

**The Effects on Gluten Strength and Bread Volume of Adding Soybean
Peroxidase Enzyme To Wheat Flour**

Ratia Kirby

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Human Nutrition, Foods and Exercise

William E. Barbeau, Chairman
Frank D. Conforti
Sean F. O'Keefe

August 9, 2007
Blacksburg, Virginia

Keywords: soybean peroxidase, gluten strength, bread-making, bread volume

The Effects on Gluten Strength and Bread Volume of Adding Soybean Peroxidase Enzyme to Wheat Flour

Ratia Kirby

ABSTRACT

Soy peroxidase enzyme obtained from isoelectric precipitation procedures was added to all-purpose flour (APF) to assess its effects on the rheological properties and consumer acceptability of yeast bread. A pH 4.8 isoelectrically precipitated fraction from soybeans was used because it produced the most precipitate and had about the same peroxidase activity as the other fractions. Gluten strength was determined using a farinograph for seven treatment groups: control (all-purpose flour), bread flour, all-purpose flour + soy flour, bread flour + soy flour, all purpose flour + pH 4.8 precipitate, all-purpose flour + 15 mg soybean peroxidase, and all-purpose flour + 25 mg soybean peroxidase. Four types of yeast bread were baked for loaf volume determination, texture analysis, and consumer acceptability: a control loaf using only all-purpose flour, a reference loaf using all bread flour, a loaf with all purpose flour + whole soy flour, and a loaf with all-purpose flour + pH 4.8 soy precipitate.

The APF+soy flour, bread flour, bread flour + soy flour, and the APF + pH 4.8 precipitate produced an improvement in the gluten strength and mixing tolerance compared to the control ($p < 0.05$). However, the improvement by the addition of the pH 4.8 precipitate cannot be attributed to the peroxidase enzyme because peroxidase needs hydrogen peroxide as a substrate and no hydrogen peroxidase could be added to the farinograph; therefore, it was concluded that the increase in gluten strength produced by the pH 4.8 soy precipitate was due to an unknown component present in the pH 4.8 fraction. No significant differences ($p < 0.05$) were found in crumb or crust texture for any of the treatment groups. The addition of pH 4.8 precipitate to APF significantly decreased ($p < 0.05$) loaf volume compared to bread made from bread flour. The results from sensory analysis showed there was no difference in preference for any of the breads. This study showed no conclusive evidence that peroxidase enzyme improved gluten strength or loaf volume of yeast bread, but further research is warranted.

Acknowledgements

First and foremost, I want to thank the Holy Trinity; God, my Lord and Savior Jesus Christ, and the Holy spirit. Without You, I am nothing, but through You, I can do all things.

Dr. William Barbeau, thank you for seeing something in me that I did not see in myself and all the encouragement and help you have given me. Thank you for all the meals and the stories. I truly enjoyed listening to them. I hope I have made you proud.

Dr. Sean O'Keefe, thank you for being on my committee and introducing me to the isoelectric precipitation experiment. Your advice and suggestions were a vital component of my research.

Dr. Frank Conforti, thank you for being on my committee and all the encouragement also. You are one of a kind and I love it.

Judy Yan and Sherry Saville, thank you for your helpfulness and patience with me when I needed to repeat an experiment a second or third time, or needed the same email a second or third time. Thank you

Mr. David Ruggio and the entire Food Analysis lab and Dr. Josep Bassaganya-Riera, thank you for allowing me to use the centrifuges.

Thanks to all my sensory panelists and all my fellow graduate students and friends. Your supports means a lot to me.

Dr. Michelle Penn, words cannot express the gratitude I have in my heart for you. Your friendship is a God send. Thank you so much for all the times you have prayed for me, comforted me, encouraged me, pushed me, fed me, and kept me company. It has been my pleasure knowing you and your wonderful family. God bless you all!

Alida Gibson, Chartis Hayes, and Amanda Betts, we have known each other from the seventh grade and on. God could not have sent me better friends than the three of you. You each hold a special place in my heart. Thank you for all the memories we have made and the ones we will make. Thank you for pushing me forward when I wanted to give up, for holding my head up, drying my tears or crying with me. But most of all, thank you for all the laughs!!!! I love each and every one of you and may God bless all your future endeavors.

To my other mother Waver, thank you for taking me in and calling me daughter. Thank you for your love and advice.

To my brothers Kelcey, Marcus, and Chris and my father James Williams, thank you for all the financial and emotional support you have given me throughout the years. And thank you for putting up with me and believing in me. I could not have done this without you. I love you.

To the rest of my family and friends, thanks for being in my life and loving me. God bless you.

I dedicate this thesis to my late mother Rosie Mary Kirby. You always believed in me and was my cheerleader. I could not have asked for a better mother than

you. You made me proud everyday and I hope I have made you proud also. I love and miss you.

Table of Contents

| | |
|--|----------|
| Abstract..... | ii |
| Acknowledgments..... | iii |
| Table of Contents..... | v |
| List of Tables | vii |
| List of Figures..... | viii |
| <i>Chapter 1: Introduction.....</i> | <i>1</i> |
| <i>Chapter 2: Review of Literature.....</i> | <i>3</i> |
| 2.1 Soybean Peroxidase..... | 3 |
| 2.1.1 Soybean Peroxidase Isoenzymes..... | 3 |
| 2.1.2 Soybean Peroxidase Characteristics..... | 6 |
| 2.1.3 Thermal Stability..... | 6 |
| 2.1.4 Soybean Peroxidase Substrates..... | 7 |
| 2.2 Wheat..... | 9 |
| 2.3 Function of Ingredients in Bread Making..... | 11 |
| 2.3.1 Flour..... | 11 |
| 2.3.2 Water..... | 11 |
| 2.3.3 Yeast and Sugar..... | 11 |
| 2.3.4 Salt..... | 12 |
| 2.3.5 Fat..... | 12 |
| 2.4 Chemical Bonds..... | 12 |
| 2.4.1 Disulfide Bonds..... | 13 |

| | |
|---|-----------|
| 2.5 Tyrosine Cross-links..... | 14 |
| <i>Chapter 3: Justification of Study.....</i> | <i>17</i> |
| 3.1 Purpose and Objectives of Study..... | 19 |
| <i>Chapter 4: Materials and Methods.....</i> | <i>20</i> |
| 4.1 Production of Soy Precipitate by Isoelectric Precipitation..... | 20 |
| 4.2 Determination of Peroxidase Activity..... | 21 |
| 4.3 Protein Determination..... | 22 |
| 4.4 Determination of Gluten Strength..... | 22 |
| 4.5 Bread Making..... | 25 |
| 4.6 Determination of Loaf Volume | 28 |
| 4.7 Sensory Evaluation..... | 28 |
| 4.8 Texture Analysis..... | 31 |
| 4.9 Statistical Analysis..... | 31 |
| <i>Chapter 5: Results and Discussion.....</i> | <i>32</i> |
| 5.1 Dough Rheology (Farinograph)..... | 34 |
| 5.2 Volume..... | 42 |
| 5.3 Texture Analysis..... | 42 |
| 5.4 Sensory Evaluation..... | 48 |
| 5.5 Summary and Conclusions..... | 50 |
| 5.6 Recommendations for Future Research..... | 51 |
| <i>References.....</i> | <i>52</i> |
| <i>Appendix A.....</i> | <i>56</i> |

List of Tables

| | |
|---|----|
| Table 1: Specific Peroxidase Activity of Different Soybean Tissues..... | 5 |
| Table 2: Treatment Groups for Farinograph Analysis..... | 24 |
| Table 3: Bread Ingredients..... | 27 |
| Table 4: Mean Peroxidase Activity of Soy Flour Fractions..... | 33 |
| Table 5: Mixing Characteristics of All Dough Treatment Groups..... | 38 |
| Table 6: Loaf Volume Comparison Between Treatment Groups..... | 44 |
| Table 7: Crumb and Crust Texture of Breads..... | 45 |
| Table 8: Hedonic Scores of Breads..... | 49 |

List of Figures

| | |
|--|----|
| Figure 1: An Example of the Sensory Evaluation Scorecard..... | 30 |
| Figure 2: Farinograms of Treatment Groups..... | 39 |
| Figure 3: Farinograms of Treatment Groups..... | 40 |
| Figure 4: Farinograms of Treatment Groups..... | 41 |
| Figure 5: Photographs of Breads Made From the Baking Trials..... | 46 |
| Figure 6: Photographs of the Crumb Texture of the Bread..... | 47 |

Chapter 1: Introduction

Wheat is the oldest and most extensively grown of all crops (1). There are three major species of wheat grown in North America; *Triticum aestivum* L., *Triticum compactum*, and *Triticum durum*. Each species have different classes of wheat. The classes include hard red winter, hard red spring, and soft red winter. Hard and soft white belong to *Triticum aestivum*. Club wheats belong to *Triticum compactum* while durum wheat belongs to the species *Triticum durum*. Durum wheats have a hard texture and are high in protein; therefore, they are most suitable for production of semolina or durum flour for pastas. Club wheats are soft in texture and low in protein, so they are useful for cake and pastry flours because of the low protein content and weak gluten strength. Neither durum nor club wheat are desirable for bread making. The hard red spring and hard red winter wheat class of species *Triticum aestivum* have the best properties for bread-making flour because they produce high-gluten flours (1).

The quality of flour and the subsequent end product, bread, is important to bakers, industry, and cereal chemists alike, hence the addition of dough improvers. Dough improvers are substances added to dough that favorably alter some aspect of the dough to produce better bread than if the improver were not added. Most of the improvers are chemicals, such as potassium bromate, potassium iodate, L-ascorbic acid, L-cysteine, and sodium metabisulfite, just to name a few (1). However, given that some of these chemical improvers,

particularly potassium bromate, have been given negative press about their suspected toxicity (2-4), enzymes have been used as a natural alternative. Enzymes, such as, lipoxygenase, catalase, and peroxidase (POX) are already present in flour and can catalyze oxidative reactions that improve the rheological properties of dough (5,6).

Chapter 2: Review of Literature

2.1 Soybean Peroxidase

Peroxidases are a class of oxidoreductase enzymes. They use hydrogen peroxide or organic hydroperoxides as oxidants. Soybean peroxidase (SBP) is a glycoprotein extracted from the seed coat of soybeans (*Glycine max.*). Soybean peroxidase belongs to class III of the plant peroxidase superfamily (7, 8). Other peroxidases in this family are horseradish (HRP), barley, and peanut (9).

2.1.1 Soybean Peroxidase Isozyme

Soybean peroxidase was found to have up to 20 different isozyms (isoenzymes) in the plant tissue by analytical isoelectric focusing, but only the seed coat exhibited a peroxidase isozyme pattern where one form predominated (10). This isozyme is called SP4.1 (soybean peroxidase 4.1) because its isoelectric point was pH 4.1. Most peroxidase activity was localized in the seed coat. SP4.1 activity increases as the seed coat matures and develops. Its activity remains very high at least 3 years after harvest. The increase in SP4.1 activity may also be attributed to the degradation of other soluble proteins during maturation. The molecular weight of SP4.1 was considered to be 37kD because the increase in SP4.1 peroxidase specific activity correlated with the buildup of a polypeptide with a molecular weight of 37kD (10). The specific activity of peroxidase in the mature seed coat is 20 times more than in any other tissue in the soybean (Table 1). Table 1 shows the specific

peroxidase activity at different locations in the soybean seed coat and soybean plant (10).

Table 1: Specific Peroxidase Activity of Different Soybean Tissues

| Soybean Tissue | Specific Peroxidase Activity (units/mg soluble protein) |
|-------------------|--|
| Mature Seed Coat | 20620 |
| Seed Coat 35 daa* | 15620 |
| Cotyledon 35 daa* | <100 |
| Pod | 807 |
| Leaf | <100 |
| Stem | <100 |

*daa = day after anthesis (flowering) (Gillikin and Graham 1991)

2.1.2 Soybean Peroxidase Characteristics

SBP contains 326 amino acids (7) with one single tryptophan at position 117 (Trp117) (9, 11). It is stabilized by a Fe (III) protoporphrin IX heme prosthetic group (6, 10), has 4 disulfide bonds, eight glycans and contains two Ca^{2+} binding sites located distal and proximal to the heme group (9). SBP is approximately 18% carbohydrate, with 5 or 6 glycosylation sites. SBP activity is measured spectrophotometrically at 403 nm because that is the wavelength of the absorbance of the prosthetic heme group(8). SBP has a high oxidation potential. In the presence of hydrogen peroxide (H_2O_2), SBP can directly oxidize veratryl alcohol and other nonphenolic aromatics (10).

2.1.3 Thermal Stability

Soybean peroxidase has a high thermal stability (6, 8-10)The thermal stability transition midpoint (T_m) for the unfolding of peroxidase polypeptide backbone at neutral pH is $T_m = 86^\circ\text{C}$ at pH 7 (4). It has a high resistance to urea denaturation and remains in its native state with addition of up to 5M guanidine HCl(12). The Gibbs Free Energy of unfolding (H_2O , 25°C) from the denaturation with guanidine HCl is $\Delta G^\circ = 43.3 \pm 2.4 \text{ kJ mol}^{-1}$ (6, 10). The thermal stability of SBP has been compared with that of thermophilic microbial enzymes(12). Soybean peroxidase has its maximum denaturation resistance and maximum conformational stability at pH 5.5. Soybean peroxidase is more stable than horseradish peroxidase because its conformational and thermal stability numbers are much higher than those of horseradish peroxidase(9).

At pH 7, SBP has 77% helical content, 16% β -strands and β -turns, and 7% other structures. The helical structures are unchanged from pH 3.7-8.0, but the β -strands and turns are pH dependent and shift to other structures. The transition of β -strands plus β -turns to other structures reaches its maximum at pH 5.5. This shift of β -strands and β -turns to other structures assists in improving the overall conformational flexibility of the enzyme, perhaps increasing the solvent accessibility to the heme active site, and thereby improving the enzyme's overall activity (9).

The unusual high thermal stability of soybean peroxidase can be explained by the heme prosthetic group. The heme group binds very tightly to SBP, more so than HRP (12). This was shown when HRP and SBP were subjected to excess apomyoglobin at 30°C in the presence of 1 mM EDTA (pH 7). HRP gave up its heme group readily, while SBP was able to hold on to its heme group. The tight binding of heme to SBP may help with retaining a catalytically active conformation because once the heme group is lost (melting point 90.5°C), SBP is deactivated and it is more susceptible to thermal denaturation (12). SBP high thermal stability makes it a great catalyst for biocatalyst and biosensor reactions (9, 11) as well as for wastewater treatment, medical diagnostics, and phenol resin synthesis (12).

2.1.4 Soybean Peroxidase Substrates

Soybean peroxidase catalyzes the oxidation of substrate (AH) to a radical product (A[•]) by hydroperoxide (ROOH) in a three-step process via two

intermediates (Compound I and Compound II) at the expense of hydrogen peroxide (H_2O_2) (13, 14).

- (i) Peroxidase + ROOH + $\text{H}_2\text{O}_2 \rightarrow$ Compound I + ROH + H_2O
- (ii) Compound I + AH \rightarrow Compound II + A^\bullet
- (iii) Compound II + AH \rightarrow Peroxidase + A^\bullet + H_2O

Some of the substrates for soybean peroxidase are phenols, nonphenolic aromatics, aromatic amines, and polycyclic aromatic hydrocarbons (15). Two of the nonphenolic aromatic substrates are veratryl alcohol and methoxybenzenes (13, 16). Soybean peroxidase catalyzes the oxidation of veratryl alcohol by abstracting an electron from its aromatic ring system, thereby creating a cation radical intermediate, which becomes converted to the product veratraldehyde. The oxidation of veratryl alcohol has a 1:1 stoichiometry for veratryl alcohol per mol of H_2O_2 consumed (16). The pH optimum for the reaction is pH 2.4 (13, 14).

Soybean peroxidase can catalyze the removal of seven phenolic compounds from wastewater(17). Wright and Nicell (1999) found that the optimum catalytic activity for the reaction was at pH 6.4, with over 90% of the maximum activity between pH 5.7 and 7.0, and more than 10% between pH 3 and 9. Soybean peroxidase catalyzes the polymerization and precipitation of the phenolic compounds from the wastewater (17).

The fact that soybean peroxidase can use nonphenolic aromatic compounds, such as veratryl alcohol, and phenolic compounds as substrates

indicates the importance of soybean peroxidase as a biocatalyst (16) and for wastewater treatment (17).

2.2 Wheat

Wheat is the oldest and most extensively grown of all the crops (1). The wheat proteins can be separated into four groups; albumins, globulins, prolamins, and glutelins. These groups are based on the solubilities of each group. The albumins are the water-soluble proteins, the globulins are soluble in 0.5-1.0 M salt, the prolamins are soluble in 60-70% aqueous ethanol, and the glutelins are soluble in dilute acid or alkali (18). There are relatively more albumins (60%) than globulins (40%) in wheat. Gliadin, which is a prolamins and glutenin, which is a glutelin are the proteins that comprise gluten proteins. These gluten proteins contribute greatly to good dough characteristics and bread quality (19). Gliadin is composed of low molecular weight (LMW) proteins, is cohesive, has low elasticity, and contains more proline, glutamic acid with glutamine, cysteine, isoleucine, and phenylalanine than glutenin. Gliadins are soluble in acids, bases, and hydrogen bonding solvents (20). Glutenins have high molecular weight (HMW) proteins and LMW, are cohesive, elastic, and have more glycine, lysine, and tryptophan than gliadins. Glutenins are suspendable in acids, bases, and hydrogen bonding solvents (20). Gluten proteins are essential for bread production because elasticity and extensibility are considered to be very important in bread making. Gliadins contribute extensibility and stickiness to

dough, whereas glutenins contribute to dough mixing time, strength, and elasticity (20).

The protein content of flours made from wheat can range from 6-20%, which depends on the variety of the wheat used. Flours with higher amounts of protein make better breads. Strong wheat flours, which contain a high percentage of protein, form elastic glutes with good gas retaining abilities and produce well-risen, well-shaped loaves of bread with good crumb grain and texture. Weak wheat flours have low protein contents, and form soft, weak, mainly nonelastic glutes with poor gas retaining abilities (21). Flour with at least 11% protein is preferred for yeast-leavened breads. Although, the protein content of flour is very important to bread quality, the quantity and quality of the gluten proteins is more important (20). When breads were made from wheat of two genotypes of different quality, Manitou wheat cultivar, was consistently better in baking quality at any protein content compared with 11-463A because of its better quality gluten (19).

To produce bread with desirable characteristics such as a high loaf volume, and a light and even crumb, depends on the ability of the dough to retain the gas produced over long periods of time especially during fermentation, and oven rising. The ability of dough to retain gas is one of the most important quality requirements for bread making. Gluten is responsible for this ability because it provides the dough with the viscoelastic properties to hold gas (19).

2.3 Function of Ingredients in Bread Making

2.3.1 Flour

Flour provides the structure of bread. It produces dough when mixed with water. It is the interaction of the flour proteins with water that produces gluten (22).

2.3.2 Water

Water is necessary to make dough. It hydrates the other ingredients and is needed to produce the gluten structure, provides the correct amount of moisture, and is needed for gelatinization of starch. Water also provides cohesion. The hardness of the water used in bread making affects the end product. Water of medium hardness (50-100ppm) is recommended for baking because the mineral salts present in it will have a strengthening effect on the gluten. Hard waters retard the fermentation process by tightening the gluten structure too much and soft waters have a tendency to produce soft, sticky dough because of their lack of gluten strengthening minerals salts (22).

2.3.3 Yeast and Sugar

Yeast produces the carbon dioxide needed to obtain a light crumb texture. It produces the carbon dioxide gas that is used as the leavening agent (23). Yeast ferments the sugars added to dough. Sugar is added as food for yeast, as a sweetening agent, a tenderizing agent, and is active in the browning of the bread (22).

2.3.4 Salt

Salt essentially has three functions in dough. Salt enhances the flavor in bread. Without salt, bread has a bland taste and flavor and usually is unpalatable to consumers unless they follow a low-sodium diet. The second function of salt is inhibition of yeast activity and control of the microflora of dough. In the absence of salt, yeast fermentation would proceed at an irregular rate and produce undesirable results. Salt also provides a strengthening and tightening effect to gluten (22).

2.3.5 Fat

The primary function of fat or shortening is for tenderness and the shortening effect they give to bakery products. Fat, or shortening, increases loaf volume, and improves the crumb grain, retention of freshness, and dough handling properties of dough (22).

2.4 Chemical Bonds

In bread making, dough is formed by the addition of water to flour. Dough is an interaction between the proteins, carbohydrate, lipids, minerals, and enzymes contained in the flour, as well as other added ingredients. Dough undergoes physical and chemical modifications during processing. Chemical bonds are produced in the dough (24). Some of the chemical bonds produced in dough are ionic bonds, hydrogen bonds, hydrophobic and covalent bonds. Ionic bonds are formed by the addition of salt to dough (salts form ions, Na⁺ Cl⁻ etc when they are dissolved in water, some these ions may then bind to oppositely

charged groups in proteins, eg. Cl⁻ ions may bind to positively charged epsilon amino groups in proteins). These bonds, by adding salt, contribute to increased dough rigidity and reduce extensibility (24).

The pentosans, although they are only 1.5% of the total flour content, can absorb 15 times their own weight in water; therefore they are the most effective hydrogen-bond forming components in wheat flour. Hydrogen bonds can also be formed by the amino acid glutamine, which is abundant in gluten proteins, by polar substances, and lipids. Hydrogen bonds affect the mobility and the plasticity of dough. The lipids in dough can also produce hydrophobic bonds. Hydrophobic bonds can supply both elasticity and plasticity to dough, as well as being important in the formation of oven spring (24).

2.4.1 Disulfide Bonds

Disulfide bonds are covalent bonds. They are composed of -S-S- linkages within and between proteins. Disulfide bonds play an important role in the structure, reactivity, and properties of gluten proteins (25, 26). They contribute to dough firmness. Inter- and intramolecular disulfide bonds are important to the aggregation of glutenin (25). Disulfide bonds also form between the monomeric gliadins proteins (26). Disulfide bonds are broken and reformed in dough, especially during mixing and baking. This interchange must occur because dough has viscous flow and therefore the bonds have to be able to break and reform to provide the required mobility. Heat causes a conformational change in

the S-S bonds in the gluten matrix. Heat rearranges S-S bonds in the monomeric proteins of gluten, in particular, the gliadins (26).

Disulfide bonds are important to dough strength. According to the results of Morel and Bonicel (1996), the addition of small amount of dithioerythritol (DTE), which reduced the amount of disulfide bonds produced, resulted in weak dough strength (27). *N*-ethyl-maleimide (NEMI), another reducing agent and thiol-blocker, caused a reduction in dough development, increased viscosity, and increased resistance, which led to the breakdown of dough with mixing (24). Because of the importance of disulfide bonds, a number of dough improvers have been added to increase the amount of disulfide interchange. Some of these dough improvers include bromate, iodate, ascorbate, and cysteine (24).

2.5 Tyrosine Cross-Links

Until recently (Tilley 2001), disulfide bonds were considered to be the only significant covalent bonds contributing to the gluten matrix (28). Tyrosine cross-links, in the form of isodityrosine have thoroughly been studied in extensin proteins and are common in plant proteins (28). Dityrosine, another form of tyrosine cross-links was first found in vivo from the insect cuticulum protein resilin, a major rubber-like protein found in the cuticle of arthropods and insects (28-31). Dityrosine bonds are also found in elastin and collagen in vertebrates (28), and also in naturally occurring proteins such as wool keratin, the human eye lens, or the adhesive discs of sea mussels (29).

Since dityrosine has been found mostly in tissues responsible for membrane integrity, it is considered to be a stabilizing cross-link in structural proteins (29). Dityrosine bonds are very strong and are mainly intermolecular (30). The proteins with dityrosine bonds have amino acid contents and secondary structures that are similar to those of the M_r glutenin proteins found in gluten. Individual high M_r glutenin subunits contain from 3-5% tyrosine that occur periodically throughout the length of their protein structures and most frequently found in repeat pairs of tyrosine residues (YY)(27); therefore, the formation of dityrosine bonds in dough is very likely.

Tilley, et al (2001), conducted a study to test the existence of tyrosine cross-links present in dough. They prepared pup loaves of bread from spring wheat variety Bronze Chief with 14.76% protein and analyzed the dough at different mixing times for the presence of tyrosine cross-links. A peak eluting at 22.5 minutes found to be dityrosine increased as mixing time increased. The addition of the oxidizing agents; ascorbic acid, azodicarbonamine (ADA), and potassium bromate increased this peak. The dityrosine bond was the only one facilitated by the addition of these agents, therefore the authors implied that perhaps these agents were, in fact, increasing dityrosine bonds in the gluten matrix, instead of forming disulfide bonds between proteins, which was the former hypothesis that did not have any direct evidence to support it (28). However, the addition of free radical scavengers, such as, cysteine and

glutathione, decreased the formation of tyrosine cross-links indicating that these cross-links occur from a free radical mechanism.

The authors indicated that dityrosine bonds are formed by the presence of an unknown enzyme that is present in the water-soluble extract of flour.

Peroxidase enzymes are present in wheat flour (24) and peroxidases can catalyze the cross-linkage of tyrosine residues in vitro (30, 31) , therefore it is hypothesized that peroxidase enzymes are responsible for the dityrosine bonds found by Tilley et al (2001). Takasaki et al (2005) supported this theory with their study. They found that the addition of hydrogen peroxide and hydrogen peroxide plus peroxidase significantly increased the formation of dityrosine bonds, more so with hydrogen peroxide plus peroxidase (32).

Chapter 3: Justification of the Proposed Study

There has been research on the effects of peroxidase on the mixing and baking characteristics of dough. Hilhorst et al (1999) examine the effects of xylanase and oxidative enzymes on the baking performance, rheology, and chemical composition of wheat dough and gluten (33). Doughs were made, from either control, or enzyme-treated doughs with added xylanase, peroxidase, glucose oxidase, xylanase-peroxidase, or xylanase-glucose oxidase. The flour used was untreated cookie type flour Kolibri with 10.5% protein. The six treatment groups were baked and the resulting crispy rolls were scored manually by experienced test bakers for dryness, stiffness, elasticity, extensibility, fermentation stability, roll shape, and crumb structure using a 10-point scale for each parameter.

The addition of peroxidase alone to commercial untreated cookie type flour Kolibri improved the handling properties of the dough and the crumb texture, but did not increase loaf volume of the baked product. However, the combination of xylanase with soy peroxidase gave the best handling properties for dough, and the best crumb structure and the largest roll volume for white tin loaves.

The authors also analyzed the gluten composition of each treatment before and after dough development. The results indicated that peroxidase only slightly changed the properties of the gluten matrix. Since the addition of peroxidase only changed the gluten structure slightly, but improved the handling

properties, the authors indicated that peroxidase probably acted on other fractions of the dough (34).

Dunnewind et al (2002) found that the addition of peroxidase increased the amount of short-term crosslinks or entanglements but that no permanent crosslinks were formed. This was shown through relaxation tests (34). The authors indicated that the entanglements could be due to the ability of peroxidases to crosslink pentosans. They indicated also that peroxidase did not affect the gluten proteins, but glucose oxidase did (34).

Hilhorst et al (2002) found that peroxidase cross-linked large arabinoxylans through ferulic acid to each other (35). They also indicated that peroxidase produces an acceptable baked product using a low protein flour and peroxidase enzyme also prevents sticky dough that is formed when xylanases are used by crosslinking the arabinoxylans (35).

Even though these articles indicate that the addition of peroxidase to dough improves mixing and baking characteristics of flour with lower protein contents, they also indicate that these benefits are due to crosslinking of arabinoxylans (33-35). However, it is now known that dityrosine bonds are formed by the addition of peroxidase (25), hydrogen peroxide, or hydrogen peroxide and peroxidase to wheat flour (32). Tilley et al (2001) and Takasaki et al (2005) found that dityrosine bonds were in fact formed in gluten proteins, which is in direct contradiction to previous studies dealing with peroxidase (28, 32). Takasaki et al (32) observed also that the addition of peroxidase/hydrogen

peroxide or hydrogen peroxide improved the dough-forming properties.

However, more research needs to be conducted to prove that dityrosine bonds do in fact improve dough rheology.

3.1 Purpose and Objectives of the Proposed Study

The purpose of this study is to determine the effects of adding soy peroxidase has on gluten strength and bread volume of yeast bread. The objectives of the study are:

1. To incorporate soy peroxidase from soy flour into bread to determine the effects on gluten strength and loaf volume.
2. To determine if soy peroxidase added to all-purpose flour can produce as desirable a product as bread flour.
3. To determine the sensory quality of breads using soy peroxidase from soy flour.

Chapter 4: Materials and Methods

4.1 Production of Soy Precipitate by Isoelectric Precipitation

Initially, purified soybean peroxidase from Sigma[®] was going to be used for the baking trials; however, since soybean peroxidase from Sigma[®] is an expensive enzyme and peroxidase is naturally present in soy flour, isoelectric precipitation of soy flour was performed to produce a more cost effective form of soybean peroxidase. The isoelectric procedure was a method adapted from Thanh and colleagues (1975)(36).

Red Mill[®] stone ground whole grain soy flour (35% protein) (Milwaukee, OR) was combined with 0.3M phosphate buffer, pH 8.0 in a ratio of 15:1 (v/w) buffer to soy flour. Phosphate buffer was used instead of Tris-HCl because phosphate buffer is a FDA approved food additive, whereas Tris Hcl is not. The slurry was constantly agitated overnight at 4°C and then centrifuged at 5000 rpm. The supernatant was decanted and the pH adjusted to 6.4 with 3N HCl, centrifuged, supernatant decanted, the pH adjusted to 4.8 with 3N Hcl, centrifuged, and the supernatant decanted. A portion of the supernatant and precipitate was retained at each pH (8, 6.4, 4.8) for assay of peroxidase activity and protein content to determine which fraction of the soy flour had the highest peroxidase activity per mg of protein. The pH 4.8 precipitate was freeze-dried to use for baking trials.

4.2 Determination of Peroxidase Activity

A microassay was utilized to determine the peroxidase activity of the supernatant and precipitate at each pH (8.0, 6.4, and 4.8) The method for determining peroxidase activity was a method adapted from Hatcher and Barker (2005)(37).

The supernatants, retained from the isoelectric precipitation procedures were filtered through a Whatman 13-mm, 1.5 μm glass microfiber syringe filter (Clifton, NJ). An aliquot of supernatant (10 μL) was diluted with 990 μL of cold Tris-HCl buffer, pH 8.0 in duplicate tubes.

Approximately 2.5 g of soy flour fraction precipitate (pH 8.0, 6.4, and 4.8) was placed in a centrifuge tube and redissolved in 25 mL of cold (4°C) sodium acetate buffer (0.1M, pH 4.2), capped, and immediately vortexed for 10 seconds to ensure suspension of all material. The centrifuge tubes were placed on a rotating mixer at 4°C and continuously tumbled for 30 minutes. The tubes were centrifuged (4300 rpm, 30 min) at 4°C and the supernatant was decanted and retained for analysis. The supernatants were filtered, diluted 1:100 with cold 0.1 M sodium acetate buffer, pH 4.2 into duplicate tubes.

All test tubes were kept in the dark to minimize light exposure. The assay was initiated by the addition of 150 μL of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate at room temperature into each test tube. The reaction was stopped after 1 min by adding 100 μL of 2M sulfuric

acid. To ensure maximum reproducibility, the stopping agent was added in the same sequence as the addition of the substrate. The samples were analyzed at absorbance 405 nm, using pH 5.0, 0.1M acetate buffer as the blank. The peroxidase activity of the samples was determined using the standard curve.

4.3 Protein Determination

A 10 mg/ml stock solution of bovine serum albumin (BSA) was prepared by adding 1 g BSA to 100 ml of 0.3M phosphate buffer, pH 8. Calibration standards ranging from 0-10 mg/ml BSA were prepared in duplicate. The samples for protein determination of the isoelectric precipitation supernatants and precipitates were prepared in the same manner as for peroxidase activity. Four milliliters of Biuret reagent was added to each tube, vortexed, and allowed to stand at room temperature for 20 minutes. The absorbance was read at 540 nm using the 0.0 mg/ml tube as the blank. Protein content was calculated using the standard curve.

4.4 Determination of Gluten Strength

Percent water absorption, arrival time, peak time, dough stability, departure time, and mixing tolerance index were measured with the farinograph. Dough hydration is adjusted so that the peak of the graph is centered on 500 BU. The arrival time indicates the interval between the first addition of water (time zero) and the point at when the top of the curve first intersects the 500 BU line. The peak time is the time interval between time zero and when the curve indicates maximum resistance. A lower peak time generally correlates with lower

flour protein and absorption. The departure time is the elapsed time from time zero to the time when the top of the curve first leaves the 500 BU line. The mixing tolerance stability is the difference between departure time and arrival time. The mixing tolerance index (MTI) is the difference in BU, from the top of the curve at its peak to the top of the curve measured at five minutes after the peak (38). The gluten strength of samples was performed using a Brabender Farinograph® (South Hackensack, NJ). Fifty grams of flour was added to the farinograph and the water was adjusted until the curve of the line centered on the 500 Brabender Unit (BU) mark. Table 2 describes the tested treatments. Each group was tested four times.

Table 2: Farinograph Treatment Groups

| |
|---|
| Treatment Groups (50g) Total |
| 1. All-purpose Flour (APF) (Control) |
| 2. Bread Flour (BF) |
| 3. All-purpose Flour + 2.81g Soy Flour (SF) |
| 4. Bread Flour + 2.81g Soy Flour |
| 5. All-purpose Flour + 2.81g pH 4.8 precipitate |
| 6. All-purpose Flour + 15mg peroxidase (POX) |
| 7. All-purpose Flour + 25mg POX |

Peroxidase (POX) was soy peroxidase enzyme purchased from Sigma.

4.5 Bread Making

Four types of bread were made; a control loaf using only all-purpose flour, a reference loaf using all bread flour, a loaf with all-purpose flour plus whole soy flour, and a flour with all-purpose flour plus the pH 4.8 soy precipitate fraction. The breads were made using a modified method of the American Association of Cereal Chemists (AACC) Basic Straight-Dough Method #10-09 (39). Red Star[®] Active Dry Yeast (10.6 grams) was allowed to ferment with 6 grams of sugar and 40 mL of water (105-115°F) for 10 minutes. The yeast and ~90 mL of water were added to the other ingredients.

The other ingredients were 200 grams of Gold Medal[®] Bakers All-Purpose Flour (Minneapolis, MN) or All Trumps High Gluten Bread Flour (Minneapolis, MN), 6 grams of sugar, 3 grams of salt, and 6 grams of Crisco[®] All Vegetable Shortening (Orrville, OH). For the experimental breads, 194.38 grams of all-purpose flour and 5.62 grams of Bob's Red Mill[®] stone ground whole grain soy flour (Milwaukee, OR) or 5.62 grams of ground, freeze dried pH 4.8 soy precipitate (from the isoelectric precipitation experiment) were used. A detailed ingredient list can be found in Table 3.

The ingredients were mixed in a Kitchen Aid mixer using the dough hook attachment for five minutes, and the resulting dough was allowed to rise for 52 minutes. The doughs were then will divided into two loaves of approximately 165 grams each, placed in pup loaf pans, allowed to rise for 33 minutes and baked in a 419°F oven for 24 minutes. The loaves of bread were allowed to cool

completely, wrapped in plastic wrap, labeled and stored at room temperature. A total of three replicates of each type of bread was made, for a total of 12 loaves. Each bread type was given a number and baked in random order each day for three days.

Table 3: Bread Ingredients

| Types of Breads | | | | |
|------------------------|-------------|-----------|-------------|--------------------|
| Ingredients | All-Purpose | Soy Flour | Bread Flour | pH 4.8 Precipitate |
| All-purpose Flour | 200 g | 194.38 g | 0 g | 194.38 g |
| Soy Flour | 0 g | 5.62 g | 0 g | 0 g |
| Bread Flour | 0 g | 0 g | 200 g | 0 g |
| Yeast | 10.6 g | 10.6 g | 10.6 g | 10.6 g |
| Salt | 3 g | 3 g | 3 g | 3 g |
| Sugar | 12 g | 12 g | 12 g | 12 g |
| Shortening | 6 g | 6 g | 6 g | 6 g |
| pH 4.8 Soy Precipitate | 0 g | 0 g | 0 g | 5.62 g |
| Water | ~ 130 ml | ~ 130 ml | ~ 130 ml | ~ 130 ml |

4.6 Determination of Loaf Volume

Loaf volume was determined by rapeseed displacement. The volume of the empty chamber filled with rapeseeds was recorded, and then the volume of the chamber with a wrapped loaf of bread filled with the rapeseeds was recorded. The volume of the loaf of bread was ascertained by subtracting the volume of the empty chamber from the volume of the chamber with the loaf of bread. The volume was measured in cubic centimeters (cc). The average of the two loaves of each type of bread made each day was used as one volume measurement.

4.7 Sensory Evaluation

The acceptability of each type of bread was determined using a 9-point hedonic scale, where 1 was extremely dislike and 9 was like extremely. Sensory evaluation was conducted in the Food Science and Technology sensory lab in the Food Science and Technology building and the Human Nutrition, Foods and Exercise sensory lab in Wallace Hall on two separate days.

Approval from the Institutional Review Board (IRB) for research involving human subjects was received (IRB # 06-454) (Appendix A). Each participant signed a consent form. Each bread sample was given a random 3 digit number. The number was placed on a plate with a scorecard for each bread sample. The samples were given to each taste panelist in a randomized order. They were given water to drink to clean their palate after each sample. Panelists tasted each sample individually and were instructed to place an "X" in the box corresponding to how acceptable the bread product was (see Figure 1). Anyone

with wheat, gluten, yeast, or soy allergies were omitted from the study. Sensory analysis was performed on freshly baked loaves of bread the day after each baking trial.

Sensory Evaluation Scorecard: Hedonic Scale

Product _____

Date _____

Please taste the samples and indicate your overall acceptability of the product by placing an "X" in the appropriate box.

1
Dislike Extremely

5
Neither like nor dislike

9
Like extremely

Comments: _____

Figure 1. An Example of the Sensory Evaluation Scorecard

4.8 Texture Analysis

Crust and crumb compression strength measurements were analyzed by the EZ Test Texture Analyzer. A round flat probe appropriate for testing bread was used with a 10 N load cell. The probe descended 10 millimeters (mm) into the bread sample for the reading. The compression strength (grams of force (gf)) and Youngs (grams of force per mm² (gf/mm²)) were recorded.

4.9 Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine the differences between breads. Difference between means was assessed using Tukey's HSD test. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Chapter 5: Results and Discussion

The purpose of the study was to determine the effects of soy peroxidase on gluten strength and bread volume of yeast bread. The first objective was to incorporate soy peroxidase from soy flour into bread to determine the effects on gluten strength and loaf volume.

The soy peroxidase was prepared by isoelectric precipitation. Table 4 shows the peroxidase activities of the supernatant and precipitates of the soy flour fractions at pH 8, 6.4, and 4.8. Ideally, the pH fraction of soy flour that had the highest peroxidase activity would have been added to bread for analysis, however, it was decided that the pH 4.8 would be used because it produced the most precipitate and had about the same amount of peroxidase activity as the other two fractions.

Table 4: Mean Peroxidase Activity of Soy Flour Fractions

| Soy Fractions | Supernatant (purpurogallin units/ml protein) | Precipitate (purpurogallin units/ml protein) |
|---------------|--|--|
| pH 8.0 | 0.159400 | 0.003900 |
| pH 6.4 | 0.085900 | 0.003580 |
| pH 4.8 | 0.049900 | 0.003020 |

The second objective was to determine if soy peroxidase added to all-purpose flour could produce as desirable a product as bread flour. Gluten strength determination, loaf volume, texture analysis, crust color, and sensory evaluation were the parameters utilized to assess this objective.

5.1 Dough Rheology (Farinograph)

The farinograph develops and overmixes a dough by combining flour and water. The resulting curve, in essence, measures dough resistance to being stretched, sheared, relaxed, and compressed against time (40). The farinograms of the treatment groups are illustrated in Figures 2, 3, and 4.

Water absorption, dough development (peak time), and dough stability, are three factors used to examine dough quality. Water absorption is considered one of the main physical factors affecting the curve characteristics of the farinogram (39). Water absorption values for bread flour alone and bread flour + soy flour were significantly greater ($p < 0.05$) than the other treatment groups (Table 5). The major components contributing to variety or class differences in absorption values are flour protein content and starch damage (40). Generally, water absorption increases as protein content increases; therefore, higher absorption values for bread flour and bread flour + soy flour is expected since they have more protein

Dough development time (peak time) indicates the time as when the dough has its optimum viscoelastic properties for gas retention and pertains to changes in the gluten matrix. Table 3 exhibits the peak time for all the treatment groups.

The long peak times for bread flour (12.50 ± 0.96 min), APF+pH 4.8 precipitate (13.83 ± 1.10 min) indicate very strong wheat flours, while the short peak times for APF±15 mg peroxidase (2.50 ± 1.10), APF+25 mg peroxidase (3.90 ± 0.85 min), and control/APF (2.40 ± 0.85 min) indicate weak flours (38).

Dough stability and mixing tolerance index (MTI), as a general rule, gives an indication of the dough's tolerance to mixing. Flours with good mixing tolerance have low MTIs; therefore, the higher the MTI value, the weaker the flour (41). Bread flour/soy flour mixture had the highest stability time. The bread flour, APF+pH 4.8 precipitate, and APF/soy flour mixture were significantly different ($p < 0.05$) than the remaining three treatments. The 15 mg POX and the 25 mg POX were significantly lower stability times than all of the treatment groups, including the control (Table 5).

The APF+pH 4.8 precipitate, APF/SF, BF, and BF/SF treatment groups appear to have a good tolerance to mixing according to their low MTI scores. The control and 15 mg peroxidase seem to be weaker doughs, whereas, the 25 mg peroxidase appears to be in the middle, indicating, perhaps, that mixing tolerance increases with increasing peroxidase concentration.

Overall, bread flour and bread flour+soy flour are strong flours with great mixing characteristics. This was expected because bread flour has a high protein content, which is formulated for bread making. All-purpose flour (control) was weak flour, which was also anticipated because of its low protein content.

According to the farinograph data, adding 15 and 25 mg purified soybean peroxidase to all-purpose flour produced the same effects as all-purpose flour alone, meaning that mixing characteristics and gluten strength were not improved; however, the farinograph data may not be an accurate representation of the effects of peroxidase on gluten strength. Peroxidase enzymes need hydrogen peroxide as a substrate for activity. The poor results were similar to that observed by Dunnewind et al (2002) and Takasaki et al (2005) who reported that there was no effect on dough rheology in the absence of hydrogen peroxide. On the other hand, in the presence of hydrogen peroxide and peroxidase, Takasaki et al (2005) saw a significant increase in peak time, dough development, and dough softening indicating that the addition of hydrogen peroxide or hydrogen peroxide/peroxidase strengthened dough (32), whereas Dunnewind et al (2002) found that peroxidase in the presence of hydrogen peroxide, produced a higher peak time, had a lower weakening slope meaning that it was more tolerant to overmixing (33). Numerous efforts were attempted to add hydrogen peroxide to the farinograph experiments including peroxidase enzymes; however, none were successful. The hydrogen peroxide appeared to break down the dough as shown in Figure 4.

Given that hydrogen peroxide is essential to the activity of soybean peroxidase enzyme and none could be added to the farinograph in the present research, the improvement in all the farinogram parameters with the pH 4.8 precipitate would be misleading if one believed the improvement came from the

peroxidase enzyme. This would not be possible because no hydrogen peroxide was added. Comparing the farinogram results of APF+pH 4.8 precipitate, 15mg POX, and 25mg POX shows that the APF+pH 4.8 precipitate improved farinogram parameters. Therefore it is safe to assume the improvement in all the farinogram parameters with the pH 4.8 precipitate was from some other component in the soy flour and not from the peroxidase enzyme present in the soy flour. It can also be concluded that the unknown component was not dityrosine bonds because hydrogen peroxide is needed to increase dityrosine bonds enough to improve farinogram parameters (32).

Table 5: Mixing characteristics of all dough treatment groups

| | Arrival Time (min) | Peak Time (min) | Stability (min) | Departure Time (min) | Mixing Tolerance Index (BU) | Breakdown (min) | Water Absorption (%) |
|----------------------------|--------------------------|---------------------------|----------------------------|----------------------------|-----------------------------|---------------------------|----------------------------|
| 15mg Peroxidase | 1.67 ± 0.23 ^b | 2.50 ± 1.10 ^b | 4.67 ± 1.46 ^d | 6.33 ± 1.41 ^d | 43.33 ± 6.11 ^{ab} | 4.83 ± 1.50 ^c | 63.77 ± 0.74 ^{ab} |
| 25mg Peroxidase | 2.10 ± 0.18 ^b | 3.90 ± 0.85 ^b | 8.40 ± 1.13 ^{cd} | 10.50 ± 1.09 ^{cd} | 35.0 ± 4.73 ^{abc} | 7.80 ± 1.16 ^c | 62.95 ± 0.64 ^b |
| Bread Flour | 3.00 ± 0.20 ^a | 12.50 ± 0.96 ^a | 22.00 ± 1.27 ^b | 25.00 ± 1.22 ^b | 15.00 ± 5.29 ^c | 20.75 ± 1.3 ^b | 66.65 ± 0.64 ^a |
| All-Purpose Flour | 1.30 ± 0.19 ^b | 2.40 ± 0.85 ^b | 12.80 ± 1.134 ^c | 15.20 ± 1.09 ^c | 56.00 ± 4.73 ^a | 4.90 ± 1.16 ^c | 62.60 ± 0.57 ^b |
| APF* + 4.8 Soy Precipitate | 1.33 ± 0.23 ^b | 13.83 ± 1.10 ^a | 20.83 ± 1.46 ^b | 22.17 ± 1.41 ^b | 11.67 ± 6.11 ^c | 25.17 ± 1.50 ^b | 62.00 ± 0.74 ^b |
| APF + Soy Flour | 1.25 ± 0.20 ^b | 11.00 ± 0.96 ^a | 20.25 ± 1.27 ^b | 21.50 ± 1.22 ^b | 18.75 ± 5.29 ^{bc} | 19.0 ± 1.30 ^b | 62.35 ± 0.64 ^b |
| Bread Flour + Soy Flour | 3.33 ± 0.23 ^a | 12.50 ± 1.10 ^a | 31.50 ± 1.46 ^a | 34.83 ± 1.41 ^a | 23.33 ± 6.11 ^{bc} | 34.0 ± 1.50 ^a | 66.60 ± 0.74 ^a |

Each value is the mean ±SD of at least four replicates. Values within a column with different letters are significantly different (p<0.01).

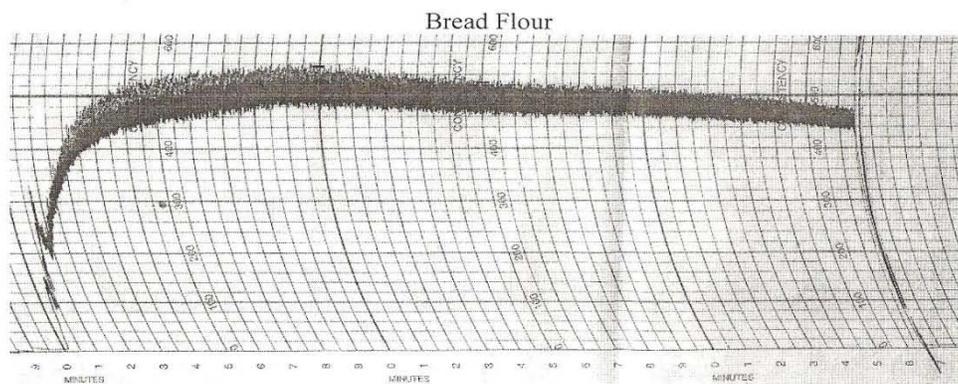
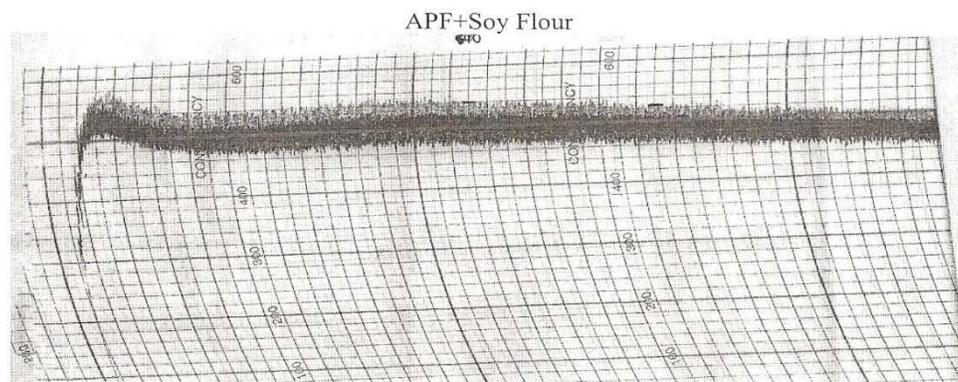
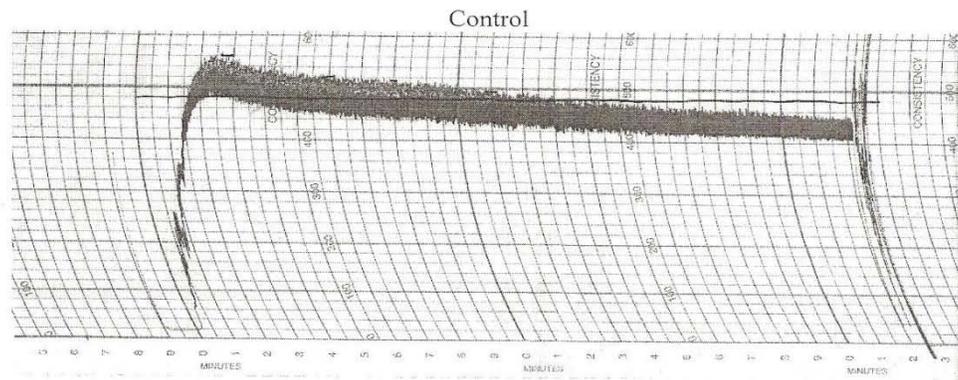
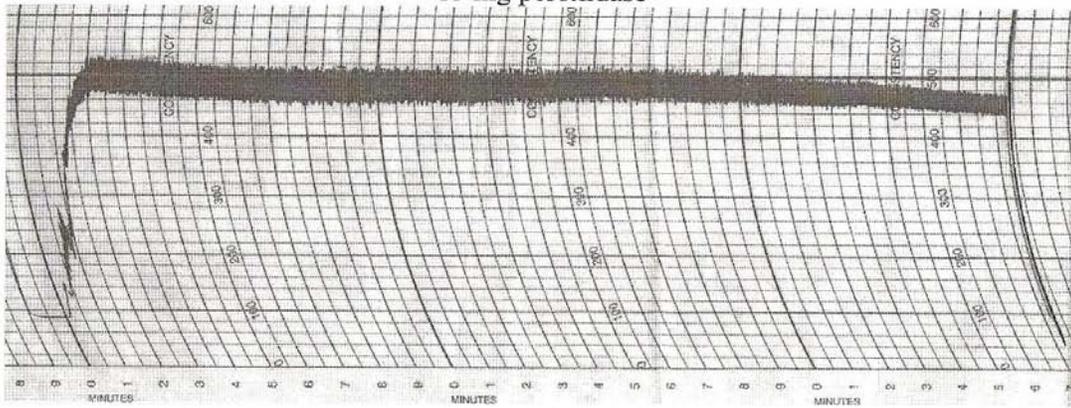
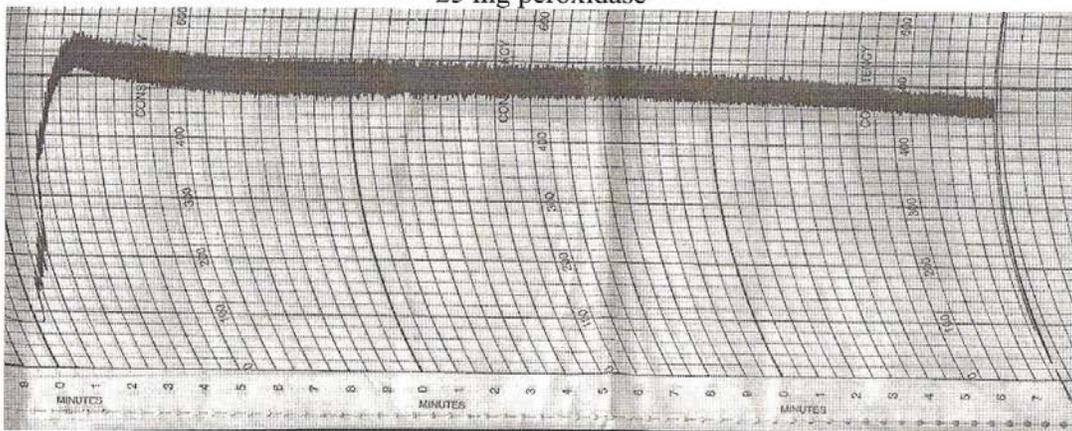


Figure 2: Farinograms of Treatment Groups

15 mg peroxidase



25 mg peroxidase



APF+ ph 4.8 Precipitate

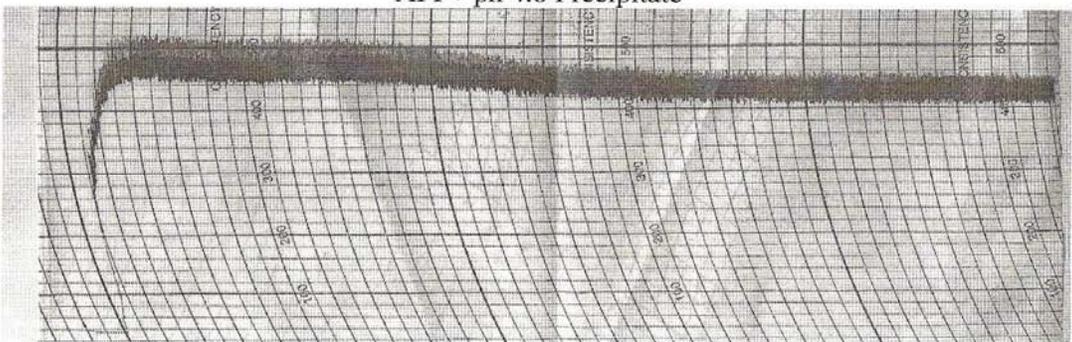
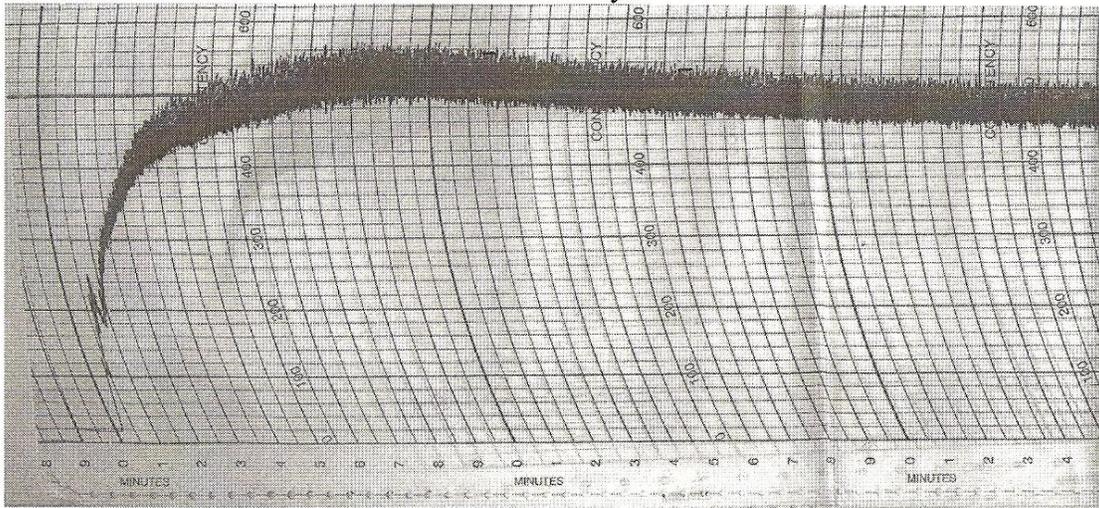


Figure 3: Farinograms of Treatment Groups

Bread Flour+Soy Flour



All-Purpose Flour + Hydrogen Peroxide Added

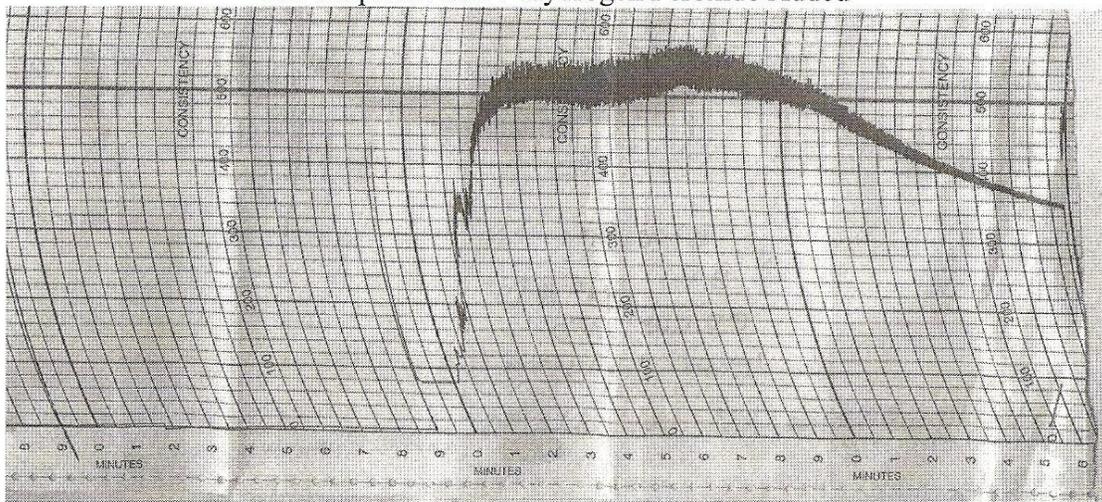


Figure 4: Faringrams of Treatment Groups

Objective Tests

Four loaves of bread were made for the objective test and for sensory analysis; bread flour, all-purpose flour (control), all-purpose flour+soy flour, and all-purpose+pH 4.8 precipitate (Figures 5 and 6). The all-purpose flour was the control, the bread flour was the reference loaf, the all-purpose+pH 4.8 precipitate was the experimental loaf, and the all-purpose flour+soy flour was made to determine if the differences from the pH 4.8 precipitate were due to the peroxidase enzyme or from some other component in the soy flour.

5.1.2 Volume

Hydrogen peroxide did not need to be added to the flour for the baking trials because yeast produces enough hydrogen peroxide for the peroxidase enzyme (34). Bread flour produced a significantly higher volume ($p < 0.05$) loaf of bread than the all-purpose flour+pH 4.8 soy precipitate (Table 6). No other significant differences were observed, but it seems that there was a trend to increasing loaf volume as protein content increased. It would have been expected that the loaf volume of the bread made with the soy flour and with the pH 4.8 precipitate would be around the same given their similar farinograph parameters.

5.1.3 Texture Analysis

Soft, tender, and resilient bread with a short crumb texture is generally preferred among American consumers (42). The texture and tenderness of the bread was analyzed in grams of force (gf). The higher the force, the less tender the product. No significant difference in crumb or crust texture occurred in any of the treatment groups (Table 7). It would have been expected that the other three

bread loafs would have produced a softer loaf of bread compared to the control loaf. Perhaps, a difference would have been detected if more than three replicates were made.

Table 6: Loaf Volume Comparison Between Treatment Groups

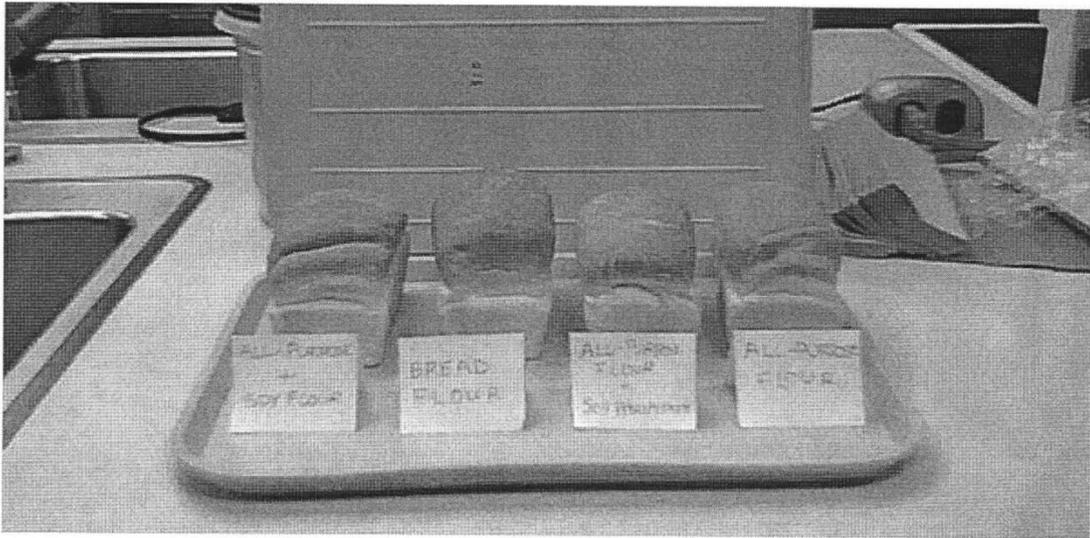
| | Mean \pm standard error |
|-------------------------|---|
| Type of Bread | Loaf Volume (cubic centimeter) (cm ³) |
| Bread Flour | 766.67 \pm 19.09 ^a |
| APF+Soy Flour | 729 \pm 47.32 ^{ab} |
| All-Purpose Flour | 712.5 \pm 12.5 ^{ab} |
| APF+4.8 Soy Precipitate | 662.50 \pm 12.50 ^b |

Each loaf volume is the mean \pm SD of 3 replicates. Means with different superscripts are significantly different at $p < 0.05$ level.

Table 7: Crumb and Crust Texture of Loaves of Bread

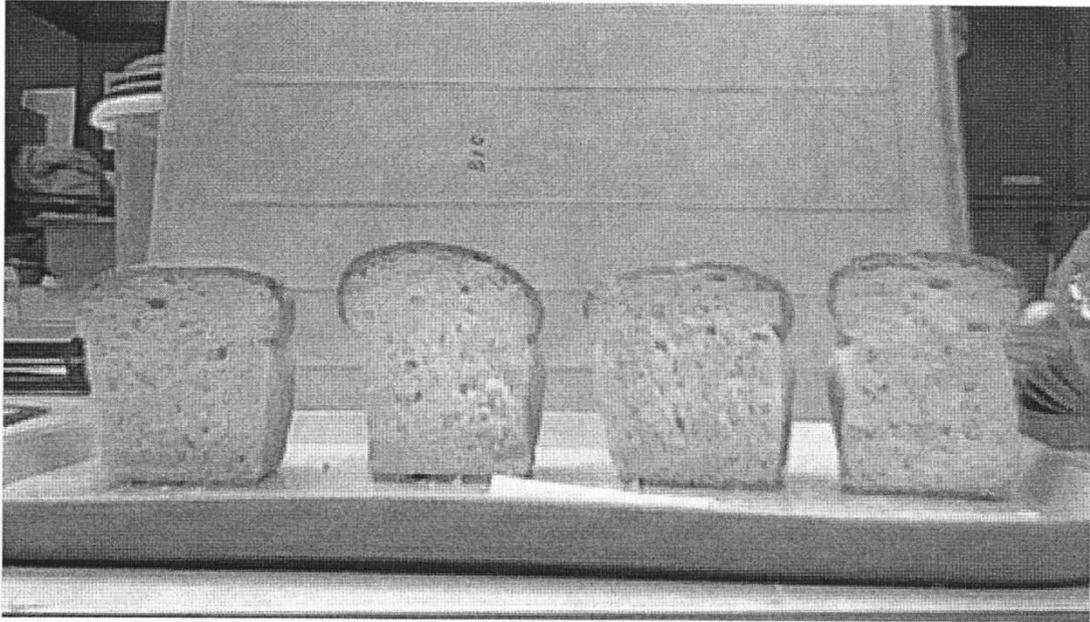
| | Mean±Standard Deviation | Mean±Standard Deviation |
|--------------------------|-------------------------------------|-------------------------|
| Type of Bread | Crumb Texture (grams of force) (gf) | Crust Texture (gf) |
| Bread Flour | 157±23.2a | 479±124a |
| APF+Soy Flour | 134±13.4a | 482±181a |
| All-Purpose Flour | 137±24.8a | 490±145a |
| APF+ 4.8 Soy Precipitate | 164±25.5a | 436±94.5a |

Each force reading is the mean ± SD of 3 replicates. Means with different superscripts within columns are significantly different at p<0.05 level.



Left to right: All-purpose flour+soy flour, bread flour, all-purpose flour +pH 4.8 soy precipitate, and control

Figure 5: Photographs of loaves of bread made from the baking trials



Left to right: All-purpose flour+soy flour, bread flour, all-purpose flour +pH 4.8 soy precipitate, and control

Figure 6: Photographs of the crumb texture of the loaves of bread

5.2 Sensory Evaluation

Any alterations to the standard bread recipe can affect the taste of the resulting loaves of bread. Sensory evaluation was conducted to determine how the treatment groups affected the sensory acceptability of the loaves of bread. A 9-point Hedonic scale was utilized to determine the acceptability of the bread loaves, where 1 was dislike extremely, 5 was neither like nor dislike, and 9 was like extremely.

Sixty-one men and women participated in the sensory evaluation. The results of the test are in Table 8. No differences in preference were determined. All treatment groups were evaluated as slightly more than 5, neither like nor dislike, with the loaf with soy flour at a 6, slightly like. This result is encouraging because no adverse flavors were produced with the addition of the pH 4.8 soy precipitate.

Table 8: Hedonic Score of Bread Loaves

| Type of Breads | Mean Hedonic Score |
|---------------------------|--------------------|
| APF + Soy Flour | 6.18 ^a |
| All-Purpose Flour | 5.90 ^a |
| Bread Flour | 5.48 ^a |
| APF + 4.8 Soy Precipitate | 5.52 ^a |

Means with different superscripts are significantly different at $p < 0.05$ level.

5.3 Summary and Conclusions

The purpose of this study was to determine the effects of adding soy peroxidase on the gluten strength and bread volume of yeast bread. The objectives were met by objective tests and sensory evaluation. Isoelectric precipitation of soy flour was used to produce an inexpensive form of soy peroxidase. However, the fraction used for the experiments was not high in peroxidase activity; therefore, none of the results from the study can be attributed to the effects of peroxidase.

The major finding of the study was that soy peroxidase needs hydrogen peroxide as a substrate for activity as evidenced by the farinograph data. Therefore, the increase in gluten strength experienced from the pH 4.8 soy precipitate was not due to peroxidase, but some other component present in that fraction, perhaps from other enzymes that may be present in that fraction. The improvement in farinograph parameters for the APF+soy flour, bread flour, bread flour+soy flour, and the APF+pH 4.8 precipitate compared to the control did not translate into increased loaf volume or increased tenderness as was expected. The addition of the pH 4.8 soy precipitate to all-purpose flour actually significantly decreased loaf volume compared to bread made from bread flour and there was no significant difference in crumb or crust texture for any of the treatment groups. Sensory evaluation indicated that there was no negative effect on preference for any of the breads.

Given all the results, it can be concluded that isoelectric precipitation of soy flour using phosphate buffer was not an effective procedure in producing a soy fraction with high peroxidase activity.

5.4 Recommendations for Future Research

1. A better procedure to produce a cost-effective form of soy peroxidase is needed.
2. A repeat of this study using purchased purified soybean peroxidase and analysis of the dityrosine content of the samples
3. Perform farinograph experiments, loaf volume, texture analysis, and sensory evaluation of each soy fraction from the isoelectric precipitation (pH 8.0, pH 6.4, and pH 4.8), and compare the results to each other and to whole soy flour. Perhaps some insight into exactly what component of soy flour exhibits its good dough handling qualities can be determined.

References

1. Orth RA, Shellenberger JA, 1988. Origin, production, and utilization of wheat. In: Pomeranz Y, Ed. Wheat: Chemistry and Technology 1. St. Paul, MN: American Association of Cereal Chemists, Inc. p 1.
2. Dupuis B, 1997. The chemistry and toxicity of potassium bromate. Cereal Food World 42:171-81.
3. Gonzalez M, 1993. Potassium bromate in bread making. Bulletin Association of Operative Millers:6191-5.
4. Poul JM, Huet S, Godard T, Sanders P, 2004. Lack of genotoxicity of potassium iodate in the alkaline comet assay and in the cytokinesis-block micronucleus test. Comparison to potassium bromate. Food Chem Toxicol 42:203-9.
5. Delcros J-F, Rakotozafy L, Boussard A, Davidou S, Porte C, Potus J, Nicolas J, 1998. Effect of mixing conditions on the behavior of lipoxygenase, peroxidase, and catalase in wheat flour doughs. Cereal Chem 75:85-93.
6. Rakotozafy L, Mackova B, Delcros, J-F, Boussard, A, Davidou, S, Potus J, Nicolas J, 1999. Effect of adding exogenous oxidative enzymes on the activity of three endogenous oxidoreductases during mixing of wheat flour dough. Cereal Chem 76:213-8.
7. Janardhanan KA, Kizhakkedathu P, Nazeerunnisa M, Behere DV, 2002. Thermal unfolding of soybean peroxidase. J Biol Chem 43:40717-21.
8. Miranda MV, Magri ML, Navarro AA, Navarro del Canizo, Cascone O, 2002. Study of variables involved in horseradish and soybean peroxidase purification by affinity chromatography on concanavalin A-Agarose. Process Biochem 38:537-43.
9. Kamal JKA, Behere DV, 2003. Activity, stability, and conformational flexibility of seed coat soybean peroxidase. J Inorg Biochem 94:236-42.
10. Gillikin JW, Graham JS, 1991. Purification and development analysis of the major anionic peroxidase for the seed coat of *Glycine max*. Plant Physiol 96:214-20.
11. Kamal JKA, Behere DV, 2001. Steady-state and picosecond time resolved fluorescence studies on native and apo seed coat soybean peroxidase. Biochem Biophys Res Commun 289:427-33.

12. McEldoon JP, Dordick JS, 1996. Unusual thermal stability of soybean peroxidase. *Biotechnol Prog* 12:555-8.
13. Nissum M, Schiodt CB, Welinder KG, 2001. Reactions of soybean peroxidase and hydrogen peroxide pH 2.4-12.0, and veratryl alcohol at pH 2.4. *Biochim Biophys Acta* 1545:339-48.
14. Henriksen A, Mirza O, Chiara I, Teilum K, Smulevich G, Welinder KG, Gajhede M, 2001. Structure of soybean seed coat peroxidase: A plant peroxidase with unusual stability and haem-apoprotein interactions. *Protein Sci* 10:108-15.
15. Munir IZ, Dordick JS, 2000. Soybean peroxidase as an effective bromination catalyst. *Enzyme Microb Tech* 26:337-41.
16. McEldoon JP, Pokora AR, Dordick JS, 1995. Lignin peroxidase-type activity of soybean peroxidase. *Enzyme Microb Tech* 17:359-65.
17. Wright H, Nicell JA, 1999. Characterization of soybean peroxidase for the treatment of aqueous phenols. *Bioresource Technol* 70:69-79.
18. Mills ENC WN, Salt LA, Robertson J, Jenkins JA, 2003. Wheat proteins and bread quality. In: Cauvain S, Ed. *Breadmaking: Improving quality* Boca Raton, FL: CRC Press LLC. P 121-31.
19. Wrigley CW, Bietz JA, 1988. Proteins and amino acids. In: Pomeranz Y, Ed. *Wheat: Chemistry and Technology 1*. St. Paul, MN: American Association of Cereal Chemists, Inc. p 165-93.
20. Halverson J, Zeleny L, 1988. Criteria for wheat quality. In: Pomeranz Y, Ed. *Wheat: Chemistry and Technology 1*. St. Paul. MN: American Association of Cereal Chemists. p 25.
21. Mailhot WC, Patton JC, 1988. Criteria of flour quality. In: Pomeranz Y, Ed. *Wheat: Chemistry and Technology 2*. St. Paul, MN: American Association of Cereal Chemists, Inc. p 70-1.
22. Pylar EJ, 1988. Baking Science and Technology. In: Ed. 1. Kansas City, MO: Sosland Publishing Co. p 183-227,300-71,443-87, 581.
23. Cauvain S, 2003. Breadmaking: an overview. In: Cauvain SP , Ed. *Breadmaking: Improving quality* Boca Raton FL: CRC Press LLC. P 8-17.
24. Pomeranz Y, 1988. Composition and functionality of wheat flour components. In: Pomeranz Y, Ed. *Wheat: Chemistry and Technology 2*. St. Paul, MN: American Association of Cereal Chemists, Inc. p 192-229.

25. Lavelli V, Guerrieri N, Cerletti P, 1996. Controlled reduction study of modifications induced by gradual heating in gluten proteins. *J Agric Food Chem* 44:2549-55.
26. Shewry PR, Tatham AS, 1995. Disulfide bonds in wheat gluten proteins. *J Cereal Sci* 25:207-27.
27. Morel M-H, Bonicel J, 1996. New investigations of disulfide bonds of wheat proteins by dithioerythritol (DTE) reduction. In: Wrigley CW, Ed. *Gluten '96, Proceedings of the Sixth International Gluten Workshop*. Melbourne, Australia: Royal Australian Chemical Institute. P 257-261.
28. Tilley KA, Benjamin RE, Bagorogoza KE, Okot-Kotber BM, Prakash O, Kwen H, 2001. Tyrosine cross-links: Molecular basis of gluten structure and function. *J Agric Food Chem* 49:2627-32.
29. Hanft F, Koehler P, 2005. Quantification of dityrosine in wheat flour and dough by liquid chromatography-tandem mass spectrometry. *J Agric Food Chem* 53:2418-23.
30. Michon T, Wang W, Ferrasson E, Gueguen J, 1998. Wheat prolamine crosslinking through dityrosine formation catalyzed by peroxidase: Improvement in the modification of a poorly accessible substrate by "Indirect" catalysis. *Biotechnol Bioeng* 63:4491-58.
31. Oudgenoeg G, Hilhorst R, Piersma SR, 2001. Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *J Agric Food Chem* 49:2503-10.
32. Takasaki S, Kato Y, Murata M, Homma S, Kawakishi S, 2005. Effects of peroxidase and hydrogen peroxide on the dityrosine formation and the mixing characteristics of wheat-flour dough. *Biosci Biotechnol Biochem* 69:1686-92.
33. Hilhorst R, Dunnewind B, Orsel R, Stegeman P, van Vliet T, Gruppen H, Schols HA, 1999. Baking performance, rheology, and chemical composition of wheat dough and gluten affected by Xylanase and oxidative enzymes. *J Food Sci* 64:808-13.
34. Dunnewind B, van Vliet T, Orsel R, 2002. Effect of oxidative enzymes on bulk rheological properties of wheat flour doughs. *J Food Sci* 36:357-66.
35. Hilhorst R, Gruppen H, Orsel R, Laane C, Schols HA, Voragen AGJ, 2002. Effects of xylanase and peroxidase on soluble and insoluble arabinoxylans in wheat bread dough. *J Food Sci* 67:497-506.

36. Thanh VH, Okubo K, Shibasaki K, 1975. Isolation and characterization of the multiple 7S globulins of soybean proteins. *Plant Physiol* 56:19-22.
37. Hatcher D, Barker W, 2005. A rapid microassay for determination of peroxidase in wheat and flour. *Cereal Chem* 82,(2):233-7.
38. Lorenz K, 1984. Interpretation of the farinogram. In: D'Appolonia BL; Kunerth WH, Ed. *Farinograph Handbook* St. Paul, MN: American Association of Cereal Chemists, Inc. p 31-2.
39. AAAC. 1983. *Approved Methods*, 10-09. American Assoc. Cereal Chem., St. Paul, MN.
40. Preston KR, Kilborn RH, 1984. Dough rheology and the farinograph. In: D'Appolonia BL; Kunerth WH, Ed. *Farinograph Handbook* St. Paul, MN: American Association of Cereal Chemists. p 38-41.
41. Shuey WC, 1984. Physical factors influencing farinograms. In: D'Appolonia BL; Kunerth WH, Ed. *Farinograph Handbook* St. Paul, MN: American Association of Cereal Chemists. p 24-30.
42. Chung OK, Park SH, Tilley M, Lookhart GL, 2003. Improving wheat quality. In: Cauvain S, Ed. *Bread making: Improving quality* Boca Raton:FL: Woodhead Publishing Ltd CRC Press LLC. p 548



Office of Research Compliance
Institutional Review Board
1880 Pratt Drive (0497)
Blacksburg, Virginia 24061
540/231-4991 Fax: 540/231-0959
E-mail: moored@vt.edu
www.irb.vt.edu

DATE: August 30, 2006

MEMORANDUM

TO: William E. Barbeau
Ratia Kirby
Erin Danielson

FROM: David M. Moore 

SUBJECT: **IRB Exempt Approval:** "The Effects of Soy Peroxidase and Soy Lipoxygenase Enzymes on the Gluten Strength and Loaf Volume of Yeast Bread", IRB # 06-454

I have reviewed your request to the IRB for exemption for the above referenced project. I concur that the research falls within the exempt status. Approval is granted effective as of August 29, 2006.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

cc: File
Department Reviewer:Kathy Hosig

Invent the Future