation because the rich protein areas of dough are located around air bubbles.

Time of Dough CO_2 Release (Tx)

Appearance of CO_2 released observed from this study started from 44.25 to 64.58 min of fermentation. Tx of fermented dough was significantly decreased by TG treatment (P<0.001). The different effect of TG to Tx began at concentration of 0.2% TG when it caused the dough to release gas sooner than the dough without TG. However, at levels of 0.4% and higher TG was not able to bring significant effect to observed Tx. The earlier releasing of gas from the dough treated with TG than the one without TG may be explained using the breakdown of time from the Farinograph data. Time to breakdown of dough also decreased after treated with TG in level 0.4% or higher. It is proposed that TG causes structure of protein network supporting dough become brittle at high dose TG. The formation of brittle protein network leads to loss of its structure (Kuraishi et al 2001). Uneven and rough structure of dough becomes permissive to gas escape.

CO_2 Lost (ml)

CO_2 release from the wheat dough treated with TG in this study ranged from 61 to 73 ml or only about 3% from total volume of gas. Statistical analysis indicated that the amount of CO_2 lost from wheat dough samples was significantly influenced by TG treatment (P<0.0001). The amount of CO_2 lost decreased with increasing levels of TG applied to the wheat flours.

The ability of TG to reduce the amount of CO_2 escape from the dough system may be related to the theory of gas cells and dough expansion postulated by Gan et al (1995). This theory suggests that the discrete gas bubbles inside dough lined with liquid film and embedded in continuous phase of protein-starch matrix soon after mixing process. During fermentation, the protein-starch matrix develops as a thin layer and ruptures as more gas expands. The developing of discontinuities of the matrix depends largely on the extensibility of gluten protein (Gan et al 1995). Maximum inflated ability of gas cells can only be achieved if gluten-starch matrix around them stretches to maximum extensibility without breaking (Sroan et al 2009). If this postulate is accepted, then one may claim that TG indirectly strengthens starch-protein matrix by strengthening the structure of protein due to its ability to create more covalent bonds. Improving of the protein-starch strength in one side may reduce the growing of gas as previously explained, but it can also delay the rupture of the matrix and reduce the CO_2 lost from dough system.

Coefficient of Retention (%)

Coefficient retention of samples ranged from 96.8 to 97.4% and was significantly influenced by TG treatment (P<0.001). The trend indicated that TG increased the coefficient of retention of the wheat dough. Regardless of the effect of TG, all samples seemed to have the criteria as a good flour since the coefficient of retention was closed to 100.

The Effect of TG on the Baking and Texture Properties

Baking Preparation

Preparation of dough was started by mixing all ingredients for 3 to 5 min until the dough homogeneously mixed that was indicated by formation of 'windowpane', a thin-smooth-transparent window when the edge of dough was gently pulled with finger. The dough was then proofed, punched, and molded before it was baked in the oven. The appearance of the dough with 0-1.6% levels of TG in several stages of preparations is displayed in Figure 4.
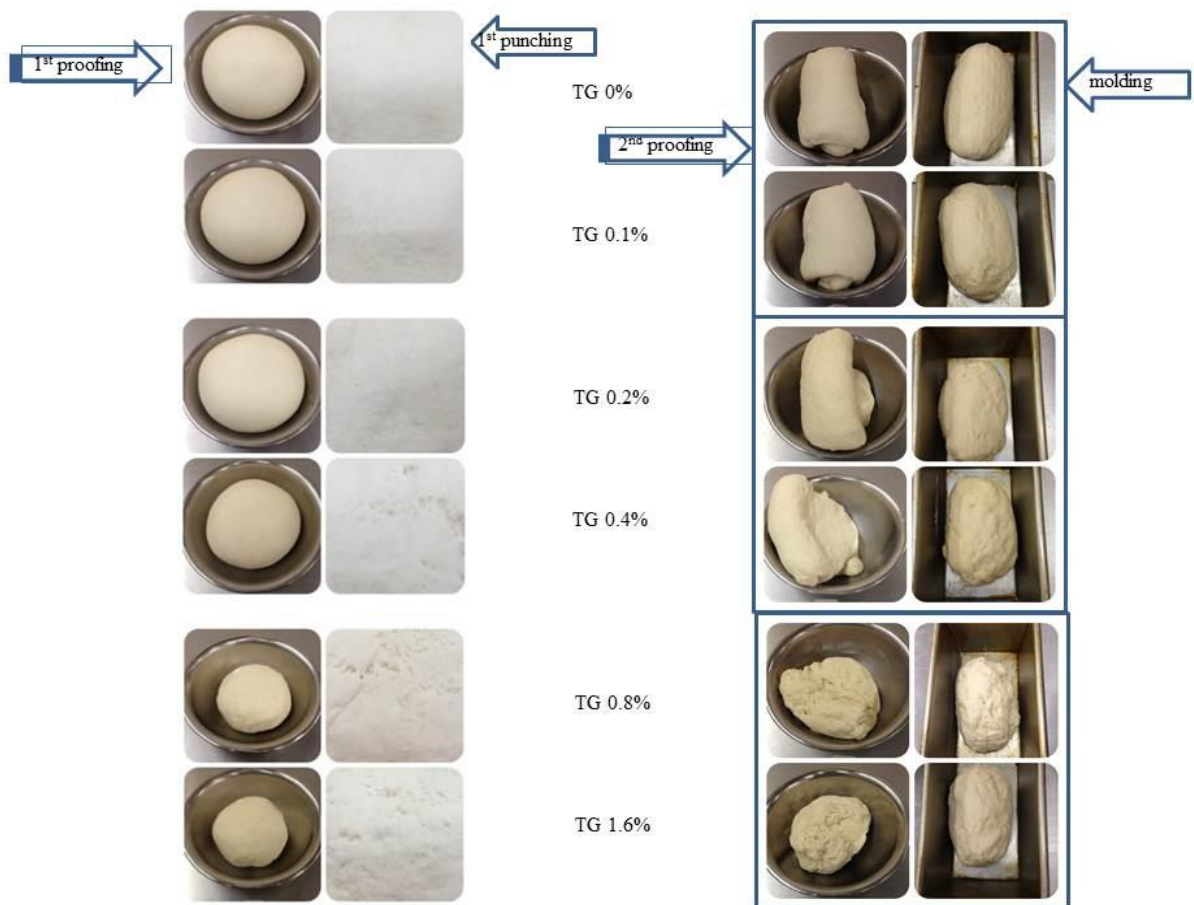
Figure 4. Example of physical appearance of dough with different levels of TG at several stages preparation.

Doughs with level of TG 0-0.2% were well developed (Figure 4). However, at level TG 0.4% and up, the dough volumes were smaller and structure of sheeted dough was coarser compared to the control. At 1st proofing, all dough surfaces still looked smooth. According to Mills et al (2003) expansion of dough at early stages of proofing is not significantly influenced by dough rheology, but the dough rheology becomes critical for dough expansion at later stages. After 1st punching, the dough with TG $\geq$0.4% formed a 'coarse' surface due to over-crosslink of gluten protein and the broken of hydrogen bonds and hydrophobic interactions of gluten protein (Steffolani et al 2008). Bauer et al (2003) suggested that increasing number of cross-link causes gluten to behave like a solid rather than viscoelastic material.

At the end of proofing time and at molding process, the effect of TG to the dough structure became more evident. The dough with TG 0.4% or more could not form a spherical shape. Bauer et al (2003) mentioned the dough structure as very dry and plastic; hence it could not stick together after molding (Basman et al 2002). Since the flours used in this study had relatively high protein content (11.0$\pm$0.5%); at level 0.4% or more, TG could create excessive cross-link and over-strong dough and retard air bubbles growth as suggested by Basman et al (2002). Excessive protein polymerization will bring detrimental effects to the bread baking quality (Renzetti et al 2010). Increase of strength conferring proteins may prevent the expansion of gluten-starch matrix because slippage of gluten protein is reduced due to increasing number of entanglements per chain (Sroan et al 2009). Expansion of gluten-starch matrix is required for responding to the gas pressure and preventing the escape of gas from the bread system. Considering these facts, baking was done by reducing TG to the levels 0, 0.1 and 0.2%.

Bread Properties

Bread loaf volume varied within a range of 453 to 673 ml where the volume was smaller with increasing of TG levels. It may be related to the protein nature of hard red winter wheat flour samples that contains Dx5 and Dy10 glutenin subunits, the good protein substrates for TG to form isopeptide covalent bonds. Treatment of the flour with TG, even at a small amount, may already significantly modify and cause too strong gluten protein. Too strong gluten protein retards gas production during fermentation, therefore it produces smaller dough volume compared to the dough control. Similar results of decreasing bread volume with increasing TG was reported by (Caballero et al 2007; Koh and Ng 2008). In contrast TG improved pastry and croissant volume (Gerrard et al 2000), wheat bread with high ferulic acid content (Koh and Ng 2008), and frozen dough (Huang et al 2008). TG strengthens the dough sheet that protects fats from leakage in croissant and pastry dough layers (Gerrard et al 2000).

Table 3. Baking properties of wheat flour treated with 3 different levels of TG[a].

TG (%)	Loaf volume (ml)	Proof height (mm)	Loaf height (mm)	Oven spring	Specific volume (cm^3/g)
0	673±10.58 a	71.64±0.61 a	91.01±1.00 a	19.36±1.11 a	5.15±0.08 a
0.1	577±15.54 b	72.01±0.91 a	83.05±1.50 b	11.04±1.36 b	4.32±0.13 b
0.2	453±10.07 c	69.13±0.78 b	72.83±1.59 c	3.38±1.26 c	3.37±0.07 c
p-value	<0.0001	0.005	<0.0001	<0.0001	<0.0001

[a] Means ± standard error from duplicate analysis. Means within the same column with the same letter are not significantly different at p=0.05.

Average height of the proofed dough with TG 0.1% was as high as the dough without TG, however data of bread loaf height (Table 6) indicated that dough with TG

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could not expand as high as dough without TG during baking. In the early stage of the baking process, yeast still produces gas until the stage where dough is continuously transformed to bread starting at 65°C (He and Hoseney 1992) when heat inactivates the yeast, denatures protein and gelatinizes starch. Therefore, the volume of bread is higher than the volume of proofed dough. However, the increasing of dough volume is dependent on gluten and dough structure where both might still be modified by TG during the early stage of baking. TG is active until a temperature of 66°C (Ajinomoto 2012), hence it still has the ability to create glutamyle-lysine bonds at the early stage of baking. The enzyme activity may be higher when temperature sifted from proofing room (37°C) to the oven before dough temperature reaches 66°C. In addition, when TG is inactive at higher temperature, according to Gerrard (2002), glutamyl-lysine covalent bonds could still be naturally formed during extreme heating process. Therefore, compared to the dough with lower TG level, dough with higher level of TG has higher prospect to have more isopeptide bonds. According to Basman et al (2003) excessive cross-link causes too strong gluten protein that could retard air bubbles growth. Gan et al (1995) postulated that polymerization of glutenin due to inter-molecular disulfide bonds may encourage rupturing of starch-protein matrix during heating by sharply increasing tensile stress leading to loss gas retention. If this postulate is accepted then one may assume that TG creates both glutamyl-lysine and disulfide bonds, and it may also contribute to higher tensile stress in gluten-starch matrix, hence it creates more rupture of the matrix and loss of gas retention.

Oven spring and specific volume that were indirectly determined by loaf volume decreased with increasing levels of TG (Table 3). At levels of 0.4% or higher, the level of

TG was too high for the wheat flour sample. Instead of bringing positive effects, TG caused detrimental effects to the baking properties. Dough became too strong and less extensible very quickly after mixing (Autio et al 2005). TG encouraged the formation of thicker gluten protein fiber, so that the gas produced by the yeast could not extend the dough (Bauer et al 2003). At low level TG helped the gluten network to be more developed, but at high level TG caused uneven distribution of protein in the bread system which most of the protein-rich area are surrounded air bubbles (Autio et al 2005). Autio et al (2005) suggested that without increasing the amount of water added in the formulation, TG will reduced the volume of bread.

Bread Crumb Structure

Bread crumb structure was positively affected by TG treatment at level of 0.1% where the coarse and non-homogenous distributed of crumb size of bread control became finer and homogenous with addition 0.1% TG (Fig. 5). Caballero et al (2007) described the crumb structure with TG as a brighter color, smaller gas cells, greater cell density and uniformity, smaller void fraction and cell wall thickness as compared to bread crumb control. However, at level more than 0.1%, TG produced smaller bread loaf volume. Similar finding was reported by Basman et al (2002), where bread from the weak wheat flour showed better crumb structure with TG at level 0.1-0.5%, while the stronger wheat flour sample produced better bread crumb structure with TG level up to 0.25%. Basman et al (2002) also found that at levels higher than 0.25%, TG decreased loaf volume and deteriorated crust structure and color. They reported that the color of the crust was lighter with increasing levels of TG due to a reduction of Maillard reaction since lysine was no longer available.

Figure 5. Example of bread crumb of wheat flour (b and c) treated with 0, 0.1 and 0.2% TG.

Bread Firmness

Bread crumb firmness was generally monitored over a seven-day storage period which represents a common bread shelf life since the firmness is function of time, temperature and formulation. Bread crumb firmness at day 1 ranged from 246.9 to 688.0 (Table 4). Statistical analysis indicated that bread crumb firmness was significantly influenced by TG (p<.0001) and there was a distinct change in the crumb texture for all breads over the storage period examined (Table 4). The overall result indicated that the modification structure of gluten protein, the continuous phase of bread, by TG led to the

increase of the firmness of the bread. This finding agrees with Caballero et al (2007) who

reported that the hardness and resilience of bread crumb increased with presence of TG.

Contrary with this finding Renzetti et al (2008) reported that TG brought positive effect

to the gluten free bread by increasing specific volume and decreasing crumb hardness and

chewiness. With the condition to not decrease the other quality, Gerrard et al (1998)

suggested that the increases of the crumb strength caused by TG beneficiates for fresh

bread which the bread crumb becomes easier to slice and withstand butter.

Table 4. Bread crumb firmness with different levels of TG evaluated on days 1, 4 and 7[a].

TG	Firmness (N)		
	Day 1	Day 4	Day 7
0	246.9±15.06 c	875.4±205.51 b	897.6±64.57 c
0.1	424.4±33.15 b	931.1± 56.59 b	1254.0±85.48 b
0.2	688.0±54.07 a	1560.3± 89.77 a	1988.9±93.27 a
p-value	<0.0001	<0.0001	<0.0001

[a] Means ± standard error from triplicate analysis. Means within the same column with the same letter are not significantly different at p=0.05.

Correlation between Dough Properties and Bread Loaf Volume

There were 10 out of 21 variables that significantly correlated (p <0.05) to the

bread loaf volume. The six variables that closely related and had high correlation

coefficient to the bread loaf volume were part of the fermentation (Hm (r = 0.84) and h (r

= 0.83)) and baking properties (bread loaf height (r = 0.86), oven spring (r = 0.82), bread

specific volume (r = 0.99) and bread firmness (r = -0.78)). Whereas, four variables that

were also significantly correlated to bread loaf volume but had a small correlation

coefficient (≤0.5) are Tx (r = 0.0009), volume of CO_2 lost (r = 0.30), coefficient of

retention (r = -0.38), and proof height (r = 0.25).

None of farinogram-mixing properties significantly correlated to bread volume. Stojceska and Butler (2012) also found no correlation among parameters of 24 farinograms to the bread loaf volume. Oliver and Allen (1992) explained why the Farinograph and Extensograph of flour-water dough did not well relate to baking properties; it is caused by differences of mixer speed and dough formulation used between Farinograph and the commercial baker. Commonly mixer of Farinograph is operated in about 60 rpm, while a commercial baker uses a higher mixer speed (for example, 300 rpm for CBP bread, Tronsmo et al (2003)). Farinograph uses a flour-water system, while commercial bread formulation contains additional ingredients such as salt, sugar, fat, yeast etc. Therefore, Oliver and Allen (1992) suggested that in order to increase correlation, Farinograph should be run using bread formulation and work input level of mixer as applied in a commercial baker.

Table 5 also indicated that among other mixing properties, stability had the highest correlation with bread loaf volume even though with a small coefficient correlation (r= - 0.25). Development time (Butt et al 2001) and stability (Osella et al 2008) had been reported to have a close association to the bread quality (specific volume). Stojceska et al (2007) also found that stepwise regression correlated dough stability with loaf volume, but then they suggested that r^2 value of the model was too low to be practically able predicting loaf volume for individual flour sample.

Correlation between gluten properties and bread loaf volume were done as well (data not shown). Compared to Farinograph dough mixing properties, in exception of stability, gluten parameters had better correlation to bread loaf volume. According to Tronsmo et al (2003) small deformation test of freshly extracted wet gluten had a greater

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potential than dough to predict bread making performance because gluten was more representative of protein-protein interactions while dough had additional contribution from starch-starch and starch-protein interaction.

Table 5. Correlation between dough properties and bread loaf volume.

Variable	Pearson correlation coefficient	Probability
Mixing properties		
Water absorption	0.18	0.21
Development time	-0.16	0.26
Stability	-0.25	0.08
Tolerance index	0.09	0.53
Breakdown time	-0.13	0.36
Fermentation properties		
Dough development curve parameters		
Hm	0.84	<0.0001
h	0.83	<0.0001
(Hm-h)/Hm	-0.27	0.055
T1	0.25	0.08
Gaseous release curve parameters		
H'm	0.02	0.87
T'	0.21	0.14
Tx	0.45	0.0009
Total volume	-0.16	0.27
Volume of CO_2 lost	0.30	0.03
Volume of retention	-0.17	0.23
Coefficient of retention	-0.38	0.006
Bread properties		
Proof height	0.25	0.04
Loaf height	0.86	<0.0001
Oven spring	0.82	<0.0001
Specific volume	0.99	<0.0001
Bread firmness	-0.78	<0.0001

Three different gluten tests were performed, including wet gluten, gluten index and the newest test gluten recovery (%). It was reported by Kieffer et al (1998) that

several gluten properties, such as gluten index and wet gluten, were valuable for predicting baking results. However, in our study that involved of TG treatment, both wet gluten and gluten index were not correlated to bread loaf volume. According to Dobraszczyk and Salmanowicz (2008) the gluten quantity and gluten quality are independent each other, therefore wet gluten (gluten quantity) alone is inadequate to predict bread volume.

The only gluten property that significantly related to bread loaf volume was gluten recovery, though the coefficient correlation was not as high as fermentation and baking properties ($r = -0.31$). This negative correlation coefficient means that increasing of gluten recovery could cause reducing loaf volume. Increasing level of TG causes increasing gluten recovery indicating that gluten becomes too strong for gas to support expanding bread. Chapman et al (2012) suggested that gluten recovery as a single measurement is not a good predictor for bread volume, but it is useful for differentiating gluten strength among wheat varieties.

A fermentation test is a better predictor for bread loaf volume compared to Farinograph mixing properties and gluten quality, since there are five parameters that significantly correlate to bread volume, Hm, h, Tx, volume of CO_2 lost and coefficient of retention. Both dough maximum height and height of dough at the end of fermentation positively relate to bread volume ($r= 0.84$ and 0.83). This is reasonable since the loaf volume was mostly developed during fermentation when yeast produced CO_2 and air bubbles grew fast. Further developing of bread loaf volume was determined during baking of the dough.

Even though the correlation was not as strong as dough height (Hm and h), bread volume also relates (r=0.45) to Tx, the time when the dough begin to release CO_2. When the dough had ability to hold CO_2 longer in its system, the bread loaf volume was higher. Volume of CO_2 gas lost weakly related to bread loaf volume (r=0.30). It is interesting that CO_2 lost positively relates to bread loaf volume. This positive correlation does not necessarily mean that the more amount of CO_2 lost, the higher bread loaf volume to be. This positive correlation occurs because TG decreases both CO_2 lost (Table 2) and bread loaf volume (Table 3). Coefficient of retention is volume of gas retention divided by total gas release, and according to Tripette and Renaud (2004), the good quality flour has coefficient retention close to 100%. Coefficient of gas retention negatively correlated to bread loaf volume (r=-0.38), again it does not mean that increasing flour quality will cause decreasing bread loaf volume. This correlation takes place because the effect of TG, where TG increases coefficient retention of flour by increasing number of covalent bonds of gluten protein. However, when the amount of covalent bonds due to increasing levels of TG is too much, it modifies the gluten and dough characters become too strong. Increasing gluten strength may prevent the expansion of gluten-starch matrix (Sroan et al 2009), leading to decrease of bread loaf volume.

Among other parameters, Hm showed the highest correlation to the loaf volume (r= 0.84). It is reasonable since the loaf volume was mostly developed during fermentation when CO_2 was produced and air bubbles grew fast. Similar finding was reported by Huang et al (2008) who observed fermentation characteristics and bread specific volume of sweet dough. The main positive correlation with bread loaf volume

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was dough expansion (dough height of Rheofermentometer) and strain hardening (Ktenioudaki et al 2011).

CONCLUSION

TG significantly influenced ($p<0.05$) most dough rheological and fermentation, and baking properties. TG at levels of 0.1 to 0.4% positively increased development time and stability of wheat dough, however a dramatic decrease of these properties took place at TG levels of 0.8% and up. TG also positively impacted the fermentation characters of dough by decreasing the amount of CO_2 release during dough fermentation, and increasing the coefficient of gas retention. However, maximum height of dough decreased with increasing level of TG.

The effect of TG was easily differentiated by baking tests. Wheat flour treated with TG at the level of 0.1 % produced higher fermented dough height and a more homogenous bread crumb structure than control. However, increasing level of TG more than 0.1% TG caused excessive protein covalent interaction and decreased Hm (height maximum of dough) and bread specific volume, and increased crumb texture.

Bread loaf volume did not correlate to rheological properties, but it correlated to the dough fermentation properties. Maximum dough height (Hm, $r = 0.84$) and dough height at the end of the test (h, $r = 0.83$) were two fermentation parameters that highly correlated to bread loaf volume of samples treated with TG. This study suggests that fermentation test is a good test to predict baking properties of wheat flour.

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CHAPTER VI

WHEAT-SOY TEMPE FLOUR: PREPARATION AND POTENTIAL AS BREAD INGREDIENT

ABSTRACT

Indonesia tempe, a fermented food using *Rhizopus* spp. inoculum has functional properties attributed to bioactive peptides, isoflavones, vitamins and increased availability of minerals. The functional compounds in tempe can broaden utilization of wheat and soy as new food ingredient and increase the consumption of these crops. The study was aimed to prepare soy-wheat tempe flour, analyze potential of soy proteins as tranglutaminase (TG) substrates, and evaluate the effect of TG and tempe flour on the rheological and fermentation properties of composite wheat-tempe dough. Tempe was prepared from wheat:soy at 4:0, 3:1, 1:1, 1:3 and 0:4 ratios, w/w. The potential of soy proteins as TG substrate was projected using the content of lysine and glutamine residues of glycinin and conglycinin that were obtained from the National Center for Biotechnology Information (NCBI) website. Dough rheological and bread baking properties were evaluated with a range of tempe flour and TG (0-40% and 0-0.1%, respectively).

With consideration to desirable fungi growth, desirable sensory characteristics, and the calculated lysine content, a wheat: soy ratio of 1:1 was selected to use for preparing tempe flour. In view of the number of lysine and glutamine residues, soy glycinin and conglycinin should be a good substrate for TG because each has 19-23 and 21-25 lysine residues and 45-76 and 33-39 glutamine residues, respectively.

Most of the mixing and fermentation properties of composite (wheat-tempe) flour were significantly influenced by tempe flour but not by TG. Increasing levels of tempe from 0 to 40% significantly increased dough development time (5.5 times) and stability (96%). Tempe flour positively reduced CO_2 release during dough fermentation. Tempe flour up to 20% did not change gaseous release of fermentation properties, but at higher levels than 20%, tempe flour had a negative effect to the total volume of fermented dough (35% decrease). Tempe flour decreased the maximum (Hm, 95%) and final height (h, 98%) of fermented dough. These observations suggest that composite flour using up to or less than 20% tempe flour can be used in bread formulation using 0.1% TG, and the interest in its functional properties and potential of tempe flour commands further research of tempe-based ingredients.

Key words: wheat, soy, tempe, transglutaminase, mixing and fermentation properties.

INTRODUCTION

Consumer's awareness of healthy food is increasing. Today, nutritional benefits are an important factor, as well as taste and convenience, in consumer purchases. Tempe, an Indonesian traditional fermented food mainly made from soybean using inoculums

Rhizopus spp. (Astuti et al 2000), is well-recognized as nutritious. Several compounds such as bioactive peptides (Hernandez-Ledesma et al 2011), GABA (gamma amino-butyric acid) (Aoki et al 2003), glucosamine, folate (Hogervorst et al 2011), vitamin B12 (Bisping et al 1993), β-carotene, ergosterol and γ-tocopherol (Denter et al 1998) are produced during tempe fermentation.

Tempe is an affordable source of protein with health benefits such as antioxidants, antibacterial properties, and support for the human brain. Bioactive peptides, isoflavones and tocopherols are considered potent as antioxidant (Hoppe et al 1997; Hernandez-Ledesma et al 2011), and folate supports human brain and health (Hogervorst et al 2011). Tempe also has HMW fraction 'arabinose' compounds that act as anti-bacterials (Kiers et al 2007; Roubos-van den Hil et al 2010). Tempe is relatively safe, because it has been consumed by various age groups for a long period of time without delivering adverse effects (Astuti et al 2000) and *R. oligosporus* is a strain that does not produce any potentially poisonous metabolites (Jennessen et al 2008).

Wheat and soybean, two crops abundantly produced in USA with values of about 62 and 82 million ton in 2012 (FAOSTAT, 2014) respectively, are good source of certain nutrition. Wheat grain contains bran, germ and endosperm, compenents that are considered as good sources of essential amino acids (9 out of 10 essential amino acids, minus lysine), minerals (iron, zinc and selenium), vitamins (folate, tocol), and beneficial phytochemicals (lignin, phenolic acids) and dietary fiber (arabinoxylans, glucans) components to the human diet (Shewry, 2009; Slavin 2010). Soybean is recognized as a good source of protein and isoflavones (Messina 1999; Villares et al 2011).

Tempe from blends of wheat and soy offers novelty and potential as a food ingredient. It is of interest to combine wheat grain and soybean and adopt the tempe fermentation method to prepare tempe flour. In the correct ratio, wheat and soy may negate nutritional deficiencies very well; wheat is deficient in lysine while soy has high lysine and isoflavone compounds. The flour could have the compounds found in tempe, broaden utilization of wheat and soy as new food ingredient, and increase the consumption of these crops.

One possible application of tempe flour is substitution into bread/ baked products formulation, because according to Delcour et al (2012) bread could be an excellent carrier of health promoting components. Substituting soy flour to bread formulation delivers benefits, such as a decreased carbohydrate and energy (calories) (Mohamed et al 2006), increased in minerals, fibers (Serrem et al 2011), isoflavone (Shao et al 2009) protein and lysine content (Shogren et al 2003; Serrem et al 2011). However, adding soy flour has negative effects on the gluten network formation and dough properties (Roccia et al 2012) leading to decrease of bread loaf volume and increase bread weight (Islam et al 2007). Regarding limitations of soy flour, so far a 10% substitution is recommended to make bread (Islam et al 2007). There are no studies in the literature on the effect of increasing levels of incorporation of soy in form of tempe flour into bread formulation.

Treatments such as soaking, boiling, fermentation, drying and milling are applied during tempe flour preparation. Heat treatments modify the conformation of protein which disrupt the secondary structure resulting unfolded structure and denaturation of protein (Nordqvist et al 2012; Jin et al 2009); decrease free SH due to crosslink between gliadin and glutenin through disulphide bonds, and lead to formation of aggregates of

wheat gluten protein (Wang et al 2009). All the preparation process may decrease baking quality if tempe flour is substituted into wheat bread formulation. Application of TG is expected to alleviate this problem. According to Ahn et al (2005), TG increases functional properties of weak wheat flour even when it is blended with barley (40%) or soy flour (20%).

According to DeJong and Koppelman (2002) cross-linking reaction will occur if TG is exposed to readily available glutamine and lysine in the protein substrate. Availability of glutamine and lysine may not be a big problem, since during fermentation protein is partially hydrolyzed resulting in smaller molecules (Wang et al 2009) and causes more available lysine (Handoyo et al 2006) and glutamine. Thus we hypothesize that it is possible to utilize TG to improve mixing and fermentation properties of composite wheat-tempe flour. This study was aimed to explore the potential of tempe flour made from wheat grain and soy bean fermentation and to evaluate the effect of transglutaminase (TG) and tempe flour on the rheological and fermentation characteristics of composite wheat-tempe dough properties.

MATERIALS AND METHODS

Materials used for making tempe flour were Billing hard red winter wheat grain (Stillwater, OK), soy bean from Jayone Foods Inc. (Paramount, CA) and tempe mix-cultures inoculum (LIPI, Indonesian Institute of Science, Bandung, Indonesia). The resulted-tempe flour had protein content of 30.4% (at 14% moisture basis). Wheat flour used in composite flour was commercial wheat flour (SH chief bakers flour, Shawnee Milling Co, Oklahoma) with protein content of 10.9% (at 14% moisture basis).

Tranglutaminase enzyme used was microbial TG, Activa TI, with activity 100U/g, obtained from Ajinomoto (Fort Lee, NJ)

Tempe Flour Preparation

An observation was conducted to determine the best ratio among 0:4, 1:3, 2:2, 3:1 and 4:0 of whole wheat:soy (w/w) to use in tempe making. The ratio was selected by considering three aspects; appearance/fungi growth, sensories (taste and aroma) analysis of tempe and calculation of lysine content.

Tempe was prepared following traditional process. Soybean was soaked in water (ratio soy:water was 1:4 w/w) overnight, boiled for 15 min, peeled, steamed for 20 min and cooled at room temperature. Wheat grain was soaked in water (ratio wheat grain:water was 1:4 w/w) overnight, coarsely ground with food chopper (KitchenAid 3 cups, St. Joseph, MI) for 30 s, boiled in water (ratio wheat grain: water was 1:2 w/w) for 10 min, steamed for 20 min and cooled at room temperature. Steamed wheat and soy were blended (ratio of wheat:soy was 1:1 w/w), inoculated with 0.1% mix-cultures tempe starter, packed in perforated-sealed plastic bag (16.5x14.9 cm), incubated at $30\pm2^{o}C$ for 36 h. Tempe was steamed for 3 min, coarsely cut with food chopper (KitchenAid 3 cups, St. Joseph, MI) to small pieces, dried in oven $60^{o}C$ for 12 h, milled in Kitchen Mill (Blendtec, West Orem, Utah) and sifted to pass a 40 mesh sieve. Tempe flour was placed inside closed plastic jar and stored at $-4^{o}C$ until needed.

Evaluation of Tempe Proteins as TG substrate

The amino acids sequences were compiled from NCBI database (http://www.ncbi.nlm.nih.gov/.). The presence and possession and position of lysine, glutamine and cysteine residues of each of the dominant proteins (glycinin and conglycinin soy) will be used to evaluate the sites that can be substrates of TG.

Rheological Measurements

Mixing properties such as water absorption (%), development time (min), stability (min), tolerance index (FU), and breakdown time (min) of wheat flour and wheat flour substituted with different levels of tempe flour and TG were measured using Farinograph-E (C.W. Brabender Instruments,Hackensack, NJ). Each analysis was set in condition using 10g sample capacity, 63 rpm, 30°C, based on AACC approved method 54-21 (AACCI, 2011).

Fermentation Characteristics

Fermentation properties of flours were tested using F3 Rheofermentometer (Chopin, France). Dough was prepared in a Chopin AlveoConsistograph kneader. Flour (250 g), dry yeast (3 g), and TG (with concentrations of 0, 0.05 and 0.1% w/w) were put inside the kneader. Prior to mixing, sodium chloride (5 g) was dissolved in deionized water. The amount of water required depended on the moisture content of flour as suggested in the Chopin protocol. At first 2 min mixing, salt solution was progressively added to the flour. After 2 min, mixing was stopped to remove flour sticking on the

kneader' wall and to make sure all dry ingredient evenly hydrated. Mixing process then continued for more 6 min to produce well developed dough.

About 315 g of the dough was placed in the bottom aluminum basket of rheofermentometer bowl and a 2000 g piston was placed over the top of the dough. The cover of rheofermentometer bowl system was connected with displacement sensor and tightly closed. Fermentation properties of dough were evaluated during 3 h under constant temperature of $28.5^{\circ}C$. The test provided data such as Hm (height of maximum dough development under constraint, in mm), h (height of the dough development at the end of test, in mm), (Hm-h)/Hm (percentage of development lowering after 3 h, in %), T1 (time to reach the maximum rise, in h and min), H'm (maximum height of the gaseous release curve, in mm), T'1 (time spent to reach H'm, in h and min), Tx (time when dough starts to release CO_2, in h and min), total volume (ml), volume of CO_2 lost (ml), volume retention (volume of CO_2 kept in the dough at the end of test, in ml) and coefficient of retention (%).

Statistical Analysis

The study was conducted in a Randomized Complete Block design. Tempe flour was substituted in wheat flour at 0, 10, 20, 30, and 40%. Levels of TG additions were 0, 0.05 and 0.1%. Analysis of Variance (ANOVA) procedures using a 0.05 significance level was performed using SAS version 9.2 (SAS Institute, Cary, NC). Mean separation of significantly different data were conducted using protected pair wise t-tests.

RESULTS AND DISCUSSION

Tempe Preparation

The appearance of tempe is reported in figure 1 and 2. Limited fungi growth was observed on the whole wheat kernel during 36 h fermentation (Fig. 1) but it was well improved (Fig. 2) by cracking the wheat grain in a food chopper (KitchenAid 3 cups, St. Joseph, MI) for 30 s prior to steaming. Fungi grew well on both cracked wheat grain and prepared soy, and produced the compact tempe cake in all selected ratios. Therefore, a sensory analysis was conducted to select tempe with the highest preferable taste and aroma according to the selected panelists from the cereal chemistry lab. Results from the panelists indicated that the taste and aroma of tempe made from ratio 1:1 wheat:soy was the most preferable (tempe made from only soy had a 'strong' taste and a bitter after taste, while tempe produced from only wheat had an unpleasant 'alcoholic' and 'acid' aroma).

Ratio	4:0	3:1	1:1	3:1	0:4
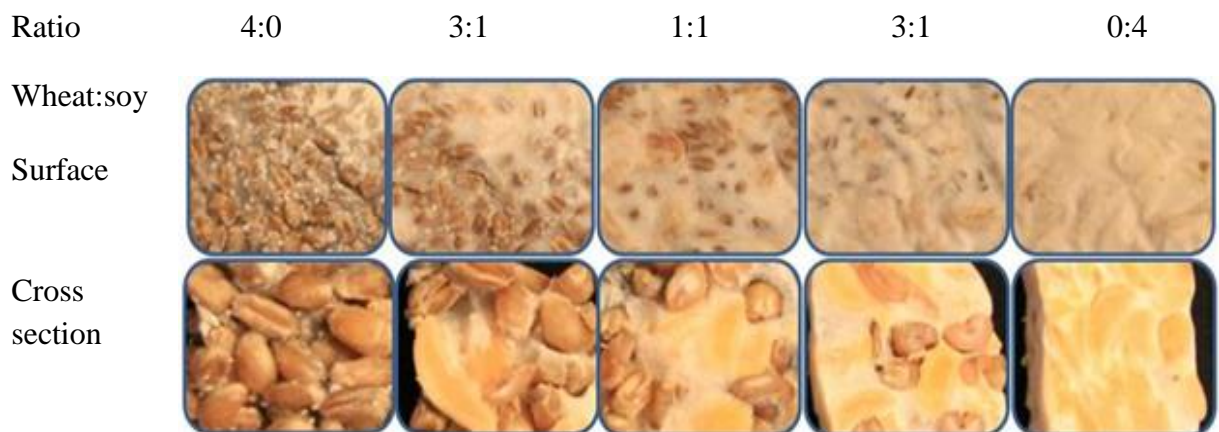
Figure 1. Tempe cake from whole wheat kernels and soy beans prepared with different ratios (w/w).
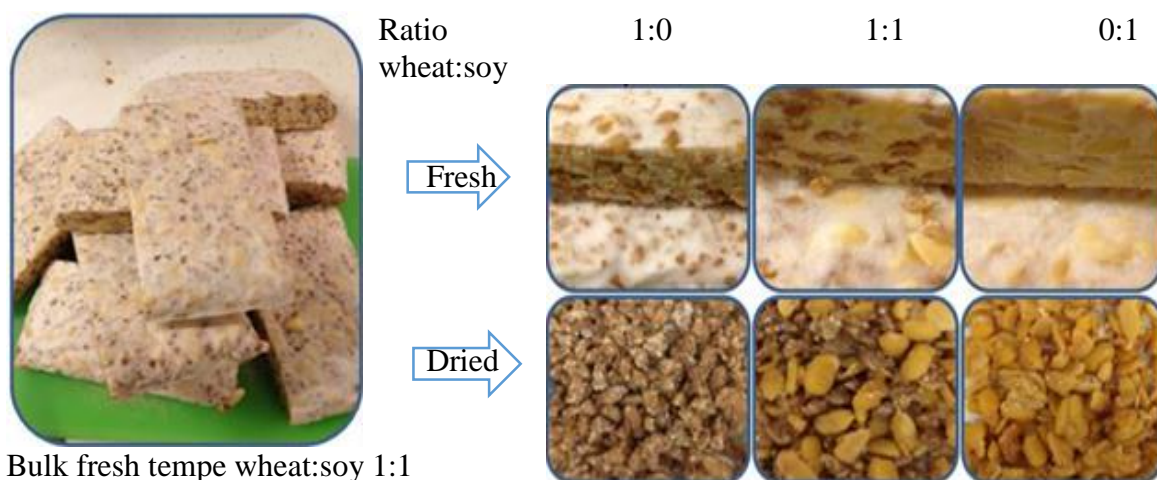
Figure 2. Bulk, fresh and dried tempe prepared from cracked wheat kernels and soybean at ratio 1:0, 1:1 and 0:1 (w/w).

The calculation of lysine content of each ratio was done using data from USDA nutrient data base (http://ndb.nal.usda.gov/ndb/foods/list). The wheat:soy ratio with 1:1 was the closest to the lysine content of FAO/WHO recommended levels of essential amino acids for adult human (Table 1). Thus, this ratio was used for preparing tempe flour.

Table 1. Calculation of lysine and methionine content with different ratios of wheat and soy compared to FAO standard.

	Wheat: soy ratio (w/w)					FAO standard
	4:0	3:1	1:1	1:3	0:4	
Lysine (mg/g prot)	25.51	34.86	44.22	53.57	62.93	45
Methionine (mg/g prot)	16.20	15.33	14.46	13.59	12.72	16

Tempe Flour Preparation

Fresh tempe has a limited shelf life, because the fermentation /enzymatic process still continues after it is harvested. Mold growth is largely complete at 46 h incubation, pH of tempe increases from 6.6 (at 46 h) to 7.1 (at 72 h) due to increasing amount of

ammonia (Sparingga and Owens 1999) released from hydrolysis of macromolecule. During tempe storage, beans become visible and color of fungal mycelium turns brown due to senescence, texture of tempe softens and ammonia odor emerges (Nout and Kiers 2005). Therefore, a treatment is required to stop both mold growth and further fermentation. Wang et al (1968) steamed fresh tempe for 5 min to terminate mold growth. In our study, fresh tempe was steamed for 3 min, coarsely cut for 30 s with food chopper (KitchenAid 3 cups, St. Joseph, MI) to small pieces, and dried in oven at 60°C for 12 h.

Tempe flour was prepared by milling the dried tempe in the Kitchen Mill (Blendtec, West Orem, Utah) with smallest dot texture setting (for producing a fine flour). Collected flour was then screened to pass through a number 40 mesh sieve. The particles that did not pass through the 40 mesh were re-ground. Figure 3 shows the flour that passes through 40 mesh sieve from the first through sixth grinding. All flours were blended to obtain the 'whole grain tempe flour' sample.
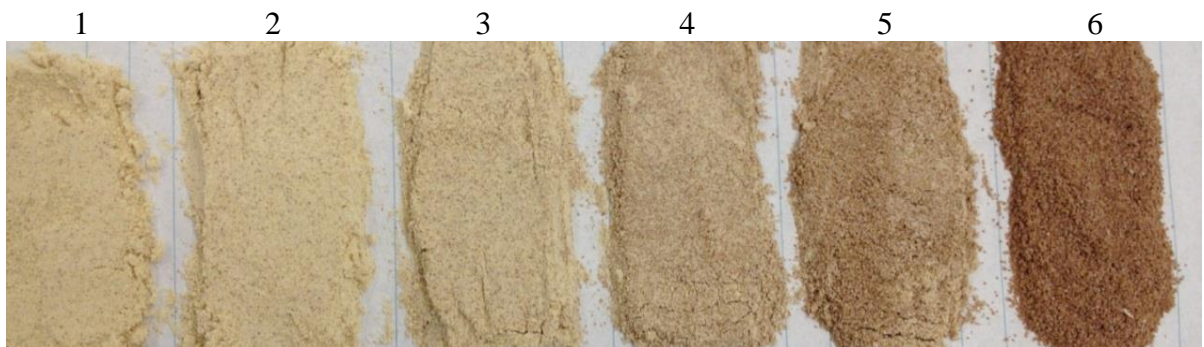
Figure 3. Tempe flours obtained from grinding and sieving through a 40 mesh sieve. Numbers represent the grinding process from first through sixth grinding.

Soy Protein as TG Substrate

Tempe flour was prepared from fermented wheat grain and soy bean 1:1 ratio, w/w; therefore it contained both wheat and soy proteins. Since the structure of wheat

proteins and its possibility as TG's substrate has been discussed previously in the dissertation (Chapter III), this chapter will discuss the amino acid composition of soy proteins as a TG substrate.

Soy contains about 35-45% protein where globulin is about 90% of total protein (Hou and Chang 2004). The protein consists of four main fractions: 2S, 7S, 11S and 15S globulin. Among them glycinin (11S) and β-conglycinin (7S) comprise about 70% of the storage protein (Maforimbo et al 2006). β-conglycinin is a trimeric protein that consists of α-subunit, α'-subunit and β-subunit with a molecular weight of 150-200 kDa. Whereas, glycinin is a hexameric protein comprised of acidic polypeptides (34-40 kDa) and basic polypeptides (20 kDa) linked by a disulfide bond (Pérez et al 2008). These proteins (11S and 7S) are soluble in water or salt solutions (Pérez et al 2008).

Table 2. Total lysine, cysteine and glutamine residue of soy glycinin and conglycinin.

	Accession	AA	Mw (kDa)	Lysine (K)	Cysteine (C)	Glutamine (Q)
Glycinin	AAA33964	516	67138	19	8	45
Glycinin	BAA74952	517	67430	19	8	47
Glycinin	CAA37044	562	73872	23	9	76
Glycinin	BAA74953	563	73811	22	8	74
Glycinin	BAA19059	517	67436	19	8	47
α-conglycinin	BAA23360	544	72824	32	1	46
β-conglycinin	BAA23361	416	55371	21	0	33

Accessions were obtained from NCBI website, AA is amino acids sequence

The selected accession of soy glycinin, with 516 to 563 amino acid sequences, contains from 19 to 23 lysine residues. Glycinin also has 8 to 9 cysteine and a moderate number of glutamine (45 to 76) residues. The α-conglycinin is composed of 32 lysine residues, one cysteine residue and 46 glutamine residues, while β-conglycinin has 21

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lysine residues, no cysteine and 33 glutamine residues. These observed results indicate that soy proteins provide enough lysine and glutamine as main substrate of TG. On the other hand, cereal protein are known to have lysine as its limiting amino acid. Therefore adding soy protein to the wheat bread formulation might provide a better substrate for TG and it also add of nutritional benefit.

Tang et al (2006) reported that majority of soy proteins β-conglycinin and acidic subunits of glycinin were effectively cross-linked by TG to form a large polymer, whereas basic subunit of glycinin was almost unchanged by TG. Marcoa and Rosell (2008) confirmed that the crosslink occurred in soy protein as indicated by the decreasing of free amino groups after TG treatment.

The inter-peptide cross-link between glutamine and lysine can also take place between two different proteins leading to the formation of hetero-polymers with totally new functionality (DeJong and Koppelman 2002). Thus, inter-molecular cross-link of glutamine and lysine of soy and wheat protein could take place. However, according to Han and Damodaran (1996) there is an additional factor, thermodynamic compatibility of mixing of protein substrates at the active site of the enzyme may affect the inter-chain cross-linking catalyzed by TG. Thermodynamic compatibility is the nature and intensity interaction of two macromolecules to approach each other, where thermodynamic compatibility of different classes of proteins is limited. Han and Damodaran (1996) also postulated that the heterogeneous cross-linking was favorable to occur between similar proteins, but not between dissimilar proteins (for example hydrophobic β-casein and hydrophilic β-lactoglobulin).

Table 2 indicates that both glycinin and conglycinin have higher lysine than 1-10 residues of gluten proteins, therefore soy proteins should be a better substrate for TG than gluten proteins. However, Köksel et al (2001) observed that the presence of soy protein isolates in the wheat flour system was not sufficient to significantly influence the action of TG. They explained by referring to the finding that gluten proteins, mainly HMW glutenin was an efficient substrate for TG (Larre et al 2000) and dynamic incompatibility as other factors that may influence heterologous crosslink (Han and Damodaran 1996). Further, Koksel et al (2001) postulated that even though soy protein isolate had high lysine content, TG might not cross-link gluten and SPI protein through lysine of SPI and glutamine of gluten protein. Most gluten proteins are hydrophobic, while most of soy proteins are hydrophilic. Therefore, most available lysine of gluten proteins might be cross-linked among gluten proteins to form large polymers, and proteins of soy might not able to participate in TG reaction.

Tempe preparation employs several physical treatments, such as soaking, heating (boiling and steaming), fermenting and drying. This sequence of treatments encourages the modification of the protein's structure and thus changing the native state. Heat treatment of soy usually results in changes in secondary and tertiary structure of the protein molecules (Maforimbo et al 2006), leading to denaturation.

Wheat protein may lose its viscoelastic properties and soy protein, according to Maforimbo et al (2008), will increase size distribution and hydrophobicity after experiencing heat treatment. Kang et al (1994) suggested that heat treatment before TG reaction would increase surface glutamine and lysine residue of glycinin, leading to an increase in the amount of resulting glutamyl-lysine cross-linkage. Pérez et al (2008)

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suggested that soy protein in an unfolded, denatured state had a higher interaction rate with gluten protein compared to soy protein in a non-denatured or native state.

Wheat and soy proteins experience further modification during the fermentation process of tempe, where the microbes in the inoculum also produce enzymes such as proteases. Proteolysis of soy proteins may provide both free lysine and increase the accessibility of TG to the substrate.

To the best our knowledge no one has reported the possibility of TG to catalyze glutamyl-lysine bonds between the soy proteins of tempe flour and wheat proteins. If the lack of hydrophobicity of soy protein is a limiting factor for hetero-polymer cross-link between soy proteins and gluten proteins as proposed by Koksel et al (2001), then increasing hydrophobicity of soy proteins by heating and fermentation process (tempe) may increase possibility of hetero-polymer cross-link with gluten proteins to occur.

Mixing Properties of Composite Flour as Affected by Tempe Flour and TG.

The effect of TG and tempe flour, on the mixing properties of composite flour are reported in Figure 4, and Tables 3 and 4. Most of the mixing properties were not significantly influenced by TG (p>0.05), with the exception of development time and stability of the composite flour with 40% tempe flour and tolerance index of composite flour with 20% tempe flour (Table 3). This result is consistent with study on wheat flour (Chapter VI –Table 1 of this dissertation); where at level of 0.1%, TG did not cause significantly difference to the farinograph mixing properties. Basman et al (2003) also reported that the mixing properties of wheat-soy flour blends were not significantly influenced by up to 0.25% of TG.
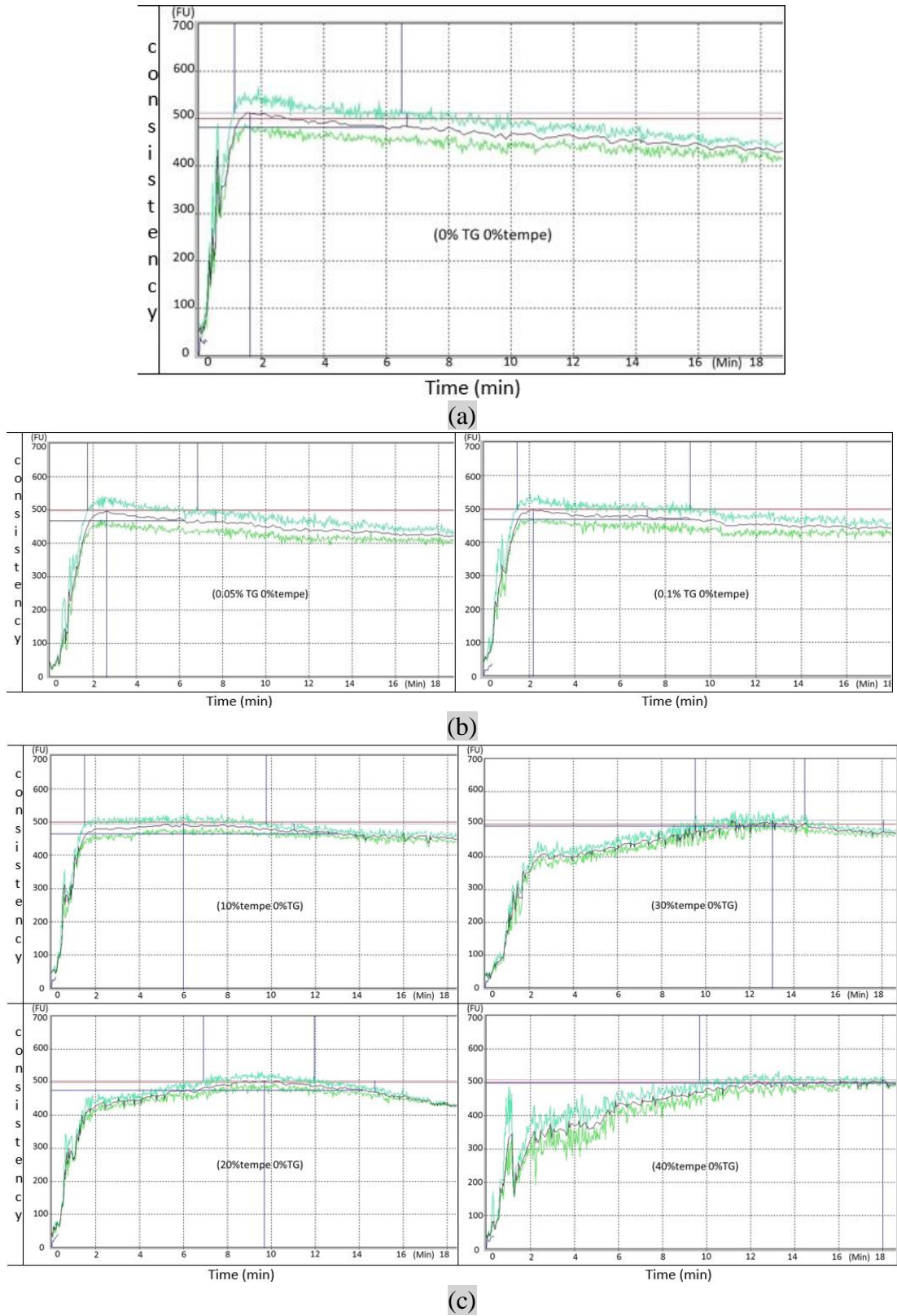
Figure 4. Example of farinograms of composite flour control (a) wheat flour with different levels of TG (b) and containing different levels of tempe flour at 0% TG (c).

Table 3. Effect of TG at 0-40% tempe flour on the mixing properties of composite flour [a].

Tempe (%)	TG (%)	Water absorption (14%)	Development time (min)	Stability (min)	Tolerance index (BU)	Breakdown time (min)
0	0	54.6±0.15 a	2.2±0.27 a	5.8±0.54 a	32.7±2.03 a	6.2±0.64 a
	0.05	53.3±1.35 a	2.1±0.44 a	4.9±0.11 a	35.7±2.03 a	5.1±1.07 a
	0.1	52.9±1.15 a	2.4±0.17 a	6.6±0.54 a	27.7±4.81 a	7.1±1.13 a
p-value		0.26	0.98	0.53	0.31	0.20
10	0	53.4±0.14 a	6.2±0.44 a	8.4±0.81 a	25.0±3.46 a	12.1±1.32 a
	0.05	53.9±0.46 a	6.8±0.70 a	7.9±0.31 a	26.3±2.03 a	12.5±0.70 a
	0.1	53.8±0.12 a	6.7±0.48 a	7.5±0.49 a	32.0±3.51 a	11.2±0.95 a
p-value		0.90	0.79	0.82	0.37	0.45
20	0	52.2±0.15 a	10.1±0.30 a	5.8±0.36 a	40.3±3.93 a	13.8±0.26 a
	0.05	52.0±0.06 a	9.6±0.10 a	6.1±0.12 a	27.3±3.53 b	14.9±0.23 a
	0.1	52.4±0.10 a	10.1±0.61 a	6.6±0.33 a	29.0±2.08 b	15.0±0.35 a
p-value		0.93	0.83	0.88	0.36	0.49
30	0	51.7±0.20 a	12.0±0.95 a	4.1±0.87 a	30.7±5.36 a	12.3±1.18 a
	0.05	53.4±2.13 a	11.8±0.52 a	4.0±0.35 a	32.0±4.58 a	12.1±0.58 a
	0.1	51.6±0.12 a	12.1±0.23 a	2.3±0.62 a	42.3±4.48 a	12.8±0.31 a
p-value		0.17	0.96	0.43	0.06	0.78
40	0	50.6±0.21 a	15.7±1.53 a	13.0±2.90 a	4.0±4.00 a	19.4±0.57 a
	0.05	50.8±0.00 a	12.6±1.37 b	15.3±1.68 a	7.0±3.61 a	20.0±0.00 a
	0.1	50.8±0.09 a	15.4±0.69 a	5.7±0.60 b	3.0±3.00 a	20.0±0.00 a
p-value		0.98	0.005	<0.0001	0.73	0.82

[a] Means ± standard error from triplicate analysis. Means in the same column and within same treatment of tempe flour followed by the same letter are not significantly different at a 0.05 level of significance.

Tempe flour significantly affected most of the composite flour mixing properties (Table 4). Water absorption ranged from 50.6 to 54.6 %, and this value was significantly influenced by tempe flour at TG levels of 0 and 0.05% (P<0.007 and P=0.04) but it was not significantly different at TG level 0.1% (P=0.06). Without TG, 20%, tempe flour already decreased water absorption of the flour system, but when the amount of TG was increased to 0.05%, it required at least 40% tempe flour to significantly decrease the water absorption of flour system. Either increasing or decreasing water absorption of wheat flour with the incorporation of non-wheat protein/flour is determined by the nature of the protein. Basman et al (2003) reported that water absorption increased in wheat

flour as the concentration of incorporated soy increases. Bonet et al (2006) reported that water absorption of wheat flour increased with the addition of lupin or gelatin, but it decreased with addition of egg protein sources.

Tempe flour was prepared through several heating process such as boiling, steaming, and drying. These heat treatments could denature protein of both wheat and soy. According to Wagner and Anon (1990) more denatured-protein has high surface hydrophobicity that promotes the formation of a protein matrix stabilized by hydrophobic interactions and has ability to retain a significant amount of water in the structure.

The development time of composite flour with different levels of tempe flour ranged from 2.2 to 15.7 min, and it was significantly influenced by tempe flour ($P<0.0001$) (Table 4). The increase in development time with increasing amounts of tempe flour can be explained by several factors. First, a higher development time indicates that the flour requires longer time to hydrate all the flour compounds (Hadnađev et al 2011) to achieve the dough consistency of 500 BU. Tempe flour and wheat flour may have different hydration rates, where tempe competes for the water with wheat flour. Because tempe ingredients have been fermented, it hydrates fast and protein of wheat flour later. Second, a longer development time occurs as a consequence of decreasing gluten content (Koksel and Scanlon 2012) due to the increasing amount of non-wheat flour (tempe flour) substituted in the composite flour. Similar findings were previously reported by Basman et al (2003) and Maforimbo et al (2008) who reported that as the level of soy flour added to wheat flour increased, the arrival/development time also increased.

Table 4. Effect of tempe flour at 0-0.1% TG on mixing properties of the composite flour[a].

TG (%)	Tempe (%)	Water absorption (14%)	Development time (min)	Stability (min)	Tolerance index (BU)	Breakdown time (min)
0	0	54.6±0.15 a	2.2±0.27 d	5.8±0.53 bc	32.7±2.03 ab	6.2±0.64 c
	10	53.4±0.15 ab	6.2±0.44 c	8.4±0.81 b	25.0±3.46 b	12.1±1.32 b
	20	52.2±0.15 bc	10.1±0.29 b	5.8±0.37 bc	40.3±3.93 a	13.8±0.26 b
	30	51.7±0.20 bc	12.0±0.95 b	4.1±0.87 c	30.7±5.36 ab	12.3±1.18 b
	40	50.6±0.21 c	15.7±1.53 a	13.0±2.90 a	4.0±4.00 c	19.4±0.57 a
p-value		0.007	<0.0001	<0.0001	<0.0001	<0.0001
0.05	0	53.3±1.35 a	2.2±0.27 d	4.9±0.12 bc	35.7±2.03 a	5.1±1.07 d
	10	53.9±0.46 a	6.8±0.44 c	7.9±0.31 b	26.3±2.03 a	12.5±0.70 c
	20	52.0±0.06 ab	9.6±0.10 b	6.1±0.12 bc	27.3±3.53 a	14.9±0.233 b
	30	53.4±2.13 a	11.8±0.52 a	4.0±1.35 c	32.0±4.58 a	12.1±0.58 c
	40	50.8±0.00 b	12.6±1.37 a	15.3±1.68 a	7.0±3.61 b	20.0±0.00 a
p-value		0.04	<0.0001	<0.0001	<0.0001	<0.0001
0.1	0	52.9±1.16 a	2.4±0.17 d	6.6±0.95 a	27.7±4.81 b	7.07±1.13 d
	10	53.8±1.16 a	6.7±0.48 c	7.5±0.49 a	32.0±3.51 ab	11.2±0.95 c
	20	52.4±0.10 a	10.1±0.61 b	6.6±0.34 a	29.0±2.08 b	14.9±0.35 b
	30	51.6±0.12 a	12.1±0.23 b	2.4±0.62 b	42.3±4.48 a	12.8±0.31 c
	40	50.8±0.09 a	15.4±0.69 a	5.7±0.60 a	3.0±3.00 c	20.0±0.00 a
p-value		0.06	<0.0001	0.02	<0.0001	<0.0001

[a] Means ± standard error from triplicate analysis. Means in the same column and within same treatment of TG followed by the same letter are not significantly different at a 0.05 level of significance.

Increasing development time with increasing levels of tempe flour might relate to the ability of the soy globulin to interact with gluten protein leading to the formation of high molecular weight aggregates as observed by Maforimbo et al (2008) using SE-HPLC. The association between soy proteins and wheat proteins occurs during mixing and resting by involving physical, covalent and non-covalent bonds (Pérez et al 2008). Increasing tempe flour in the composite flour provides more soy protein to interact with gluten and form large aggregates, therefore it requires a longer time to hydrate these aggregate proteins compared to only gluten protein. Increasing development time was also reported by Sanz Penella et al (2008) when wheat bran was incorporated in wheat

flour. They explained that the interaction between fiber of the bran and protein prevented

hydration of the protein which resulted of in a longer development time.

The stability time of composite flour with different levels of tempe flour ranged

from 2.4 to 15.3 min and was significantly influenced by tempe flour (p<0.0001 at TG

level 0 and 0.05%, and p=0.0186 at TG 0.1%) (Table 4). The stability changed with the

substitution of 0-30% tempe, while a sharp increase in stability was observed with 40%

tempe except with the sample containing 0.1% TG. The increasing stability might relate

to the quality of gluten. Bonet et al (2006) reported that soy increased the quality of

gluten as measured by gluten index. However, this is contrary to what was observed by

Hadnađev et al (2011), who found that stability of non-wheat flours were lower than

those of wheat flour alone.

The tolerance index of wheat flour with several levels of tempe flour ranged from

3.0 to 35.7 BU. Statistical analysis suggested that the tolerance index was significantly

influenced by tempe (P<0.0001). Similar to stability, this variable also showed great

modification when wheat flour was substituted with 40% tempe flour.

Breakdown time ranged from 5.1 to 20 min, and it was significantly affected by

tempe flour (P<0.0001) (Table 4). The overall breakdown time increased as increasing

amounts of tempe flour substituted into the wheat flour system. This may occur as a

consequence of the formation of high molecular weight (HMW) aggregate/polymers

between soy globulin and gluten protein (Maforimbo et al 2008) during early mixing. The

early mixing process encourages the formation of covalent interactions, such as glutamyl-

lysine and or disulfide, until the dough is developed. These covalent interactions,

according Bonet et al (2006), possibly occur between both wheat-wheat protein (homogenous) and between soy-wheat proteins (heterogeneous).

Increasing tempe flour substituted in the wheat flour increases the possibility of the presence of the high molecular weight aggregate/polymer. On the other hand, the breakdown of the dough is due to further mixing process that disrupts inter-chain interactions such as covalent glutamyl-lysine and disulfide.

Fermentation Properties of Composite Flour as Affected by TG and Tempe Flour

F3 Rheofermentometer provides two curves, dough development and gaseous release curve. Dough development curve informs data such as dough maximum height (Hm), dough height at the end of test (h), the time required for reaching the maximum height (T1), and the lowering of the dough development percentage after 3 h time frame compared to T1 (Hm-h)/Hm (Ktenioudaki et al 2011; Tripette and Renaud 2004). Figure 5 is an example of the dough development curve consisting of commercial wheat flour as affected by TG (a) and tempe flour (Figure b).
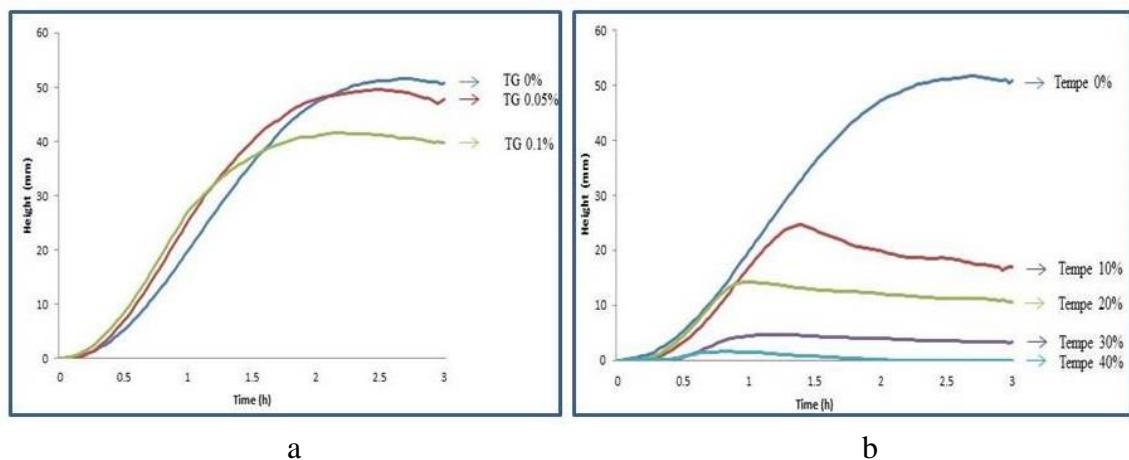

a   b

Figure 5. Example of the dough development characteristics of wheat dough as affected by TG (a) and tempe flour (b).

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Fermentation Properties of Composite Flour as Affected by TG

TG at levels of 0-0.1% did not significantly affect (p>0.05) dough development

characteristics of composite flour, with the exception of Hm (p<0.0001) and h (p<0.0001)

of wheat flour with 0% tempe flour (Table 5).

Table 5. Dough development parameters during fermentation of composite flour as affected by TG at 0 to 40% tempe flour[a].

Tempe (%)	TG (%)	Hm (mm)	h (mm)	(Hm-h)/Hm	T1
0	0	49.9±1.11 a	49.2±1.31 a	1.4±0.73 a	173.0± 4.92 a
0	0.05	49.9±1.10 a	48.7±0.87 a	2.5±0.86 a	150.5± 3.91 a
0	0.1	40.9±0.75 b	40.1±0.85 b	1.9±1.20 a	148.0±16.09 a
p-value		<0.0001	<0.0001	0.99	0.23
10	0	25.4±0.40 a	18.9±1.13 a	25.7±3.35 a	78.0± 4.77 a
10	0.05	23.2±1.89 a	16.6±1.83 a	28.6±2.19 a	87.5± 0.87 a
10	0.1	26.7±1.85 a	20.2±1.47 a	24.2±1.24 a	78.5± 5.29 a
p-value		0.08	0.053	0.94	0.25
20	0	13.5±0.38 a	9.2±0.70 a	32.2±3.25 a	69.0± 1.32 a
20	0.05	15.2±0.66 a	10.1±0.39 a	33.4±0.87 a	61.0± 3.91 a
20	0.1	13.2±0.90 a	9.6±0.57 a	26.7±1.45 a	68.5± 5.89 a
p-value		0.36	0.81	0.86	0.39
30	0	4.8±1.33 a	3.0±1.20 a	42.9±12.54 a	66.5± 7.76 a
30	0.05	4.5±0.61 a	2.6±0.47 a	41.9± 7.54 a	52.5± 4.82 a
30	0.1	4.4±0.78 a	2.1±1.20 a	58.5±16.86 a	56.0± 0.87 a
p-value		0.97	0.82	0.37	0.08
40	0	1.7±0.87 a	0.3±0.27 a	91.9± 8.07 a	57.5± 4.44 a
40	0.05	2.4±1.17 a	1.3±0.72 a	65.1±18.72 a	57.5± 7.50 a
40	0.1	3.3±0.92 a	1.5±0.73 a	64.2±18.20 a	51.5± 3.50 a
p-value		0.60	0.67	0.07	0.70

[a] Means ± standard error from triplicate analysis. Means in the same column and within same treatment of tempe flour followed by the same letter are not significantly different at a 0.05 level of significance.

Dough height (Hm) ranged from 1.7 to 49.9 mm and TG significantly influenced

the dough height (p<0.0001) only in wheat flour (0% tempe flour). Dough heights of

wheat flour at 0 and 0.05% TG were similar and decreased at 0.1% TG. A similar pattern

was also observed in the height of dough at the end of the 3 h test (h) where h decreases

with 0.1% TG. The wheat flour used in this study was a hard red winter wheat with a protein content of 11%. The glutenin subunits in hard red winter wheat identified as Dx5 + Dy10 subunits are associated with gluten strength (Payne et al 1987; Shewry et al 2003). Therefore, at 0.1%, TG already created covalent cross-linkage that decreases dough expanse.

TG did not significantly modify dough Hm of composite flour containing 10% tempe flour (Table 5). However, it is interesting to note a trend to increase dough height with increasing level of TG (Figure 6). The p value was higher than 0.05 (p=0.08) and therefore TG might influence Hm at a level higher than 0.1%. This suggests that heterogeneous covalent bonds between glutamine wheat protein and lysine of soy protein may occur when TG is added at levels higher than 0.1%.
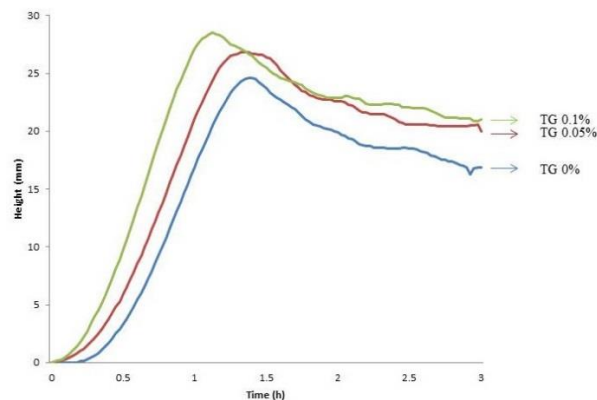

Figure 6. Effect of TG on the dough development curve of composite flour containing 10% tempe flour.

Gaseous Release Curve

The second curve provided by the F3 Rheofermentometer is gaseous release curve that provides seven parameters of fermentation: H'm, T'1, Tx, total gas volume, CO_2 lost,

volume of CO_2 retention and retention coefficient. Figure 7 displays an example of gaseous release curves of the wheat flour as affected by TG with 0% tempe flour.
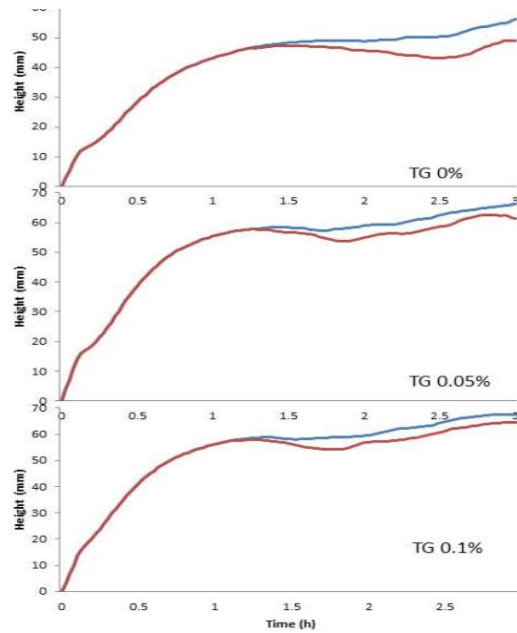

Figure 7. Example of gaseous release curves of commercial wheat flour treated with different levels of TG (0-0.1%) at 0% tempe flour.

Table 6. Gaseous release of composite dough as affected by TG at 0 to 40% tempe flour[a].

Tempe (%)	TG (%)	H'm (mm)	Tx (min)	Total volume (ml)	CO_2 lost (ml)	Volume of retention (ml)	Coefficient retention (%)	T'1 (min)
0	0	61.5±3.06 a	83.0±17.19 a	1313.7± 82.51 a	68.7±16.91 a	1244.7± 95.28 a	94.6±1.65 a	180.0±0.00 a
	0.05	64.3±1.31 a	70.0± 8.76 a	1405.7± 41.03 a	55.0±1.73 a	1350.7± 40.74 a	96.1±0.15 a	177.0±3.00 a
	0.1	67.1±0.40 a	69.0± 3.97 a	1486.7± 15.43 a	61.7±0.33 a	1425.3± 15.76 a	95.8±0.07 a	174.0±3.12 a
p-value		0.47	0.75	0.21	0.28	0.16	0.06	0.71
10	0	64.3±3.35 a	69.0±11.72 a	1314.3± 90.12 a	58.0±4.73 a	1256.7± 86.83 a	95.6±0.29 a	180.0±0.00 a
	0.05	65.0±0.20 a	77.3± 5.25 a	1346.3± 9.60 a	52.7±1.76 a	1293.7± 9.39 a	96.1±0.15 a	180.0±0.00 a
	0.1	70.7±3.32 a	66.8± 0.75 a	1479.0± 78.05 a	60.7±5.81 a	1418.7± 74.18 a	95.9±0.27 a	180.0±0.00 a
p-value		0.31	0.90	0.21	0.63	0.20	0.78	1.00
20	0	60.5±0.49 a	66.0± 1.50 a	1259.3± 8.01 a	55.7±2.40 a	1203.7± 5.61 a	95.6±0.15 a	180.0±0.00 a
	0.05	62.7±3.00 a	108.0±46.50 a	1321.0± 89.84 a	55.7±2.91 a	1268.7± 86.79 a	95.8±0.11 a	179.5±0.50 a
	0.1	57.8±4.77 a	-	1200.3±122.11 a	52.0±7.00 a	1148.3±115.70 a	95.7±0.26 a	160.5±19.5 b
p-value		0.55	0.12	0.44	0.88	0.44	0.96	0.02
30	0	57.4±8.73 a	-	1139.3±133.36 a	43.0±8.02 a	1096.7±125.55 a	96.3±0.26 a	180.0±0.00 a
	0.05	51.5±2.38 a	-	1073.7± 61.58 a	47.0±4.04 a	1026.3± 58.65 a	95.6±0.25 a	180.0±0.00 a
	0.1	51.7±1.95 a	-	1096.7± 49.24 a	45.3±2.85 a	1051.3± 46.40 a	95.9±0.09 a	179.0±1.00 a
p-value		0.33		0.79	0.89	0.74	0.57	0.99
40	0	45.6±2.00 a	-	952.0± 44.30 a	38.7±3.28 a	913.0± 41.94 a	95.9±0.18 a	180.0±0.00 a
	0.05	41.0±1.67 a	-	843.0± 29.14 a	31.7±2.19 a	811.3± 27.70 a	96.2±0.18 a	179.0±1.00 a
	0.1	44.4±1.73 a	-	930.7± 41.25 a	39.0±3.00 a	891.7± 38.26 a	95.8±0.12 a	180.0±0.00 a
p-value		0.56		0.49	0.62	0.52	0.82	0.99

[a] Means ± standard error from triplicate analysis. Means in the same column and within same treatment of tempe flour followed by the same letter are not significantly different at a 0.05 level of significance.

Gaseous release characteristics of commercial wheat flours treated with different levels of TG at given tempe flour is displayed in Table 6. Most of gaseous release parameters were not significantly influenced by TG at all tempe flour levels (p>0.05), with the exception of T'1 at 20% tempe flour (P=0.02).

Fermentation Properties of Composite Dough as Affected by Tempe Flour

Statistical analysis showed that most of dough development parameters during fermentation were significantly influenced by tempe flour, except of Tx (p= 0.20-0.90) and coefficient retention (p=0.15 – 0.99). The mean and standard error of the dough development parameters as affected by tempe flour at given TG are displayed in Table 7.

Table 7. Dough development during fermentation of composite flour as affected by tempe flour at 0 to 0.1% TG[a].

TG (%)	Tempe (%)	Hm (mm)	h (mm)	(Hm-h)/Hm	T1
0	0	49.9±1.10 a	49.2±1.31 a	1.4± 0.73 c	173.0± 4.92 a
	10	25.4±0.40 b	18.9±1.12 b	25.7± 3.35 bc	84.5± 4.77 b
	20	13.5±0.38 c	9.2±0.70 c	32.2± 3.25 b	65.0± 1.32 c
	30	4.8±1.33 d	3.0±1.19 d	42.9±12.54 b	61.0± 7.76 c
	40	1.7±0.87 d	0.3±0.27 d	91.9± 8.07 a	50.5± 4.44 c
p-value		<0.0001	<0.0001	<0.0001	<0.0001
0.05	0	49.9±1.10 a	48.7±0.87 a	2.5± 0.86 c	150.5± 3.91 a
	10	23.2±1.89 b	16.6±1.83 b	28.6± 2.18 bc	84.0± 0.87 b
	20	15.2±0.66 c	10.1±0.39 c	33.4± 0.87 b	62.0± 3.91 c
	30	4.5±0.61 d	2.6±0.47 d	41.9± 7.54 ab	58.5± 4.82 c
	40	2.4±1.17 d	1.3±0.72 d	65.1±18.72 a	57.0± 7.50 c
p-value		<0.0001	<0.0001	0.001	<0.0001
0.1	0	40.9±0.75 a	40.1±0.85 a	1.9± 1.19 b	148.0±16.09 a
	10	26.7±1.85 b	20.2±1.47 b	24.2± 1.24 b	75.5± 5.29 b
	20	13.2±0.90 c	9.6±0.57 c	26.7± 1.45 b	71.5± 5.89 bc
	30	4.4±0.78 d	2.1±1.20 d	58.5±16.86 a	55.5± 0.87 c
	40	3.3±0.92 d	1.5±0.73 d	64.2±18.20 a	59.0± 3.50 bc
p-value		<0.0001	<0.0001	0.0002	<0.0001

[a] Means ± standard error from triplicate analysis. Means in the same column and within same treatment of TG followed by the same letter are not significantly different at a 0.05 level of significance.

Maximum dough height (Hm) of wheat flour substituted by different levels of tempe flour ranged from 1.7 to 49.9 mm. The dough expansion decreased with increasing amount of tempe flour substituted to wheat flour (Figure 5b). For example, about a half of Hm decreased when 10% tempe flour was substituted for wheat flour and it decreased more than 90% when 40% of tempe flour was substituted for wheat flour. The height of the dough at the end of fermentation (h) also decreased and the trend of h as affected by tempe flour was similar to Hm. Lowering development dough (Hm-h)/Hm increased as increasing tempe flour incorporated in wheat flour. Commonly, good fermentation tolerance is indicated by small percentages of (Hm-h)/Hm because the dough has a well maintained height during fermentation. However, incorporation of tempe flour to replace some portions of wheat flour resulted in a higher (Hm-h/Hm) percentage. For example, the addition of 10% tempe flour resulted in about a 90% increase of observed (Hm-h/Hm). This indicates that replacing wheat with tempe flour produces a weaker dough that has a lower ability to maintain the dough height during fermentation compared to control. T1 is the time taken by dough to reach maximum height, and it as the tempe flour increased, the dough reached its maximum height faster.

The Hm represents the expansion of dough due to production of gas (CO_2) during fermentation. Hm and all the fermentation parameters quality decreased when tempe flour was substituted, even at 10%, to the wheat dough (Table 7). This occurrence can be explained from 3 points of view.

First, in the absence of TG, substitution of tempe flour may interfere the balance ratio between gluten and starch and other components in the composite dough. According to Koksel and Scanlon (2012) the ratio between gluten, starch and water strongly

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determines dough development and dough ability to entrain gas during mixing and hold gas during fermentation.

Tempe was prepared from 50% soy and 50% whole grain wheat. Soy protein has no gluten, and the gluten protein present in the whole wheat was modified (denatured) by heating and hydrolysis during tempe processing. Therefore, the presence of tempe flour dilutes the gluten of the dough, which, according to Dube et al (2007), leads to a reduction of baking (including fermentation) capacity. Decreasing the gluten content, according to Koksel and Scanlon (2012), will increase the dough density and decrease air entrainment into the dough during mixing.

Low fermentation characteristics of composite flour may be a result of the different nature of two sources of the proteins. Wheat proteins are dominated by gluten, which favors hydrophobic interactions. However, tempe has soy proteins that, according to Maforimbo et al (2006), are mostly dominated by globulin. Blending wheat flour with tempe flour causes each of the proteins to compete with absorption of water, and because of its nature as globulin, soy proteins has higher water absorption than gluten proteins. According to Pérez et al (2008) the water that is bound by soy protein is no longer available for gluten development, which leads to weakening of the dough and affects the dough flowing properties. Insufficient protein hydration causes insufficient gluten network formation during dough processing. This condition, according to Dube et al (2007), produces gluten with lack of elasticity and contributes to the reduced dough quality. It commonly occurs during manufacture of bread with low wheat protein content (Dube et al 2007).

In the presence of TG, the decrease of fermentation properties as tempe flour increases could be related to lysine content of soy protein. Amino acids sequence showed that soy proteins contained more lysine (about 20 residues per mol protein, Table 1) compared to the wheat proteins (about 6-7 residues per mol protein in glutenin). The presence of soy proteins obstructs the ability of TG to catalyze crosslinking of gluten proteins due to a better compatibility of soy proteins with the active site of TG than wheat proteins (Basman et al 2002). The microstructure of dough made from wheat-soy flour with TG seems dense due to the formation of a more compact and homogenous protein network than the control wheat dough (Bonet et al 2006). In addition, Autio et al (2005) suggested that mixed-proteins polymers were not evenly distributed in dough system because protein strands were not extended as much as gluten proteins.

Gaseous release curve provides information of the amount of gas produced by yeast during dough fermentation and the ability of dough to retain the gas during 3 h test. Figure 8 illustrates one example of gaseous release curve as affected by different levels (0-40%) of tempe flour substituted to the wheat flour.

Statistical analysis suggested that four of the seven gaseous release parameters of wheat dough were significantly influenced by tempe flour. These were maximum height (H'm) ($p=0.025$, $p<0.0001$ and $p<0.0001$ at given TG 0, 0.05 and 0.1%), total volume ($p=0.003$, $p<.0001$ and $p<.0001$ at TG 0, 0.05 and 0.1%), CO_2 lost ($p=0.01$, $p=0.04$ and $p=0.05$ at TG 0, 0.05 and 0.1%) and CO_2 retention ($p=0.004$, $p<.0001$ and $p<.0001$ at TG 0, 0.05 and 0.1%). Whereas, three others parameters (Tx, coefficient retention and T'1) were not significantly modified ($p<0.05$) by tempe flour. Means and standard error of each gaseous release properties are displayed in Table 8.
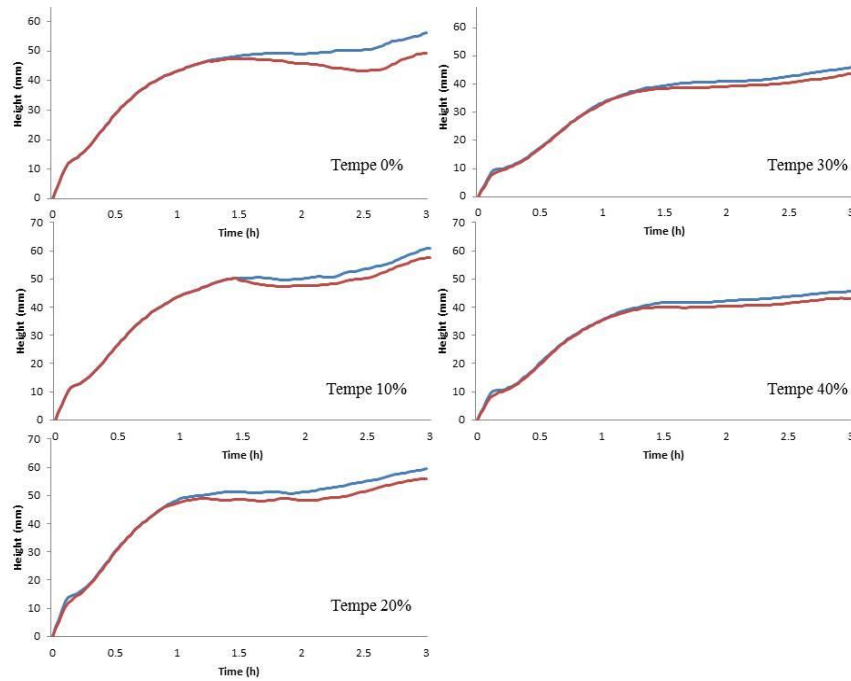
Figure 8. Example of gaseous release curves of composite dough as affected by substitution tempe flour at 0% TG.

Table 8. Gaseous release parameters of composite dough as affected by tempe flour at 0 to 0.1% TG[a].

TG (%)	Tempe (%)	H'm (mm)	Tx (min)	Total volume (ml)	CO_2 lost (ml)	Volume of retention (ml)	Coefficient retention (%)	T'1 (min)
0	0	61.5±3.06 a	83.0±17.19 a	1313.7±82.51 a	68.7±16.91 a	1244.7±95.28 a	94.6±1.65 a	180.0±0.00 a
	10	64.3±3.35 a	69.0±11.72 a	1314.3±90.12 a	58.0±4.73 ab	1256.7±86.83 a	95.6±0.29 a	180.0±0.00 a
	20	60.3±0.49 a	-	1259.3± 8.01 a	55.7±2.40 abc	1203.7±5.61 a	95.6±0.15 a	180.0±0.00 a
	30	57.4±8.73 a	-	1139.3±133.36 ab	43.0±8.02 bc	1096.7±125.56 ab	96.3±0.26 a	180.0±0.00 a
	40	45.6±1.99 b	-	952.0±44.30 b	38.7±3.28 c	913.0±41.94 b	95.9±0.18 a	180.0±0.00 a
p-value		0.003	0.50	0.003	0.009	0.004	0.15	1.00
0.05	0	64.3±1.31 a	70.0±8.76 a	1405.7±41.03 a	55.0±1.73 a	1350.7±40.74 a	96.1±0.15 a	177.0±3.00 a
	10	65.0±0.20 a	77.3±5.25 a	1346.3±9.60 a	52.7±1.76 a	1293.7±9.39 a	96.1±0.15 a	180.0±0.00 a
	20	62.7±2.99 a	66.0±1.50 a	1324.0±89.84 a	55.7±2.91 a	1268.7±86.79 a	95.8±0.12 a	179.5±0.50 a
	30	51.5±2.38 b	-	1073.7±61.48 b	47.0±4.04 ab	1026.3±58.65 b	95.6±0.25 a	180.0±0.00 a
	40	41.0±1.67 c	-	843.0±29.14 c	31.7±2.19 b	811.3±27.69 c	96.2±0.18 a	179.0±1.00 a
p-value		<0.0001	0.90	<0.0001	0.04	<0.0001	0.86	0.34
0.1	0	67.1±0.40 a	69.0±3.97 a	1486.7±15.43 a	61.7±0.33 a	1425.3±15.76 a	95.8±0.07 a	174.0±3.12 b
	10	70.7±3.32 a	66.8±0.75 a	1479.0±78.05 a	60.7±5.81 a	1418.7±74.18 a	95.9±0.27 a	180.0±0.00 a
	20	57.8±4.77 b	108.0±46.5 a	1200.3±122.11 b	52.0±7.00 ab	1148.3±115.70 b	95.7±0.26 a	180.0±0.00 a
	30	51.7±1.95 bc	-	1096.7±49.24 bc	45.3±2.85 ab	1051.3±46.40 bc	95.9±0.09 a	179.0±1.00 a
	40	44.4±1.73 c	-	930.7±41.25 c	39.0±3.00 b	891.7±38.26 c	95.8±0.12 a	180.0±0.00 a
p-value		<0.0001	0.20	<0.0001	0.04	<0.0001	0.99	0.003

[a] Means ± standard error from triplicate analysis. Means in the same column and within same treatment of TG followed by the same letter are not significantly different at a 0.05 level of significance.

The maximum height of the gaseous release curve is attributed to the growth of air bubbles during fermentation. Figure 8 and T'1 data (Table 8) indicated that the maximum height was reached nearly at the end of the test. This means that gas was still produced until the end of the 3 h fermentation. H'm was affected by tempe flour at all given TG levels ranged from 41.0 to 70.7 mm. H'm decreased at increasing levels of tempe flour, however the decreasing of H'm begun to take place when 40% tempe flour was substituted to the wheat flour without TG, or 30% tempe flour at 0.05% TG, or 20% tempe flour at 0.1% TG. A similar trend as H'm was found in total volume of gas and volume of gas retention (Table 8). It indicates that tempe flour substitution of up to 20% of the wheat flour at any TG levels (0-0.1%) does not disturb gas production during fermentation, gas development inside the dough, or the ability of the dough to retain gas.

CONCLUSION

Tempe was prepared from wheat and soy through sequences of traditional process as including soaking, boiling, inoculation, and fermentation. With these preparations, wheat grain was still too hard for fungal mycelia to penetrate and grow well on it. Cracking the wheat grain prior to steaming improved the wheat-soy tempe cake. Considering the desirable tempe performance and sensory properties, and lysine content closest to WHO standard lysine requirement for adults, a ratio 1:1 of wheat:soy was selected to use for preparing tempe flour.

Two major components of soy proteins, glycinin and conglycinin each had 19-23 and 21-32 lysine and 45-76 and 33-46 glutamine residues. Considering the number of lysine and glutamine, soy proteins of tempe flour should be a good substrate for TG.

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TG at levels 0-0.1% did not significantly modify ($p < 0.05$) both mixing and fermentation properties of the composite flour. However, it is interesting that at 10% tempe flour, treatment with 0.1% TG produced the highest dough height compared to those with 0 and 0.05% TG.

Substituting tempe flour in the wheat flour significantly influenced most of the mixing characters of composite flour. It increased development time, stability and breakdown time. Tempe flour also significantly modified fermentation properties. However, tempe flour up to 20% did not change gaseous release properties although higher levels modified them sharply. As positive effect, tempe flour decreased CO_2 loss during fermentation. This observation suggests that composite flours using up to or less than 20% tempe can be used in bread formulation using 0.1% TG.

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CHAPTER VII

CONCLUSION AND FUTURE STUDIES

CONCLUSION

Evaluation of wheat gluten proteins as TG substrate was done by comparing the content and position of lysine and glutamine residues using amino acid sequences reported at the NCBI website. It was observed that among gluten proteins fraction, HMW-GS possess the highest (4-10) lysine residues, for example Dx5 subunit on average has 6 lysine, 299 glutamine and 5 cysteine residues, while Dy10 subunit has 7 lysine 206 of glutamine and 7 cysteine residues. In theory HMW-GS has the highest probability to participate in the formation glutamyl-lysine covalent bonds catalyzed by TG. Formation of these covalent bonds, both inter- and intra-molecular, increases the molecular size of gluten protein polymer along with increase proximity of chains that encourages formation of disulfide bonds. We propose the formation of closed loop strands due to combination of inter- and intra-molecular crosslink. The more crosslinks formed in a system, the smaller the closed loop would be. This closed loop model could help to understand an increase in covalent crosslinks of gluten via TG.

In general, properties of wheat flour including rheological, gluten, fermentation and baking properties were significantly influenced by TG ($p<0.05$). For analysis with test duration time relatively short such as mixing properties (20 min) and gluten quality (5-7 min), TG did not significantly influence these parameters until the levels of application were at least 0.8%. For analysis that took a longer testing time such as fermentation (3 h) and baking properties (4h 29 min), TG brought varying effects. Some parameters were significantly modified by TG at levels of 0.1-0.2%, for example dough maximum height during fermentation, bread specific volume and bread crumb textures. This study suggests that the effects of TG are related to the preparation and duration time of the test.

Gluten index and gluten elastic recovery increased and wet gluten decreased with increasing levels of TG. When TG levels was increased from 0 to 1.6%, wet gluten decreased 2.5% (standard method) and 5.3% (modified method) and gluten index increased 4.6%. The largest impact of TG levels was on elastic recovery of gluten where the 1 min mixing yielded an increase in recovery ranging from 3.6 to 10.5% and the modified method consisting in extended mixing and resting yielded an increase range from 8.7 to 39.9%.

Compared to standard method, modified method of gluten extraction significantly produced higher wet gluten (7 to 11%) and higher increasing of gluten recovery (10.5% of standard method vs 39.9% of modified method). These results suggest that the modified method is a good alternative for measuring the effect of time dependent additives, like TG, on gluten properties and thus avoid underestimating the full potential of TG.

TG at levels of 0.1 to 0.4% positively increased development time and stability of wheat dough, however a dramatic decrease of these properties took place at TG levels of 0.8% and up. TG also positively impacted to the fermentation characters of dough by decreasing amount of CO_2 release during dough fermentation, and increasing the coefficient of gas retention. However, the maximum height of the dough decreased with increasing level of TG.

Wheat flour treated with TG at the level of 0.1 % produced fermented dough height higher and bread crumb structure more homogenous than control. However, increasing level of TG more than 0.1% TG decreased Hm (height maximum of dough) and bread specific volume, and increased crumb texture.

Bread loaf volume did not correlate to rheological properties, but it correlated to the dough fermentation properties. Maximum dough height (Hm, r = 0.84) and dough height at the end of the test (h, r= 0.83) were two fermentation parameters that highly correlated to bread loaf volume of samples treated with TG. This study suggests that fermentation test is a good test to predict baking properties of wheat flour.

Tempe was prepared from wheat and soy through sequences of traditional process including soaking, boiling, inoculation, and fermentation. By these preparations, wheat grain was still too hard for fungi's mycelia to penetrate and grow well on it. Cracking the wheat grain prior to steaming improved the wheat-soy tempe cake. Considering to the desirable tempe performance and sensories, and lysine content closest to WHO standard lysine requirement for adults, ratio 1:1 of wheat:soy was selected to use for preparing tempe flour.

The potential of tempe flour as TG substrate was predicted by examining the content of lysine and glutamine of wheat protein and soy protein. Two major components of soy proteins, glycinin and conglycinin each had 19-23 and 21-32 lysine and 45-76 and 33-46 glutamine residues. Considering the number of lysine and glutamine, soy proteins of tempe flour should be a good substrate for TG.

TG at levels 0-0.1% could not significantly modify ($p<0.05$) both mixing and fermentation properties of the composite flour. However, it is interesting that at 10% tempe flour, treatment with 0.1% TG produced the highest dough height compared to those with 0 and 0.05% TG.

Substituted tempe flour to the wheat flour significantly influenced most of the mixing characters of composite flour. It increased development time, stability and breakdown time. Tempe flour also significantly modified fermentation properties. However, tempe flour up to 20% did not change gaseous release properties but higher levels modified them sharply. Positively, tempe flour decreased CO_2 lost during fermentation. This observation suggests that composite flours using up to or less than 20% tempe can be used in bread formulation using 0.1% TG.

FUTURE STUDIES

Study I was aimed to evaluate the content of lysine, glutamine and cysteine of selected gluten proteins and their possible bonding as affected by TG. Multiple alignment of amino acids sequences provided in NCBI database indicates that all gluten proteins possess lysine and glutamine with variation in number and position. Glutamine was abundantly (about 30% of total amino acids) available in all of gluten proteins. The

number of lysine residue was limited. Lysine content of gluten proteins varied from 4 to 10 (HMW-GS), 1 to 2 (LMW-GS), 0 to 2 (α/β- gliadin), 1 to 3 (γ- gliadin) and 1 to 3 (ω-gliadin). Considering to possession of glutamine and lysine, theoretically all of gluten proteins are able to form isopeptidic glutamyl-lysine covalent bonds catalyzed by TG. However, Gerrard et al (2001) and Mujoo and Ng (2003) suggested that LMW-GS and gliadins were not involved in the reaction catalyzed by TG. It seems that lysine residues of LMW-GS and gliadins are not accessible by TG. Accessibility of TG to LMW-GS and Gliadin may increase after the proteins experience of mixing, fermentation and heat process during bread making. A study is proposed to determine whether bread making process can increase accessibility of TG to LMW-GS and gliadins. Study can be done by comparing LMW-GS and gliadins extracted from 1) wheat flour, 2) dough after mixing, 3) dough after fermentation, 4) bread.

Most gluten proteins, except of ω-gliadin, contain 4 to 9 cysteine residues that according to Gujral and Rosell (2004), Bonet et al (2005) potentially form inter- and intra-molecular disulfide bonds due to increase of proximity of chains caused by TG. An investigation is required to prove whether disulfide bonds occur due to TG activity. It is important to determine whether these two types of covalent bonds (glutamyl-lysine and disulfide) work synergistically or one is the dominant to determine the gluten/dough properties.

The second study was aimed to evaluate gluten properties as influenced by TG and to compare two gluten extraction methods, standard and modified. The modified method provided a longer time for TG interaction with gluten proteins due to additional 4 min mixing time and 10 min resting dough prior to standard gluten extraction. TG

significantly increased gluten index and gluten elastic recovery and decreased wet gluten. Compared to standard method, the modified method produced higher wet gluten, gluten index and gluten elastic recovery. This result indicates that dough preparation and resting also influences the modification of gluten proteins by TG. This result conveys an idea to further explore the optimum dose and the time reaction (mixing and resting) of TG in different types of wheat flours (weak, medium, strong) as guidance for example for extracting gluten.

The third study was aimed to determine the effect of TG to the dough rheological and fermentation properties of wheat flour with $11\pm0.5\%$ protein content. At levels up to 0.4%, TG increased polymerization of gluten protein by formation of covalent bonds; and positively increased Farinograph development time and stability; decreased amount of CO_2 release during dough fermentation, increased the coefficient of gas retention and produced a more homogenous bread crumb structure. However, TG decreased maximum height of dough and bread volume and increased crumb texture. These observations suggest that TG contributes both positive and negative effects to the dough and bread properties. It is proposed a further study to use wheat flours with protein content lower than 11% (9 to 10%) treated with TG alone, and combine TG with other additives/enzymes (such as arabinoxilanase) to improve bread volume and reduce bread crumb texture.

The fourth study was aimed to explore the potential of tempe flour made from wheat grain and soy bean and to evaluate effects of TG and tempe flour on the rheological and fermentation characteristics of composite dough properties. The study was conducted by replacing 0-40% of wheat flour by tempe flour and adding TG (0, 0.05

and 0.1%) to the composite (wheat-tempe) flour. The results indicated that TG up to 0.1% did not significantly influence to the mixing and fermentation properties, and application of tempe flour as wheat-substituted ingredient in bread formulation was possible under 20%. Improved results may still be possible because: 1) level of applied TG was too small and 2) TG may not (optimally) catalyze formation of inter-molecular crosslink between tempe proteins and wheat proteins, or TG may prefer to catalyze gluten proteins than tempe proteins. Therefore, future studies are suggested to 1) apply TG in a wider ranges of concentration (0 to 1.6%), and to perform baking test as a preliminary test to narrow down the TG levels used. 2) to react TG with tempe flour for a certain duration prior to using it to formulate a composite flour (bread formulation).

The interest in tempe flour functional properties commands further research of tempe-based ingredients. The particle of 'whole tempe flour' is coarse due to chemical/ structural changes caused by a combination of couple heat treatment and fermentation. Wheat-soy tempe flour may contain more resistant starch and denatured proteins than the common whole wheat flour. These two components have been known to have functional value for human nutrition, because resistant starch is a source of fiber and denatured proteins are easier to digest than non-denatured proteins. Future study is required to quantify resistant starch and denatured protein content. Product development studies will also be necessary to utilize more tempe flour in the food industry.

Flour sample preparation and storage

Four commercial wheat flours were obtained from different milling supplies in Oklahoma. Protein, ash and moisture content of the flours are displayed in Table 1. Wheat flours were labeled and stored inside double plastic bag in the freezer soon after it was received to kill insects' eggs until it was used. Whenever needed, the flour was taken off from freezer and moved to the room temperature at least for 24 h before it was ready to use/ analyze.

Table 1. Characteristics of four commercial wheat flours.

Flour	Name	Protein content (%)[a]	Ash (%)[a]	Moisture (%)
1	Soft	9.1	0.55	12.6
2	Chief	10.8	0.51	13.8
3	Spring	13.1	0.56	14.0
4	Tortilla	11.8	0.49	13.0

[a] based on 14% moisture content

In order to obtain six different flours samples (A, B, C, D, E and F) with similar protein content (11.0±0.5%), the flours were blended inside a V-20 Flour Mixer for 5 minutes. The calculation of blending followed the formulation:

M (flour 'blends') with P (11.0±0.5) = M(flour 'x')*P(flour 'x') + M(flour 'y')*P(flour 'y')

Where, M is mass of flour in g

       P is protein content of flour in %

       'x', 'y' are randomly representation of either soft, chief, spring or tortilla flour.

Summary of blending formulation are displayed in Table 2. The flour samples then were put inside jars with tight lid and brought to the lab to be analyzed. The protein, moisture and ash contents of flour were determined using the NIR system (FOSS NIR Systems Inc, Laurel, MD 20723)

Table 2. Formulation for blending the flours to obtain 6 new flours (A, B, C, D, E, and F)

Flour	Mixed from flour	Amount (kg)
A	Spring + Chief	3.04 + 6.96
B	Soft + Spring	3.17 + 6.83
C	Soft + Tortilla	1.16 + 8.84
D	Soft + Chief + Tortilla	5.20 + 1.80 + 3.00
E	Soft + Spring + Tortilla	3.90 + 3.10 + 3.00
F	Chieft	10.00

VITA

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