INCORPORATION OF A COMMERCIAL HYDROLYZED WHEY PROTEIN ISOLATE WITH ANGIOTENSIN-CONVERTING ENZYME – INHIBITION ACTIVITY INTO BREAD

By

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To the Faculty of Washington State University:

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Chair

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INCORPORATION OF A COMMERCIAL HYDROLYZED WHEY PROTEIN ISOLATE WITH ANGIOTENSIN-CONVERTING ENZYME – INHIBITION ACTIVITY INTO BREAD

Abstract

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Functional foods are foods which provide a health benefit beyond basic nutrition. Angiotensin-converting enzyme – inhibitors are one treatment for the management of hypertension. A commercial hydrolyzed whey protein isolate that has angiotensin-converting enzyme – inhibitor activity was added to wheat bread and the effect on the rheological properties of the dough and the physical properties of bread measured. In addition, the angiotensin-converting enzyme – inhibition activity of the final product was evaluated.

Ten, 20, and 30% commercial hydrolyzed whey protein isolate were added on a flour replacement basis to wheat dough and the effect on mixing properties observed using a mixograph. The addition of commercial hydrolyzed whey protein isolate shortened the mixing time to make an optimal dough. Incorporating commercial hydrolyzed whey protein isolate after the gluten had

begun to develop improved the overall dough properties. Incorporation of 30% commercial hydrolyzed whey protein isolated did not produce an acceptable dough.

To further test the functionality of the commercial hydrolyzed whey protein isolate in bread 10, 20, and 30% were added at two addition times: at the beginning of the mixing process and after two minutes. Breads with commercial hydrolyzed whey protein isolate had a significantly ($p < 0.05$) lower loaf volume and baked loaf weight than the control bread. Additionally, incorporation of commercial hydrolyzed whey protein isolate at all levels resulted in significantly ($p < 0.05$) darker crust than the control as determined by CIE- LAB values.

The angiotensin-converting enzyme – inhibition activity of the final product was tested to determine if the peptide was able to withstand the harsh conditions of processing including fermentation and heating. Angiotensin-converting enzyme – inhibition activity was present in the final bread samples, suggesting that the peptide can withstand bread food processing.

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INTRODUCTION

Functional foods, defined as foods which provide a health benefit beyond basic nutrition, are increasing in prevalence to meet the health needs of consumers. One way to create a functional food is to add an ingredient with a specific biological function to an established food product. Many food proteins contain sequences of amino acids with biological activity known as strategic zones. The processing of these proteins to increase the availability of strategic zones may result in increased potency of biological activity of the processed protein, when compared to the native protein. Whey proteins contain sequences of amino acids that act as angiotensin-converting enzyme-inhibitors; the hydrolysis of whey proteins may increase the ability of whey to act as an ACE-inhibitor. ACE-inhibitors disrupt the pathway in the rennin-angiotensin system, interfering with the conversion of angiotensin I to angiotensin II. Angiotensin II is also a vasoconstrictor and increases blood volume. A commercial hydrolyzed whey protein isolate (CHWPI) is available that is known to act as an ACE-inhibitor.

The addition of CHWPI to a product, such as bread, may result in a functional food. Hypertension is a chronic condition, thus products which are designed to help manage hypertension must be able to be consumed daily. Wheat bread was selected as the delivery system for CHWPI because it is a

commonly consumed product in the United States and can be easily consumed everyday.

There are many aspects to consider when formulating a functional food. The ingredient which makes the food “functional” should not adversely affect the processing, physical, or sensory aspects of the product. Additionally, the processing which a product goes through should not affect biological activity of the functional ingredient. In the formulation of a functional food, the amount of the functional ingredient that needs to be consumed to get a health benefit should be considered.

This research took into consideration the many challenges of creating a functional food. The objective of this research was to determine if commercial hydrolyzed whey protein isolate could be incorporated into bread to create a functional food. To examine how commercial hydrolyzed whey protein isolate functions in bread this research was divided into three different stages.

The first stage of the research examined the effect that CHWPI had on the rheolology of wheat flour bread dough. A series of studies using a mixograph was carried out using different levels of CHWPI to determine the maximum amount of CHWPI that could be incorporated into bread dough as well as the proper water absorption. In the second stage a controlled bread bake was carried out to determine the effect that CHWPI had on the physical properties of bread. The objective of the third stage of the research was to determine if the ACE-inhibiting activity of CHWPI survived the bread-making process.

CHAPTER I

LITERATURE REVIEW

LITERATURE REVIEW

INTRODUCTION

Consumers are becoming increasingly aware of the connection of diet to their health. The knowledge of nutrition among the general population has lead to the development of foods that enhance ones health or meet the specific nutrition requirements required by individuals. One way to deliver a specific health benefit in a familiar manner is the incorporation of beneficial ingredients into existing products (Fitzgerald and Murray, 2006, Mazza, 1998).

As research has begun to associate specific benefits with the consumption of certain foods, ingredients, and components, the market for foods with features that provide heath benefits have increased. Currently, there is no standard definition, nor is there a singular term to indicate a food product or ingredient in a food product that has a health benefit(s) beyond basic nutrition. Some common terms that are used in the food industry are: functional foods, functional ingredients, nutraceuticals, bioactive foods, bioactive ingredients, bioactive components, physiologically-active foods and designer foods (Clydesdale, 2005, Mazza, 1998).

The term “functional food,” which is used for both ingredients and whole products, is one of the more common terms. Currently, the term “functional foods” is defined by the food industry. The Institute of Food Technologists in the

IFT Expert Report Functional Foods: Opportunities and Challenges document, defines functional foods as “food and food components that provide a health benefit beyond basic nutrition (for the intended population)…[They] provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development and/or other biologically active components that impart health benefits or desirable physiological effects”(Clydesdale, 2005). The U.S. Food and Drug Administration (FDA) currently has no legal definition for the term “functional foods.” However, the FDA has sought definitions of the term “bioactive food components”. Several definitions were submitted in response to the Federal Register Notice (Table 1). Some groups disagreed with the term “bioactive food components” and preferred a term that consumers would be more likely to understand such as “functional food component”. The common theme among all the definitions submitted is that the ingredient or food have a specific beneficial effect on the body (Clydesdale, 2005, Saldanha, 2005).

The sales of functional foods are growing within the retail food sector. The market size for functional foods ranges from $2 – 200 billion in the United States depending on the classification of “functional foods”. Americans are seeking out functional foods due to the increasing idea of taking a holistic approach to the care of ones body. Spending on functional foods has increased from $20.30 per person in 1998 to $37.40 per person in 2003. The market for functional bakery products looks promising. The share of the bakery market that is dedicated to functional foods is increasing steadily along with an increase in bakery and

snacks overall, and is projected to keep on growing (Table 2). The increase in the value of sales is due to the innovation in the bakery product industry, leading to a higher price point per unit. The addition of whey protein to bakery products, which increases the protein of the product is one of the more common ways to create functional bakery products and add value (Shortt and O'Brien, 2004).

One type of functional foods are bioactive peptides. Bioactive peptides can come from a number of food sources; the most common are from dairy proteins such casein and whey proteins. Bioactive peptides are small, ranging from two to twenty amino acids residues. The size of the peptides allows them to reach the target organ or system. Bioactive peptides may be very specific, performing one function, or they may perform two or more functions. Bioactive peptides can act as antihypertensive agents, antioxidants, antimicrobials, or opioids. In addition, they can help regulate the immune system and help the body absorb minerals (Meisel, 2004, Meisel, 2007).

Hydrolyzed whey proteins fall into the class of bioactive peptides. Whey proteins contain a sequence of amino acids embedded inside the primary protein structure that act as an angiotensin-converting enzyme-inhibitor (ACE-inhibitor). ACE-inhibitors may be beneficial to individuals with hypertension. Approximately a third of the western population has hypertension, which is characterized by higher than normal blood pressure. Hypertension increases the risk of many diseases including heart attack and stroke. However, for an ACE-inhibitor to be effective it needs to be active in the carrier food. Thus, it must be able to

Table 1. Definitions of "Bioactive Food Components" from Response to the Federal Register (Clydesdale, 2005)

Group / Individual	Proposed Definition
American Dietetic Association	Bioactive food components are physiologically active constituents in foods or dietary supplements derived from both animal and plant sources, including those needed to meet basic human nutrition needs, that have been demonstrated to have a role in health and to be safe for human consumption in intended food and dietary supplement uses.
American Herbal Product Association (AHPA)	Bioactive food components are constituents in foods or dietary supplements, other than those function to meet basic nutritional needs, that effect changes in health status or changes in the structure or function of the body.
Chris Hawkes, USDA/ ARA Western Human Nutrition Center, University of California at Davis	Any compound that occurs naturally in foods commonly consumed in the United States in quantities sufficient or likely to cause detectable biological effect in humans
Food Products Association (FPA)	Bioactive food components are those food substances that contribute beneficially to supporting health promotion and disease risk reduction in the context of the diet
Grocery Manufacture's of America (GMA)	A bioactive food component is a nutrient, food, food component, or a combination of food components that affects the structure or function or imparts a physiological benefit in the body to improve health.
ILSI North America	Physiologically-active food components: Food components demonstrated to result, directly or indirectly in a consistent positive physiological response linked to health promotion or reduction in risk of disease, as measured through utilizing appropriate methodology and biomarkers.
Institute of Food Technologists	Bioactive food components are substances in foods, including dietary supplements, that have biological activity that directly affect the structure or function of the body
National Yogurt Association	Constituents in foods or dietary supplements, including those need to meet basic nutritional needs that are responsible for changes in health stats beyond nutrition
Robert E. Levin, University of Massachusetts	Bioactive components of foods are individual chemical components that either influence the physiology and metabolism of the body directly, or have an indirect effect, by altering the metabolism or other chemical components in the diet. Such bioactive components of the diet being either detrimental or of benefit to the human body under otherwise normal conditions of dietary intake.
The American Society for Nutritional Sciences (ASNS); Them American Society for Clinical Nutrition (ASCN)	Bioactive food components are dietary constituents that elicit physiological effects beyond those associated with essential human nutrition

withstand the harsh environments of processing, packaging and storage. Additionally, it should not negatively alter the physical or sensory properties of the carrier food. In the case of whey proteins, the amino acids at the C-terminal are mostly responsible for their function as an ACE-inhibitor. These amino acids can possibly be affected by processing due to oxidation of amino acids or the Maillard browning reaction. The effects of food processing on ACE-inhibitors has not been well documented (Lopez-Fandino, et al., 2006).

Bread is a widely consumed, affordable food in the United States. The consumption of bread has been a common thread throughout much of human civilization. Consumption of bread has been traced back to the ancient Egyptians. In many societies, throughout history and up to present times, bread has been considered a staple food. Bread can be produced from different flours including rye, corn

Table 2. The future of functional bakery and snack products in the United States (Global Market Information Database, 2007)

Year	Total bakery product and snack value sales ($ million)	% of total bakery product and snack sales that are functional
1998	69662.1	1.2
1999	72347.8	1.4
2000	75232.0	1.5
2001	77778.9	1.6
2002	79474.9	1.6
2003	81798.0	1.7
2004*	82492.7	1.8
2005*	82665.1	1.9
2006*	82765.3	2.1
2007*	83070.5	2.2
2008*	83222.5	2.3

*projected years

and wheat. The most popular flour and that which is considered to yield the best loaf of bread is wheat flour. Today, the supermarket is filled with different varieties of bread from white to hearty wheat. Due to the prevalence of bread in the diet it would be a good delivery system for a functional food that benefited a chronic condition, as it the case with bioactive peptides that act as ACE-inhibitors (Crowley, et al., 2002, Faridi and Faubion, 1990, Wood, 1998).

BREAD

The most basic bread formula contains flour, yeast, salt, and water. This formula can be varied seemingly infinitely by modification of the basic formula and the addition of other ingredients. Breads made with additional ingredients are categorized as variety breads and may be labeled home-style, farm-style, or country. Variety breads typically attempt to mimic homemade bread with a coarser texture. According to the Code of Federal Regulations, bread is a product resulting from the baking of yeast-leavened dough that has not less than 62% total solids. Of the various breads produced in the United States, the most common is the white pan bread. White pan bread must meet certain specifications, including a final moisture of no more that 38%. According to the standard of identity, bread must consist of flour, bromated flour, phosphated flour or a combination as well as a moistening ingredient that may be water, milk, egg products, or a nutritive carbohydrate sweetener and yeast. In addition,

ingredients or additives can be incorporated into bread to achieve variation in flavor and texture or functionally of the loaf (Table 3) (Faridi and Faubion, 1990).

All yeast bread undergoes three basic processing steps. The first step is to make the dough, which is followed by a fermentation step, and finally the baking step. The most popular method of making bread in the United States is the sponge and dough method, which accounts for about 61% of all bread made. Other methods include the straight-dough method, which is used in many retail bakeries and for specialty breads, the continuous method, no-time method, and Chorleywood process. The continuous method is not employed widely commercially due to the low quality bread that it produces. The Chorleywood

Table 3. Optional ingredients to incorporate into bread according to 21CFR136.110

Specified Ingredients	Limit of addition
Nonwheat flours, nonwheat meals, nonwheat grits, wheat and nonwheat starches	May be used in any combination of two or more, so long as the total quantity does not exceed 3 parts for each 100 parts by weight of flour used
Ground dehulled soybeans (may be heat treated, and oil removed, must maintain enzymatic activity)	Must not exceed 0.5 part for each 100 parts by weight flour used
Yeast nutrients, calcium salts	Must not exceed 0.25 part for each 100 parts by weight of flour used; monocalcium phosphate must not exceed 0.75 part per 100 parts by weight flour used; no limit for calcium propionate
Azodiocarbonamide	Must not exceed 0.0045 part for each 100 parts of flour used
Dough strengtheners and conditioners	Combinations must not exceed 0.5 part for each 100 parts by weight flour used
Spices, spice oil, spice extract	No specified limit
Other ingredients	May be added if do not adversely affect the basic identity, physical, nutritional characteristics

process, a method by which the dough is mechanically developed, is not common in the United States (Faridi and Faubion, 1990, Hoseney, 1986, Wood, 1998)

The straight-dough system is the simplest of all methods (Figure 1). All ingredients of the bread formula are mixed together at the same time, exposing all ingredients to the same amount of fermentation time. The straight-dough system is ideal for making bread with low protein flours. The advantages of this method are that it is simple and produces a chewier loaf with a coarser structure than other methods. However, this method produces a loaf with less flavor then the sponge-dough method (Hoseney, 1986, Pomeranz, 1987).

The sponge and dough method is a two step method to create the loaf in which the sponge is created first, allowing the yeast to become active, and then the dough is formed. The sponge consists of the majority of the water from the formula, the yeast, and a small amount of flour. In the sponge the actual ratio of flour to water varies depending on the producer. The sponge is allowed to set for a number of hours, which lends a characteristic flavor to the bread. Next, the dough is formed by mixing the rest of the flour and water into the sponge. A disadvantage of this method is that an extra step is required (Hoseney, 1986, Wood, 1998).

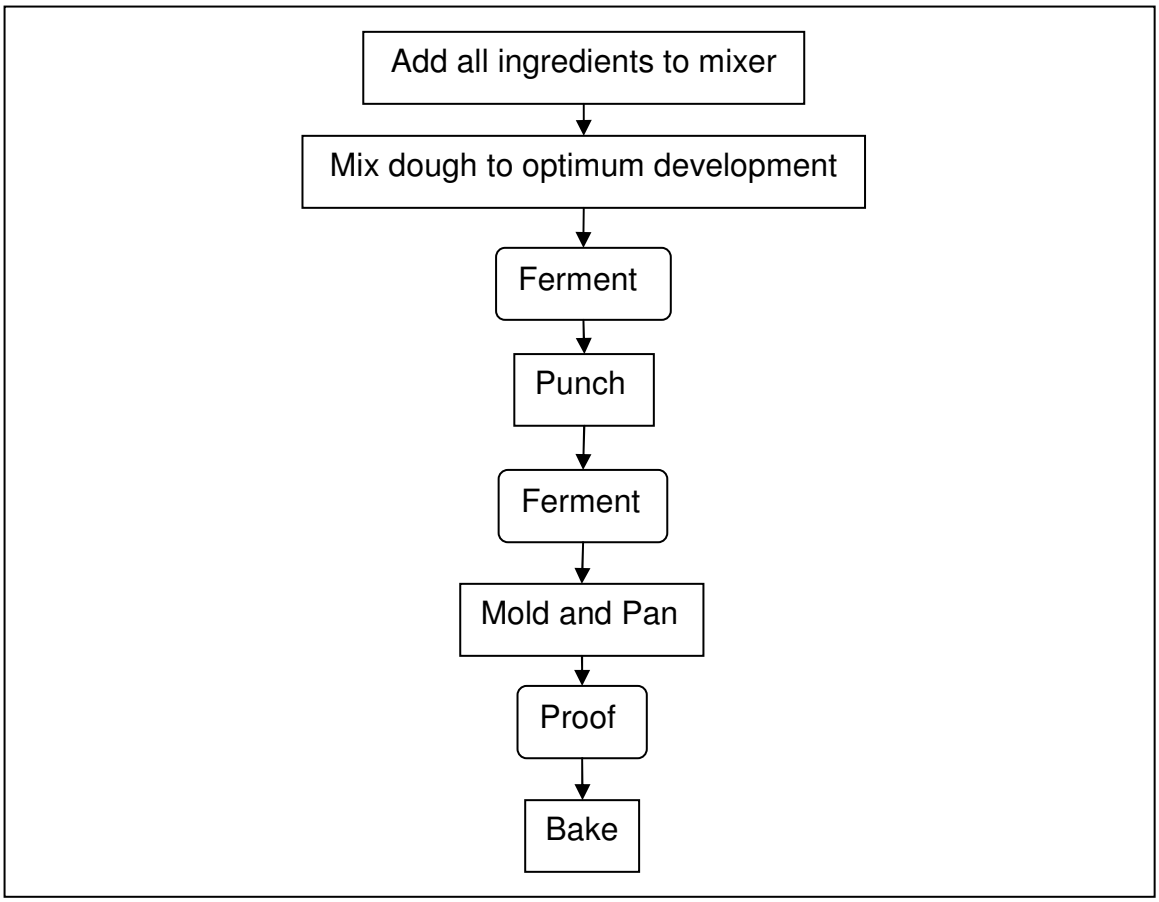

Figure1. Straight-dough process

Flour

Wheat is classified according to color, kernel and varietal characteristics. Based on these characteristics there are eight classifications of wheat: Hard Red Spring, Hard Red Winter, Soft Red Winter, Durum, Soft White, Hard White, Unclassified, and Mixed. The selection of wheat to be milled into flour for a particular application should take into consideration of its hardness, protein level, and protein quality. Flour is made up largely of the endosperm. Flour consists of

starch, proteins, fat, sugar, minerals, moisture, and cellulose. Flour for making bread is milled from hard wheat, which has a high protein content and thus is stronger (Faridi and Faubion, 1990, Hoseney, 1986).

The gluten protein in wheat flour allows a cohesive dough to form that can retain the gas produced by yeast. Gluten proteins have a low charge density which allows them to interact with each other and form a dough. There are hydrophobic interactions between gluten proteins, which allow the structure of the dough to form. Gluten can be fractionated into two parts: glutenins and gliadin. Glutenins are multi-chained, larger molecular weight proteins. The elastic nature of bread dough is believed to be due to the nature of glutenin. The smaller, single-chained, gluten proteins are gliadins. The single-chained gliadins are believed to be the contributing proteins in flour to the highly viscous nature of bread dough (Faridi and Faubion, 1990, Hoseney, 1986).

Water

The proper amount of water is essential to optimizing loaf volume, crumb structure, and overall attributes of the bread. Several factors affect the amount of water to be added to bread dough. These factors include the water absorption of the flour (optimal amount of water that flour can hold), the method used to process the dough and the desired physical characteristics of the loaf. The addition of too much water results in a sticky dough that may be difficult to

handle. The moisture content of the bread baked from dough containing too much water may be susceptible to spoilage by microorganisms and exceed legal levels. Dough that is too dry will not have enough moisture to allow the flour to hydrate, thus full development potential of gluten will not be achieved. Additionally, lack of moisture in the dough will result in bread that stales quickly and crumbles easily (Faridi and Faubion, 1990, Pomeranz, 1987).

Salt

Salt is added to bread dough at the addition level of up to 4% to enhance the flavor of the final product as well as to improve the handling property of the dough. The addition of salt to bread dough helps the loaf retain moisture after baking. Salt is also used in bread dough to decrease the rate of gas production by the yeast, thus leading to the desired crumb structure. It also has the ability to strengthen the gluten structure of the bread, which is important for mechanical dough making processes. The level of salt addition will have an effect on the final product. At lower levels, salt may be used to allow the production of gas by the yeast (Faridi and Faubion, 1990, Pomeranz, 1987, Wood, 1998).

Sugar

Bread can be made without the addition of sugar, as is the case with the traditional baguette. However, many bread formulas do contain sugar for the desirable sweetness that it lends to the final product. The amount of sugar in the bread formula depends on regional preferences. An additional benefit is that the addition of sugar in the form of sucrose will be hydrolyzed to glucose and fructose, which can then be used by the yeast during fermentation. Thus, the addition of sugar will increase the fermentation rate, producing bread with a higher loaf volume. Dough formulations with large amounts of sugar will have a decreased water activity, which will result in slower yeast fermentation (Wood, 1998).

DOUGH DEVELOPMENT AND RHEOLOGY

The initial stage in bread making involves combining the ingredients into a dough. Dough formation is not spontaneous; it requires the input of energy. In the industrial setting this is done with a mechanical mixer. An ideal dough can withstand the rigors of mechanical mixing and produce a palatable bread loaf. Some of the factors that contribute to properly developed dough include flour quality, the amount of water that is added, and the energy that is used to develop

the dough. The optimization of these factors is key to producing a bread loaf with maximum volume, ideal crumb structure, and color (Faridi and Faubion, 1990, Hoseney, 1986).

The mixing process is key to the development of bread dough; it provides energy to combine flour, water, salt, shortening, yeast and other ingredients into dough. Mixing of the dry ingredients with water, or other liquid, leads to hydration of the system. Additionally, breakage of disulfide bonds that are part of the gluten protein of flour occurs, creating weaker dough. During the process of mixing the gluten proteins begin to align, which creates resistance. The amount of air that is incorporated during mixing is important, as yeast can not produce new gas cells. Instead, yeast uses the nitrogen gas in the air incorporated into the bread dough as nuclei for expansion of gas cells. Mixing is essential to fermentation, as it allows the yeast to come into contact with the sugar. The addition of yeast to dough changes the rheology of the dough, making it more elastic (Faridi, 1985, Faridi and Faubion, 1990, Hoseney, 1986).

Proper dough development is achieved when maximum resistance to mixing is reached. During the mixing process there is an optimum point in which all ingredients are hydrated and incorporated into the continuous phase of the dough. Additional mixing beyond the optimum point will cause a deterioration in quality of the final bread loaf, resulting in dough that is mechanically broken down, characterized by sticky, difficult to handle dough (Calderon-Dominguez, et al., 2004).

It has been postulated that the breakdown of the dough during mixing is related to shear thinning that occurs during mixing; the breakdown of the dough is also recognized to be an oxidation process. Chemical agents that are added to the dough, especially reducing agents such as cysteine and sodium bisulfite shorten mixing time. Reducing agents shorten mixing time by breaking disulfide bonds in gluten proteins. The hydrophilic proteins are able to hydrate easier than the larger, and generally more hydrophobic proteins, consequently the mixing time is decreased. Another factor that can affect the mixing time is the pH of the bread dough. A lower pH leads to a shorter mixing time while a higher pH increases the mixing time. High levels of salt will decrease the affect that pH of the dough has on the mixing time (Hoseney, 1986).

Rheology, the study of deformation of matter upon the application of force, can be used to study and optimize dough formulas. The rheological properties of dough are important indicators of the quality of the final baked product. Dough rheology is dependent on a number of factors, including the composition of the dough itself, the addition of additives such as oxidants and the pH of the dough. Additionally, the rheology of the dough changes during and after fermentation, at which point it becomes more elastic (Hlynka, 1964, Hoseney, 1986).

Dough testing devices attempt to simulate, measure, and record the deformation that takes place during processing. Physical dough testing devices are used to evaluate and compare new varieties of wheat, as well as for quality control and basic rheological studies. In the process of making bread,

deformation is present at nearly every stage, though too different degrees. The most extreme deformation takes place during the initial mixing stage. There are different instruments that have been developed to measure the deformation of bread dough in order to optimize the dough. The mixograph, farinograph, and extensigraph are some of the instruments used to test and compare bread dough. The mixograph and farinograph both record the torque required to mix dough, which provides quantitative information regarding the rheological properties of the dough (Dobraszczyk and Morgenstern, 2003, Faridi, 1985, Faridi and Faubion, 1990, Hlynka, 1964, Hoseney, 1994, Pomeranz, 1987, Rha, 1975).

A mixograph is an instrument used to test dough development, water absorption, and mixing time. The mixograph is used to compare the strength of flours of different varieties of wheat. Doughs that are stronger and more resistant to mixing generally make better breads. A mixograph records the rate of dough development, the maximum resistance of dough to mixing and how prone to overmixing a dough is. The output of the mixograph is a mixogram, a chart which corresponds to the force needed to mix the dough over a period of time (Faridi, 1985, Faridi and Faubion, 1990, Pomeranz, 1987).

The mixograph is essentially a high speed mixer with a recording device attached which produces a force deformation curve of the dough as it is mixed. There are five basic parts to the mixograph: (1) mixing pins, (2) mixing bowl, (3) swivel base, (4) tension spring, and (5) kymograph / dynonometer. The four

mixing pins on the head of the unit lower into the mixing bowl which has three vertical pins. The bottom mixing bowl is stationary; the mixing action is performed by the pins on the head of the unit which form the dough by rotating it along a single plane: pulling, folding and re-pulling the dough. The swivel base swings in proportion to the amount of force that is necessary to mix the dough. The tension spring provides resistance to rotation and can be adjusted depending on the materials being tested. The output is recorded by a kymograph / dynanometer which is an arm that is attached to pivot that records the movement of the swivel base using an ink pen (Faridi, 1985, Faridi and Faubion, 1990, Hlynka, 1964, Pomeranz, 1987, Rha, 1975).

The output of the mixograph, called a mixogram, is a record of the power it takes to pull the dough at a constant speed. The mixogram is a curve that monitors the development of the dough over time: each bold vertical line on the mixogram is equivalent to one min of mixing, the length of the curve along the horizontal axis indicates how long the test was performed. Information about cohesiveness and elasticity of the dough can be gained by examining the width of the curve. A curve that is compressed throughout may indicate that the dough has too much water. On the other end of the spectrum a curve that is wide and spiky suggests a dry dough or non-uniform distribution of ingredients (Faridi, 1985, Faridi and Faubion, 1990, Hlynka, 1964, Pomeranz, 1987, Rha, 1975).

How fast the curve of the mixogram reaches a peak corresponds to dough strength during dough development. Initially, there is an excess amount of water

in the system, so there is little resistance to mixing. This is represented on the mixogram as a compressed curve low on the chart. Next, the flour particles begin to hydrate. The water penetrates the surface of the flour particles, the mixing action disperses hydrated particles into the developing dough matrix, allowing a new part of the flour particle to become hydrated. The dough is fully developed when the flour particles are fully hydrated which is indicated by a peak on the mixogram. The peak is also called “point of most resistance,” the “optimum dough development time,” or the point where the dough has minimum mobility. For commercial bread manufacture the peak should not appear to early or late. There needs to be sufficient time to incorporate all the ingredients into the dough, but extra energy, which costs money in commercial manufacturing, should not have to be spent too achieve proper gluten development. When the peak is achieved at the desired time, the water absorption for the particular dough can be extrapolated. After the peak on the mixogram, a decline can be seen. Dough which resists change after the reaching the peak is said to have good mixing tolerance, which is ideal for rougher processing techniques, or making hardier breads. However, dough which develops quickly and deteriorates rapidly is not suitable for commercial bread manufacture. Another way to measure tolerance to overmixing is to compare the area under the curve. A mixogram that has a larger area under the curve represents a dough with a greater tolerance to overmixing (Faridi, 1985, Faridi and Faubion, 1990, Hlynka, 1964, Pomeranz, 1987, Rha, 1975).

The mixograph is a widely used instrument to evaluate the bread making potential of wheat varieties and for quality control. A mixograph is easy to use, fast and practical for everyday use. The information can be used to predict which flours will perform best in industrial processing conditions. However, there are some limitations regarding the use of the mixograph. Most notably, is the inability to compare mixograph data from one laboratory to another laboratory. The lack of ability to compare mixograms is due to the lack of standardization of methods due to differences in water absorption, which depends on many extrinsic factors such as the flour used, as well as the temperature and humidity of the environment. Additional factors that influence mixogram results are slight differences in the mixing speed of the mixograph, modifications in the flour composition and the addition of additives, which may not affect the final bread loaf but may affect the results of the mixograph. However, it has also been noted that results within labs are extremely reproducible, which is why the mixograph is trusted for quality control. Another disadvantage when using the mixograph is that to achieve the optimum levels of water for dough mixing, a trial and error approach is employed, which is not always efficient. However, the results of a mixogram give guidance to the best product, but can not always predict the end product quality, as the mixogram does not include the processes such as fermentation and baking which the bread will undergo (Dobraszczyk and Morgenstern, 2003, Hlynka, 1964, Pomeranz, 1987).

THE EFFECT OF ADDITIVES ON DOUGH

There are many different dough additives available to the baking industry. These additives are incorporated to improve the functionally of the dough during processing or increase the stability or palatability of the final product. The category of improvers can generally be divided into: oxidizing agents, reducing agents, pH regulators, emulsifiers, and enzymes. Oxidizing agents such as calcium peroxide, potassium bromate, calcium bromate, potassium iodate, and calcium iodate may improve the dough by increasing firmness or allowing the dough to expand. The enzyme α-amylase may be added to bread dough if flour is deficient in this enzyme. α-amylase is added to the bread to prolong the shelf life of bread by increasing the softness of the crumb. Improved browning of the crust is another benefit of adding α-amylase. A softer crumb may be achieved by addition of emulsifiers such as monoglycerides, diglycerides, and propylene glycol mono- and diglycerides. The use of reducing agents such as L-cysteine reduce the amount time required for mixing (Pomeranz, 1987, Wood, 1998).

It has been shown that the addition of additives affect dough properties in various ways. Lang, Neises, and Walker (1992) investigated how various additives at different absorption levels of water (58, 61, 64, 67, and 70%) affected dough properties. The work was carried out on a 35 g mixograph following AACC method 54-40A, except that the mixograph was modified by connecting it to a computer. The results of the mixograph were analyzed using a BASIC

program for peak time, peak height, and area under the curve. In general, higher amounts of water increased the time to reach the peak, which was expected. The additives that were analyzed fell into six general categories: vital glutens, oxidants, reductants, surfactants, salts, and others. Three different types of vital glutens were added at the levels of 2.5 and 5%. The addition of all three types of vital gluten increased the time to peak, peak height, and area under the curve at increasing concentrations. The results of this experiment suggest that vital gluten may be a used to give a dough greater tolerance to mixing. The oxidants added were: ascorbic acid (AA), potassium iodate (KIO_3), azodicarbonamide (ADA), and potassium bromate ($KBrO_3$). The results of the addition of oxidants can not be generalized. Mixograms with the addition of $KBrO_3$ showed little effect on peak time, peak height, and area under the curve when compared to the control. The addition of AA increased the time to the peak when increasing levels were added, however the peak height was decreased with addition of increasing amounts of AA. The area under the curve was higher for the lower amount of AA added. The addition of ADA at increasing concentrations decreased the time to reach the peak. The addition of the oxidant KIO_3 increased peak height, time to peak and increased the area under the curve. L-cysteine was the reductant that was analyzed in the study. The addition of L-cysteine at increasing levels decreased the time to reach the peak at all levels of moisture. However, it produced a negligible effect on peak height. Addition of L-cysteine slightly decreased the tolerance to mixing. Two different surfactants

were analyzed for their effect on dough: sodium stearoyl lactylate (SSL) and sucrose esters (SE). No significant changes in dough properties resulted from the addition of SE. The addition of SSL increased the time to the peak with increasing concentrations. Of the several salts tested all except $CaCl_2$ increased the mixing time to the peak. This research demonstrates how the addition of various ingredients can improve dough functionality. The addition of salt or SSL may be beneficial to dough which has a short mixing time. Mixing tolerance may be increased by the addition of vital gluten or KIO_3.

Ingredients which are added to improve the nutritional properties of the dough may also affect the rheological properties. Koh and colleagues (2005) investigated the effect of the addition of amino acids and peptides on dough. Dough properties were examined using a 10 g mixograph following AACC procedure 54-40 (AACC 2000). Amino acids and peptides were added dry on a flour basis and mixed in with the flour prior to starting the mixograph. Cysteine had the most pronounced effect on the mixing time. The addition of 1% cysteine rapidly increased dough development and the dough quickly deteriorated after the peak, showing little tolerance for mixing. In contrast, dough with 1% cystine took longer to develop than the control, and did not show the reduced tolerance that cysteine did. The addition of 1% of histidine, arginine, and lysine slightly prolonged the time it took to reach optimum development and showed a slight increase in mixing tolerance. Two commercial peptides, bonito (Pepuccino, Nihon Shokuhin Kako Co., Fuji, Japan) and corn (Senmi peptide, Senmi Ekisu

Co., Ohzu Japan) were also evaluated for their effect on mixing properties. Addition of bonito peptide and corn peptides at 1% resulted in a dough that had little tolerance for mixing after the optimum time was reached (Koh, et al., 2005). When adding ingredients for nutritional purposes it is important to consider the rheological properties of the dough. The peptides may have undergone too much hydrolysis to be incorporated into a dough, a longer peptide may not have exerted such a negative effect.

PROOFING

The proofing stage allows fermentation in the bread dough. The fermentation that occurs during the processing of bread is anaerobic. For fermentation to proceed in bread dough proper conditions must be present, including proper temperature (70-90°F), adequate moisture, and food for the yeast. Fermentation by yeast in bread dough produces carbon dioxide, ethanol, and flavor compounds. The products of fermentation result in the characteristic flavor and texture of bread (Pomeranz, 1987).

After proofing, the dough is punched down. The punching and re-mixing that the bread dough undergoes divides the gas cells produced by the yeast into smaller cells which results in a more uniform crumb structure (Hoseney, 1994).

BAKING

During baking the crust and crumb of the bread reach different temperatures. The crust reaches 100°C during the first 10 min of baking, and may get as high as 150-200°C. The browning that occurs on the crust is a result of caramelization of sugars as well as Maillard browning, in which there is reaction between sugars and proteins. At the end of baking the crumb will barely reach 100°C, at this point steam will form in the crumb which will set the final volume of the loaf (Pomeranz, 1987).

WHEY PROTEINS

Whey is a by-product of the cheese-making process. The remaining liquid after the casein and fat have been separated during the production of cheese is whey. Casein can be coagulated by rennet or acid and the resulting whey from these processes has slightly different properties. Whey that is collected from coagulation of casein by rennet is termed sweet whey, while whey that collected to due to coagulation of casein by acid is called acid whey or quark whey. Fresh whey consists of water, dry matter, lactose, protein, citric acid, minerals, vitamins, and a trace amount of lactic acid. Sweet whey contains very little calcium, while acid whey contains calcium lactate. Whey may also vary in

composition due to the cheese from which the whey comes, heat treatment, and any other treatments such as purification or concentration (Erdogdu-Arnoczky, et al., 1996, Spreer, 1995).

Whey was once considered a waste product of the cheese making industry. However, it now is processed further and used to add nutritional and functional properties to a variety of products. Whey proteins make up approximately 20% of all milk proteins. Whey protein concentrate is a product derived from whey with a protein content of 25% or higher. The physical functionality of the whey protein concentrate will vary from product to product depending on the processing conditions (Mazza, 1998).

Whey protein consists of a mixture of proteins, the most of abundant of which are β-lactoglobulin and α-lactalbumin. Other components of whey include albumin, immunoglubins, lactoferrin, and milk growth factor. Each of the individual proteins that make up whey differ in amino acid composition and sequence, which leads to different properties. β-lactoglobulin is the most abundant protein in whey. It is a globular protein that exists, below 40°C, in the form of a dimer with two identical subunits. At temperatures above 40°C β-lactoglobulin dissociates, exposing two disulphide bridges and a free thiol group. α-lactalbumin is the smallest and the most heat resistant of the whey proteins (Mazza, 1998, Shortt and O'Brien, 2004, Spreer, 1995).

The physical functionality of whey protein is affected by a variety of processing steps including heat treatment, the pH at which the treatment takes

place, demineralization, and hydrolysis by proteolytic enzymes. The hydrolysis of protein by enzymes can change the functionality of whey protein. Hydrolysis of protein generally results in increased solubility, decreased viscosity, and decreased tendency for gelation. The tendency for protein to foam during whipping is increased due to hydrolysis, while foam stability is decreased. Hydrolyzed proteins have increased thermal stability (Kester and Richardson, 1984).

The benefits of adding whey protein as an ingredient can go beyond basic nutrition, as whey protein is known to contain bioactive peptides. Milk proteins including whey protein, contain bioactive compounds within the sequence of the protein. Amino acids sequences within proteins that have been identified to have biological activity, such as angiotensin-converting enzyme- inhibition (ACE-inhibition), are known as strategic zones. This sequence of amino acids in whey proteins that act as an ACE-inhibitor can be released by enzymatic proteolysis during food processing or during the digestive process (Lopez-Fandino, et al., 2006, Mazza, 1998, Meisel, 2004).

ANGIOTENSIN-CONVERTING ENZYME-INHIBITION

Hypertension is defined by a rise in systolic and diastolic blood pressures above the normal levels. Constriction of systemic arteries by vasoconstrictors can cause an increase in blood pressure. Prolonged hypertension increases and

individuals' risk of heart disease, cerebral hemorrhage, and renal failure (Ganong, 1995).

Angiotensin-converting enzyme (ACE) is a type I transmembrane protein. It is a metallocarboxydipeptidase. ACE functions in the rennin-angiotensin system (RAS) by hydrolyzing angiotensin I to angiotensin II. The RAS controls systemic blood pressure, electrolyte and fluid balance as well as blood volume. Antiogentensin II is a vasoconstrictor. Another function of ACE is to inactivate bradykinin by cleaving the C-terminal dipeptide. An ACE-inhibitor decreases the amount of vasoconstrictor in the blood by inhibiting the formation of angiotensin II and not allowing ACE to bind to braydkinin and kallidin (Fitzgerald and Murray, 2006, Lopez-Fandino, et al., 2006).

Methods have been developed to measure ACE-inhibition *in vitro* as well as *in vivo*. Spectrophotometric, fluorometric, radiochemical, high performance liquid chromatography, and capillary electrophoresis have all been used to determine the ACE-inhibition in a sample. *In vivo*, spontaneously hypertensive (SHR) rats have been used to test ACE-inhibition. The potency of an ACE-inhibitor is generally expressed as the amount it takes to get 50% inhibition, or IC_{50} (Fitzgerald and Murray, 2006, Lopez-Fandino, et al., 2006).

The ability of a peptide to function as an ACE-inhibitor is influenced by the tripeptide sequence at the C-terminus. Peptides which inhibit ACE are small, ranging in size from two to twenty amino acids in length. More inhibition is

achieved when hydrophobic resides are at the C-terminal positions (Meisel, 2004, Meisel, 2007).

ACE inhibiting peptides have been found in a variety of food proteins such as soy protein hydrolystate, and chickpea hydrolystate. Many sequences of peptides that act as ACE-inhibitors have been isolated from dairy products (Table 4). Bioactive peptides are peptides that have a specific biological function. In whey protein biological active peptides are a sequence of amino acids imbedded within the larger protein and are released when the protein is hydrolyzed during digestion. Bioactive peptides are small enough to across the digestive epithelial barrier and enter the blood stream (Wu and Ding, 2002, Yust, 2003).

INCORPORATION OF BIOACTIVE PEPTIDES AND PROTEINS INTO BREAD

Protein can be added to food products for a number of reasons. Commonly protein is added as an emulsifier, to bind water or fat, form a foam or gel, or to alter the flavor, appearance, or texture of a product. In bakery products, dairy ingredients are also added to improve physical properties. The addition of various dairy proteins have reportedly resulted in positive results such as increased water absorption, reduced staling rate, and increased crust color. Negative results such as dough slackening and volume depression have also been reported. It has been noted that the addition of protein supplements high in

TABLE 4. List of peptides with ACE-inhibitory activity isolated from dairy products (Chen, et al., 2007, Ferreira, et al., 2007, Fitzgerald and Murray, 2006, Hernandez-Ledesma, et al., 2004, Meisel, 2004)

Source	Peptide Sequence
Milk	TTMPLW
Milk	YPFPGPIPNSL
Milk	YPFPGPI
Milk	AVPYPQR
Milk	YQQPVL
Milk	YIPIQYVLSR
Milk	YG
Milk	YGLF
Milk	YLLF
Milk	ALKAWSVAR
Cheese: Crescenza	LVYPFPGPINSLPQ
Cheese: Gouda	RPKHPIKHQ
Cheese: Gouda	YPFPGPIPN
Cheese: Manchego	KKYNVPQL
Cheese: Manchego	VRYL
Cheese: Manchego	VRGPFP
β-lactoglobulin	ALPMHIR
β-lactoglobulin	GTW
α-casein	GAW
β –casein	DKIHPF
β –casein	YPFPGPIPN
β –casein	EMPFPK
β –casein	HLPLP
β –casein	LPLP
β –casein	SKVLPVPQ
β –casein	PPQSVLSLSQSKVLPVPQ
β –casein	GPV
β –casein	LLYQQPVRGPFPIIV

Amino acids in the peptide sequence are represented by the following letters: A - Alanine, F - Phenylalanine, G – Glycine, H - Histidine, I - Isoleucine, K - Lysine, L - Leucine, M - Methionine, N - Asparagine, P - Proline, Q - Glutamine, R - Arginine, S - Serine, T - Tyrosine, V - Valine, W - Tryptophane, Y – Tyrosine

lysine decrease loaf volume and have had a negative effect on crumb formation, as well as decreased shelf life (Korhonen, et al., 1998, Pomeranz, 1987).

As indicated before, protein may be added as a functional food component, as in the case when bioactive peptides are added to a food product. When adding bioactive proteins to bread it is important to consider how processing may affect the peptides. The structure of a protein is dependent on environmental factors such as pH, temperature, and the presence of fat, and air. Whey proteins interact with the components of the food system. The application of heat to a product can result in the denaturation of a protein or precipitate and/or the formation of Maillard reaction products. Fermentation may also affect the activity of the peptide (Korhonen, et al., 1998, Mangino, 1984).

In addition to the effect on dough, the effect that amino acids and various peptides and proteins have on bread was studied by Koh and colleagues (2005). The specific volume of the bread (specific volume = bread volume/bread weight) and the color of the crust and crumb were measured. Addition of 1% tryptophan, cystine, methionine, or phenylalanine resulted in breads with a significantly ($P < 0.05$) higher specific volume than the control. The addition of bonito and corn peptides also resulted in a bread loaf with a higher specific volume. The addition of amino acids and peptides to the dough resulted in darker crusts. Presumably this is due to the availability of free amines to undergo Maillard browning. This study highlights the positive impact the addition of some amino acids and peptides can have on improving dough and bread properties. It also showed that

some amino acids (isoleucine, histidine, glycine, arginine, glutamic acid, aspartic acid, and lysine) have negative effects on loaf volume (Koh, et al., 2005).

The initial processing of the dairy protein will influence how it performs in a bakery application. Erdogdu-Arnoczky and colleagues (1996) investigated the effect that untreated and heat treated dairy ingredients has on bread during the process. Nonfat dry milk (NFDM), acid whey powder (AWP), and acid casein (AC) were included in the study. Freshly prepared NFDM, AWP and AC underwent two different heat treatments at 80 and 90°C for 10 min. The freshly prepared NFDM, AWP and AC were compared to commercial acid whey protein concentrate (CAWPC) and commercial acid casein (CAC). Dough properties were analyzed using a mixograph, incorporating the dairy ingredients at a level of 4% on a flour basis. The addition NFDM, AWP, and AC resulted in a decrease of time to reach the peak on the mixograph as well as a quick deterioration after the peak, which indicates low tolerance to mixing. Heat treatment of NFDM and freshly prepared AWP prolonged the mixing time and increased the tolerance. In the case of NFDM treatment at 80°C for 10 min, dough properties were similar to the control while heating for 90°C for 10 min did not show an additional improvement. CAWPC slightly decreased the optimum mixing time and tolerance when compared to the control. It was also reported that the non-heat treated NFDM and AWP resulted in dough that was hard to work with after the peak mixing time, this corresponds to the low tolerance to mixing reported in the mixograms (Erdogdu-Arnoczky, et al., 1996).

In addition to analyzing dough properties, Erdogdu-Arnoczky and others examined the effect that 4% by weight NFDM, AWP, AC and CAWP (flour basis) had on bread. Two different baking tests were preformed: fixed and optimized. In the fixed test the amount of water and mixing time remained constant throughout all treatments. In the optimized procedure, the mixing time and water absorption was varied depending on the dairy ingredient added to get an optimum result. In the optimized procedure the addition of non-heat treated AWP and AC decreased both the loaf height and volume when compared to the control. When AWP and AC was heat treated for 80°C for 10 min the loaf volume was higher than the control. However, further heat treatment at 90°C for 10 min resulted in a reduction of the loaf volume, though not as much as the AWP and AC without heat treatment. Inclusion of CAWP increased the height and volume of the bread (Erdogdu-Arnoczky, et al., 1996).

The addition of whey protein to bread has been studied because of its ability to increase the nutrition of bread, as well as to extend the shelf life. Kadharmestan and others (1998) examined the effect that untreated, heat and high hydrostatic pressure treated commercial whey protein concentrate had on wheat bread. The commercial whey protein concentrates were added on a replacement basis of 5 and 10% and compared to a control bread with no whey added. A 10 g mixogram was used according to AACC method 54-40A to determine the optimum moisture absorption and mixing time for each treatment. The dough was baked in accordance with AACC method 10-10B, after which

volume was measured using rapeseed displacement and texture analyzed using TA-XT2. During the baking process it was reported that the untreated commercial whey protein concentrate resulted in dough with undesirable properties, such as being too wet, sticky and extensible. The heat treated and high hydrostatic pressure treated commercial whey protein concentrate produced a better dough. All of the breads with added commercial whey protein concentrate showed a significant decrease in volume when compared to the control. However, the untreated commercial whey protein concentrate depressed the volume the most. Bread with a lower volume was also harder. Bread with a higher amount of all types of whey protein concentrate resulted in a darker crumb (Kadharmestan, et al., 1998).

CONCLUSION

There are various treatments to control hypertension including diet modification, exercise, and pharmacological agents, such ACE-inhibitors. Due to the prevalence of bread in the diet it may be an ideal delivery system for a functional food ingredient that benefits a chronic condition, such as hypertension. The incorporation into a product of a functional food, such as whey protein, should not adversely affect the carrier product or the physiological properties of the functional food.

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CHAPTER II

MATERIALS AND METHODS

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MATERIALS

For all experiments, Hard Red Spring (HRS) wheat flour milled from grain harvested in 2006 (supplied by the Western Wheat Quality Laboratory, Pullman, WA) was used as the standard flour. Commercial hydrolyzed whey protein isolate (CHWPI) (BioZate 1 Lot # LE 001-5-919 supplied by DAVISCO Food Inc., Le Sueur, MN) was used.

EXPERIMENTAL DESIGN

The experiments were designed to meet the objectives of the project: to examine the rheological properties of dough with CHWPI, to examine the physical features of bread with CHWPI, and to analyze the final bread with ACE-inhibition activity. Initially, the mixograph was used to study the effect the level of CHWPI incorporation had on the dough as well as to optimize the water absorption when 10, 20 and 30% CHWPI was baked into bread. The second part of the study involved baking bread with 10, 20, and 30% CHWPI. The bread produced during this part of the experiment was analyzed for physical attributes and then freeze dried to test for ACE-inhibition activity. Figure 1 shows the flow of experiments used to determine the rheological properties of dough with

CHWPI as well as the ACE-inhibition activity, total nitrogen, and free amines in the bread and the measurement of bread quality.

MIXOGRAPH METHOD

A 10 g mixograph was used to determine optimum water absorption, mixing time, as well as to examine mixing properties (AACC, 1983). The mixograph was run at 23°C. To warm up the mixograph, at least three mixograms using standard flour were run before the samples.

HRS and CHWPI were weighed separately in metal weigh dishes (+/- 0.01g). CHWPI was added to the flour on a replacement basis at the levels of 10%, 20% and 30% so that the total weight of dry ingredients in the mixograph bowl equaled ten grams. HRS and CHWPI were mixed while dry with a flat-bottomed tongue-depressor. Using the tongue-depressor, a well was made in the flour between the three prongs of the bowl. Room temperature deionized water was dispensed from a 10 ml self-leveling burette into the well in the mixing bowl. Immediately after the addition of deionized water, the bowl was placed on the mixograph, and the timer and mixograph was started. The dough was allowed to mix for ten min.

In initial experiments with CHWPI, it was noticed that the mixing tolerance was substantially reduced. So, to determine the maximum amount of CHWPI that could be added to the dough and minimize the interference of CHWPI on

dough development, CHWPI was added after the dough had begun to develop. HRS and water were dispensed as previously stated. After two min the mixograph was stopped and CHWPI was added to the dough. The mixograph was allowed to run for the completion of the ten min period. For 30% CHWPI it was thought that the dough could benefit from additional water. A pipette was used to add water (2 or 4 ml) after two min along with the CHWPI.

All combinations of CHWPI and water absorption were run in at least duplicate.

BREAD BAKING

Pup loaves (100 g flour) were baked to evaluate the physical functionality of CHWPI in wheat bread as well as determine the ability of CHWPI to act as an ACE-inhibitor in a final baked bread loaf. The procedure for baking pup loaves was adapted from the AACC method for optimized straight-dough bread-making (AACC 10-10B). The procedure was slightly modified to allow for the addition of CHWPI. The bread formula consisted of HRS flour, CHWPI, liquid hydrogenated shortening, sugar, salt, malt solution, and yeast (Table 1). Commercial hydrogenated shortening (All-Vegetable Crisco, Orrville, OH) sugar (Safeway, Pleasanton, CA) and iodized salt (Safeway, Pleasanton, CA) were used. Active dry yeast (Red Star Insta-Blend, Milwaukee, WI) and malt was provided by the Western Wheat Quality Laboratory. The malt solution was prepared by mixing

60 g of Ross malted wheat flour with 500 ml distilled water, shaking for 15 min and then centrifuged. The supernatant was removed and diluted 1:1 with distilled water to give a concentration of 300 mg malt / 5ml water.

Additional mixographs were carried out before the bread bake in the same room where the bake took place to ensure optimal water absorption for the environmental conditions. From these mixographs, the milliliters of water added to the bread dough was determined by subtracting 28.7 from the absorption value of the flour and CHWPI mixture (Table 1)

The day before the baking, HRS and CHWPI were weighed (+/- 0.1g) into numbered tins to equal a total weight of 100 g. If CHWPI was to be added after 2 min of mixing, the flour and CHWPI were weighed into separate tin containers. In addition three controls, consisting of only HRS, and four internal standards (made with flour that is regularly used by the Western Wheat Quality Laboratory) were weighed out. The internal standards were used as a check that the baking went as expected. If the internal standards did not rise as expected then one of the ingredients, such as yeast or an external factor, such as temperature could have contributed to the poor quality of the bread loaves. The back of a metal spoon was used to make a ring in the flour mixture around the edge of the can. In the indention, 3 g of heated (55-60℃) liquid hydrogenated shortening was added using a glass pipette.

On the day of the baking, the tins of the pre-weighed ingredients were transferred to a mixing bowl, using a 1 inch brush to facilitate the transfer as

needed. Yeast (1.8 – 2.0 g) was weighed and added to the mixing bowl. A tongue depressor was used to mix the yeast and flour and make a well in the bottom of the mixing bowl. Sugar, salt, malt and water were delivered by burettes to reach the pre-determined absorption level, called the bake absorption (Table 1). The sugar-salt solution consisted of 0.6% sugar and 0.15% salt. A 0.3% solution of malt extract was used. Doughs were mixed between two to four min to achieve optimum development.

After mixing, the dough was removed from the bowl and formed into a loaf by hand. The dough was then placed into a stainless bowl, covered with plexiglass and placed into the fermentation cupboard (30°C). The dough was punched down after 90 min, sheeted and panned. The dough was allowed to proof until the internal standards and controls reached a proof height of 7.7 cm. When the desired proof height was reached, the bread was baked on a four min schedule at 425°F. Three loaves of each level of CHWPI (10%, 20%, 30% and as well as 10%, 20% and 30% added at 2 min) incorporation were baked.

PROOF HEIGHT

The proof heights (+/- 0.1 cm) of the loaves were measured immediately before baking using a proof height gauge. The measurement was taken in the loaf pan. The highest point of the dough was measured.

LOAF WEIGHT

The loaf weight was determined by weighing each loaf immediately after removal from the oven (+/- 0.1 g).

LOAF VOLUME

The volumes of the loaves were measure by the rapeseed displacement method (AACC 10-05, 9th ed.) The volumes of the loaves were measured immediately after weighing.

PREPARATION OF BREAD FOR ANALYSES

The pup loaf was stored 24 h after baking in a polyethylene zip-type bag (Unisource Worldwide Inc., Norcross, GA) at room temperature. A guide (Progressive Bread Keeper, Kent, WA) was used to cut slices approximately 1.5 mm thick from the center of the loaf. Four slices were cut from the center of each pup loaf. One slice was used for moisture determination of the pup loaf. Two slices were analyzed for texture. One slice was used for color determination of crumb. The slices that were analyzed for texture as well as the slice analyzed for color were also used for nitrogen and ACE-inhibition analysis. Each bread loaf was sliced immediately before analysis.

Three slices from each bread loaf were used for nitrogen and ACE-inhibition analysis. The crust was cut away from the crumb using a paring knife. The samples were placed in plastic Petri dishes, frozen until solid at -20 ℃ and then freeze dried (Virtis Freezemobile 24 with Unitop 600L, Gardiner, NY) at a 20 ℃, plate temperature. All samples were weighed before and after freeze drying (+/- 0.01g).

The freeze dried samples of bread were ground using a motor and pestle. The ground samples were used for total nitrogen determination using a Leco instrument.

For the ACE-inhibition and 2,4,6- Trinitrobenzene Sulfonic Acid (TNBS) assay of the bread samples an extract was required. Two g (+/- 0.1 g) of a ground freeze dried bread sample consisting of crumb or crust was weighed into a 30 ml test tube along with 10 ml deionized water. The mixture was allowed to sit for ten min and then vortexed (VWR Mini Vortexer MVI, Batavia, IL) at 1600 RPM for thirty seconds (this procedure was repeated twice). Next the bread and water mixture was centrifuged for ten min at 26,712 RCF (Beckman J2-HS, Fullerton, CA) at 30℃. A 500 ul pipette was used to draw off the supernatant which was stored in microfuge containers. To clarify the extract further for the TNBS assay, the extract was placed in the microfuge (Galaxy 14D VWR, Pittsburgh, PA) for 10 min at 12,000 RPM. The supernatant was removed and diluted with deionized water to be within the absorbance range of the standard curve.

MOISTURE OF BREAD

The moisture of each loaf of bread was determined by using the two-step procedure as outlined in AACC Method 44-15A. The bread was prepared using AACC Method 62-05.

The initial weight of the slice was taken and then left to dry twenty h on paper at ambient room temperature. The weight of the dried slice was then weighed before grinding in a food processor. Approximately two g of ground sample were placed on a weighed aluminum dish and weighed (+/- 0.0001g). The samples were dried in a 130°C oven (Precision Scientific, Winchester, VA) for 60 min. The samples were then left in a desiccator to cool before being weighed. The % total moisture (dry weight basis) of the samples was determined by the following equation (from AACC Method 44-14A):

$$\% \text{ Total Moisture} = A + [(100 - A)\, B] / 100$$

A = percent moisture loss on air-drying

B = percent moisture loss as determined by oven-drying

TEXTURE EVALUATION

A Texture Analyzer (TA-XT2 Sable Microsystems, Godalming, Surrey, UK) was used to perform a texture profile analysis on the crumb of baked bread. Texture Expert Exceed Software (v. 2.64) was used to run the texture profile

analysis (TPA) test. A test speed of 1.0 mm/s, a distance of 50% and a load cell of 25 kg was used with the TPA macro. A cylindrical probe with a diameter of 25 mm was used.

COLOR EVALUATION

The color of the crust and crumb was measured using a Minolta Spectrophotometer (CM-2002 Tokyo, Japan). L*, a*, and b* values were taken the day after baking. Three measurements of crust color were taken from the top, center of the loaf before slicing. After slicing, one slice of bread was used to take measurements of the crumb color. The slice of bread was placed on a white sheet of paper before the reading was taken. Readings were taken in triplicate. The average L* value and chroma value of the crust and crumb were compared. The formula $(a^{*2} + b^{*2})^{1/2}$ was used to determine the chroma value.

ANGIOTENSIN-CONVERTING ENZYME (ACE) INHIBITION

Sodium borate buffer (pH = 8.3) consisting of 0.1 M sodium tetraborate (CAS # 1303-96-4 Fisher Scientific, Fair Lawn, NJ), 0.1 M boric acid (CAS# 10043-35-3 JT Baker Chemical Co., Phillipsburg, NJ), 0.3 M sodium chloride (CAS # 7647-14-5 JT Baker Chemical Co., Phillipsburg, NJ) was used. N-[3-(2-Furyl) acryloyl]-Phe-Gly-Gly (FAPGG) (CAS# 64967-39-1 Sigma, St. Louis, MO)

was made up to 1.6mM using sodium borate buffer. One unit antiogensin converting enzyme from rabbit lung (CAS# 9015-82-1 Sigma, St. Louis, MO) was diluted with 4 ml sodium borate buffer.

The assay was read using a microplate scanning spectrophotometer (Powerwave X-I Bio-Tek Instruments, Winooski, VT). Temperature was controlled at 37°C. Readings were taken at 340nm every 5 min for a period of 30 min after a lag time of 5 min. The uninhibited control consisted of 153 ul 1.6mM FAPGG, 25ul ACE enzyme, and 97 ul sodium borate buffer. For samples, 153 ul 1.6 mM FAPGG, 25ul ACE enzyme, 72ul sodium borate buffer, and 25ul bread extract were added to each well. The assay was run in triplicate.

ACE-inhibition of the samples was calculated according to the following formula:

% ACE-inhibition = [1 – (slope in presence of inhibitor / slope of control)] *100

(Shalaby, et al., 2006).

TOTAL NITROGEN DETERMINATION

Total nitrogen was determined according to the Crude Protein – Combustion Method (AACC 46-30, 9th eddition Vol. 2) using a Leco instrument (Leco Corp., St. Joseph, MI). Freeze dried, ground bread samples were used. Each sample was run in triplicate.

FREE AMINE DETERMINATION

Free amines, expressed as glycine equivalents, in the bread samples was determined by modifying the Pierce 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) assay (Pierce, 1999).One-tenth molar sodium borate buffer (pH = 8.3) was used to dilute TNBSA (28997 Pierce, Rockford, IL) to 0.01% w/v. A 10% (w/v) solution of sodium dodecyl sulfate (CAS# 151-21-3 Sigma, St. Louis, MO) was made with deionized water.

For the assay, 25ul of bread extract and 60ul of TNBSA solution and 95ul sodium borate buffer were mixed in a microplate scanning spectrophotometer (Powerwave X-I Bio-Tek Instruments, Winooski, VT) for 5 seconds at an intensity of 4. To account for the absorbance due to the bread, a control was run at the same time as the TNBSA assay with 25ul bread sample and 155ul sodium borate buffer. After an incubation period of two h at 37°C, 60ul of 10% SDS solution and 30ul 1N HCl (CAS# 7647-01-0) were added to all cells. Absorbance was read at 335 nm.

Glycine (CAS# 56-40-6 Sigma, St. Louis, MO) was used to make a standard curve for the determination of the amount of free amines in the samples. The concentration of glycine used for the standard curve was 0 – 0.1 ug/ml. The bread samples were diluted with deionized water, so that the samples would be in the linear range of the standard curve. The absorbance due

to the bread alone was subtracted from the absorbance of the bread sample with TNBSA to get the final absorbance.

STATISTICAL ANALYSIS

Data were analyzed using one way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p=0.05$). All statistical analyses were run using SAS 9.1 for Windows.

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Figure 1. Flow chart of experiments

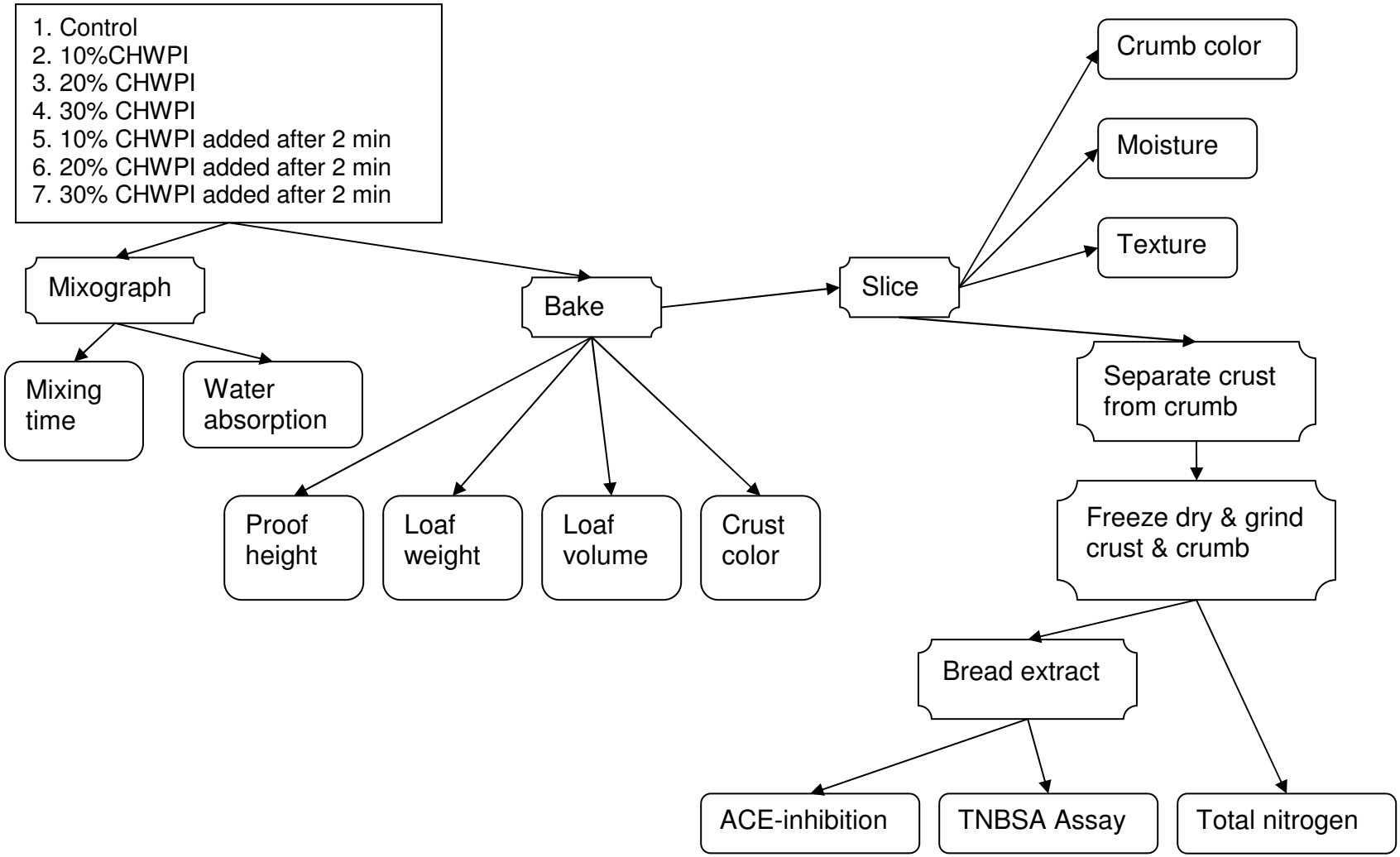

Table 1. Bread formula

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Flour (g)	CHWPI (g)	Bake Absorption (0.6 % sugar & 0.15% salt solution; 0.3% malt solution) (ml)	Actual Absorption (water added + water in sugar, salt and malt solution) (ml)	Hydrogenated shortening (g)	Yeast (g)
0	0	100		35.5	64.2	3	1.8 – 2.0
10	0	90	10	21.5	50.2	3	1.8 – 2.0
10	2	90	10	21.5	50.2	3	1.8 – 2.0
20	0	80	20	16.5	45.2	3	1.8 – 2.0
20	2	80	20	16.5	45.2	3	1.8 – 2.0
30	0	70	30	18.5	47.2	3	1.8 – 2.0
30	2	70	30	18.5	47.2	3	1.8 – 2.0

CHAPTER III

RESULTS AND DISCUSSION

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INTRODUCTION

There are different reasons to incorporate dairy ingredients into bread; one reason is to improve the nutritional properties of the bread. Wheat, and subsequently wheat bread, is low in the essential amino acid lysine. Addition of a dairy protein into bread will increase the total amount of protein in the bread as well as balance the amino acids in the loaf. Another reason to add dairy ingredients into bread is for the physical functional properties that the ingredient provides. For example, sodium caseinate may be added to a bread formula as an emulsifier, thickener and foaming agent (Crowley, et al., 2002). The increase in water absorption is a common reason to add dairy ingredients to bread (Gallagher, et al., 2003). Dairy proteins may also be added to create a functional food, as is the case with an ACE-inhibiting CHWPI.

In the development of a functional food, the consumer must be able to consume enough of the food to receive a benefit. Therefore, it was decided to determine the maximum amount of CHWPI that could be incorporated in a bread dough.

The affect that CHWPI had on dough rheology was evaluated, as well as the maximum amount of CHWPI that could be incorporated. Next bread was baked to determine how the CHWPI would perform in an actual loaf. Finally, to

assure that the product retains its ACE-inhibiting properties after baking the final product was tested for ACE-inhibiting activity.

DOUGH RHEOLOGY

Bread formulas often contain various additives. These additives may be incorporated to improve dough handling properties, prolong shelf life, increase palatability, or increase nutritional properties. It is well established that the addition of ingredients may affect the dough in various ways (Erdogdu-Arnoczky, et al., 1996, Harper and Zadow, 1984, Lang, et al., 1992, Miller, et al., 1997). The first step taken in creating bread with functional properties was to evaluate the effect that CHWPI had on bread dough. A mixograph was used because of its widespread, longstanding use in the baking industry, and the ability of the mixograph to provide practical information such as water absorption, dough cohesiveness, and mixing tolerance that can not be obtained by other means (Song and Zheng, 2007). This experiment sought to maximize the amount of CHWPI that could be added to bread dough in addition to optimizing factors such as water absorption and time of addition of CHWPI.

Whey may be beneficial to bread dough or exert a negative effect depending on how the whey is processed. It has been shown that partial denaturation of whey protein concentrate will increase the quality of bread compared to native whey protein, while whey protein that has been subjected to

heat treatments resulting in more than 90% denaturation will negatively affect the end product (Harper and Zadow, 1984). The incorporation of whey protein that has been treated with high hydrostatic pressure has been shown to increase the mixing tolerance of dough when compared to untreated whey protein concentrate. In baked bread, whey protein treated with high hydrostatic pressure improved the volume of bread when compared to bread made with untreated whey protein (Kadharmestan, et al., 1998).

The levels of addition of CHWPI were selected to provide a functional, physiological benefit *in vivo*. Studies have shown that an intake of 20 g of CHWPI have significantly reduced blood pressure in individuals. This study sought to incorporate the maximum amount of CHWPI into bread to determine if it would be possible to put enough CHWPI into the bread loaf to provide a physiological benefit. Based on an incorporation level of 10% CHWPI and a serving size of 25 g, each slice of bread would contain approximately 2.5 g of CHWPI per slice. At this 10% level of incorporation it would not be feasible to obtain 20 g of CHWPI a day by eating only bread. However, the bread could be included as part of a diet that included CHWPI from various sources (FitzGerald, et al., 2004, Pins and Keenan, 2004).

The results of this study confirm that dairy additives affect dough properties (Figures 1-4, Table 1). CHWPI was added to dough at different levels and the water content and time of addition of CHWPI was varied to achieve the best possible dough (Figure 1 B-I, Figure 2 B-I, Figure 3 B-I). The optimum

mixing point, as indicated by the mixograph was 3.5 min for the control dough with absorption of 60% (Figure 1 A, Table 1). The addition of CHWPI affected the optimal water absorption, mixing time, and mixing tolerance.

The conditions required to make an optimal dough varied with the amount of CHWPI added (Figure 4). The amount of water required for an optimal dough, as indicated by the mixogram, was the highest in the control, and decreased with increasing amounts of CHWPI. The optimum mixing time also varied (Table 1). Dough without CHWPI (control) and with 10% CHWPI at 55% water absorption had an optimum mixing time of 3.5 min. The optimum mixing time is the point where the dough reaches the most resistance during mixing, at this point optimum gluten development is achieved (Faridi and Faubion, 1990). When 20% and 30% CHWPI was incorporated 2 min after mixing began there was an improvement in the dough.

The addition of 10% CHWPI at the beginning of the mixing process (Figure 1 B - E) resulted in dough requiring less water than the control dough (Figure 1 A). The ideal absorption for dough with 10% CHWPI is 55% (Figure 1 D). The dough made with 10% CHWPI had approximately the same optimum mixing time as the control dough. After optimum mixing time mixograms of the dough made with 10% CHWPI show a narrower tail when compared to the control. The mixograms with 10% CHWPI indicate that the dough is not as tolerant to overmixing as the control dough, this is shown by the deterioration in

the mixogram curve after the peak, which is shown by a quick decline in the mixogram curve, when compared to the control (Figure 1 A - E).

Mixing tolerance is important for commercial bread manufacture, as dough must be strong enough to withstand mechanical mixing and sheeting (Pomeranz, 1987). To determine if mixing tolerance could be increased and improve the gluten development of the dough, ten percent CHWPI was added after mixing began (Figure 1 F - I). The addition of 10% CHWPI after two min of mixing increased the amount of time to the optimum mixing point, to between 3.75 -4.25 min depending on the water absorption, as well as increasing the mixing tolerance (Table 1). This was not surprising, since the gluten had time to develop before the CHWPI was added. The optimum water absorption for dough made with 10% CHWPI added at two min was 55% (Figure 1 G).

When 20% CHWPI was added at the beginning of mixing, visual inspection revealed that the dough did not form properly. This observation was substantiated by the mixograms (Figure 2 B, C). At a water absorption of 48% the dough with 20% CHWPI reached the optimum mixing time earlier than the control and quickly deteriorated (Figure 2 B). At this level of water absorption (48%) and CHWPI addition, a ball of dough did not form around the pins; after ten min of constant mixing the dough was observed to be pushed up the sides of the lower mixing bowl. The addition of 20% CHWPI after two min was more successful (Figure 2 D - I). Dough that was made with 20% CHWPI added at two min had a higher mixing tolerance than dough made with 20% CHWPI added

initially. The optimum amount of water absorption for dough made with 20% CHWPI added after two min is 50% (Figure 2 F) with a mixing time of 4.5 min (Table 1). Although this is longer than the control, the CHWPI was fully incorporated at 3.5 min, so the dough could be mixed for the same time as the control.

When 30% CHWPI was added initially to the dough the optimum mixing time was reduced dramatically, to 1.75 min, as was the mixing tolerance (Figure 3 B). Adding 30% CHWPI after two min (Figure 3 C – I) increased the optimum mixing time because it gave the dough a chance to form and allowed the gluten to develop, but the mixing tolerance was not increased. After a full ten min of mixing, which is beyond the optimal mixing point of the dough, the dough with 30% CHWPI did not form a ball and instead stuck to the sides of the bowl. The texture of the dough was very tough and pasty. Predicting the outcome of bread from dough can be difficult. However, one of the factors that is an indicator is the cohesiveness of the dough (Armero and Collar, 1997). Since the dough made with 30% CHWPI was not cohesive, it was not expected to make a good bread loaf.

An interesting observation is how water interacts in dough at all levels (10, 20 and 30%) of CHWPI. The optimal amount of water is essential to making an ideal bread loaf. Water not only hydrates the gluten proteins, allowing a continuous network to form, it also performs many other functions (Hoseney, 1986). The amount of water required for an optimal loaf of bread will vary

depending on the temperature, the water absorption of the flour, the method used to make the bread, the desired moisture level of the bread, and any additives in the formula (Pomeranz, 1987).

The water absorption of the optimally mixed doughs with CHWPI is less than that of the control. This finding has been reported by other researchers. Kenny and others (Kenny, et al., 2000) found that the addition of commercial whey proteins to wheat dough decreased the water absorption of the dough. The experimenters added 4% of three different commercial whey protein concentrates to wheat flour bread dough. The optimal water absorption was determined with a farinograph. The dough with 4% commercial whey protein concentrates had a water absorption 1% less than that of the control. Kardharmestan and others (Kadharmestan, et al., 1998) found that when 10% of wheat flour was replaced by untreated commercial whey protein concentrate the water absorption decreased when compared to the control. However, when the commercial whey protein concentrate was treated with heat or high hydrostatic pressure the water absorption of the dough increased. In contrast, other researchers have noted that the addition of fermented dairy ingredients to dough increases the water absorption significantly (Gelinas, et al., 1995).

CHWPI did not react to water in the same way as wheat flour; in a flour and water dough the addition of excessive water will cause the mixogram curve to become compressed (Figure 5). However, when CHWPI was introduced into the system the mixogram curve became “spikier” as more water was added

(Figure 1 B-I, Figure 2 B-I, Figure 3 B-I). This was observed with all levels (10, 20, and 30%) of addition of CHWPI to the dough mixture.

The interaction that amino acids and peptides have on the rheology of wheat dough has been studied by other researchers. Koh and colleagues added a number of amino acids to wheat flour dough and examined mixing properties (Koh, et al., 2005). The mixograph patterns of doughs with the addition of 10% CHWPI were similar to those mixographs with wheat flour and 1% aspartic acid. The addition of 1% cysteine to wheat flour dough also resulted in a shorter optimal mixing time and reduced mixing tolerance after the peak. Aspartic acid is a hydrophilic, polar amino acid. The amino acid cysteine contains sulfur, is polar and uncharged. Of the hydrophobic amino acids added, the mixogram with added leucine has a pattern most similar to that of CHWPI. Leucine, which is present in whey peptides with ACE-inhibiting activity and one of the most abundant amino acids in CHWPI, was incorporated into wheat dough at the level of 1%. The mixogram with 1% leucine shows a slightly compressed curve after the optimum mixing time, which is similar in pattern, though not as pronounced as the mixograms with CHWPI. The less pronounced pattern can be attributed to the small amount (1%) of leucine added to the dough, compared to the dough with 10% CHWPI.

The amino acid sequence in the ACE-inhibitor contained in CHWPI determines how effective the ACE-inhibitor will be. However small, polar peptides will affect dough properties. Koh and colleagues (2005) added various

amounts of bonito and corn peptides (no information was given regarding the ACE-inhibiting effect of these two peptides) to wheat flour and observed the dough mixing properties. The mixograms of these two peptides show different trends. As more bonito peptide was added to the dough the amount of time required for optimum development increased. In contrast, when corn peptide was added to dough the optimal mixing time decreased. Both of the peptides decreased the mixing tolerance of the dough. The addition of CHWPI at increasing amounts resulted in behavior more like the corn peptide, with a shortened optimal mixing time and reduced mixing tolerance. The small size of both the corn peptide and CHWPI may be what is common in affecting the dough properties. The insoluble nature of the peptides may be what is affecting the dough development.

Garlic is a non-dairy additive that results in the significant breakdown of dough, as reported by Miller and colleagues (Miller, et al., 1997). The addition of 0.15% garlic powder resulted in reduced mixing tolerance of the dough. The authors compared the breakdown of the dough with garlic to that of cysteine and found that the mixograms were not similar. As a result, the authors concluded that the effect that garlic had on dough was not due to the thiol compounds in the garlic interacting with the disulfide bonds of the gluten. The garlic dough did produce a similar mixogram to dough with fumaric acid. The addition of fumaric acid to dough slightly reduced dough mixing time and substantially reduced mixing tolerance. The mixograms of dough with garlic and fumaric acid are

similar in pattern to that of the mixograms with CHWPI. The authors hypothesized that the breakdown of the dough with garlic was due to unidentified α,β–unsaturated carbonyl compounds, not due to breakage of the disulfide bonds. The addition of CHWPI may react with dough in a number of different ways. The small, polar size of the peptide may interact with the water absorption of the dough, or there may be some breakage of disulfide bonds of gluten due to the presence of amino acids with thiol groups.

Other factors, such as stickiness and viscosity of the dough, affect both how well the dough can be processed commercially as well as the quality of the final product. Dough that is too viscous is hard to sheet and will not maintain the desired loaf shape expected by consumers. Dough that is not viscous enough, bulky dough, will be too round when shaped, which is also an undesirable attribute. Dough that is too sticky will not sheet properly, however if dough is not moist enough it will not have proper development including desired crumb properties, and low loaf volume (Faridi, 1985).

Based on the cumulative factors of cohesiveness, stickiness, viscosity, and mixograms it was hypothesized that a loaf of bread with 10% CHWPI could produce a satisfactory loaf of bread, while the addition of 20% would present some quality issues. It was predicted that incorporation of 30% CHWPI would not produce a satisfactory loaf of bread.

PHYSICAL PROPERTIES OF BREAD

Physical dough testing devices provide useful information on the rheology of bread dough. However, it is widely recognized that these devices alone can not predict the quality of a baked product. Sometimes there are unforeseen reactions that take place during fermentation and baking which do not show up during the rheological testing (Pomeranz, 1987). Some of the physical parameters that can be affected by the addition of dairy ingredients are crust color, bread flavor, as well as the structure and texture of the bread (Bilgin, et al., 2006).

After dough is mixed the opportunity to add ingredients which affect the rheology of the final product has passed (Faridi, 1985). Characteristic flavors and textures are developed when the dough is allowed to proof. During fermentation, air bubbles, which were incorporated during mixing expand (Dobraszczyk and Morgenstern, 2003). Thus, in bread that has undergone proper fermentation, bubbles can be seen in the final crumb, giving the loaf an airy appearance. Any ingredients or processing steps which interfere with proper fermentation will result in changes in the final product that are not desirable. The final loaf with CHWPI had a smaller volume than the control bread. Additionally, the loaves with all amounts of CHWPI were denser and had very small air bubbles in the crumb. Thus, CHWPI may interfere with fermentation.

Many changes take place during baking. Initially, when bread begins to bake, the increase in temperature increases the activity of enzymes, yeast and bacteria. As the temperature continues to rise the bacteria and yeast will be killed and the enzymes inactivated. At the high temperatures inside the loaf, the starch gelatinizes, proteins coagulate and the texture and volume of the bread is set. Caramelization and Maillard browning reactions occur on the crust during baking (Pomeranz, 1987). The addition of an ingredient such as CHWPI may affect the volume and color of the final product.

The measurements taken during and after the bread bake reflect a difference in the treatments (Table 2). The water absorption of the dough is the amount of water needed to make the best possible dough, this was determined by the use of a mixograph. The control dough had the highest water absorption followed by the dough with 10% CHWPI. There is a slightly higher water absorption for the dough with the 30% than the 20%. These results differ slightly from the previous mixograms. However, they were run using a different mixograph in a different facility so a variation in water absorption not unusual (Pomeranz, 1987).

The height of the loaf just before it is put into the oven is the proof height. The control dough had the highest proof height and was significantly higher than the loaves made with CHWPI. The proof height of the doughs made with various amounts (10, 20, and 30%) of CHWPI did not significantly differ from each other (Figure 6, Table 2). The loaf volume, which was measured by rapeseed

displacement immediately after baking, followed the same trend as the proof height (Table 2). The control bread had a significantly larger volume than the breads with 10, 20, and 30% CHWPI. Proof height and loaf volume are indicators of gas expansion and gas retention in the dough and final bread. The incorporation of CHWPI in the dough appears to inhibit the dough from rising to its full potential. It should be noted that by adding CHWPI, the amount of gluten available in the dough is reduced, so a smaller loaf volume should be expected. Although the proof heights and loaf volumes of breads with CHWPI did not differ significantly from each other, visually there appeared to be a difference. The bread with 30% CHWPI had the appearance of a jelly roll after baking, indicating that the dough didn't rise.

The weight of the control bread is significantly different from bread with 10, 20 and 30% CHWPI (Table 2). The specific volume of the bread is the ratio of the volume of the bread to the weight. The specific volume of the bread can be used as an indicator of the quality of the bread. Bread that has a low specific volume is too dense, and did not rise properly. The specific volume followed the same trends as that of the loaf volume and weight. The specific volume of the control bread was significantly higher than that of the breads with 10, 20 and 30% CHWPI (Table 2, Figure 7). The specific volumes of the breads with CHWPI were not significantly different from each other. Specific volume depression due to the addition of dairy ingredients has been reported by other researchers (Gelinas, et al., 1995, Kenny, et al., 2000). The addition of 6% (by

weight) of fermented milk or a combination of milk and whey resulted in bread loaves that had a significant lower volume that that of the control (Gelinas, et al., 1995).

There is no significant difference in the total loaf moisture among all the treatments ($P < 0.05$) (Table 2, Figure 8). However, the initial dough absorption of the treatments (0, 10, 20, and 30% CHWPI) was different (Table 2).

The brown color on the crust of the bread is mainly a result of Maillard browning reactions. Sucrose, the main sugar in bread formulas is converted to reducing sugars by yeast, and can then react with amino acids in the gluten proteins to form Maillard browning reaction products (Hoseney, 1986). The addition of CHWPI to a bread formula adds additional amino acids to the formula, so it is expected that the darkness of the color of the crust would increase.

The crust of the bread with CHWPI was visually darker. This was reflected in the lightness values (Table 3). The control bread was significantly lighter than all bread except the bread with 10% CHWPI added at 2 min. All other breads were not significantly different from each other, though to the naked eye the bread with more CHWPI appeared to be darker.

The crumb from bread with added CHWPI appeared to be more yellow in color than the control bread. However, according to the CIE Lab values there was no significant difference in the lightness among the treatments (Table 3). A significant difference in the chroma was not found between the control bread and the bread with 10% CHWPI added. Crumb from breads made with 20% or more

CHWPI were significantly different than the control. However, there was not a significant difference in the chroma between the 20% and 30% levels of addition of CHWPI (Table 4).

Texture is an important physical feature of many food products, including bread. Bread is available in a wide range of textures due to the ingredients in the bread formula, the processing steps and consumer preferences. Texture is associated with quality. Consumers associate certain textures with freshness and quality of the overall product. Bread with a dry crust and too firm of crumb resembles stale bread, which is not desirable by consumers (Faridi and Faubion, 1990, Hoseney, 1986, Szczesniak, 2002).

Texture consists of a group of properties that can be detected through vision, hearing, touch, and kinesthetics. Texture is categorized as a sensory property, which by definition means that only humans can perceive texture. However, the need for quality control of food products has lead to the development of instruments that can quantify texture. Texture instruments quantify a number of different properties that, when combined, describe texture. Although no instrument that measures texture will be able to predict consumer feelings about a product, texture instruments do provide measurement of the products and can be used for comparison purposes. Additionally, texture instruments can be used to analyze specific texture attributes about a product. Some of the properties that texture instruments can measure are independent of each other, such as hardness, cohesiveness, adhesiveness, viscosity, and

elasticity. Other texture measurements are dependent on at least one other texture measurement, such as brittleness, chewiness and gumminess. Correlation between hardness results of texture profile analysis and sensory panels have been positive in many products including bread (Bourne, 2003, Friedman, et al., 1963, Gambaro, et al., 2004, Gambaro, et al., 2006, Szczesniak, 1987, Szczesniak, 2002).

The texture profile analysis is widely used in the baking industry to quantify a number of different texture attributes including, fracturability, cohesiveness, springiness, chewiness, and resilience (Table 5). A texture profile analysis test is a two cycle compression test, during which stress is applied to a sample and the deformation of the sample is charted. The resulting chart of the deformation is called a texture profile (Figure 9 – 10). Information about a products springiness, gumminess, chewiness, and resilience can be gained from the texture profile. Hardness of the bread can also be determined by measuring the force needed to compress the sample by 50% (Table 6, Figure 11) (Friedman, et al., 1963).

The measure of the recovery of a product to the products' original form after it has been compressed determines how springy the product is. Springiness is a measure of how well the product "springs" back to its original form after it has been compressed (Meretei and Fekete, 2003, Shah, et al., 2006, Szczesniak, 2002). The springiness of the control bread did not significantly differ from the bread with 30% CHWPI. Bread made with 10% and 20% CHWPI

(added at time = 0 and 2 min) are not significantly different from each other, but are different from the control bread and the bread with 30% CHWPI (Table 5). The control bread and the bread with 30% CHWPI are characterized as more "springy" than the bread with 10% and 20% CHWPI.

Cohesiveness of the product is how well the product holds together. Cohesiveness is measured by the ratio of the first area under the curve of the texture profile to the area of the second curve (Meretei and Fekete, 2003, Shah, et al., 2006). The most cohesive bread was the control bread, which differed significantly from the bread with 10% CHWPI and the 20% CHWPI, but not the bread with 30% CHWPI.

Chewiness is the product of gumminess and springiness (Meretei and Fekete, 2003, Shah, et al., 2006). There is not a significance difference in the chewiness between the control bread and the 10% CHWPI bread. The control bread and bread with 10% CHWPI are less chewy than the bread with 20% and 30% added CHWPI. There is no significant difference between the bread with 20% and 30% CHWPI. Descriptive words for chewy can range from tender to chewy to tough (Szczesniak, 2002).

The resilience of a food is similar to springiness. Resilience is how fast is takes a product reach its original state after compression; resilience can also be considered "instant springiness." Bread made with 10% CHWPI and bread made with 30% CHWPI significantly differed from each other, but not from the control

bread or bread with 20% CHWPI. The least resilient bread was 10% CHWPI, while the most resilient was the control bread.

The hardness of the crumb was determined by force required to compress a slice by 50%, this is represented on the texture profile curve by the height of the first curve (Shah, et al., 2006) (Table 6, Figure 11). The softest bread was the control bread, followed by bread with 10% CHWPI, then 30% CHWPI. The hardest bread contained 20% CHWPI. Incorporation of ingredients has been noted to cause a change in the firmness of bread. The addition of 6% fermented milk or a combination of milk and whey caused a significant increase in firmness of breads (Gelinas, et al., 1995). Kenny and others added three different whey protein concentrates to bread, and found that the resulting loaves varied in their firmness. One whey protein concentrate (with 79% protein that had been modified to improve gelation) increased firmness to an unacceptable level, while the other two whey protein concentrates slightly impacted the firmness of the crumb (Kenny, et al., 2000).

ACE-INHIBITION, NITROGEN AND FREE AMINE ANALYSIS

For a functional food to have the desired benefit, the functional ingredient must be able to withstand the processing that it undergoes in any one food product. In bread, steps that could affect the functionality of a protein are dough mixing, fermentation, and baking (Korhonen, et al., 1998, Mangino, 1984).

CHWPI, the functional ingredient that was added to the bread, is an ACE-inhibitor thus, the ACE-inhibition of baked bread was tested. Due to more exposure to heat it was hypothesized that the crust of the bread would have less ACE-inhibition than the crumb. To test this hypothesis the ACE-inhibition of both the crust and the crumb were tested and compared. The total nitrogen of the bread samples was tested to validate the amount of protein added. In addition free amines of the bread samples were analyzed to determine how heating played a role in affecting the peptides.

The amount of total nitrogen in the bread followed the expected trend of more nitrogen in the bread with higher amount of added CHWPI (Table 7). No significant difference was seen between the percent total nitrogen in the crust when compared to the crumb at each of the levels. A significant difference is present in the percent of total nitrogen between breads with no CHWPI (control) and 10% CHWPI when compared to breads with 20% and 30% CHWPI.

The percent total nitrogen of CHWPI was determined to be 14.103 ± 0.064. Based on the percent total nitrogen in the control bread and the percent total nitrogen of the CHWPI the expected total nitrogen values were determined. The calculated percent total nitrogen value for bread with 10% CHWPI was 3.29% total nitrogen and for 20% CHWPI was 4.48 % total nitrogen. At these levels (10 and 20 %) the measured total nitrogen was similar to the calculated. The calculated value for bread with 30% CHWPI was slightly higher (5.67%) than the actual value. The discrepancies between the calculated nitrogen levels and

actual nitrogen levels in the bread with 30% CHWPI have been considered. One explanation for the lower nitrogen in the bread with 30% CHWPI into the bread is that during the process of incorporation the CHWPI may have gone into the air do to the low density of the product. However, the amount of CHWPI that could have been lost this way would not fully explain the difference. Another possibility is that there was an error in weighing the CHWPI, however, it seems unlikely that the ingredients for all twelve breads with 30% CHWPI were weighed incorrectly.

The ACE-inhibition activity was measured using a spectrophotometric assay (Table 8, Figure 12). The substrate, 2-furananacryloyl-L-phenylanylglyclglycine (FAPGG), was hydrolyzed by ACE to furanacryloyl-L-phenylalanine (FA-Phe) and glycylgylcine (Gly-Gly). An ACE-inhibitor, such as CHWPI, prevents the hydrolysis of FAPGG to FA-Phe and Gly-Gly; this can be seen in as a smaller decrease of optical density overall, which corresponds to ACE-inhibition (Murray and FitzGerald, 2007, Shalaby, et al., 2006).

The percent angiotensin-converting enzyme – inhibition (ACE-inhibition) followed the expected trend: as more CHWPI was added to bread the percent ACE-inhibition increased (Table 8, Figure 12). The control bread actually increased ACE activity, so it was not surprising to find a significant difference between the control bread and all levels of CHWPI (10%, 20%, and 30% and at time = 0 and 2 min).

The increase in ACE activity by the control bread was unexpected (Table 8, Figure 12). The proteins in the wheat, in their native form may not have ACE-

inhibiting activity. However, when hydrolyzed, wheat gluten has been reported to have ACE-inhibiting activity. Gluten, gliadin and glutenin were hydrolyzed with pepsin, molsin F, rapidase, orientase 5A, protease M, and pepsin-protease M and analyzed for ACE-inhibitory activity. *In vivo*, the hydrolyzed gliadin hydrolystates had the highest ACE-inibitory activity. The bioactive peptide of the gliadin produced by protease M was isolated by ion exchange chromatography and sequenced using an automatic protein sequencer. The sequence of the peptide was Ile-Ala-Pro, which has been identified as an ACE-inhibitor by other authors. The purified peptide from gliadin was injected into spontaneously hypertensive rats to determine if the peptide had ACE-inhibitory activity *in vivo*. When compared to a control of saline, the purified hydrolyzed peptide from gliadin significantly reduced blood pressure at 1.5, 3, and 5 h (Motoi and Kodama, 2003). It may be that the control bread has increased ACE activity *in vitro*, but when the gluten is digested by the body there will be an ACE-inhibitory effect.

ACE-inhibition activity is present in both the crust and the crumb of the bread with added CHWPI. Generally, there is not a significant difference between the ACE-inhibition activity of the crumb and that of the crust, the exception being bread with 10% CHWPI added at two min (Table 8). The finding of no significant difference of ACE-inhibition activity in the crust and the crumb of the bread was unexpected. It was expected that that the small peptide ACE-inhibitors would be consumed as part of the Maillard reaction.

One way to explain why there is no difference between the ACE-inhibitory activity of the crust and the crumb is that the peptides that are part of the ACE-inhibiting activity of the CHWPI are being used as part of the Maillard browning reaction, and the products of the Maillard browning reaction have ACE-inhibiting properties. The Maillard browning reaction is a series of reactions that take place between reducing sugars and free amino acids or free amino groups. The addition of heat, as in the case of baking bread, increases the rate at which the Maillard browning reaction occurs. There are a series of steps in the Maillard browning reaction that take place, leading to a number of products that can be produced (Fennema, 1996).

Melanoidins, high molecular weight compounds, are one of the products of the Maillard browning reaction. Rufian-Henares and Morales (2007) examined the ACE-inhibiting properties of aqueous melanoidins prepared by reacting glucose with an amino acid and then isolating the melanoidins by ultra-filtration. Three different fractions of melanoidins were isolated: melanoidins, pure melanoidins and bound melanoidin compounds. The Cushman and Cheng assay was used to determine the ACE-inhibition of the melanoidins (Cushman and Cheung, 1971). All the melanoidins are ACE-inhibitors, with the pure melanoidins being the most potent ACE-inhibitor. This study also looked at the color of the melanoidins and the ACE-inhibiting activity. There was significant negative correlation between color and ACE-inhibiting activity. Thus, the compounds that are responsible for the characteristic brown color for which the

Maillard reaction is identified are not responsible for the ACE-inhibiting activity (Rufian-Henares and Morales, 2007). This study may explain why the crust of the bread had the same ACE-inhibiting activity as the crumb of the bread. Although the ACE-inhibiting peptides are potientally being used in the Maillard reaction, the products of the Maillard reaction are ACE-inhibitors.

According to the CIE Lab values (Table 3), (with the exception of the bread with 10% CHWPI added at 2 min), breads with CHWPI were significantly darker than the control bread. Due to the higher amount of protein in the bread, it can be surmised that the darkening of the crust is due to the Maillard reaction. To check this assumption, free amines, which are used up in the Maillard browning reaction were measured in the crust and crumb extracts of the bread samples.

The 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) assay was used to measure the free amines on a glycine equivalent basis (Pierce, 1999). The free amines in the bread reacted with TNBSA by nucleophilic substitution, an orange colored solution was formed, the absorption of which was proportional to the amount of free amines in the sample. The crust and the crumb of bread with no CHWPI, 10, 20, and 30% CHWPI (added at time = 0 and 2 min) were assayed. A trend of lower free amines in the crust when compared to the crumb was not consistent throughout all treatments, as would be expected due to the browning observed.

Overall, there is a significant difference ($p<0.05$) in free amines (calculated as glycine equivalents) among the crust and the crumb samples. When the crust and crumb samples were analyzed independently, there is a significant difference among the crumb samples of bread with different amount of added CHWPI (0, 10, 20, and 30%) but not among the crust samples (Table 9). The differences in the crumb are found between the control bread, which has less free amines ($p<0.10$) than the bread with 20% CHWPI (added at time = 0 and 2 min) and 30% CHWPI (added at time = 0). The TNBSA assay did not support the theory that free amines were consumed in the crust as part of the Maillard reaction. A high standard deviation among the crust samples, could explain why no significant difference was found among these samples.

CONCLUSION

The development of functional foods is a growing area. However, incorporation of ingredients into everyday food items to reach physiological benefits is not always straightforward. The inclusion of an ACE-inhibiting peptide such as CHWPI into a product such as bread needs further research before it would be acceptable to consumers.

Incorporation of CHWPI into wheat dough at addition levels of 10, 20, and 30% affects the rheology of the dough. Optimum mixing time is decreased and the dough is weaker overall. Adding CHWPI after the gluten had started to

develop increased the optimum mixing time of the dough and improved mixing tolerance.

Incorporation of 10, 20, and 30% CHWPI into bread affected the physical features of the bread loaf. Overall, incorporating of CHWPI caused a decrease in loaf volume, and baked loaf weight. The crust of the bread with CHWPI was significantly darker than that of the control bread.

The ACE-inhibition of CHWPI was present in the final product, suggesting that this peptide is tolerant to both fermentation and baking.

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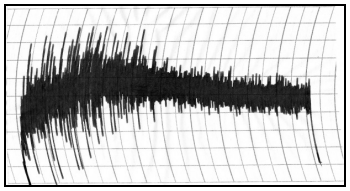

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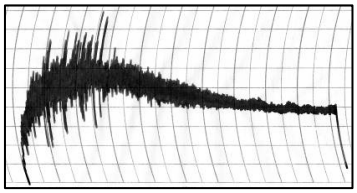
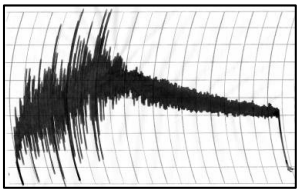


B. 51% abs



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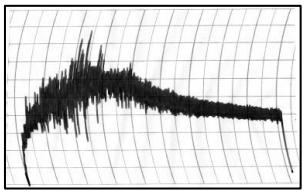

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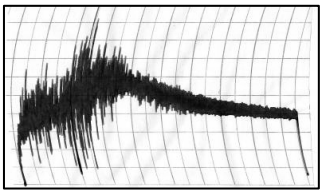
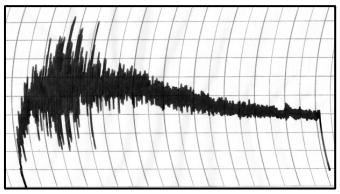


G. 55% abs



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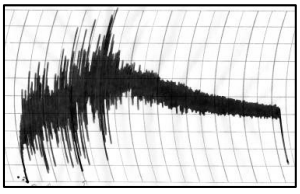

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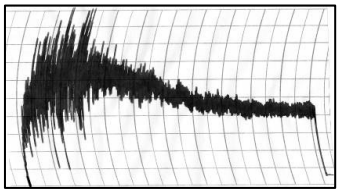
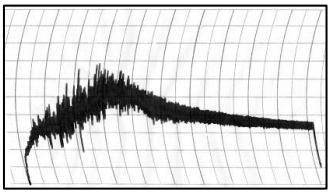
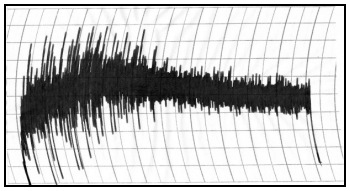


E. 57% abs

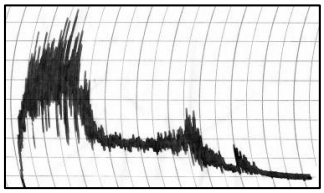


I. 59% abs

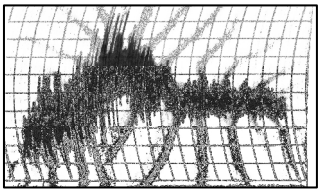
Figure 1. Mixograms with 10% CHWPI added, except for control (A), 10% CHWPI added at time = 0 minutes, water absorption varied (B-E) and 10% CHWPI added at time = 2 minutes (F-I)



A. Control, 60% abs



B. 48% abs



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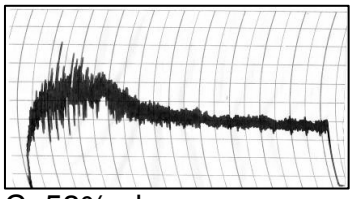
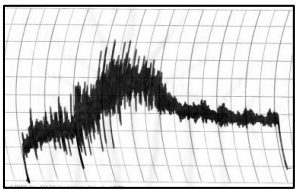
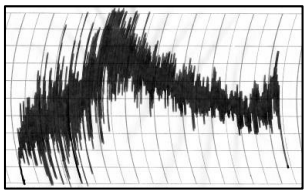


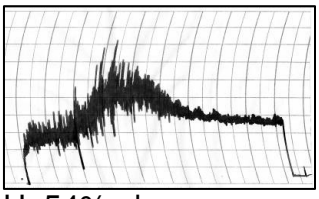
C. 53% abs



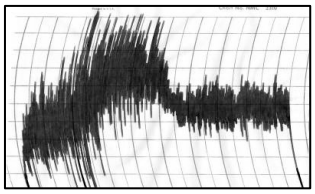
G. 52% abs



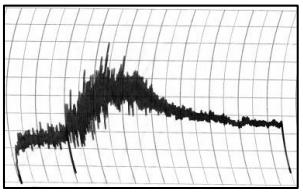
D. 44% abs



H. 54% abs

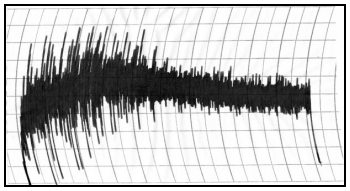


E. 48% abs

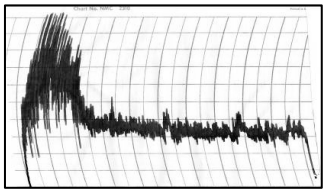


I. 56% abs

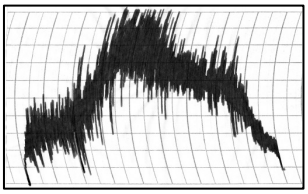
Figure 2. Mixograms with 20% CHWPI added, except control (A), 20% CHWPI added at time = 0 minutes, water absorption varied (B-C) and 20% CHWPI added at time = 2 minutes with varied water absorption (D-I)



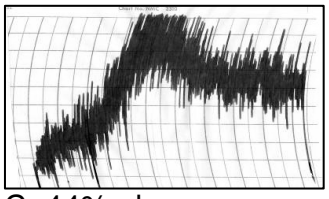
A. Control, 60% abs



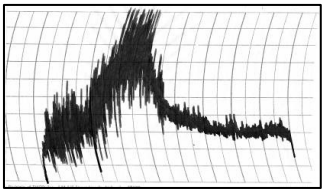
B. 44% abs



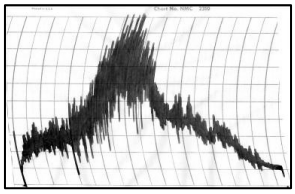
F. 46% abs (initially 44%, after 2 min 2%)



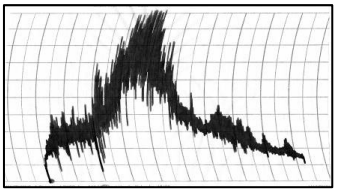
C. 44% abs



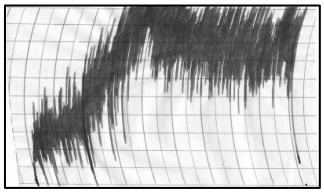
G. 48% abs (initially 44% after 2 min 4%)



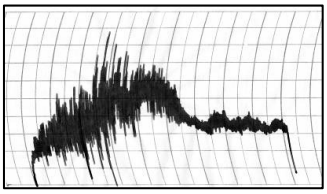
D. 48% abs



H. 48% abs (initially 46% after 2 min 2%)

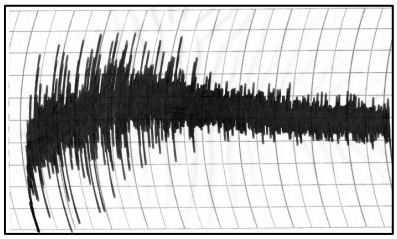


E. 42% abs (initially 40%, after 2 min 2% added)

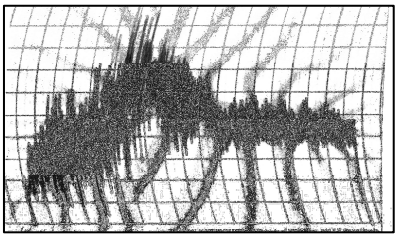


I. 50% abs (initially 46% after 2 min 4% added)

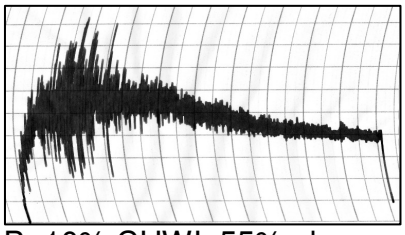
Figure 3. Mixograms with 30% CHWPI added, except control (A), 30% CHWPI added at time = 0 minutes, water absorption varied (B) and 30% CHWPI added at time = 2 minutes with varied water absorption (C-I)



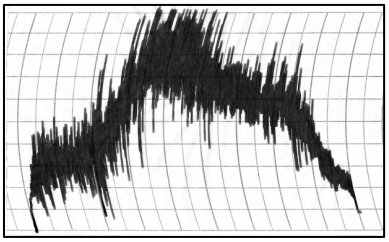
A. Control, 60% abs



C. 20% CHWPI added after 2 min, 50% abs

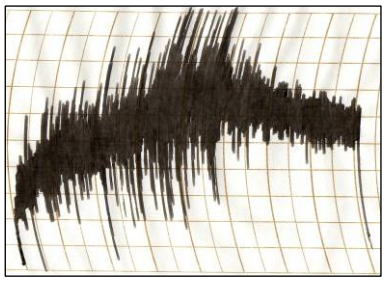


B. 10% CHWI, 55% abs

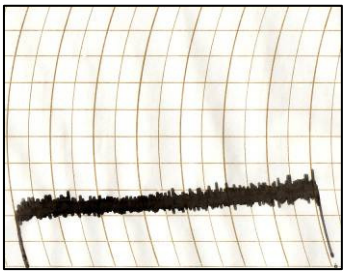


D. 30% CHWPI added after 2 min, 46% abs (initially 44%, after 2 min 2%)

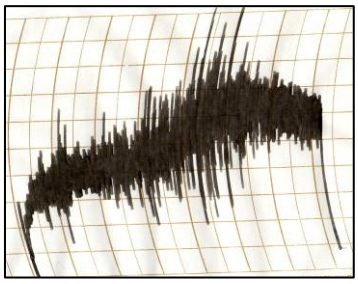
Figure 4. Mixograms of dough with the optimum absorption of each level of CHWPI (0, 10, 20 and 30%)



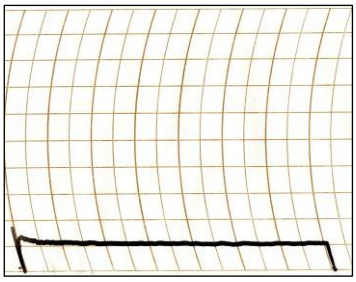
A. 75% abs



C. 90% abs



B. 80% abs



D. 120% abs

Figure 5. Mixograms response to water absorption (commercial flour)

Table 1. Mixing times of wheat flour dough with different levels of CHWPI and water absorption

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Water Absorption (%)	Mixing Time (min)
0 (Control)	0	60*	3.5
10	0	51	2.75
10	0	53	3.0
10	0	55*	3.5
10	0	57	3.5
10	2	53	4.0
10	2	55	3.75
10	2	57	4.25
10	2	59	4.0
20	0	48	2.0
20	0	53	3.0
20	0	44	4.25
20		48	4.5
20	2	50*	4.5
20	2	52	4.5
20	2	54	4.25
20	2	56	4.25
30	0	44	1.75
30	2	44	5.0
30	2	48	4.75
30	2	42 (initially 40%, after 2 min 2 % added)	4.75
30	2	46 (initially 44%, after 2 min 2%)	4.75
30	2	48 (initially 44% after 2min 4%)	4.0
30	2	48 (initially 46% after 2 min 2%)	4.5
30	2	50 (initially 46% after 2 min 4%)	4.5

* Indicates ideal water absorption

Table 2. Results of bread bake

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Dough abs (ml)	Proof ht (cm)	Loaf vol (cm)	Specific vol (ml/g)	Baked loaf wt (g)	% Total loaf moisture
0 (Control)	0	64.20	7.7 ± 0.1[a]	787 ± 31[a]	5.243[a]	150.03 ± 1.62[a]	35.84 ± 1.03[a]
10	0	50.20	5.9 ± 0.2[b]	490 ± 13[b]	3.449[b]	142.07 ± 2.27[b]	32.08 ± 0.28[a]
10	2	50.20	6.00[b]	475 ± 66[b]	3.337[b]	142.33 ± 0.70[b]	31.55 ± 0.29[a]
20	0	45.20	5.3 ± 0.1[b]	417± 78[b]	2.938[b]	141.83 ± 6.49[b]	36.78 ± 1.99[a]
20	2	45.20	5.6 ± 0.2[b]	408 ± 38[b]	2.848[b]	143.40 ± 6.32[b]	32.52 ± 0.40[a]
30	0	47.20	5.7 ± 0.6[b]	465 ± 46[b]	3.202[b]	145.20 ± 3.56[b]	30.73 ± 7.22[a]
30	2	47.20	5.6 ± 0.1[b]	445 ± 33[b]	3.246[b]	137.10 ± 8.88[b]	31.94 ± 0.56[a]

Values shown are means followed by standard deviation (n=3 for all treatments except 10% CHWPI at 2 min, n=1). Values in the same column that share the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

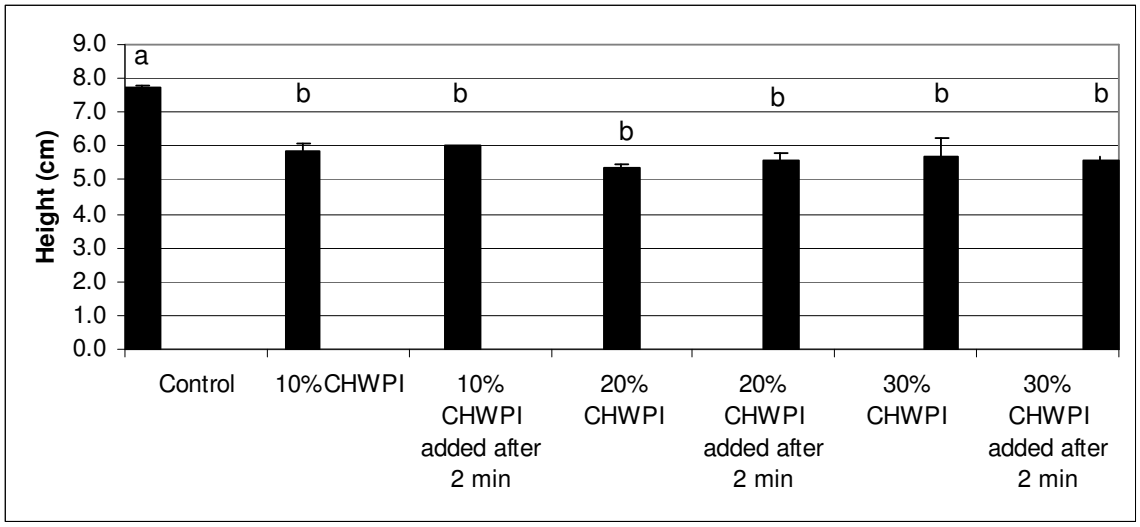


Figure 6. Proof height of bread loaves with different amount of CHWPI (0, 10, 20, and 30% added at time = 0 and 2 minutes) (n=3 for all treatments except 10% CHWPI at 2 min, n=1). Values shown are means followed by standard deviation. Values sharing the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

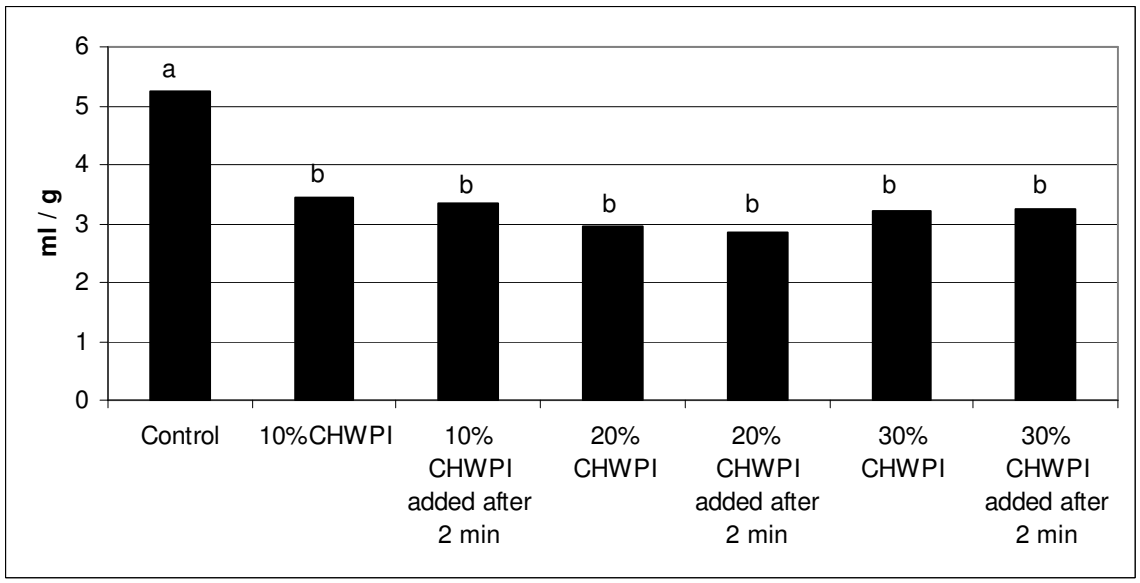


Figure 7. Specific volume (ml/g) of bread loaves with different amount of CHWPI (0, 10, 20, and 30% added at time = 0 and 2 minutes) (n=3). Values shown are means followed by standard deviation. Values sharing the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

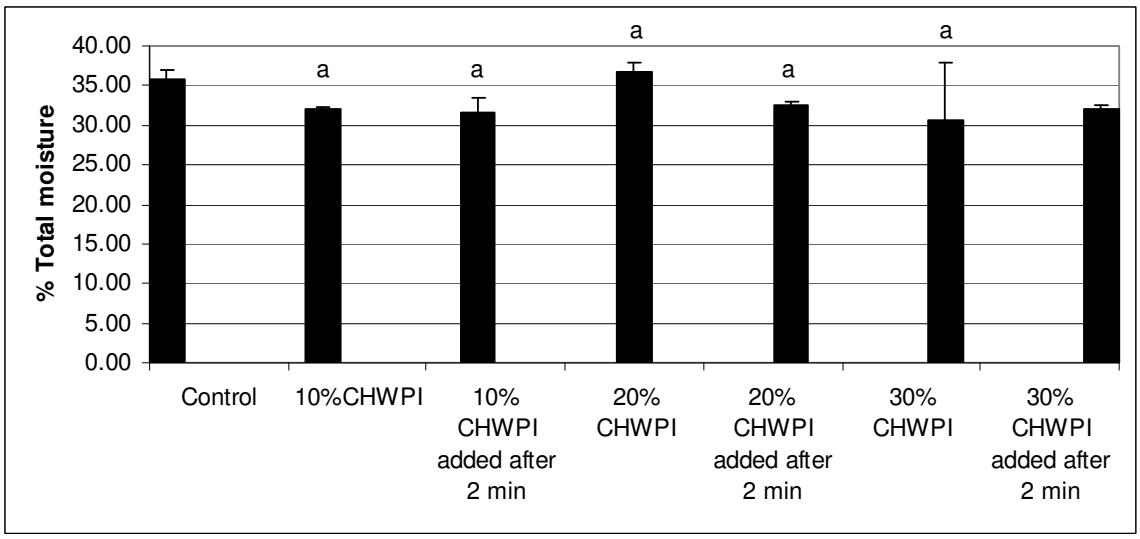


Figure 8. Total moisture of bread loaves with different amount of CHWPI (0, 10, 20, and 30% added at time = 0 and 2 minutes) (n=3); Values shown are means followed by standard deviation. Values sharing the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

Table 3. CIE-Lab values of crust

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	L*	Chroma
0 (Control)	0	52.62 ± 1.40 a	32.57 a
10	0	33.67 ± 1.96 b	17.46 b
10	2	38.16 ± 1.34 a,b	20.14 b
20	0	31.42 ± 1.42 b	18.02 b
20	2	30.37 ± 0.39 b	17.59 b
30	0	30.40 ± 2.63 b	14.06 b
30	2	29.36 ± 0.44 b	14.49 b

L* = Lightness (0 = black, 100 = white); Chroma = saturation; Values shown are means followed by standard deviation (n=9). Values in the same column that share the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

Table 4. CIE-Lab values of crumb

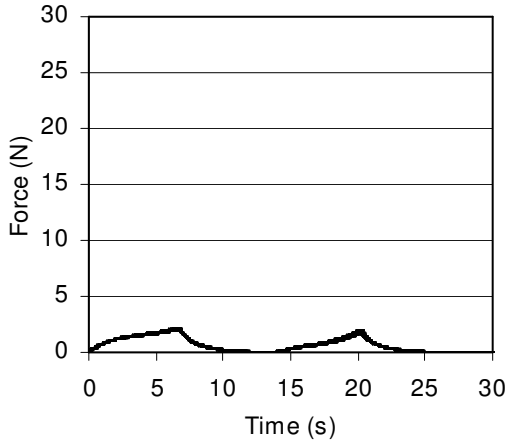
Per Cent (%) CHWPI added	Time of CHWPI addition (min)	L*	Chroma
0 (Control	0	75.77 ± 1.71 a	13.07 a
10	0	75.12 ± 1.81 a	15.98 a
10	2	75.81 ± 2.79 a	16.05 a
20	0	75.04 ± 2.90 a	18.14 b
20	2	77.63 ± 2.46 a	18.70 b
30	0	73.08 ± 2.84 a	18.03 b
30	2	75.66 ± 2.17 a	18.21 b

L* = Lightness (0 = black, 100 = white); Chroma = saturation; Values shown are means followed by standard deviation (n=9). Values in the same column that share the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

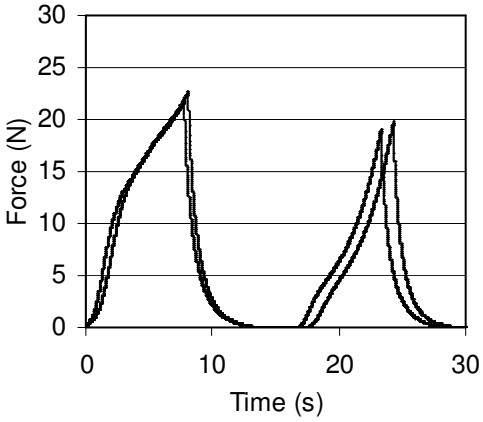
Table 5. Texture profile analysis of crumb

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Springiness (ratio)	Cohesiveness (ratio)	Chewiness (N/cm)	Resilience (ratio)
0 (Control)	0	0.923 ± 0.014^{a}	0.634 ± 0.011^{a}	1.191 ± 0.085^{a}	$0.296 \pm 0.010^{a,b,c}$
10	0	0.870 ± 0.011^{b}	0.496 ± 0.019^{b}	3.066 ± 0.148^{a}	$0.174 \pm 0.011^{b,c}$
10	2	0.863 ± 0.023^{b}	0.485 ± 0.028^{b}	3.775 ± 1.537^{b}	0.168 ± 0.015^{c}
20	0	0.861 ± 0.024^{b}	0.562 ± 0.034^{c}	7.602 ± 2.518^{b}	$0.206 \pm 0.024^{a,b,c}$
20	2	0.865 ± 0.007^{b}	0.569 ± 0.015^{c}	9.725 ± 2.203^{b}	$0.208 \pm 0.015^{a,b}$
30	0	0.882 ± 0.025^{a}	$0.619 \pm 0.013^{a,d}$	5.767 ± 2.171^{b}	0.244 ± 0.015^{a}
30	2	0.881 ± 0.009^{a}	$0.587 \pm 0.008^{a,c,d}$	6.917 ± 2.388^{b}	0.225 ± 0.009^{a}

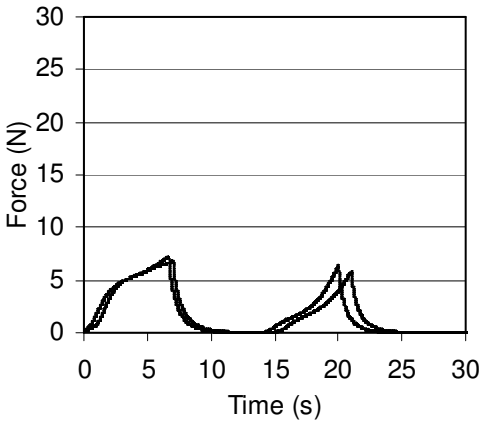
Values shown are means followed by standard deviation (n=6). Values in the same column that share the same letter indicate no significant difference using Tukey's test ($P < 0.05$)



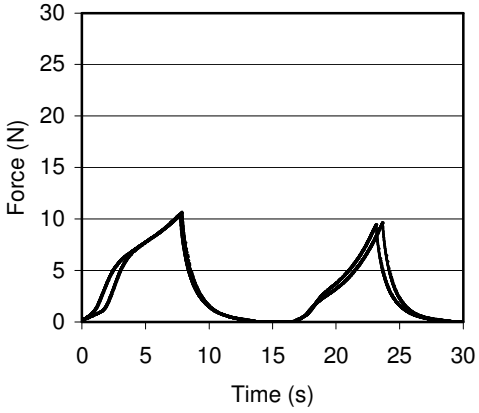
A. Control



C. 20% CHWPI

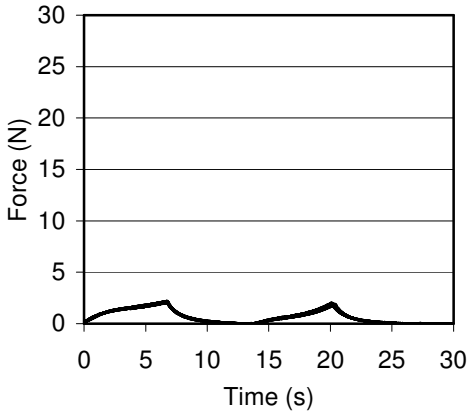


B. 10% CHWPI

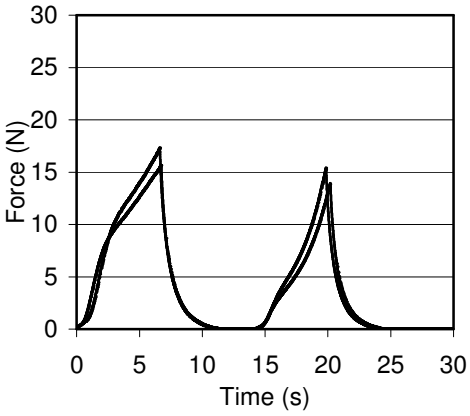


D. 30% CHWPI

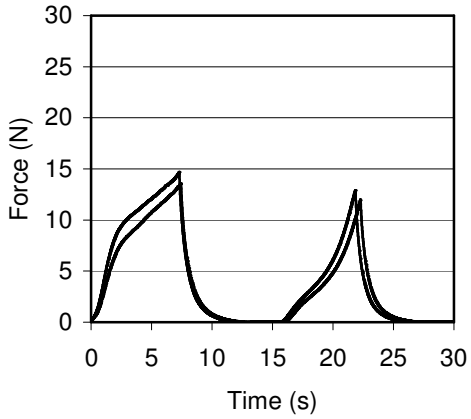
Figure 9. Representative texture profiles from texture profile analysis of bread crumb with various levels of CHWPI (0, 10 20, and 30%) added at time = 0 (n=2)



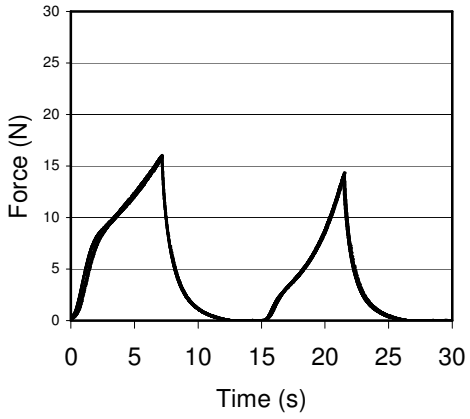
A. Control



C. 20% CHWPI added after 2 min



B. 10% CHWPI added after 2 min



D. 30% CHWPI added after 2 min

Figure 10. Representative texture profiles from texture profile analysis of bread crumb with various levels of CHWPI (0, 10, 20, and 30%) added at time = 2 minutes (n=2)

Table 6. Hardness of crumb

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Hardness (N)
0 (Control)	0	1.870 ± 0.122[a]
10	0	6.174 ± 0.399[a]
10	2	7.954 ± 3.522[b]
20	0	14.256 ± 5.467[a]
20	2	17.603 ± 4.228[b]
30	0	9.620 ± 3.797[b]
30	2	11.969 ± 4.254[b]

Values are means followed by standard deviations (n=6). Values sharing the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

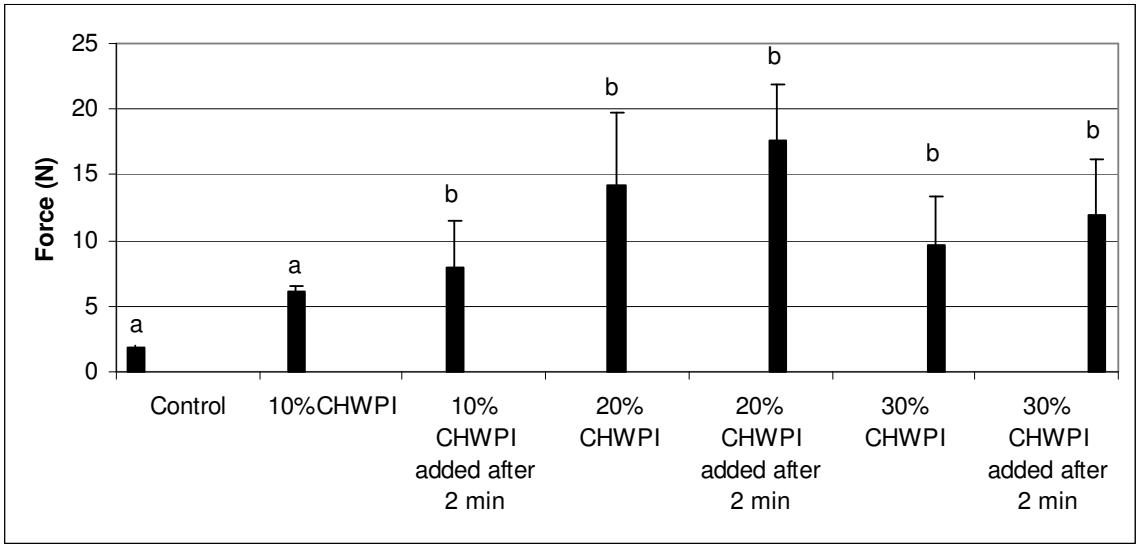


Figure 11. Hardness of crumb determined by texture profile analysis (n=6); Bars represent means. Values sharing the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

Table 7. Per cent (%) total nitrogen

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Crumb	Crust
0 (Control)	0	2.098 ± 0.045^{a}	2.039 ± 0.037^{a}
10	0	3.318 ± 0.743^{a}	3.143 ± 0.070^{a}
10	2	3.229 ± 0.098^{a}	3.162 ± 0.083^{a}
20	0	4.333 ± 0.055^{b}	4.282 ± 0.089^{b}
20	2	4.652 ± 0.410^{b}	4.565 ± 0.442^{b}
30	0	5.403 ± 0.044^{b}	5.305 ± 0.108^{b}
30	2	5.178 ± 0.443^{b}	5.129 ± 0.451^{b}

% Total nitrogen was calculated on a dry weight basis. Values are means followed by standard deviation (n=27). Values that share the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

Table 8. Per cent (%) ACE-inhibition

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Crumb	Crust
0 (Control)	0	-9.65 ± 9.82[a]	-7.37 ± 4.17[a]
10	0	39.32 ± 5.50[b]	32.25 ± 2.86[b,c]
10	2	37.31 ± 6.02[b]	27.12 ± 2.31[c]
20	0	61.19 ± 5.21[d,e]	60.24 ± 3.17[d,e]
20	2	57.78 ± 8.78[d,e]	63.83 ± 6.28[d,e,f]
30	0	64.69 ± 4.22[d,e,f]	71.19 ± 4.48[f]
30	2	67.80 ± 8.36[d,f]	69.13 ± 5.67[d,f]

Values are means followed by standard deviations (n=27). Values that share the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

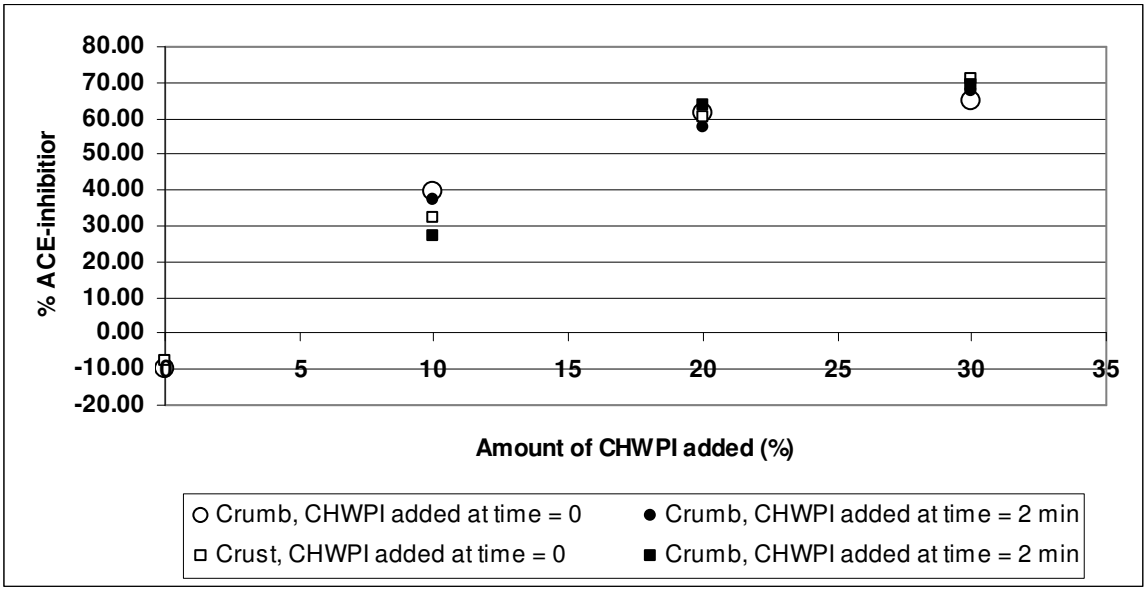


Figure 12. Comparison of % ACE-inhibition in the crust and crumb of bread samples (CHWPI added at 0, 10, 20, and 30% at time = 0 and 2 minutes)

Table 9. Free amines*

Per Cent (%) CHWPI added	Time of CHWPI addition (min)	Crumb	Crust
0 (Control)	0	0.081 ± 0.033[a]	0.070 ± 0.013[a]
10	0	0.234 ± 0.073[b,c]	0.361 ± 0.015[a,b]
10	2	0.172 ± 0.009[b,c]	0.078 ± 0.007[a]
20	0	0.149 ± 0.019[a,c]	0.146 ± 0.004[a,b]
20	2	0.106 ± 0.021[a,c]	0.125 ± 0.080[a,b]
30	0	0.195 ± 0.035[b,c]	0.189 ± 0.048[b]
30	2	0.187 ± 0.060[b,c]	0.117 ± 0.058[a,b]

Values are means followed by standard deviations (n=27). *Data is expressed as glycine equivalents (ug/ml) in bread extract. Values that share the same letter indicate no significant difference using Tukey's test ($P < 0.05$)

CHAPTER IV

FUTURE RESEARCH

FUTURE RESEARCH

Future research into the development of bread with the incorporation of a whey peptide that is an ACE-inhibitor can be divided into two main sections: improvement of the bread itself and verification of the ACE-inhibition effect *in vivo.*

The development of bread with CHWPI will require additives to improve the dough handling properties and the physical appearance of the bread. By adding CHWPI to bread, the amount of gluten in the bread dough was diluted. The addition of gluten to the bread formula with CHWPI may result in dough with better gluten formation and a loaf with a bigger volume. In addition there are many different additives that could be considered that fall into the broad category of dough conditioners. Dough conditioners work in a variety of ways to increase machine-ability of the dough, increase the tolerance of the dough to different ingredients, and improve the volume, grain and texture of the loaf. The category of dough conditioners is subdivided into dough strengtheners and crumb softeners, depending on the dominate function of the additive. Although the addition of CHWPI resulted in some negative physical features of the bread, adding one or more dough conditioners may overcome some of the problems caused by protein supplementation (Morrison, 1978, Schuster and Adams, 1984).

Emulsifiers reduce the surface tension between two immiscible phases, allowing an emulsion to form. In bread, emulsifiers are added for a number of reasons, including improving dough strength, crumb and grain structure, symmetry of the loaf, as well as to increase gas retention. Additional benefits such as a longer shelf life due to reducing staling, and a thicker crust can also be achieved with the addition of emulsifiers. A single emulsifier will not provide all of the listed benefits, so a combination of emulsifiers may be need to achieve the desired result. Some common emulsifiers that are used in bread are lecithin, diacetyl tartaric acid esters of monoglycerides (DATEM), sodium stearoyl-2-lactylate (SSL), calcium stearoyl-2-lactylate (CSL), polysorbate, and ethoxylated monoglycerides (EOM). Lecithin is used to increase loaf volume, and improve texture, which would be beneficial in bread with CHWPI. SSL and CSL strengthen dough by stabilizing the gluten network. To improve dough with added CHWPI, a combination of DATEM and SSL or CSL could be used to strengthen the dough and increase the volume of the loaf (Schuster and Adams, 1984, Stampfli and Nersten, 1995).

Oxidants and reducing agents such as ascorbic acid, potassium bromate, l-cysteine, and calcium bromate are used in the baking industry to improve dough strength and volume and are another option to improve the properties of a bread with CHWPI (Hoseney, 1986).

A variety of different emulsifiers, oxidizing and reducing agents would need to be tested to determine which combination would produce the most

acceptable loaf of bread with CHWPI incorporated. To determine the best additive, a small scale bake should be carried out with the additives incorporated into the bread formula. The final product should be validated with sensory tests, including an acceptability test, to determine if consumers like the product.

This study looked at the effect of processing on the effect of ACE-inhibitors and measured the recovery of products with ACE-inhibition activity by *in vitro* assay. Now that it has been determined that CHWPI maintains ACE-inhibitory activity throughout the bread making process, the next step is to determine if the desired physiological effect can be achieved *in vivo*. Lee and others carried out a randomized, double blind, placebo-controlled study, with a total of fifty-four subjects, over 12 weeks with a drink made with whey protein peptides. Subjects drank either a milk beverage or a beverage with whey protein peptides for 12 weeks. The whey protein peptides had been shown to have ACE-inhibition effect *in vitro,* however no significant difference was seen between the placebo and the treatment group *in vivo*. The authors suggested that the dose of ACE-inhibitors may not have been large enough, thus a dose-response study would be appropriate (Lee, et al., 2007). However, other researchers have found that the ingestion of twenty grams of hydrolyzed whey protein decreased systolic and diastolic blood pressure after one week. The reduction in blood pressure was steady over a six week period (Pins and Keenan, 2004). These studies highlight the need to examine the effects of ACE-inhibitors *in vivo*.

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