EFFECTS OF L-CYSTEINE AND TRANSGLUTAMINASE ON THE RHEOLOGICAL PROPERTIES OF GLUTENS FROM DIFFERENT WHEAT CULTIVARS

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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January, 2015

ABSTRACT

Bakery products with desirable texture possess the proper balance between elasticity and viscosity. These two rheological properties are generally contributed by the polymeric glutens formed by glutenins and gliadins with the addition of water. However, the native glutenins and gliadins in wheat flour might not possess the chemistry that could contribute to the desirable rheological properties. Therefore, an additive should be used to modify the flour.

In this study, addition of L-cysteine, a reducing agent, caused flour dough to be less elastic or more extensible. The extent of this effect is more profound in the strong wheat cultivars (Hollis (HRS), Norpro (HRS) and Trego (HW)) than in weaker wheat cultivar (Stephens (SW)) and commercial bread flour. Microbial transglutaminase at 16,000 ppm caused a significant increase in elasticity as agreed from both extension test and creep-recovery tests. However, added together with L-cysteine, microbial transglutaminase masked the rheological effect of L-cysteine.

BIOGRAPHICAL SKETCH

Monthakan Boonpermpol was born in Thailand on May 4, 1990. In 2008, she got scholarship from Agricultural Research Development Agency (Public Organization, Thailand) to study Food Science in the U.S. In 2012, she received her Bachelor of Science in Food Science and Technology from University of California at Davis (UC-Davis) with highest honors. During her study at UC-Davis, she did an internship at the Department of Viticulture and Enology and worked as a student research assistant at the Department of Biological and Agricultural Engineering at UC-Davis.

Right after her graduation in 2012, Ms. Monthakan went to Cornell University to pursuit her Master's degree in Food Science. In her M.S. program, she majored in Food Engineering with a minor in Food Chemistry. She is a member of the Institute of Food Technologists, American Association of Cereal Chemists, and Phi Tau Sigma Honorary Society of Food Science.

To my parents who are patiently waiting for me in Thailand

& my beloved one who always gives all the supports I need at Cornell

ACKNOWLEDGMENTS

The author would like to thank Dr. Steven J. Mulvaney who served as the chairman of her committee or a major advisor, and Dr. Dennis Miller who served as a minor advisor.

The author would like to thank Agricultural Research Development Agency (Public Organization, Thailand) for funding support, Perten Instrument AM, Sweden, for their generous donation of the equipment, and Dr. Hongquan Liang, Dongjun Zhao, Stephen Chapman and Lena Halabi for their previous work in this lab. The author also would like to thank Dr. Patricia Rayas-Duarte and her students at Oklahoma State University for their assistance on running additional experiments.

Additional thanks go to the author's parents and friends for their moral supports. Special thanks to my former lab mates: Agnes Lim and Susanna Kahn.

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LIST OF ABBREVIATIONS

AACC	American Association of Cereal Chemists
ANOVA	Analysis of Variance
BLV	Bread of loaf volume
DATEM	Diacetyl Tartaric Acid Ester of Monoglyceriedes
GI	Gluten Index
GIPSA	Grain Inspection Packers and Stockyard Administration
GMP	Glutenin macropolymer
HMW-GS	High molecular weight-glutenin subunits
HRW	Hard red winter
HRS	Hard red spring
HDWH	Hard white
SWH	Soft white
Zeleny	Zeleny sedimentation volume

LIST OF SYMBOLS

G_1	Shear modulus of instantaneous elasticity in 3-element, 4-element and 6-element models
G_2	Shear modulus of elasticity in Kelvin-Voigt model analogs in 3-element model and element model (Burger's model)
G_{2a}	Shear modulus of one of the Kelvin-Voigt model analogs in 6-element model
G_{2b}	Shear modulus of another Kelvin-Voigt model analog in 6-element model
μ_2	The viscous coefficient of the dashpot in the Kelvin-Voigt model in 3-element model and 4-element model
μ_{2a}	The viscous coefficient of one of the dashpots in the Kelvin-Voigt model in 6-element model
μ_{2b}	The viscous coefficient of another dashpot in the Kelvin-Voigt model in 6-element model
μ_3	The viscous coefficient of the Maxwell model in 4-element model (Burger's model) and 6-element model
γ	Recoverable shear strain (%)
σ_0	Applied stress (Pa)
N	Newton

CHAPTER 1

BACKGROUND

1.1 Overview: Chemistry of Gluten

Although wheat flour consists of less than 20% protein, the unique viscoelastic properties of dough are due to the protein fraction rather than to the starch (Brady, 2013). It has been well-established that wheat flour contains glutenin and gliadin. Glutenin and gliadin are storage proteins in wheat endosperms. When water is added to wheat flour, glutenin and gliadin develop to be a network structure known as gluten. Gluten is responsible for elasticity, extensibility, and cohesiveness of bread dough as well as other functional properties of wheat, barley, and rye in many food products (Wieser 2007). Gluten provides a structure that is viscous enough to retain the gas produced by yeast, and elastic enough to retain its original shape. Proteins in wheat endosperm consist of 10% albumins, 5% globulins, 45% prolamins, and 40% glutelins (Sluimer 2007). As shown in Figure 1, wet gluten can be extracted from a mixed wheat flour dough by washing out the water-soluble albumin proteins and starch and the salt-soluble globulin proteins. The gliadin proteins are soluble in 70% ethanol, and therefore they are considered as prolamins. The insoluble fraction in 70% ethanol is called glutenin as shown in Figure 1 (Osborne, 1907).

Gliadins and glutenins are different in terms of structure and size but still share some common features. Gliadins are single-chain polypeptides, which primarily form intramolecular disulfide bonds. Glutenins are aggregated proteins that are linked by interchain disulfide bonds. After reduction of disulfide bonds, the resulting glutenin subunits show a solubility in aqueous alcohols similar to gliadins. (Hamer et al., 2009) The gliadin proteins are relatively smaller molecules with molecular weights generally between 28,000 Da and 55,000 Da, while the glutenin

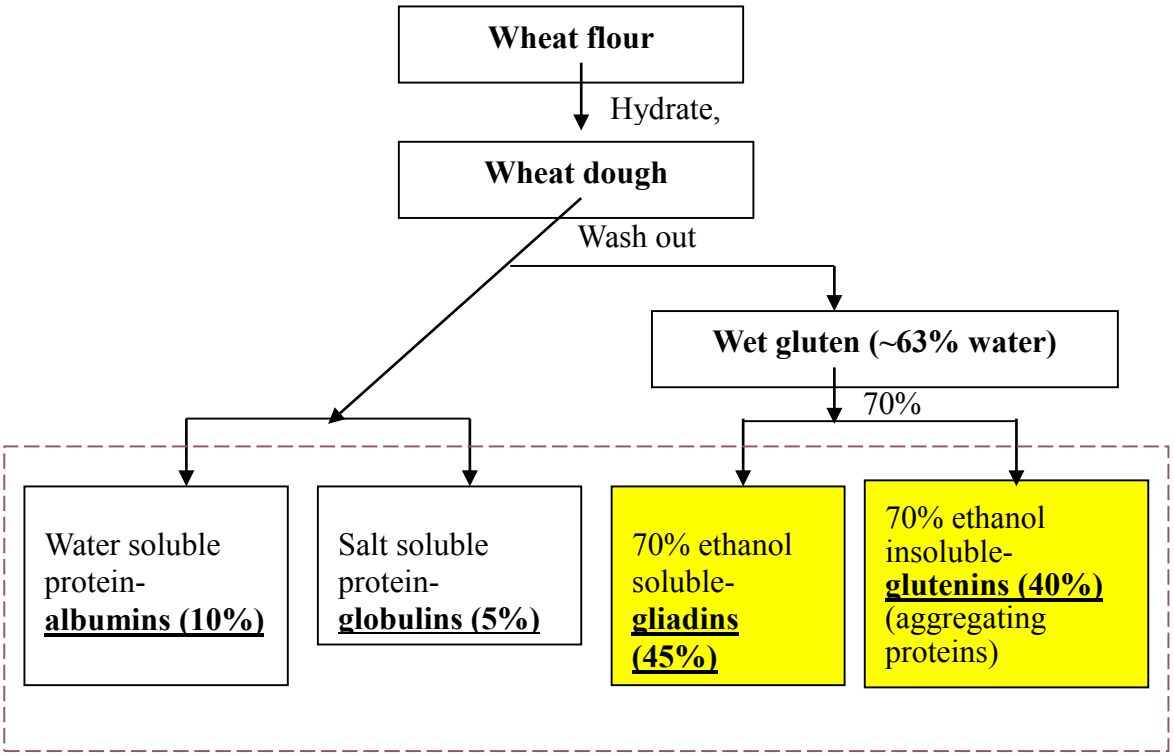


Figure 1. Extraction scheme of gliadins and glutenins from wheat flour (Osborne, 1907)

proteins consist of a fraction with molecular weight in the range of 32,000 Da to 45,000 Da, so called "Low-Molecular-Weight (LMW) subunits," and a fraction of larger molecules with molecular weights in the range 67,000 Da to 125,000 Da so called, "High-Molecular-Weight (HMW) subunits." These glutenin proteins are aggregated together through intermolecular disulfide bonds into much larger protein complexes with molecular weights ranging from 500,000 Da up to 10,000,000 Da. Both of these proteins (glutenins and gliadins) have very low contents of charged amino acids but very high contents of glutamine and proline. Due to the high proline content, neither of these types of proteins generally have well-defined tertiary conformations (Brady, 2013).

Gliadins are thought to have only intramolecular disulfide bonds, so-called "monomeric" proteins. The gliadins are classified into four groups of monomeric proteins, called alpha (α), beta (β), gamma (γ), and omega (ω) fractions based on their different mobilities in gel electrophoresis (Woychik et al., 1961). Three fractions are categorized as sulfur-rich α, β, and γ gliadins. Besides the high quantity of sulfur due to the high number of cysteine and methionine (25-30%) residues, these fractions have similar molecular weights in the range of 25-40 kDa. Later electrophoretic studies established that α and β gliadins were only one group now called α/β-type gliadins (Wringley et. al., 2006). The fourth fraction is categorized as sulfur-poor ω gliadin due to low number of cysteine and methionine residues (less than 11%); however, the ω gliadin fraction consists of more than 80% of total amino acids as glutamine, proline and phenylalanine. The combined glutamine and proline content of these four types of gliadins ranges from 50 to 75%. They also contain a significant proportion of phenylalanine, ranging from 5% in the α/β and γ gliadins to 9% in the ω gliadins. Furthermore, the molecular weight of ω gliadin is higher than the

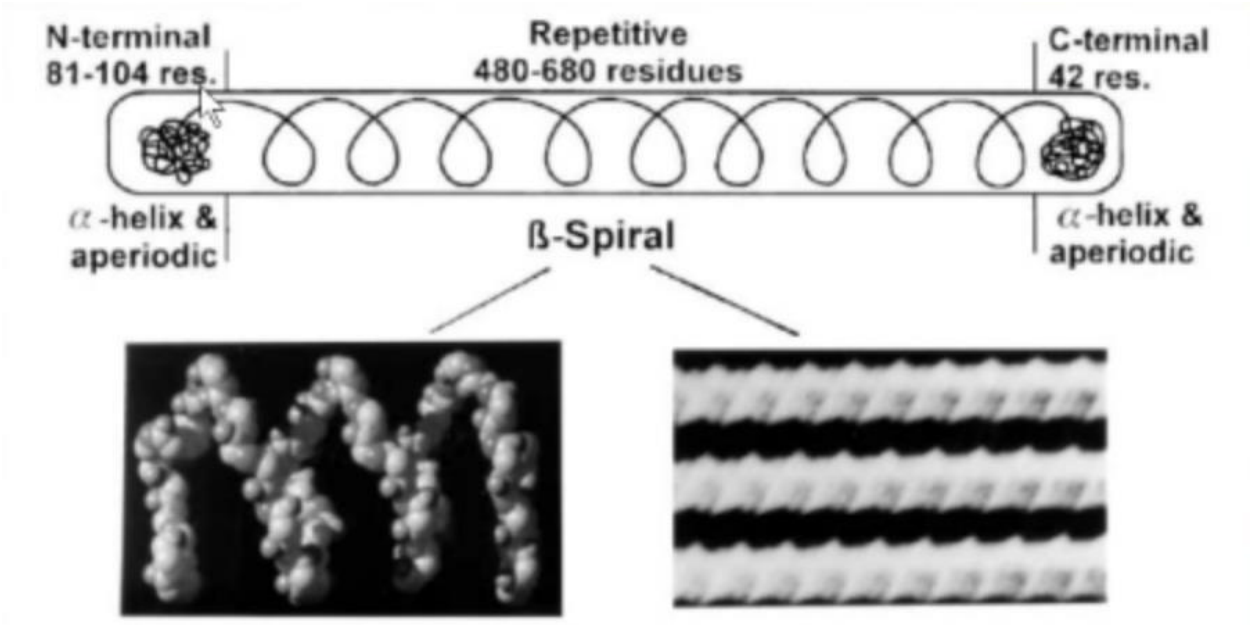


Figure 2. The chemical structure of glutenin consisting of α-helices at the two ends and β-spiral in the middle regions (Shewry et al., 2001)

molecular weights of α, β, and γ gliadins (Lasztity, 1996; Shewry et al., 1986). With proline content in the range of 16-26%, the gliadins are unlikely to have globular conformations, but they have been reported to have beta-turns (β-turns) in their N-terminal domains and alpha-helices (α-helices) and beta-sheets (β-sheets) in their C-terminal domains (Wringley et al., 2006). The α/β gliadins have six cysteines in their C-terminal regions, and the γ gliadins have eight, resulting in three and four intramolecular disulfide bonds, respectively.

In contrast, the glutenin fraction comprises aggregated proteins linked by interchain disulfide bonds. As mentioned before, glutenins are classified as either HMW or LMW subunits. Native glutenins are composed of a backbone formed by HMW subunit polymers and of LMW subunit polymers attached to HMW subunits (Hamer et al., 2009). HMW subunits are sulfur-poor, but LMW subunits are sulfur-rich. HMW subunits contain glutamine, proline, and glycine as more than 50% of the total amino acids. As shown on Figure 2, the middle segments of HMW consist of 480-680 residues and have beta-spiral (β-spiral) conformation. The N-terminal regions consist of 81-104 residues, and the C-terminal regions consist of 42 residues. Both C- and N-terminal regions have α-helices. (Shewry et al., 2001) Like the gliadins, the glutenins are relatively high in glutamine and proline residues in repetitive segments with sequences such as Glu-Gln-Gln-Pro-Pro-Phe-Ser. The C-terminal regions are similar to those of alpha/beta and gamma-gliadins. The repetitive sequences with large proline compositions are thought to lead to a series of repeated Beta-turns, producing a coiled or helical-like structure resembling a weak spring. Such a spring-like structure would contribute significantly to the elasticity of gluten doughs. Extending these coils would decrease their entropy, favoring recoil, in a manner similar to the role of entropy in the elasticity of rubber. As already noted, one of the most important features of the glutenin proteins in determining their functional behavior in doughs is that they are cross-linked by their

disulfide bonds into much larger covalent complexes. With an upper bound in molecular weight approaching 10 million, these molecules are among the largest of all proteins.

1.2 Relationships between Structures and Functions of Gluten in Foods

Non-covalent bonds such as hydrogen bonds, ionic bonds and hydrophobic bonds play vital roles in the aggregation of glutenins as well as the overall structure of gluten networks, and therefore physical properties of dough. If only gliadins are present, when they are mixed with water, the resulting dough is sticky, viscous and extensible. It has been well-established that gliadins have little elasticity and are not as cohesive as glutenins. Gliadins function as a plasticizer for the glutenins in the dough. (Hamer et al., 2009)

Furthermore, disulfide bonds have been hypothesized to contribute to the aggregation of glutenins and therefore the dough properties. Due to its ability to form disulfide linkages, cysteine are crucial in the functional properties of dough. Most of the cysteine residues exist in an oxidized form as intramolecular and intermolecular disulfide bonds between glutenin subunits, linking the chains together. Due to higher number of disulfide bonds, glutenin is generally more elastic than gliadins. (Dobraszczyk & Morgenstern, 2003)

In addition, to explain the structure and function of gluten as a network, three current hypotheses have been proposed to explain the glutenin-gliadin network contributing to the viscoelasticity of flour dough:

1. Gluten as a structured loop train network (Belton, 1999)
2. Gluten as a particulate gel network or the hyper-aggregation model (Hamer & Van Vliet, 2000; Don et. al. 2003)
3. Gluten as an entangled polymer network or the polymeric gel model. (Singh & MacRitchie, 2001; MacRitchie & Lafiandra, 1997)

1.2.1. The structured-loop train network hypothesis

The structured-loop train network model was proposed by Belton (1999) to explain the elastic properties of gluten. The model postulates that, hydrogen bonds in gluten play a significant role in the formation of loop and train regions. Alberti et. al. (2002) hypothesized that the junction zones (trains) of the gluten plastic network involved segments containing glutamine residues close to hydrophobic residues. Glutamine side chains can form hydrogen bonds with other glutamine side chains. Stretching of the gluten first extends the loops and then causes the proteins to slide over one another. Therefore, the elastic restoring energy can be stored due to the drive to reestablish the loop-train equilibrium of the glutenin chains. An emphasis is given to the role of the high molecular weight glutenin subunits (HMW-GS) in this model. The loops between HMW-GS in gluten are composed of a repeated sequence, proline-glycine-glutamine-glycine-glutamine-glutamine (PGQGQQ). The prolines that introduce a kink prevent the forming of the train region and act as an initial point for water to incorporate the formation of loops (Alberti et. al., 2002).

The changes from β-sheet to β-turn structures as detected by Fourier Transform Infrared (FTIR) spectroscopy indicate the changes between "loops and trains" of the gluten structure (Hamer et al., 2009). The hypothesis proposes that, at the low hydration state, many protein-protein interactions occur through hydrogen bonding of glutamine residues in the β-spiral structures. As more water molecules are interacted with gluten, the gluten network is plasticized, allowing the orientation of the β-turns in adjacent to β-spirals to form structures that resemble 'interchain' β-sheets. Further hydration could cause the breaking of some hydrogen bonds between glutamine residues to be replaced by hydrogen bonds between glutamine and water, which then leads to the formation of loop regions. However, the new hydrogen bonds formed between glutamine and water do not result in the complete substitution of the hydrogen bonds

between glutamine residues. The outcome is an equilibrium between hydrated 'loop' regions and hydrogen-bonded 'train' regions, with the ratio between these two states depending on the hydration state. (Shewry et al. 2002)

In conclusion, this model emphasizes the role of hydrogen bonds between glutamine residues of HMW-GS to establish loops and trains. The equilibrium between the loops and the trains may contribute to the elasticity of gluten, as an extension of the dough will cause loops to extend and unzipping of the trains, which could be a mechanism of how the elastic energy is stored in gluten (Shewry et al. 2002). However, hydrogen bonds alone may not be strong enough to serve as crosslinks for elastic effects, which are usually formed via stronger bonds, such as ionic bonds provided by calcium cations in the gelation of alginates or pectins. Another possibility for the junction zones of gluten network could be disulfide bonds (Lee & Mulvaney 2003).

1.2.2. The particle-gel or particulate gel model

The particle-gel model is based on the presence of large glutenin aggregates (Hamer and Van Vliet, 2000; Don et al., 2003). Lefebvre et al. (2003) pointed out that the gluten network may be viewed as an aggregated network of insoluble colloidal particles. The evidence that supports this hypothesis is the presence of the glutenin macropolymer (GMP), which has the highest molecular weight and thus lowest solubility. The GMP can be separated as an insoluble gel layer from the dispersion of defatted gluten in 1.5% SDS solution by ultracentrifugation. The amounts and gel properties of GMP as a particle gel or network have been correlated with dough properties analyzed by viscometry and confocal scanning laser microscopy (CSLM). The interaction between glutenin particles observed by CSLM are quite large in the range of meso-scopic level (on the order of 0.1-100 μm); it has been proposed that they originate from protein bodies, as observed in immature wheat endosperm.

In conclusion, the particulate gel model shows that gluten, through a high molecular weight compound, takes the shape of a tight globule by linking neighboring glutenin molecules through physical and disulfide bond interactions (Don et. al., 2003).

1.2.3. The entanglement model

In polymer science, entanglements are topological constraints or transient crosslinks. This model is similar to the loop-train model proposed by Belton (1999). In the theory of Termonia and Smith (1988), the extensional properties of polymers are determined by two main kinetic processes; firstly, the breaking of, such as, secondary bonds and, secondly, slippage of chains through entanglements. Each process has its activation energy. Once non-covalent bonds between entanglement nodes are broken, and chains are stretched, the only way in which chains can move relative to one another is if they slip through entanglements. The extensional behavior is then determined by the relative rates of the sample elongation and of chain slippage (Hamer et al., 2009).

1.3. Mixing Flour into Dough

The hydrogen bonding capability (Belton, 1999, Alberti et al., 2002), reversible disulfide crosslinks (Lee and Mulvaney, 2003) and possible hydrophobic interaction close to juncture zones on the same molecules are crucial to the rheological behaviors or functional properties of gluten in flour dough, which allows them to be kneaded, shaped, and rolled.

Disulfide cross-linking and the rapid exchange of these linkages are thought to contribute to elasticity and extensibility. When force is applied to the polymers with disulfide bonds, if the force is more than the strength of disulfide bonds, the bonds are broken, and the energy can be dissipated as heat. As a result, the polymers can be extensible or flow. On the other hand, if the

force applied on polymers is less than the strength of disulfide bonds, the disulfide bonds are still connected, or the rapid exchange of these linkages still exist. As a consequence, the polymers can store energy and act as an elastic material. The importance of these disulfide bonds to the quality of dough is demonstrated by the effects of reducing agents, which significantly weaken dough, and oxidizing strengthen the dough.

The changes while mixing include changes in the solubility, the composition and the size of the glutenin network. Wheat proteins are poorly soluble in water due to the low amount of hydrophilic amino acids. However, mixing can change the solubility of gluten proteins as measured by their extractability in water, acid solutions, or detergent solutions. The glutenin polymers are difficult to dissolve due to their large size; therefore, mixing, which applies mechanical force that helps break down the large structure of glutenins, led to decreases in the size of the glutenin aggregates (Mecham et al., 1965). MacRitchie (1975) also demonstrated that the solubility of these aggregates increased with increasing mixer speed and attributed this to high shear strain rates being able to cause scission of disulfide bonds. In other words, the rate of deformation is proportional to the intensity of mixing.

1.4. Rheology and Texture in Bakery Products: Overview

Consumers find texture more important in solid foods than liquids (Matsumoto, 1977). Therefore, texture measurement is very important in bakery products. One major goal of instrumental tests, including rheological measurements in food, is to relate the results with sensory perception. Rheology is the study of flow and deformation. Rheological tests could be considered as an objective way to quantify texture. In summary, studies in rheology have two related objectives:

(1) Prediction of perception from instrumental rheological measurements. Considerable practical advantages in using instruments instead of sensory panels include convenience and reduction in time for quality assurance.

(2) The development of an understanding of the relationship between food structure and perception. Rheology is an important tool that can be used to help achieve this understanding. (Rao, 2013)

In bakery products, especially in bread, gluten development is the result of deformation of dough during mixing. Shear and elongation are two modes of deformation used to describe mixing. Shear is created when two parallel layers move at different speeds in the direction provided by a force. Shear is the dominant motion when pieces of dough rub against one another. Elongation occurs when two parallel layers move at different speeds parallel to the direction of the force. Elongation is the main type of deformation when dough is stretched.

It is not clear to what extent these two types of deformation have on gluten development. Shear obtained by the difference in speed of mixing arm and bowl is the dominant motion in most mixers. Increasing the friction as well as the rate of shear by an increase in the speed difference between mixing arm and bowl can lead to faster gluten development. In most commercial mixers, deformation by shear is the dominant mode. Elongation by the repeated sheeting of dough is also known to lead to good gluten development. Hand mixing is done by stretching and folding, which is also an elongation process. Therefore, both types of deformation could lead to well-developed gluten and desirable final products. (Sluimer, 2007)

Many instruments have been established to relate the rheological properties of dough during the mixing process of dough as well as to predict its final products as summarized in Table 1.

Table 1. Traditional methods to measure the rheological properties of dough and bread

Method	Measured Material	Parameters
AACC Method 38-12A (Wet gluten, dry gluten, water-binding capacity, and gluten index)	Gluten	Water-binding capacity; Gluten index
AACC Method 54-10 (Extensigraph method, general)	Mixed dough	Resistance to extension; Extensibility; Area under curve
AACC Method 54-21 (Farinograph method for flour)	Dough mixing	Dough development time; Tolerance index; Stability; Time to breakdown
AACC Method 54-30A (Alveograph method for soft and hard wheat flour)	Mixed Dough	Maximum overpressure, P; Abscissa at rupture, L; Index of swelling, G; Curve configuration ration, P/L; Deformation energy of the dough, W.
AACC Method 54-40A (Mixograph method)	Dough Mixing	Peak time; Peak resistance; Area under curve, W.
AACC Method 10-11 (Baking quality of bread flour-sponge-dough, pound-loaf method)	Bread	Loaf volume

1.5 Non-traditional Method: Creep-recovery Test

A creep-recovery test is a type of deformation testing usually for viscoelastic materials. In this test, the initial stress is applied, and the resulting strains are measured over time. The test was chosen for dough because the long time or low frequency rheology is most relevant to real dough processing and bread rising conditions (Rao, 2007). This test allows clear separation of viscous and elastic behaviors of a food material (Lee & Mulvaney, 2003). Several models are used to explain the behaviors of the material during a creep-recovery test. These models are based on a set of two elements: a spring and a dashpot. A spring represents an ideal elastic material or a Hookean material, following Hooke's law of elasticity that can store and energy and returns to the original shape when the force is removed. On the other hand, a dashpot or a fluid body represents a viscous material or a Newtonian fluid that dissipate energy when the force acts on it. As shown in Figure 10, the Maxwell model consists of a spring connected in a series with a dashpot, while the Kelvin-Voigt model consists of a spring connected in parallel with a dashpot.

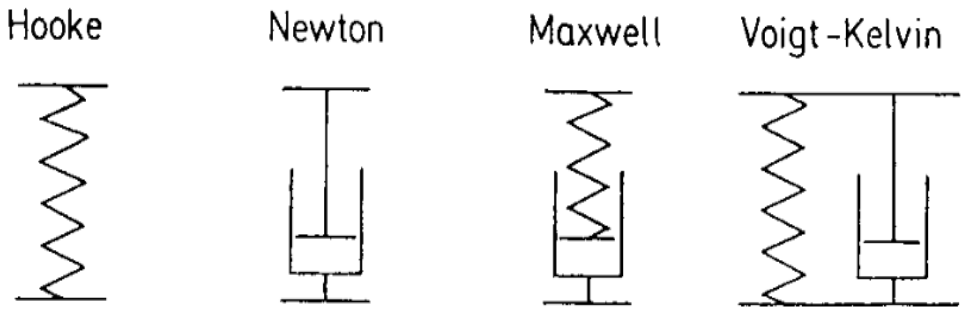


Figure 3. Mechanical analogs (from left to right:) A spring analog (Hooke's model), a dashpot analog (Newton's model), a spring-dashpot analog connected in series (Maxwell model) and a spring-dashpot analog connected in parallel (Kelvin-Voigt model) (Hess, 2012)

Further variations include a 3-element model, a 4-element model, and a 6-element model. The 3-element model consists of spring in a series with a Kelvin-Voigt model. A combination of Maxwell model and Kelvin-Voigt model is called Burger's model or the 4-element model. The addition of another Kelvin-Voigt model leads to the 6-element model (Figure 4).

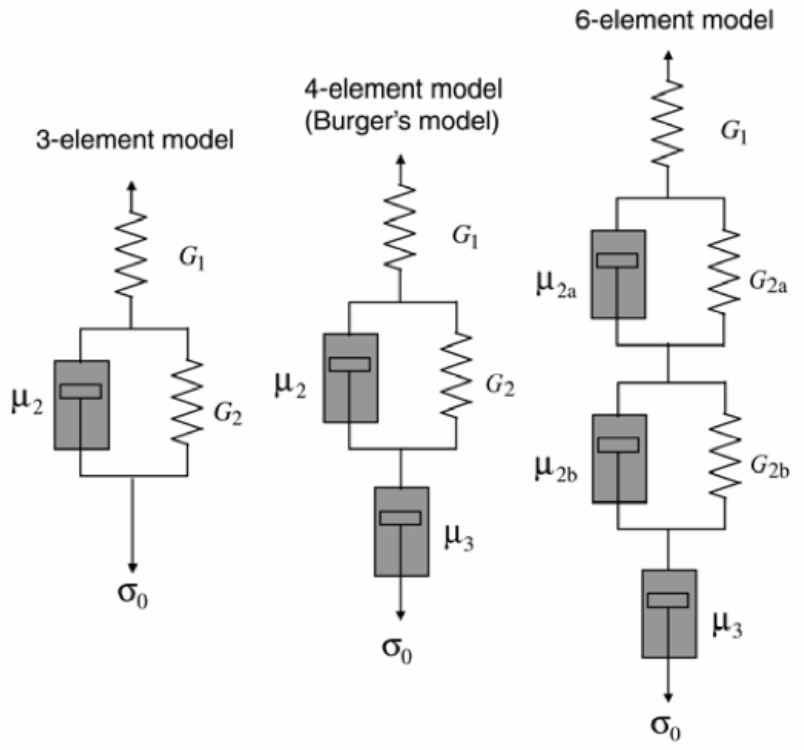


Figure 4. More complex mechanical analogs consisting springs and dashpots and representing elastic properties and viscous properties, respectively: 3-element model (left), 4-element model (middle), and 6-element model (right). (Chuang & Ye, 2006).

According to Steffe (1992), equations of total strain describing these mechanical analogs are defined as:

For the 3-element model,

$$\gamma(t) = \frac{\sigma_0}{G_1} + \frac{\sigma_0}{G_2}\left(1 - e^{-\left(\frac{G_2}{\mu_2}\right)t}\right)$$

For the 4-element model (Burger's model),

$$\gamma(t) = \frac{\sigma_0}{G_1} + \frac{\sigma_0}{G_2}\left(1 - e^{-\left(\frac{G_2}{\mu_2}\right)t}\right) + \frac{\sigma_0}{\mu_3}t$$

For the 6-element model,

$$\gamma(t) = \frac{\sigma_0}{G_1} + \frac{\sigma_0}{G_2}\left(1 - e^{-\left(\frac{G_{2a}}{\mu_{2a}}\right)t}\right) + \frac{\sigma_0}{G_3}\left(1 - e^{-\left(\frac{G_{2b}}{\mu_{2b}}\right)t}\right) + \frac{\sigma_0}{\mu_3}t$$

where, on the right side of the equation, the first term (from left) represents an instantaneous elastic strain. The second or third term represents a retarded elastic strain or Kelvin-Voigt model. The last term represents a viscous flow strain.

1.5. Flour Additives: Overview

Many types of flour additives are commonly used in the baking industry to adjust wheat flour to obtain desirable dough handling properties and final products. These include bleaching agents, oxidants (oxidizing agents), reductants (reducing agents), pH adjusting agents, emulsifiers and nutrient fortificants as summarized in Table 2.

Oxidizing agents are compounds added to dough formulations to cause the gluten network more resilient. Generally, this implies that dough is made more elastic and its gas holding ability

is promoted by facilitating the formation of disulfide bonds between glutenin subunits due to the potential of oxidizing agents to remove electrons from another reactant in a redox reaction (Figure 5). Commonly used oxidants include azodicarbonamide and calcium peroxide. This addition has been shown to increase the loaf volume of bread.

On the other hand, reducing agents are compounds used to inhibit the formation of disulfide bonds between gluten subunits and consequently make doughs mix faster and handle more easily. Commonly used reducing agents include the amino acid cysteine, sodium metabisulfite, and potassium sorbate. These chemicals react with the sulfhydryl groups of cysteine in the gluten protein and therefore prevent its reaction with other cysteine side groups bound in other chains in the gluten. (Sluimer, 2006).

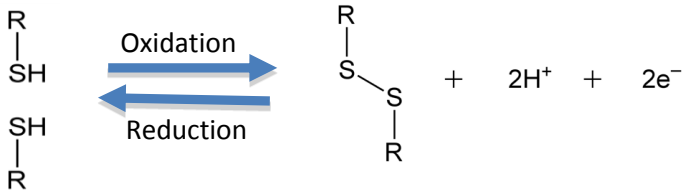


Figure 5. Formation and disruption of disulfide bonds by oxidation and reduction of sulfhydryl groups.

Table 2. Summary of Common Chemical Flour Additives

Oxidizing agents	**Reducing agents**	**Bleaching agents**	**pH adjusting agents**	**Emulsifiers**
Ammonium persulphate Ascorbic acid Azodicarbonamide Calcium bromate Calcium iodate Calcium peroxide Chlorine Pottasium Bromate Potassium iodate	Potassium sorbate L-cysteine Sodium metabisulfite	Acetone peroxide Ammonium persulphate Benzoyl peroxide Chlorine (gas) Chlorine dioxide (ClO_2)	Ammonium Chloride (acidifier) Ammonium phosphate, monobasic	DATEM

In conclusion, the chemistry of gluten, the mixing conditions and the types of additives influences the rheological properties of the bread dough. This thesis is aimed to be a continued work from previous studies in a food engineering lab, focusing on food texture. One of the objectives of this study is to evaluate the rheological responses of glutens from different wheat cultivars (different chemistry) as modified by the additions of L-cysteine by using a non-traditional method (the Compression-Recovery (CORE) instrument) to compare the result against an extension test (TA-XTPlus equipped with SMS/Kieffer Extensiblity Rig.) It is hypothesized that, with L-cysteine addition, the strength of the dough made with strong wheat cultivars could be reduced more significantly than the strength of dough made with weak wheat cultivars. Another objective of this thesis is to evaluate dough rheological effects by adding the combination of L-cysteine and microbial transglutaminase, which is a non-traditional additive, by using the extension test and a creep-recovery test. It is hypothesized that the cross-links caused by microbial transglutaminase could mask the reduction of strength caused by the addition of L-cysteine.

CHAPTER 2

EVALUATING THE RHEOLOGICAL RESPONSES OF GLUTENS FROM DIFFERENT WHEAT CULTIVARS AS MODIFIED BY ADDITION OF L-CYSTEINE

2.1 Introduction

The two most important reasons to include a reducing agent in a bread recipe are to decrease dough resilience (the capacity of a material to store energy, reflecting the elasticity of the dough) and to reduce mixing time (Sluimer, 2007).

The undesirable shrinkage of elastic dough on continuous production lines, e.g., for croissants, after sheeting, could be avoided by the addition of reducing agents. In contrast to the addition of oxidizing agents, as discussed in the previous chapter, reducing agents during dough mixing results in weaker doughs and increased solubility of the glutenin proteins. Graveland et al. (1985) reported that during dough mixing the SDS-insoluble gel protein is broken down to an SDS-soluble form as a consequence of the reduction of disulfide bonds. Adding a reducing agent cysteine makes the dough more extensible and less resilient (Lambert and Kokini, 2001).

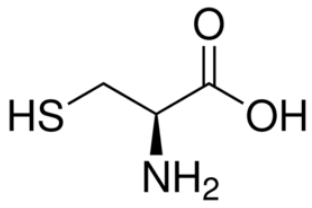


Figure 6. The chemical structure of L-cysteine.

The addition of cysteine accelerated the dough formation, visible as a faster (increase) rate of the resistance to deformation. Because its molecular mass is much smaller than that of naturally occurring thiol compounds in flours, its mobility is greater, and therefore its activity in the

interchange reaction (as shown in Figure 5) is higher, which means the thiol group of L-cysteine (Figure 6) can interact with gluten with disulfide linkages, which make the gluten more elastic at the beginning of the mixing process. This mechanism could explain the faster formation of gluten while mixing at the beginning (Sluimer, 2007). However, as the mixing is continued, the thiol group of cysteine acts as a disruptor of disulfide bonds among glutens, resulting in more extensible dough.

To summarize, L-cysteine or L-cysteine hydrochloride could function in foods as a flour improver, acting as a reducing agent to facilitate mixing. L-cysteine breaks the disulfide bonds of the native (without prior drying) gluten structure enabling faster dough development in the mixer and making the finished dough much easier to roll out into thin sheets (more extensible). That is to say, L-cysteine could be used to improve the machinability of bread and biscuit dough. Other reducing agents, having similar functions to L-cysteine, include sulfur dioxide, sodium metabisulphite, glutathione, inactive yeast, and protease.

It is hypothesized that, with L-cysteine addition, the strength of the dough made with strong wheat cultivars could be reduced more significantly than the strength of the dough made with weak wheat cultivars. This work is expected to provide additional information to what have been previously done in the food rheology/texture lab at Cornell of how different wheat cultivars (Hollis, Trego, Norpro, and Stephen) respond to an additive, such as L-cysteine as compared to commercial bread flour (Pillsbury®) by using TA_XTPlus equipped with SMS/Kieffer Extensibility rig and Compression-Recovery ("CORE") test, a novel instrument.

2.2 Materials

Four wheat cultivars (Hollis, Norpro, Trego, and Stephens) were obtained from certified seed representing three US wheat classes (out of six US wheat classes): Hard Red Winter (HRW), Hard Red Spring (HRS), and Hard White (HW). They were milled at Grain Inspection Packers and Stockyard Administration (GIPSA) into flours. The cultivars were characterized in terms of their flour protein, high molecular glutenin subunit (HMW-GS) composition, Zeleny sedimentation values, wet gluten content, gluten index (GI) and bread loaf volume (Table 2).

A commercial bread flour (Pillsbury BEST® Bread Flour) was purchased from a local supermarket.

NaCl (purity ≥ 99.5%, formular weight = 58.44) was purchased from Fisher Scientific (Lot 096144).

L-cysteine (purity ≥ 97%, molecular weight = 121.16) was purchased from Sigma-Aldrich, Inc. (Batch MKBJ6322V).

Mineral oil (Mineral Oil U.S.P, Rite Aid Corporation, Harrisburg, PA) was purchased from RiteAid (Ithaca, NY). The active ingredient is mineral oil, and the inactive ingredient is D-Alpha Tocopherol (Vitamin E) as a stabilizer.

DI water was used throughout all the experiments.

2.3 Methods

2.3.1 Sample preparation

As shown in Figure 7, a Glutomatic machine (Perten Instrument AB, Huddinge Sweden) was used to prepare gluten. Ten grams of flour samples from different wheat cultivars was

transferred to the Glutomatic washing chambers with the 88 micron polyester sieve in place. This flour sample without the addition of L-cysteine was served as a control. 0.002 grams of L-cysteine was added (in 10 g of flour to achieve 200 ppm). The wash chamber was shaken gently to spread out the sample evenly. 4.8 ml of the 2% sodium chloride solution from the dispenser was added. The test chamber was shaken gently so that the water was spread evenly over the sample. The wash chamber (with the sieve holder magnet facing inwards) with the sample was brought into the working position, and the Glutomatic machine was started. When the Glutomatic machine stopped, the washing chamber was removed, and the gluten was carefully taken out without stretching or tearing it. The mixing hook and the washing chamber should be checked to make sure that no gluten was left. Throughout all experiments, the temperature was maintained at 22 ± 2 ^{o}C.

Figure 7. Glutomatric washing machine (developed by Perten Instruments AB, Huddinge, Sweden)

2.3.2. Wet gluten (%) and gluten index

The procedure was based on the approved method 38-12.02 (AACC 2000). The wet gluten from step **2.3.1**. was put into gluten index cassettes (Figure 8) within 30 seconds and then centrifuged at 6000 ± 5 rpm for 1 minute. The gluten index cassettes were removed from the

centrifuge. A spatula was used to remove the retained gluten passed through the sieves (Figure 8). The gluten was weight to nearest 0.01 g on a balance and recorded as "wet gluten remaining on sieve (g)." The gluten was left on the balance. Tweezers were used to remove the rest of the gluten (on top of the sieve). The rest of gluten was added to gluten on balance. The total weight was recorded as "total wet gluten." Wet gluten content and gluten index were calculated as the followings:

Wet gluten content, % (14% moisture content) = $\frac{total\ wet\ gluten\ (g)\ x\ 860}{100 - \%sample\ moisture}$

Gluten index = wet gluten remaining on sieve (g)/total wet gluten x 100

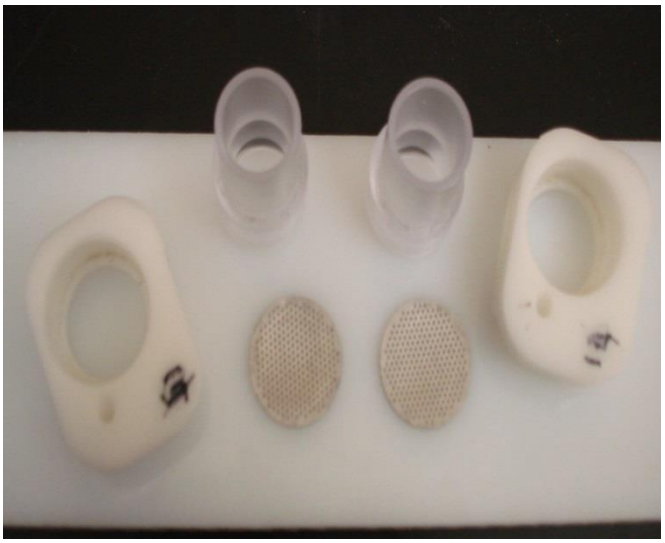


Figure 8. Gluten index cassettes with sieves

2.3.3. Protein content

The distribution ratio of polymeric to monomeric proteins was analyzed using a modified method based on Wang et al. (2007) and Fu & Kovacs (1999). During the extraction of protein

fractions, all the steps of protein was precipitated with acetone (40 and 80%) at -20 ^{o}C. The time was extended from 24 to 48 hr. The total protein (dry weight basis) on each fraction was determined using approved methods 46-30 (AACCI 2000) based on Dumas nitrogen combustion in a LECO FP-528 nitrogen analyzer (LECO Corporation, St Joseph, MI). Samples EDTA was used as standard, and the protein to N ratio was 5.7. The value of 5.7 was calculated from the amount and nitrogen content of gliadin and glutenin in wheat by Osborne (1907). The proteins contain about 17.5% of nitrogen (100 ÷ 17.5 = 5.7) (Tkachuk, 1969). This work was done in Dr. Patricia Raya-Duarte's laboratory at Oklahoma State University (OSU).

2.3.4. Sedimentation test (Zeleny sedimentation number)

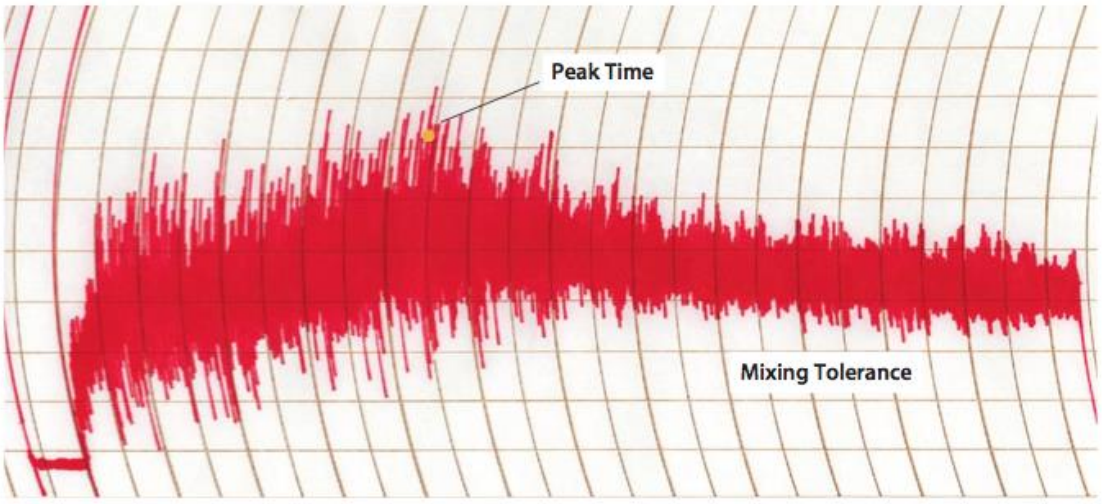
This test were done at Greats Plain Analytical Laboratory (Kansas City, MO). Using the approved method 56-61A hand mixing procedure (AACCI 2000), the volume (in ml) of sediment in a cylinder after mixing lactic acid into a flour suspension for 5 minutes was read as the sedimentation value. From bread baking standpoint, quality and quantity (strength) of gluten in wheat flour can be predicted by this test.

2.3.5. Mixograph

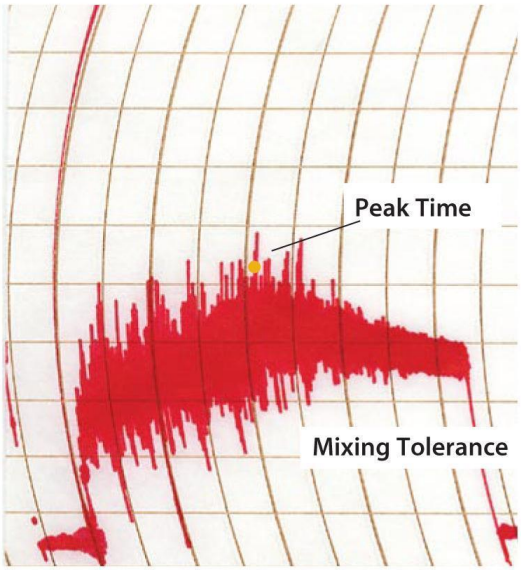
Samples were run on a 35-g Mixograph (National Manufucturing Div., TMCO, Inc., Lincoln, NE). Samples of 35 grams of flour on a 14 percent moisture basis were weighted and placed in a mixograph bowl. Water was added to the flour samples and the bowl (shown in Figure 9) is inserted in the mixograph. The machine was turned on, and the flour and water were mixed together to form a dough. While the dough was being mixed, the mixograph recored a curve on a

graph paper (as shown in Figure 10). In Figure 10, the horizontal axis represents the percentage of torque, while the vertical axis represents time. The mixograph measures the resistance of a dough against the mixing action of pins as shown (Figure 10). Strong gluten flour has a longer peak time and wider mixing tolerance than weaker gluten flour (Figure 10). This work was done previously in Dr.Mulvaney's laboratory at Cornell University.

Figure 9. 35-g Mixograph (National Manufucturing Div., TMCO, Inc., Lincoln, NE)



Strong Gluten Flour



Weak Gluten Flour

Figure 10. Examples of mixograms showing a comparison of strong and weak gluten flour

2.3.6. HMW-GS composition

The allelic variation of HMW-GS was determined in one dimensional sodium dodecyl polyacrylamide gel according to the method described by Pfluger et al (2001) with the following modifications: gliadins were not extracted and a resolving gel of 12% acrylamide was used (Wang et. al., 2006). HMW-GS alleles were identified using standard cultivars and the numbering method of Payne and Lawrence (1983) and Shan et al. (2007). This work was done in Dr. Patricia Raya-Duarte's laboratory at Oklahoma State University (OSU).

2.3.7. Bread loaf volume

Pup loaves were baked at CII Laboratory Services (Kansas City, MO). AACC approved method 10-10B, an optimized straight dough method, was used (AACC, 2000). 100 g of wheat flour was mixed with 2.0 g dry yeast, 1.5 g sodium chloride, 6.0 g sugar and water that reaches an adsorption at 500 Brabender unit in a farinograph. The first fermentation time at 30 ^{o}C (85% relative humidy) was 30 min. The dough was punched and then was fermented for another 30 min. The cycle was repeated for the last (third) fermentation (30 min). Then, the dough was divided, rounded, molded and proofed at 35 ^{o}C for 55 min. Finally, the loaves were baked at 200 ^{o}C for 20 min. Loaf volume was measured by a rapeseed displacement (AACC approved method 10.05).

2.3.7. Extension testing (TA_XTPlus with SMS/Kieffer Extensibility Rig)

A texture analyzer (TA-XT2 model, Texture Technologies, Scarsdale, NY) with a 5 kg load cell and SMS/Kieffer extensibility rig was used (Figure 11). This rig was developed at the Kurt Hess Institute in Munich by Dr. Kieffer as an improvement to the extensibility measurements provided by the Brabender Extensograph. The rig comprised a dough sample preparation press and mould, a spring-loaded test rig and a test hook. A prepared sample from the preparation press was securely located in the jig so that the hook, positioned underneath, can move vertically through it. The specification of this instrument was as the followings:

Manufacturing Material: Aluminium, Stainless Steel & Delrin

Maximum Operating Temperature: 100 ^{o}C

Maximum Applied Load (Tension): 1 kg

TA Settings:

Mode: Measure force in tension (including maximum force & distance to break (extension))

Pre-Test Speed: 2.0 mm/s

Test Speed: 3.3 mm/s

Post-Test Speed: 10.0 mm/s

Distance: 75 mm

Trigger Force: Auto-0.5 N

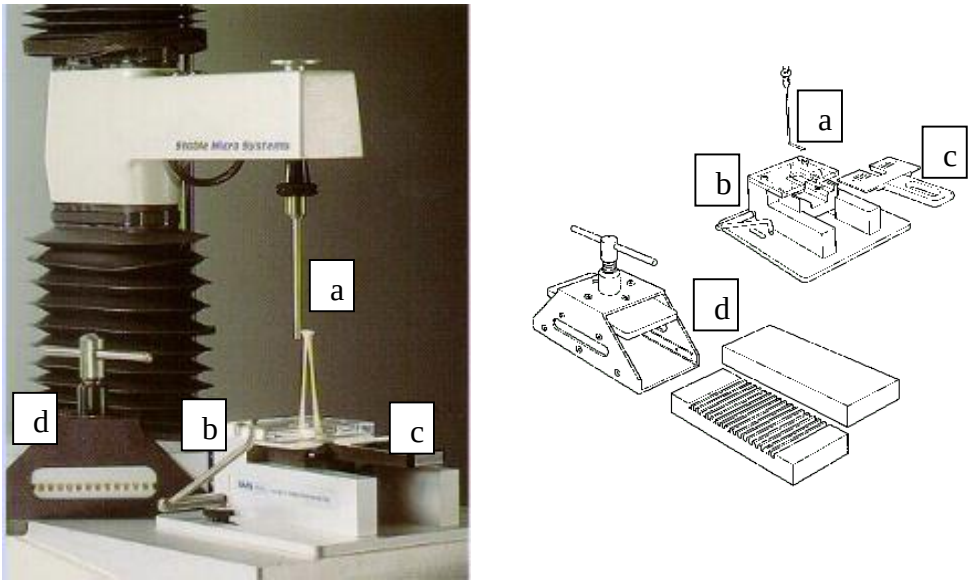


Figure 11. Schematic representation of texture analyzer (TA_XT2 Model, Texture Technologies, Scarsdale, NY) with Kieffer Extension Rig: (a) hook (with PTFE sleeve), (b) spring-loaded test rig, (c) sample platform, (d) sample preparation press and mould

2.3.6.1. Sample Preparation

A small amount of mineral oil was applied to both sides of the teflon dough form to avoid the sample adhesion. 10 g. of the prepared gluten sample was placed onto the grooved base of the form (Figure 12). The upper block of the form on top of the sample was positioned and pushed down firmly until the two blocks come together. Excess dough was cleanly removed from sides, using a knife/spatula and clamp the dough form in the form press for 40 minutes (this cuts the sample into strips, allows the gluten dough to relax and prevents loss of moisture). Any excess gluten dough was scraped off and forced out from the sides of the form. The dough press was loosened, and the upper form block was carefully slid backwards over the grooved base to uncover the first gluten dough strip. To remove the strip of gluten dough from the grooved base, the spatula was dipped in oil and were carefully slid under the sample. The first and last few strips may not be of full length, so these strips should be discarded.

Figure 12. Sample preparation press and mould

2.3.6.2. Test set-up of SMS/Kieffer extensibility rig

The Kieffer rig was positioned on the machine base. The hook probe must be covered with a plastic sleeve to prevent it from shearing through the sample. The hook probe was lowered to

just above the upper surface of the spring loaded clamp. The strip of gluten dough was placed onto the grooved region of the sample plate and the spring loaded clamp lever was held down. The sample plate was inserted into the rig. The handle slowly was released, and then the extension test could be initiated (Figure 13).

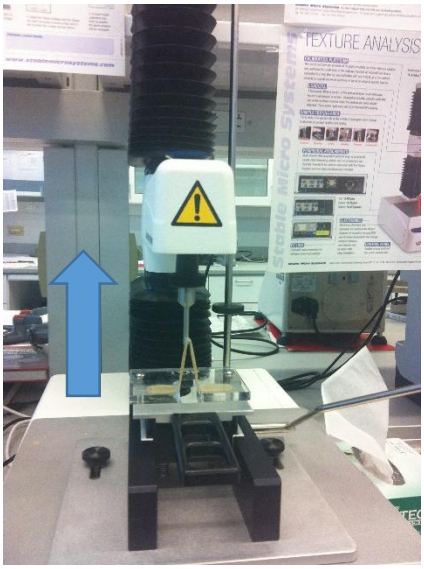


Figure 13. Texture analyzer (TA-XT2-Plus) with SMS/Kieffer extensibility rig in action

2.3.7. Compression-Recovery Testing ("CORE")

The "CORE" stands for compression-recovery and is a novel instrument supplied by Perten Instruments AB (Huddinge, Sweden). It has a sensitive 50 Newton load cell allowing the probing of the weak network structures in dough and gluten. It consists of a force transducer and a height sensor. The instrument can help determine the elasticity of gluten by measuring the ability of a gluten sample to recover freely after being subject to a biaxial compression force of 8 N for 5 seconds. The CORE has a compression phase followed by a recovery phase. A unique feature of

the CORE is that compression is applied to a cylindrical sample until a force set point is reached and is held constant for 5 seconds.

After gluten was removed from the Glutomatic washing instrument, it was placed in a shaper (Figure 15) and centrifuged in the shaper for 5 minutes using a Perten Centrifuge 2015 at 6000 ppm (Perten Instruments AB, Huddinge, Sweden). Reasonably cylindrically shaped samples were obtained using this procedure. After the samples had been removed from the shaper, they were immediately tested on Compression and Recovery (CORE) analyzer (Figure 14) to minimize moisture loss. However, before the testing could be initiated, a calibration step is required. At this step, an object with known weight and height was used to calibrate the instrument. After the calibration, the samples could be tested by loading the samples onto the lower plastic plate of the CORE analyzer. The test run consisted of compression-holding-recovery cycle. The gluten sample was compressed for 5 seconds and followed by holding the probe for 5 seconds. Finally, the probe was moved back to the original position, and this "recovery" period lasted for 55 seconds to allow the sample to relax. Thickness of the sample was recorded over time as a graph. The recovery index was calculated as the percentage of the final thickness and the original thickness of the gluten sample. Five replicates were performed for each sample

Figure 14. CORE instrument supplied by Perten Instruments AB (Huddinge, Sweden)

Note: the arrow shows the direction of the movement of the probe.

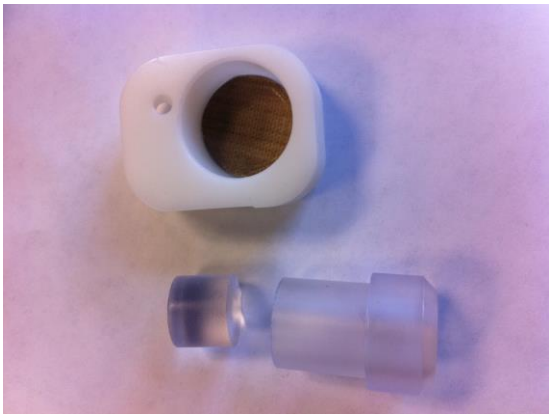


Figure 15. Gluten sieve cassette with additional (brown) plate

In summary, the setting of CORE was the followings:

Target Force: 0.5 N

Hold time start: 0 sec

Velocity at the starting point: 20 mm/sec

Target force compression: 8 N

Minimum distance: 1 mm

Target force recovery: 0.15 N

Compression time: 5 sec

Recovery time: 55 sec

Velocity compression: 4 mm/sec

2.3.8. Statistical analysis

All cultivars were tested in at least triplicates using independent samples. One-way analysis of variance (ANOVA) with a significance level of $\alpha = 0.05$ was performed to compare mean values of four wheat cultivars and a commercial flour to determine statistically significant differences for the parameters obtained from Perten Instruments AB (Huddinge, Sweden) the extension test (Kieffer) and the compression-recovery (CORE) tests. Tukey-Kramer HSD (honestly significant difference) test was performed for comparisons of means for each treatment ($\alpha = 0.05$). All analzses were conducted by statistical software JMP® (SAS Institute Inc., USA).

2.4. Results and Discussion

Table 3. Physicochemical properties of different wheat cultivars (Stephens, Trego, Norpro and Hollis): wheat class, protein content **(%**±SD**)**, Wet Gluten (%), Gluten Index, Gluten Moisture Content (MC) (%), Zeleny Sedimentation Value (Zeleny), High-Molecular-Weight Glutenin Subunit (HMW-GS) gene at *Glu-D1* locus and Bread Loaf Volume (BLV)

Cultivar	**Wheat Class**	**Protein Content (%**±SD**)**	**Wet gluten (%)**	**Gluten Index**	**Gluten MC (%)**	**Zeleny**	**HMW-GS at *Glu-D1* locus**	**BLV (mL)**
Stephens	SWH	11.62±0.03	35.5±1.1	42.7±0.3	66.0±0.0	22	2,12	675.00±7.22
Trego	HDWH	10.34±0.04	26.8±1.1	97.6±1.2	64.5±0.2	38	5,10	743.75±20.01
Norpro	HRS	12.04±0.1	31.5±1.0	88.6±1.0	66.0±0.7	36	5,10	787.50±6.25
Hollis	HRS	13.01±0.04	32.6±1.1	96.3±0.7	65.3±0.1	50	5,10	881.25±23.59

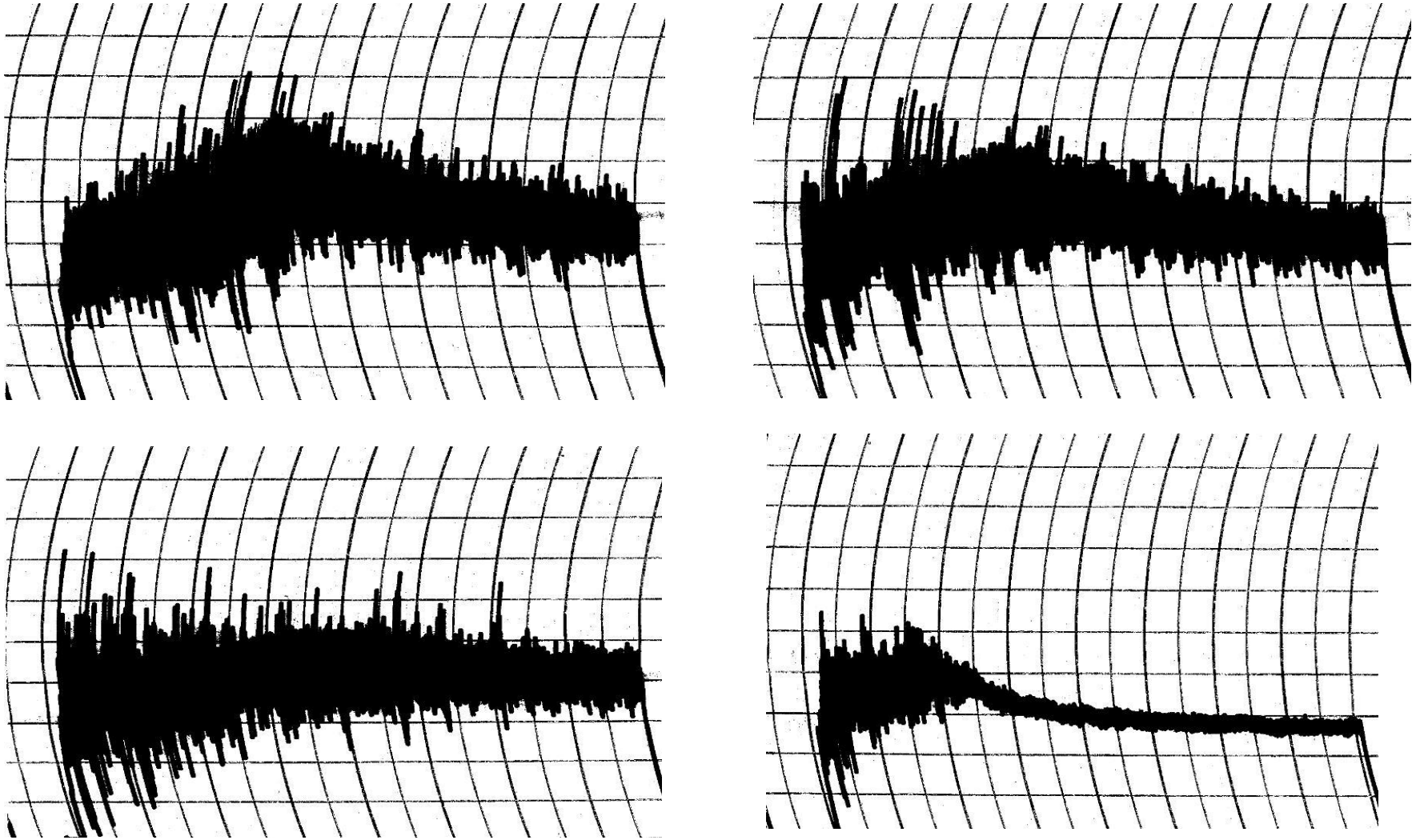


Figure 16. Mixograms (the vertical axes are % torque (not shown) and the horizontal axes are time (minute per each space) (not shown)) of different wheat cultivars: Hollis (left above), Norpro (right above), Trego (left below) and Stephen (right below)

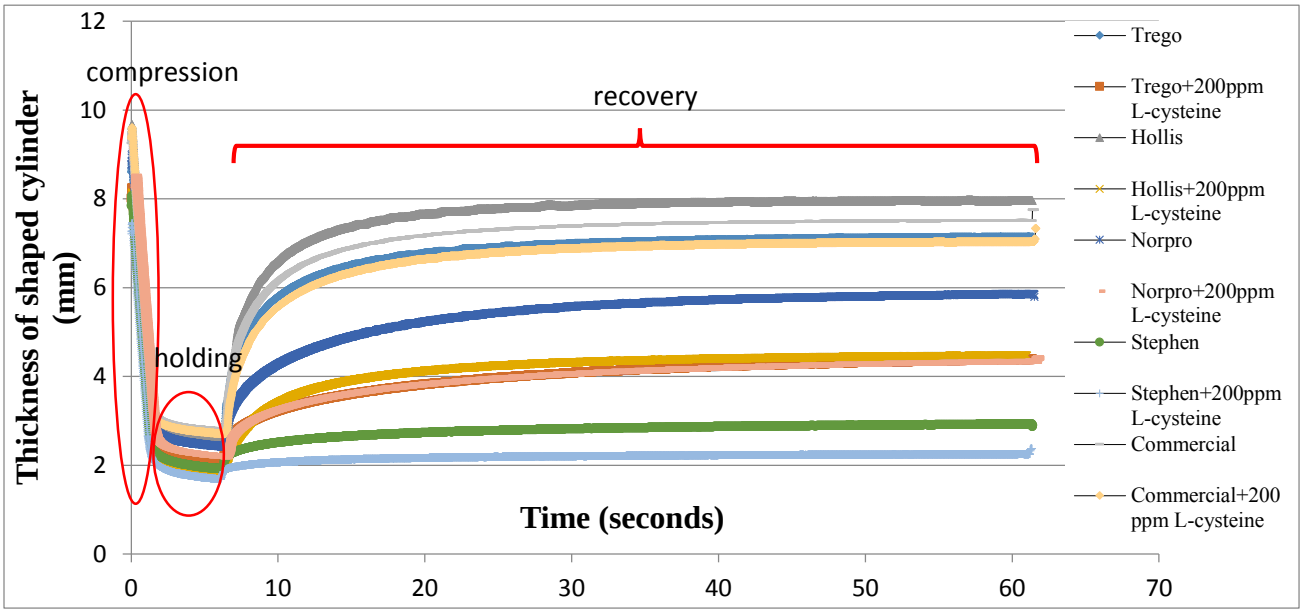


Figure 17. CORE Result: Thickness (mm) during compression (for 5 seconds) – holding (for 5 seconds)- recovery (for 55 seconds) cycle for four different wheat cultivars (Hollis, Norpro, Trego, and Stephen) and commercial flour (Pillsbury®) with and without 200 ppm (mg/kg flour) L-cysteine measured by the CORE instrument at 5 N.

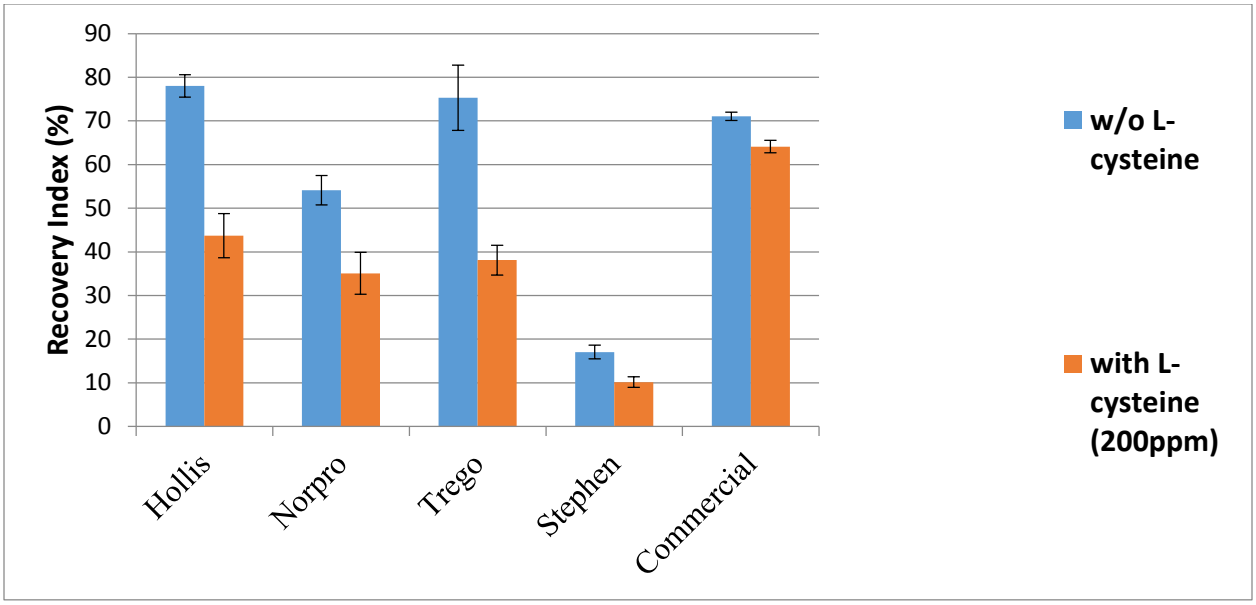


Figure 18. Recovery Index (%) vs. Four Different Wheat Cultivars (Hollis, Norpro, Trego and Stephen) and commercial flour (Pillsbury®) with and without 200 ppm L-cysteine measured by the CORE instrument at 5 N. Recovery Index (%) = $\frac{the\ final\ thickness\ x\ 100}{the\ original\ thickness}$. Five replicates were performed for each sample (Error bars represent ± standard deviations. The mean comparison was summarized in the following table.)

Table 5. Summary of recovery index (%) means of wheat cultivars (Hollis, Norpro, Trego and Stephen) and commercial flour with and without 200 ppm L-cysteine measured by the CORE instrument at 5 N.

Level	**Grouping (by Tukey-Kramer HSD test)**						**Least Square Mean of Recovery Index (%)**
Hollis	A						78.00
Trego	A						75.31
Commercial	A	B					71.05
Commercial + 200 ppm L-Cysteine		B					64.10
Norpro			C				54.13
Hollis + 200 ppm L-Cysteine				D			43.72
Trego + 200 ppm L-Cysteine				D	E		38.12
Norpro + 200 ppm L-Cysteine					E		35.09
Stephen						F	17.06
Stephen + 200 ppm L-Cysteine						F	10.15

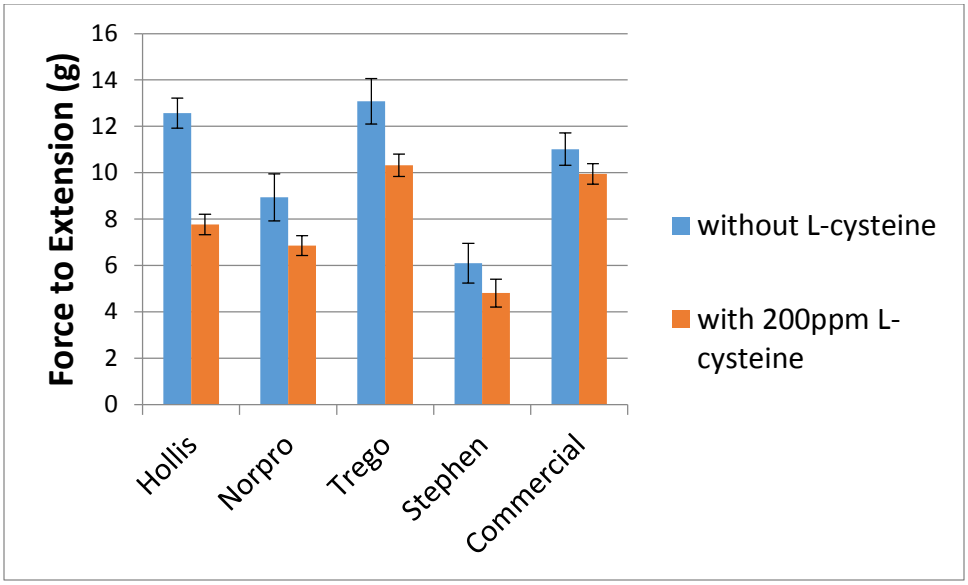


Figure 19. Maximum force to extension (N) of four different wheat cultivars (Hollis, Norpro, Trego, and Stephen) and commercial flour with and without 200 ppm L-cysteine measured by the TA-XTPlus equipped with SMS/Kieffer extensibility rig (five replicates). Error bars represent ± Standard Deviations (SD). The mean comparison was summarized in the following table.

Table 6. Summary of force to extension (N) means of wheat cultivars (Hollis, Norpro, Trego, and Stephen) and commercial flour with and without 200 ppm L-cysteine measured by the TA-XTPlus equipped with SMS/Kieffer extensibility rig. (Grouping represents the grouping of mean comparison by Tukey-Kramer HSD (honestly significant difference) test at $\alpha = 0.05$.

Level	**Grouping (by Tukey-Kramer HSD test)**								**Mean of force to extension (N)**
Trego	A								13.08
Hollis	A	B							12.80
Commercial		B	C						10.94
Trego + 200 ppm L-Cys			C	D					9.95
Commercial + 200 ppm L-Cys			C	D					8.94
Norpro				D	E				7.77
Hollis + 200 ppm L-Cys					E	F			6.85
Norpro + 200 ppm L-Cys						F	G		6.10
Stephen							G	H	0.62
Stephen + 200 ppm L-Cys								H	0.49

As shown on Table 3, loaf volume ranged from poor (650 cc, Stephen) to good (700 to 800 cc, Norpro and Hollis) to excellent (>800 cc, Hollis). Protein content (%) of flour might not be able to predict bread loaf volume (BLV), which is generally a main indicator of bread quality. As shown by the study of Bockstaele et al. (2008), the Pearson correlation between the protein content and the bread loaf volume among 17 wheat cultivars is 0.750 ($P < 0.01$). In our study, Hollis had the highest loaf volume (881.25±23.59%), Norpro (787.50±6.25%), Trego (743.75±20.01%) and Stephens (675.00±7.22%) were lower BLV, respectively, while Stephens (11.62±0.03%) had higher protein content than Trego (10.34±0.04%), suggesting that protein content did not correlate well with bread loaf volume.

As shown on Table 3, BLV correlated well with HMW-GS that can be used to categorize wheat into strong or weak cultivars. Stephen contained "2,12" HMW-GS gene on *Glu-D1* locus, while Hollis, Norpro and Trego contained "5,10" HMW-GS gene at the same locus. This is accordant with the study by Popineau et al (2001), showing that the "1D" HMW-GS gene expressed at "5,10" HMW-GS gene resulted in higher dough strength than "2,12" HMW-GS gene due to the presence of an additional cysteine residue available for formation of interchain disulfide bonds led to the formation of more highly cross-linked gluten. As shown on Figure 16, the mixogram of Stephen has the least number of lines, showing less resistance to mixing than other three wheat cultivars. These results indicate that Stephen could be categorized as weak cultivar, while Hollis, Norpro and Trego as strong cultivars.

In addition, Zeleny sedimentation (Zeleny) values and BLV have a strong correlation (Correlation coefficient (R) = 0.853 ($P < 0.001$)) as shown by Dobraszczyk & Salmanowicz (2007). Zeleny values are the volumes of gluten can swell in the presence of lactic acid. The higher the Zeleny sedimentation value, the stronger the gluten strength. As shown in Table 3,

the result shows that Stephens flour swell the least, while Hollis swelled the most, and Norpro and Trego (38) fell between the two. This trend is similar to that of BLV (Table 3).

As shown by Chapman (2011), recovery index (%) measured with the CORE analyzer correlated well with the gluten strength (Pearson correlation = 0.855 at 0.01 level (two-tailed)). Recovery index is a parameter that reflect how much gluten can recover to its original length. In Table 5 and Figure 18, the recovery index (%) of Hollis was grouped together with the recovery index (%) of Trego and commercial bread flour (the same letter "A"), while the recovery index of Norpro, another strong cultivar, was less than the two ("C" group) but still more than the weak cultivar, Stephen ("F" group). The grouping of the recovery indexes could be correlated with glutenin macropolymer yield (GMP %). In Chapman's study (2011), Trego has the highest GMP% (19.05), while Hollis, Norpro and Stephens have 12.22%, 8.64% and 8.18%, respectively. This observation is supported by Don et al. (2003), who hypothesized that gluten is a particulate gel network that consists of the aggregation of glutenin macropolymer, influencing dough mechanical properties—the higher the aggregation, the higher the gluten strength as shown by the recovery index.

As shown in Figure 18 and Figure 19, the addition of L-cysteine to flour samples had an influence on the elastic recovery of the vital gluten (gluten directly extracted from flour) obtained from different wheat cultivars. After addition of L-cysteine, Hollis, Norpro and Trego (strong cultivars) showed a tremendous decrease in recovery index and thicknesses during compression-recovery cycle (grouped as "D," "E" and "D, E" respectively) (Table 5 and Figure 18), while the weak cultivar, Stephen, showed no significant difference between with and without L-cysteine addition. This result suggests that the strong cultivars have more strength or elasticity to lose than the weaker cultivar. At the molecular level, this result suggests that the strong cultivars possess

more effective disulfide bonds available for L-cysteine to disrupt those bonds; as a consequence, a network that contributes to the gluten network and therefore the elasticity of strong cultivars were more affected than a weak cultivar.

As shown in Figure 18, while the recovery indexes (%) of strong wheat cultivars (Hollis, Norpro and Trego) were greatly reduced by the addition of L-cysteine, the recovery indexes (%) of weak cultivars (Stephen) and commercial bread flour were less affected, although the recovery index of commercial bread flour was similar to those of strong wheat cultivars. This result could be due to the presence of ascorbic acid as a dough conditioner in the commercial bread flour. As discussed in the previous chapter, ascorbic acid during mixing process can interact with oxygen from the air and be converted to dehydroascorbic acid, which acts as an oxidizing agent as opposed to the function of L-cysteine, which is a reducing agent. Therefore, the presence of ascorbic acid could buffer the reduction potential of L-cysteine.

On Table 5 and Table 6, the force to extension from TA-XTPlus equipped with SMS/Kieffer extensibility rig also shows slightly different trend from the recovery index obtained from the CORE analyzer. These differences are due to the nature of the instrument; one is in a compression mode whereas the other is in an extensibility mode. However, compared to Kieffer extension test, the CORE analyzer could better distinguish flour into different wheat cultivars as shown by the grouping of each sample based on Tukey-Kramer HSD test.

CHAPTER 3

EVALUATING THE EFFECTS OF THE COMBINATION OF L-CYSTEINE AND MICROBIAL TRANSGLUTAMINASE ON RHEOLOGICAL PROPERTIES OF GLUTENS BY CREEP-RECOVERY AND EXTENSION TESTS

3.1 Introduction

The use of enzymes in food processing has played a significant role in improving the quality of products. Enzymes produced by microorganisms, about 10-100 mg/kg of flour, have a rapidly growing application in wheat flour (Sluimer, 2007). For instance, enzymes like fungal amylases (to replace malt flour), proteases (to soften the gluten), lipases (to improve surface activity of endogenous lipids), and oxidase (to oxidize sulfur groups) are used to influence the whole baking process (Hozova et al., 2002; Schaffarczyk et al., 2014).

Transglutaminase (protein-glutamine gamma-glutamyltransferase, EC 2.3.2.13) is an enzyme capable of catalyzing acyl-transfer reactions introducing covalent cross-links between amide groups of proteins (i.e. glutamines) and various primary amines (i.e. lysines) as shown in Figure (Nonaka et al., 1989). Therefore, many protein functionalities can be specifically modified by transglutaminase (Motoki & Kumazawa, 2000).

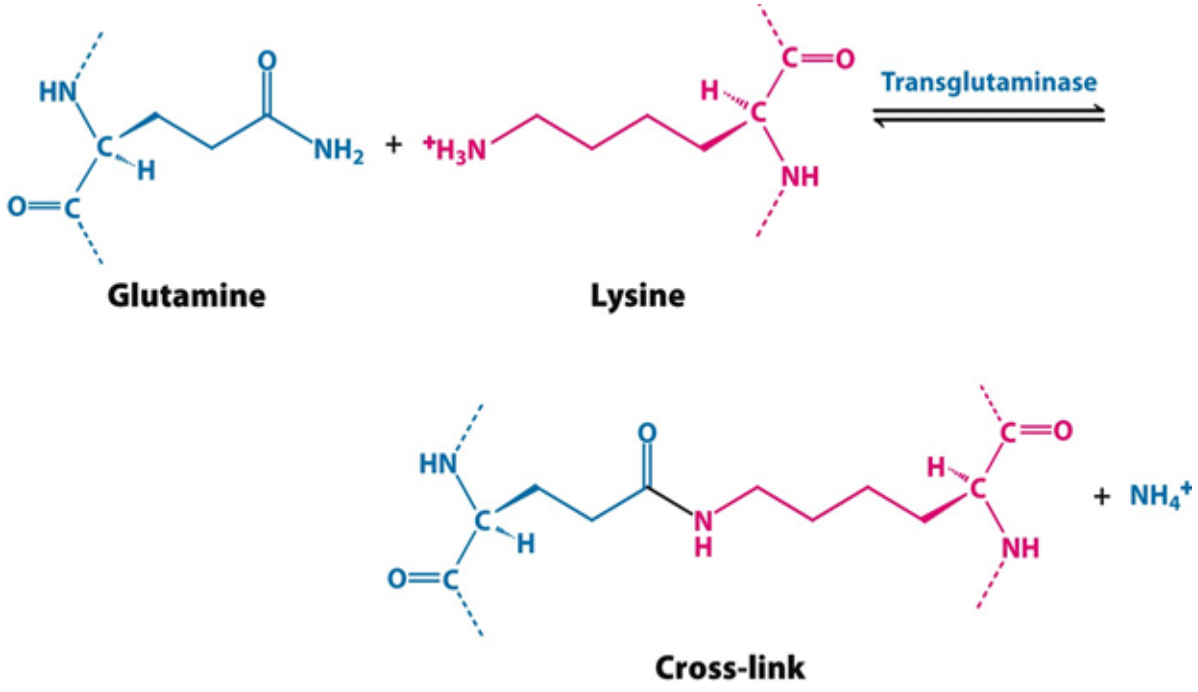


Figure 20. A general mechanism of how transglutaminase catalyzes a cross-link reaction (Berg et. al.)

The mechanism for the influence of transglutaminase in wheat dough systems has been characterized as a cross-linking reaction between the carboxyamide of a glutamine fraction and a primary amine of a lysine protein (Figure 20). Despite the low lysine content of gluten, these cross-links are formed, creating a gel-like network that is both heat and acid-resistant, leading to numerous advantages in the performance of the wheat dough (Yamazaki et al. 2001).

As shown on the previous chapter, the addition of L-cysteine leads to more extensible dough. Therefore, it is interesting to see how microbial transglutminase would interact with L-cysteine. In this study, the effects of the combination of L-cysteine and Microbial Transglutaminase on rheological properties of glutens measured by Creep-Recovery Test (Rheometer) and Extension Test (TA-XTPlus equipped with SMS/Kiffer Dough & Gluten Extensitability Rig) were evaluated. It was hypothesized that the rheological effects caused by

cross-links introduced by transglutaminase would counterbalance the extensible effects caused by the addition of L-cysteine.

3.2 Materials

Crude gluten powder (Lot 127H0169, 80% protein, 7% fat and EEC No. 232-317-7) was obtained from Sigma Chemical Company (St. Louis, MO USA).

NaCl (purity ≥ 99.5%, formular weight = 58.44) was purchased from Fisher Scientific (Lot 096144).

L-cysteine (purity ≥ 97%, molecular weight = 121.16) was purchased from Sigma-Aldrich, Inc. (Batch MKBJ6322V).

Microbial transglutaminase in a powder form, commercially known as ACTIVA® TI, was obtained from Ajinomoto Food Ingredients LLC (Chicago, IL USA). The enzyme contained 100 units of enzyme activity per gram of powdered preparation (U/g). The enzyme was in powder form and was stored in properly sealed bags at room temperature. Open bags were frozen for later use.

Mineral oil (Mineral Oil U.S.P, Rite Aid Corporation, Harrisburg, Pa) was purchased from RiteAid (Ithaca, NY). The active ingredient is mineral oil, and the inactive ingredient is D-Alpha Tocopherol (Vitamin E) as stabilizer.

DI water was used throughout all the experiments.

3.3. Methods

3.3.1. Sample preparation

Samples were prepared by using the same method as section 2.3.1. In addition, 0.02, 0.04, 0.08, 0.12 and 0.16 g of transglutaminase were added to 10 g of flour samples to achieve the concentrations of 2000, 4000, 8000, 12000 and 16000 ppm, respectively. 0.001, 0.002 and 0.003 L-cysteine were added to 10 g of flour samples to achieve the concentrations of 100, 200 and 300 ppm, respectively.

3.3.2. Stress sweep testing

Stress sweep tests were done with a TA AR1000-N rheometer (TA Instruments, New Castle, DE), using parallel plate geometry (25mm plate diameter and 2.5 mm plate gap) in shear mode as shown on Figure 21. The following method is based on Liang (2006). The temperature of the Peltier plate was set to 25 ^{o}C. Stress range for gluten was set from 10-400 Pa at a frequency of 6.28 rad/s. When the parallel plate reached the test temperature, the sample was loaded onto the lower plate of the rheometer and secured with a very thin layer of glue (QuickTite, Loctite North America, Rocky Hill, CT), to minimize slippage. Then the upper 25mm crosshatched diameter plate was lowered until the gap between plates reached 2.5 mm. Excess sample was trimmed off. Exposed edge of the sample was covered with a thin layer of mineral oil (Mineral Oil U.S.P, Rite Aid Corporation, Harrisburg, Pa). In addition, humid air (80% relative humidity) generated in a water bath at 30 ^{o}C was circulated around the sample during the test to help minimize moisture loss during measurements. After loading, the samples were allowed to relax in the rheometer until the initial loading stress on the samples relaxed to zero. Storage modulus (G'), loss modulus (G") and complex modulus (G*) were obtained during the testing.

Figure 21. TA AR1000-N rheometer (TA Instruments, New Castle, DE), using parallel plate geometry

3.3.3. Creep-recovery testing

Creep-recovery tests were done with the same rheometer and parallel plate geometry in shear mode as described above in stress sweep testing. The loading of samples and the following procedure were the same as described in the above section. A shear stress of 40 Pa was preliminary chosen (Liang, 2006) and confirmed in this study as shown on the result from stress sweep testing because at shear stress of 40 Pa, no significant change of storage modulus (G') and shear modulus (G") occurred. In addition, creep time at 100 seconds and recovery time at 1,000 seconds were chosen.

3.3.4. Statistical analysis

All cultivars were tested in at least triplicates using independent samples. One-way analysis of variance (ANOVA) with a significance level of $\alpha = 0.05$ was performed to compare mean values of four wheat cultivars and a commercial flour to determine statistical significant differences for the parameters obtained from extension test (Kieffer) and compression-recovery (CORE) tests. Tukey-Kramer HSD (honestly significant difference) test was performed for comparisons of means for each treatment ($\alpha = 0.05$). All analyzes were conducted by statistical software JMP® (SAS Institute Inc., USA). Modeling the resulting creep-recovery curves were done by using MATLAB (Mathworks., USA)

3.4 Results & Discussions:

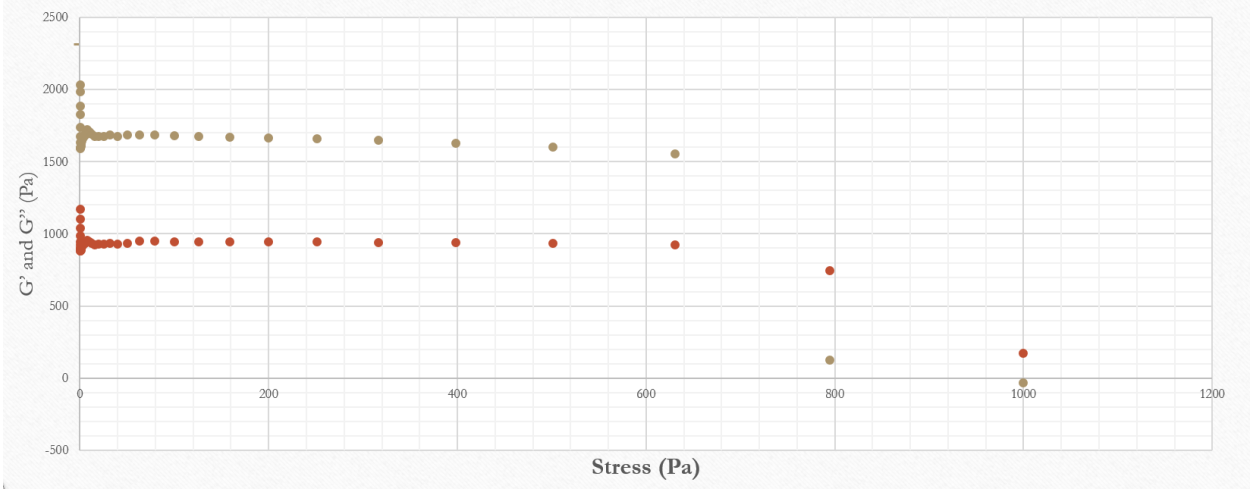


Figure 22. Stress sweep result of pure Sigma® gluten (as a control) as measured by TA AR1000-N rheometer with parallel plate geometry (25 mm plate diameter and 2.5 mm plate gap) in shear mode

(G' (tan dots on the figure) represent elastic or storage modulus (Pa). G'' (red dots on the figure) represent viscous or loss modulus (Pa).)

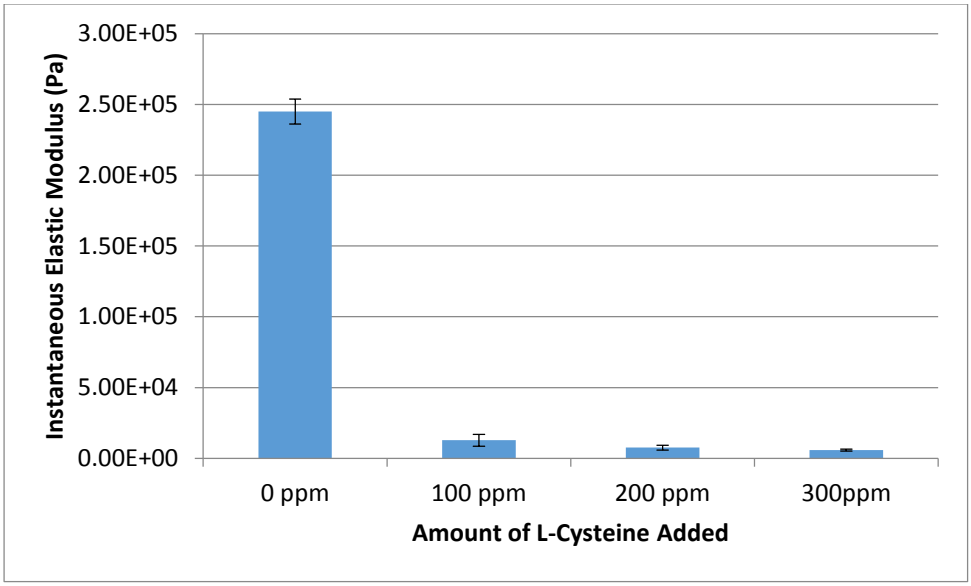


Figure 23. Instananeous elastic modulus (G_1, Pascal) of Sigma® Gluten mixed with L-cysteine at different concentrations (0, 100, 200 and 300 ppm) measured by the creep-recovery test with five replicates (n=5) (Error bars represent ± standard deviations.)

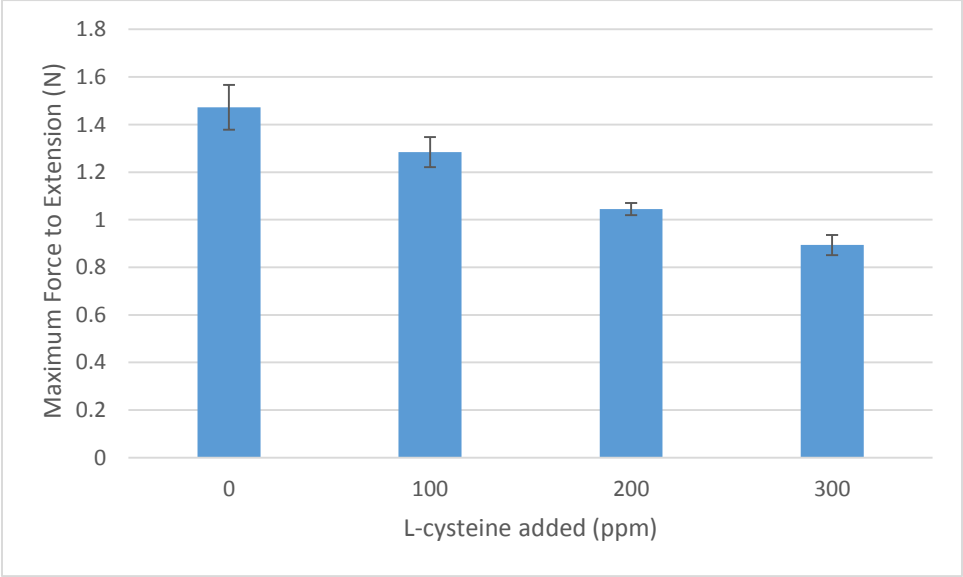


Figure 24. Force to extension (N) of Sigma® Gluten mixed with L-cysteine at different concentrations (0, 100, 200 and 300 ppm) measured by the extension test with five replicates (n=5) (Error bars represent ± standard deviations.)

Table 7. Summary of mean comparison of instantaneous elastic modulus (G_1, Pascal) of Sigma® Gluten mixed with L-cysteine at different concentrations (0, 100, 200 and 300 ppm) measured by the creep-recovery test with five replicates (n=5)

L-cysteine (ppm)	**Grouping (by Tukey-Kramer HSD test)**		**Least Square Mean of G_1 (Pa)**
0	A		2.417×10^5
100		B	1.280×10^4
200		B	7.649×10^3
300		B	5.848×10^3

Table 8. Summary of mean comparison of force to extension (N) of Sigma® Gluten mixed with L-cysteine at different concentrations (0, 100, 200 and 300 ppm) measured by the extension test with five replicates (n=5)

L-cysteine (ppm)	**Grouping (by Tukey-Kramer HSD test)**				**Least Square Mean of Force to Extension**
0	A				1.47
100		B			1.20
200			C		1.06
300				D	0.92

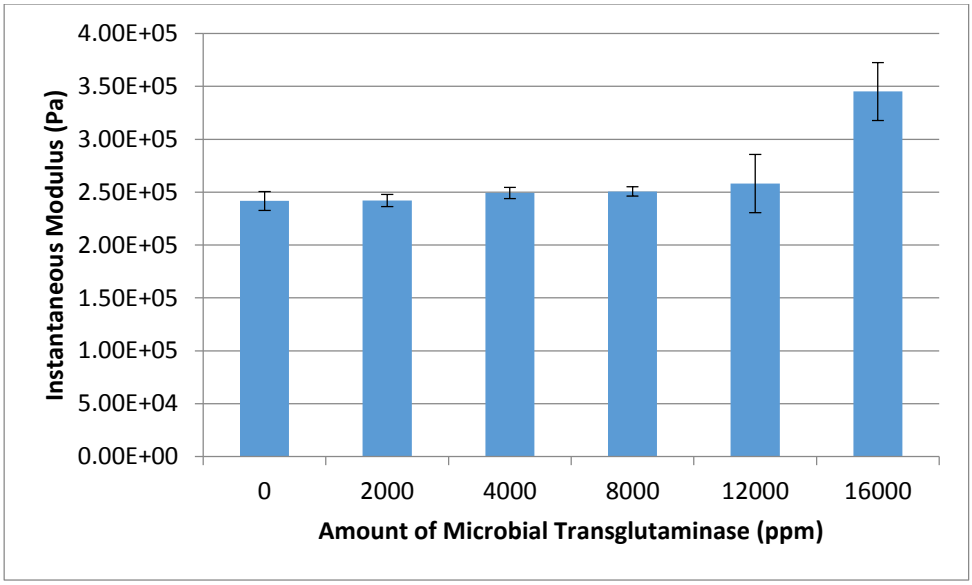


Figure 25. Instananeous elastic modulus (G_1, Pascal) of Sigma® Gluten mixed with microbial transglutaminase at six different concentrations (0, 2000, 4000, 8000, 12000 and 16000 ppm) measured by the creep-recovery test with five replicates (n=5) (Error bars represent ± standard deviations.)

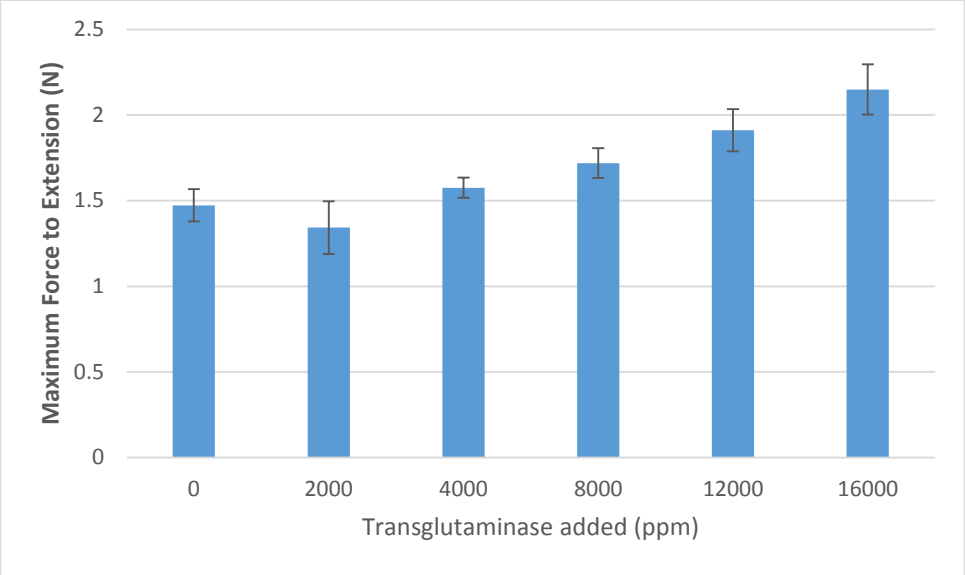


Figure 26. Instananeous elastic modulus (G_1, Pascal) of Sigma® Gluten mixed with microbial transglutaminase at six different concentrations (0, 2000, 4000, 8000, 12000 and 16000 ppm) measured by the extension test with five replicates (n=5) (Error bars represent ± standard deviations.)

Table 9. Summary of mean comparison of instantaneous elastic modulus (G_1, Pascal) of Sigma® Gluten mixed with microbial transglutaminase at six different concentrations (0, 2000, 4000, 8000, 12000 and 16000 ppm) measured by the creep-recovery test with five replicates (n=5)

Microbial Transglutaminase (ppm)	**Grouping (by Tukey-Kramer HSD test)**		**Least Square Mean of G_1 (Pa)**
16000	A		3.453×10^5
12000		B	2.580×10^5
8000		B	2.510×10^5
4000		B	2.490×10^5
2000		B	2.420×10^5
0		B	2.417×10^5

Table 10. Summary of mean comparison of force to extension (N) of Sigma® Gluten mixed with microbial transglutaminase at six different concentrations (0, 2000, 4000, 8000, 12000 and 16000 ppm) measured by the extension test with five replicates (n=5)

Microbial Transglutaminase (ppm)	**Grouping (by Tukey-Kramer HSD test)**			**Least Square Mean of Maximum Force to Extension** (N)
16000	A			2.15
12000	A	B		1.91
8000	A	B	C	1.72
4000	A	B	C	1.58
0		B	C	1.47
2000			C	1.34

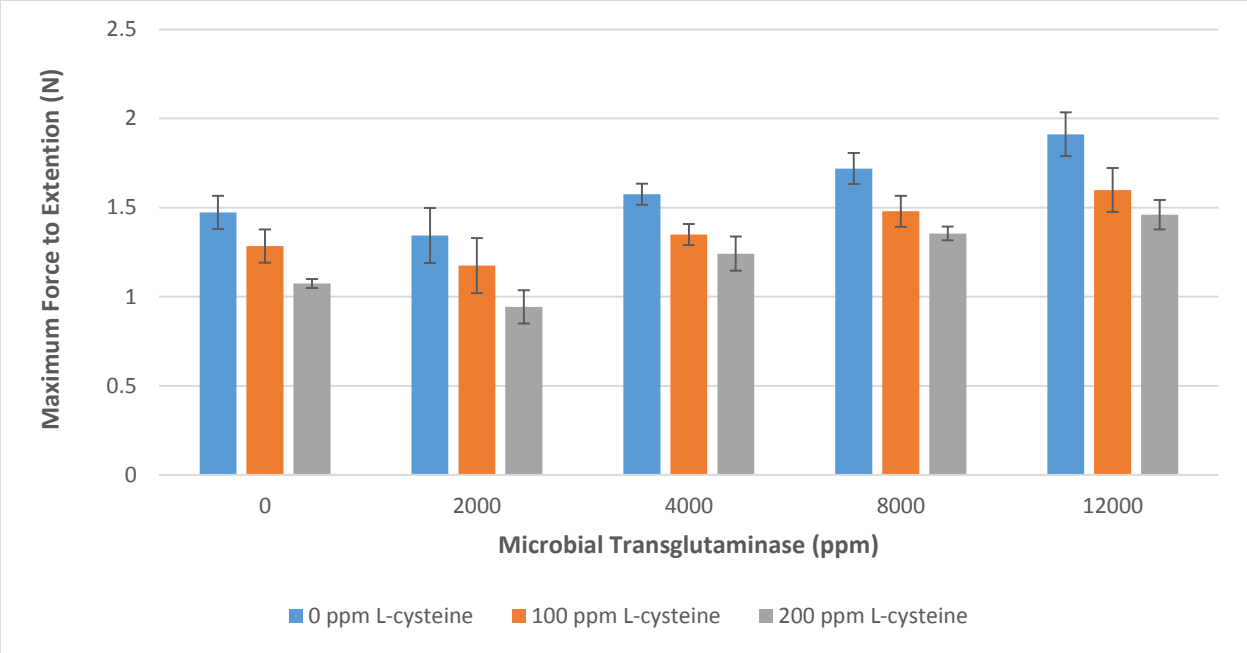


Figure 27. Force to extension (N) of Sigma® Gluten mixed with L-cysteine (0, 100 and 200 ppm) and microbial transglutaminase (0, 2000, 4000, 8000 and 12000 ppm) measured by the extension test with five replicates (n=5) (Error bars represent ± standard deviations.)

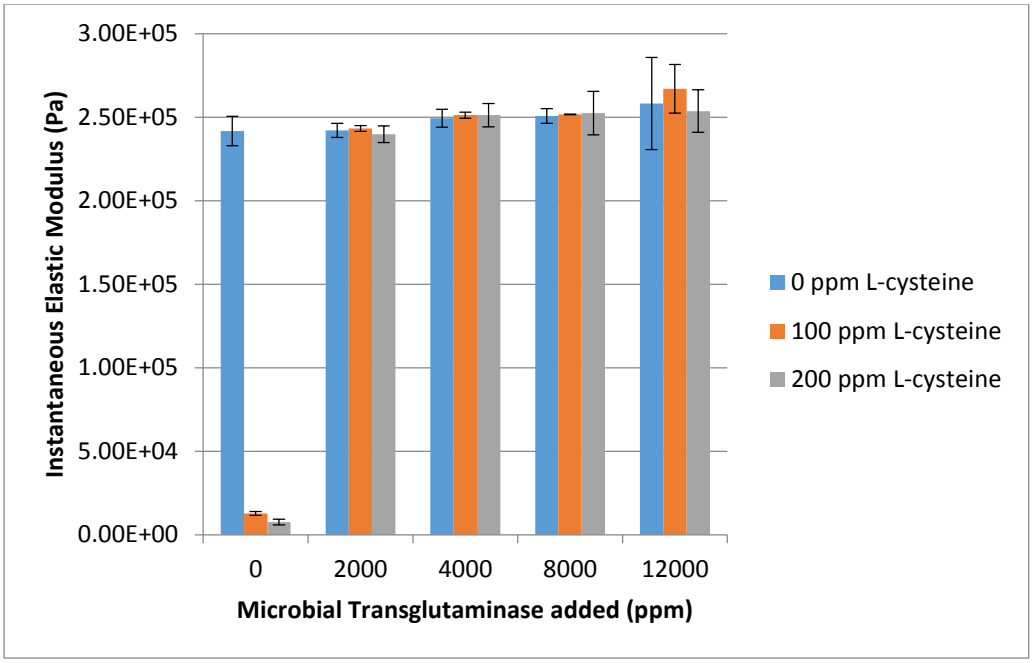


Figure 28. Instananeous elastic modulus (G_1, Pascal) of Sigma® Gluten mixed with L-cysteine (0, 100 and 200 ppm) and microbial transglutaminase (0, 2000, 4000, 8000 and 12000 ppm) by the creep-recovery test with five replicates (n=5) (Error bars represent ± standard deviations.)

Table 11. Summary of mean comparison of force to extension (F) of Sigma® Gluten mixed with L-cysteine (0, 100 and 200 ppm) and microbial transglutaminase (0, 2000, 4000, 8000 and 12000 ppm) measured by the extension test with five replicates (n=5)

L-cysteine (ppm)	**Transglutaminase (ppm)**	**Grouping (by Tukey-Kramer HSD test)**								**Least square mean of F (N)**
0	12000	A								1.87
0	8000		B							1.73
100	12000			C						1.57
0	4000			C						1.56
0	0			C	D					1.47
100	8000			C	D					1.45
200	12000			C	D					1.43
200	8000				D	E				1.33
100	4000				D	E				1.32
0	2000				D	E				1.32
100	0					E	F			1.26
200	4000					E	F			1.22
100	2000						F	G		1.15
200	0							G	H	1.02
200	2000								H	9.24

Table 12. Summary of mean comparison of instantaneous elastic modulus (G_1, Pascal) of Sigma® Gluten mixed with L-cysteine (0, 100 and 200 ppm) and microbial transglutaminase (0, 2000, 4000, 8000 and 12000 ppm) measured by the creep-recovery test with five replicates

L-cysteine (ppm)	**Transglutaminase (ppm)**	**Grouping (by Tukey-Kramer HSD test)**		**Least square mean of G_1 (Pa)**
100	12,000	A		2.673×10^5
0	12,000	A		2.580×10^5
200	12,000	A		2.537×10^5
100	8,000	A		2.517×10^5
200	4,000	A		2.513×10^5
100	4,000	A		2.510×10^5
0	8,000	A		2.510×10^5
0	4,000	A		2.490×10^5
200	8,000	A		2.443×10^5
100	2,000	A		2.433×10^5
0	2,000	A		2.420×10^5
0	0	A		2.417×10^5
200	2,000	A		2.400×10^5
100	0		B	1.280×10^5
200	0		B	7.649×10^4

From a preliminary study, the 6-element model better explains the behavior of gluten modified by L-cysteine and microbial transglutaminase ($R^2 > 0.995$) than the 4-element model.

As expected, addition of L-cysteine significantly decreased the elasticity of gluten dough as reflected by the maximum force to extension (the Kieffer extension test) (Table 8 and Figure 24) and the instantaneous elastic modulus from the creep-recovery test (Table 7 and Figure 23). This decrease in elasticity was due to the ability of L-cysteine to disrupt the disulfide intermolecular and intramolecular linkages of gluten network. However, the results from both tests were slightly different in terms of the grouping of the least square means. The values of maximum force to extension obtained from the Kieffer extension test was grouped into four different groups (as shown by different letters ("A," "B," "C" and "D")), while the instantaneous elastic modulus obtained from the creep-recovery test was grouped into two different groups of 0 ppm (shown as group "A") and 100 ppm, 200 ppm and 300 ppm (shown as group "B"). This difference may be due to the direction of the force acting on the dough or the mode of the instrument: shear (the creep-recovery test) vs. extension (the Kieffer extension test).

As shown on Table 9 and Table 10, the addition of microbial transglutaminase at 16,000 ppm significantly increased the instantaneous elastic modulus of gluten as compared to the addition at 12,000 ppm, 8,000 ppm, 4,000 ppm, 2,000 ppm and 0 ppm. This result from both the Kieffer extension test and the creep-recovery test were concurrence. However, there were slight differences between the results of these two different tests in terms of the grouping. In the creep-recovery test, only microbial transglutaminase at 16,000 ppm was grouped as "A," and the rest was grouped as "B." On the other hand, the Kieffer extension test gave the result that was grouped into A, B, C with some overlaps. For instance, the maximum force to extension of microbial transglutaminase at 16,000 ppm as "A," 12,000 ppm as "A" and "B," 8,000 and 4,000 ppm as "A,"

"B" and "C," 0 ppm as "B" and "C" and 2,000 ppm as "C." However, it can be concluded that the maximum force to extension affected by the addition of microbial transglutaminase at 16,000 ppm ("A") and 2,000 ppm ("D") were significantly different than the addition at 2,000 ppm. From this experiment, it can be concluded that adding at least 4,000 ppm of the microbial transglutaminase could lead to more elastic dough.

The deviation of the effect introduced by addition of microbial transglutaminase at 0 ppm and 2,000 ppm (Figure 25 and Figure 26) could be caused by too low amount of the microbial transglutaminase to cause the cross-linking reactions. The slightly lower force to extension as a result of adding 2,000 ppm than the control could be due to incomplete mixing, leading to inhomogeneous mixture of the microbial transglutaminase (in powder form), gluten and water.

Based on data shown in Figure 27 and Figure 28, it can be concluded that the addition of microbial transglutaminase resulted in masking the rheological effect caused by L-cysteine. In the creep-recovery test (Table 12), compared to the gluten dough without addition of microbial transglutaminase (group "B"), the dough with the addition of the transglutaminase (group "A") increased the instantaneous elastic modulus significantly. After addition of microbial transglutaminase, there was a tremendous increase in instantaneous elastic modulus as shown in Figure 28. A possible reason is that the cross-links between protein side chains introduced by microbial transglutaminase caused proteins to become cross-linked, and therefore might be so stable that the interaction of SH-group of L-cysteine would be inaccessible.

Again, there are slight differences of the results from the two different tests (the creep-recovery test versus the Kieffer extension test) in terms of the grouping (Table 11 and 12). While the creep-recovery test gave the results that could be grouped into only "A" and "B," the Kieffer extension test gave the results with eight groups ("A"-"H" with overlaps). This difference is due

to the nature of the mode of the instrument: shear (creep-recovery) versus extension (Kieffer) which lead to different responses of gluten polymers that align differently in the dough system.

In the Kieffer extension test, simultaneous addition of 100 ppm L-cysteine and 8,000 ppm microbial transglutaminase, 200 ppm L-cysteine and 12,000 ppm microbial transglutaminase, and pure gluten are significantly the same (grouped "C" and "D," see Table 12). These examples of the same groups of the means of the maximum force to extension reflect that microbial transglutaminase can counterbalance the effect of L-cysteine.

Furthermore, the simultaneous addition of 100 ppm L-cysteine and 12,000 ppm microbial transglutaminase significantly resulted in the same maximum force to extension as the addition of only 4,000 ppm microbial transglutaminase (as grouped "C.") With slight deviation, the same grouping as "D" and "E" occurred among 200 ppm L-cysteine and 8,000 ppm microbial transglutaminase, 100 ppm L-cysteine and 4,000 ppm microbial transglutaminase, and only 2,000 ppm microbial transglutaminase (Table 11.) Likewise, the addition of only 100 ppm L-cysteine and the simultaneous addition of 200 ppm L-cysteine to 4000 ppm transglutaminase were also considered as no significant difference (grouped as "E" and "F"). This result strongly indicates that the addition of microbial transglutminase could mask the viscous effect caused by L-cysteine through forming cross-linking in the dough system and therefore increase its elasticity.

CHAPTER 4

CONCLUSIONS AND SUGGESTED FUTURE WORKS

As hypothesized, the addition of L-cysteine and microbial transglutaminase to flour samples had an influence on the elastic recovery of the vital gluten (gluten extracted directly from flour). Addition of L-cysteine decreased elasticity (strength) of gluten due to its reducing potential, while microbial transglutaminase increased it due to the formation of cross-links between lysines and glutamines of the gluten network.

The impact of adding L-cysteine was more profound in strong cultivars (Hollis and Norpro (HRS) and Trego (HDWH)) than in a weaker cultivar (Stephen (SWH)) because strong wheat cultivars might have greater number of effective disulfide bonds linking the gluten network than the weak cultivar, and therefore more bonds were likely to be broken through the interactions with L-cysteine. This finding could be confirmed by further study, looking into the 3-D structure of glutenins for each wheat cultivar in the future. Additionally, although the recovery index (%) and the maximum force to extension (N) of commercial bread flour were comparable than those of strong wheat cultivars (Hollis, Norpro and Trego), the effect of adding L-cysteine on the commercial bread flour was less profound than on those strong cultivars. This could be due to the presence of ascorbic acid, which, during the mixing process, can be converted to dehydroascorbic acid, an oxidizing agent that could compete with disulfide bonds of glutenins by reacting with L-cysteine, a reducing agent instead.

The addition of microbial transglutaminase at 16,000 ppm significantly increased the elasticity of gluten as compared to the addition at 2,000 ppm and 0 ppm. Both the creep-recovery test and the extension test agreed on this result.

Besides, the addition of microbial transglutaminase resulted in masking the rheological effect affected by the addition of L-cysteine. A possible explanation is that the cross-links between protein side chains introduced by microbial transglutaminase causes gluten protein to become so rigid that the disruption introduced by L-cysteine is shielded. Additional work should be done to see whether adding microbial transglutaminase before the addition of L-cysteine could cause the same result as adding both microbial transglutaminase and L-cysteine simultaneously as shown in this study.

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