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Impact of high-pressure processing on soy and egg proteins

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To my family, for always supporting me

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ABSTRACT

Iowa is the largest soybean and egg producer in the U.S., producing over 500 million bushels of soybeans and more than 14 billion eggs per year (USDA 2009; Iowa Egg Council 2008). There is, therefore, a unique opportunity to contribute to the development of Iowa economy by identifying ways to improve value of these products. The objective of this research was to assess the effects of high-pressure processing (HPP) on soymilk and its potential for tofu production, and on the egg protein phosvitin. In the first study, soymilk pH was adjusted to 6.0 or 6.5 and a thermal or pressure pre-treatment was applied. Tofu was produced under pressure or by thermal treatment. Quality parameters including particle size and stability of soymilk, tofu yield, moisture and crude protein content, and water holding capacity and texture of the different tofu were measured. Hydrolysis of glucono-δ-lactone (GDL) in water and soymilk was performed and rates of hydrolysis under thermal and pressure treatments were determined. Finally, microstructures of the tofu were compared in order to characterize effect of the different soymilk pre-treatment and method of tofu production.

Change of soymilk particle size was dependent upon the pre-treatment applied. After pressure (400 MPa, 5 min) or thermal treatment (85 °C, 30 min), the particle size of the pH 6.5 soymilk decreased by 12-fold when compared to the control, while after pressure treatment at 600 MPa the soymilk particle size increased 2-fold. All combination of pH, pre-treatment, and method of production resulted in formation of a tofu, except the pH 6.0 soymilk that was submitted to a thermal pre-treatment prior to pressure treatment to generate tofu production. Pressure-produced tofu, regardless of pressure level (400 and 600 MPa),

produced higher yields than thermal produced tofu. Several advantages of using HPP to produce tofu exist, including a shorter processing time and the elimination of a pressing step.

In the second study, native phosvitin was pressurized in combination with a high temperature (65 °C) with the pH adjusted to 2.3, 7.0 and 11.0. Circular dichroism and size exclusion chromatography were performed to determine structural changes due to pressure, temperature and pH changes. Phosphatase treatment for up to 18 h was used to dephosphorylate phosvitin prior to *in vitro* digestion that simulated gastrointestinal digestion. SDS-PAGE was used to characterize phosvitin peptides formation. Extent of dephosphorylation and changes in angiotensin converting enzyme inhibitory activity and antioxidant activity were monitored after HPP and enzymatic treatments.

Structure of phosvitin was maintained after treatment at 600 MPa, initial temperature of 65 °C, processing temperature 83 °C, for 2 or 30 min, illustrating high stability of the protein in these conditions. The percent of helices and β sheets of the phosvitin increased by 8.1 and 22.6%, respectively, when the pH was adjusted from neutral (7.0) to acidic (2.3). The percent of phosphate released increased as the phosphatase treatment time increased, reaching 62.8% after 18 h. Changes in the phosvitin peptide profile were observed after phosphatase treatment (18 h) followed by protease treatment (pepsin and pancreatin, 3 h each), with the formation of peptides of 29, 27 and 21 kDa. Antioxidant activity of these peptides increased by 71.0% compared to native digested phosvitin, suggesting that not only is the amount of phosphate important, but the amino acid sequence and the peptides obtained as well.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Soybeans are an inexpensive, accessible source of protein for the majority of the world. Of the available soy food products, soymilk and tofu are two of the most popular. Soymilk is a colloidal solution that is produced from grinding and straining soybeans with a specific water-to-bean ratio (Guo and others 1997). There are several processing methods to produce soymilk, among which the traditional Chinese method involves heating the slurry to improve both the nutritional value and shelf-life of the soymilk, after straining. Tofu is a soybean curd produced by coagulating soymilk, followed by molding and pressing of the curd to remove the whey, and obtain a tofu with desirable textural properties (Wilson 1995). Several factors can influence the quality of soymilk and tofu. These factors include, but are not limited to, the soybean type, nature of coagulant, percent solids and method of production. Controlling these parameters is important as the yield, color, flavor, and texture can be affected (Wilson 1995; Cai and Chang 1997; Khatib and others 2002; Hou and Chang 2004).

Phosvitin is a highly phosphorylated protein that can be found in the egg yolk. It has a very distinct amino acid sequence, with ~50% of the residues being serine (Byrne and others 1984). Because of its amino acid sequence, phosvitin has very strong metal chelating abilities and some amphiphilic properties (Castellani and others 2004). However, because 95% of the iron found in the yolk is bound to phosvitin, consumption of phosvitin may have a negative nutritional impact (Ishikawa and others 2007). Because of its unique structure, phosvitin is resistant to many processing conditions including high temperatures (autoclaving), protease

action, and high pressure applied at room temperature (Albright and others 1984; Goulas and others 1996; Castellani and others 2004).

High-pressure processing (HPP) is a novel technology that has gained considerable interest as an alternative to thermal processing. While thermal processing can improve the shelf-life and safety of a food product, it can also decrease the nutritional value of foods and can produce off flavors. For example, thermal treatment of soymilk promotes undesirable, cooked flavors that can decrease consumption of soymilk (Kwok and Niranjan 1995). HPP, on the other hand, has the potential to improve the shelf-life of foods while maintaining their fresh attributes including color and nutrient content (He and others 2001). Commercial production of pressure-treated foods includes ready-to-eat meats, seafood, guacamole, salsa and fruit juices.

Traditionally, production of soymilk and tofu is time and energy consuming and therefore costly. Furthermore, the thermal treatments used create unwanted browning and off flavors. Recent data has shown that tofu can be produced under pressure. However, no studies have investigated the quality of the resulting tofu. As quality is a major driving force behind the products consumers buy, we must investigate these parameters to determine if tofu production with pressure is a practical option. In addition, none of the studies have investigated the effect of soymilk pH or pre-treatment on the tofu produced. As soymilk pre-treatment is a typical step in tofu production and pH can change with storage time, these are important factors that need to be considered.

Phosvitin has the potential to be used as a natural source for many functions including antioxidant, antimicrobial, angiotensin converting enzyme inhibition, and emulsifying properties. However, as several studies have indicated, in its native state, phosvitin is

resistant to many processing conditions and can even be nutritionally negative. Novel processing techniques, used alone or in combination, could be used to better understand which processing conditions favorably modify its structure. Limited information has shown that enzymatic digestion of phosvitin might be an efficient mean to obtain bioactive peptides, but information on the properties of these peptides is scarce.

The goal of this research was to determine how HPP affects soy proteins and egg phosvitin. In particular, effect of HPP and thermal treatments on several soymilk and tofu quality attributes was compared. We hypothesized that HPP could provide a faster, more efficient means to modify soymilk protein and produce tofu, leading to a final product with improved quality attributes and novel textures when compared to traditionally-made soymilk and tofu. Pressure level should affect the outcome, as different levels affect proteins differently. Secondly, HPP in combination with thermal treatment could alter the highly stable structure of phosvitin. Following this, phosphatase and protease treatments could produce bioactive peptides which may potentially have improved biological and chemical functions.

Thesis Organization

This thesis starts with a general introduction that recognizes the research problem and covers the thesis organization. Chapter 2 is the literature review and contains background information on the research problem. The following two chapters are journal manuscripts, each covering a separate aspect of the research. These manuscripts will be submitted to Journal of Food Science and Journal of Agricultural and Food Chemistry, respectively. The manuscripts follow the Journal of Food Science format and include an abstract, introduction,

materials and methods, results and discussion, acknowledgements, and references, with

tables and figures following the text. Results of the first study were presented orally and by

poster presentation at the Institute of Food Technologists (IFT) annual meeting on June 6-10,

2009. The result of the 1st study was awarded 3rd place in the student poster competition

session for the Non-thermal Processing Division of IFT. The final chapter provides general

conclusions on the research followed by acknowledgements.

CHAPTER 2. LITERATURE REVIEW

Soymilk and tofu

Soymilk processing

Soybeans are widely known as a high quality, cholesterol free, low-cost protein source and, therefore, are the principal vegetable proteins commercially available across the globe (Zhang and others 2004). One of the most widely-produced soy products currently on the market is soymilk. Soymilk is a colloidal dispersion usually obtained from ground and strained soaked soybeans and, therefore, contains many of the water-soluble soybean components (Guo and others 1997). Typically, soymilk composition consists of 94.0% moisture, 3.0% protein, 1.0% fat, 1.0% soluble carbohydrates and 0.3% ash (Wilson 1995). The two major proteins found in soybeans are glycinin (11S) and β-conglycinin (7S), making up approximately 40% and 30% of the total protein, respectively. The ratio of these proteins varies among varieties and can affect the quality of the soymilk and tofu. Additionally, soybean storage conditions can also affect the quality of soy products (Murphy and others 1997).

Soymilk has been a common beverage in Asian countries for many years. In recent years, Western countries have seen an increase in soymilk sales and consumption as it is a popular alternative for persons who are lactose intolerant or vegetarians, and for consumers seeking a healthier lifestyle (Lakshmanan and others 2006). Soymilk sales increased from $100 million in 1995 to over $1 billion in 2008 (Soyatech, Inc 2009). Nevertheless, soymilk sales only make up 1-2% of the dairy beverage market, and therefore an exponential amount of growth can still occur (Wrick 2003). Even though soymilk is a dairy-free alternative, consumption of soymilk in the Western diet has still been hindered by the beany flavors and

odors associated with soy products. These flavors are developed when unsaturated fatty acids are oxidized by lipoxygenase, an enzyme which is released during grinding of the soaked beans. Because these flavors are unpleasant in Western countries, the soy industry has gone to great lengths to reduce or eliminate these flavors. While genetic modifications of soybean for the production of lipoxygenase free lines have been developed, reduction of the objectionable flavors were mainly done through processing modifications with additional heat treatment to inactivate the enzyme, bacterial fermentation, or addition of flavorings, in hopes of producing an acceptable product with bland flavors (Blagden and Gilliland 2005). The use of full fat soybean flakes as the starting material instead of whole beans, could also led to the production of soymilk with improved sensory attributes (Lusas and Rhee 1995). Quality of soymilk is also important because it is the base for tofu production, which is another popular soyfood product in Asian and Western countries.

The Nutrition Labeling and Education Act (NLEA) has been credited as a major contributor to the growth of soy products. This health claim for soy foods was approved by the U.S. Food and Drug Administration (FDA) in October 1999. Under the NLEA, consuming 25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. Requirements for this claim include that foods contain at least 6.25 grams of soy protein per the FDA's recommended amount commonly consumed. Foods that currently meet the NLEA requirements include tofu, tempeh, soy beverages, and soy-based meat alternatives (Wrick 2003). Other health benefits of consuming soy protein include the prevention of breast cancer and lowering the risk of prostate cancer (Peeters and others 2003; Messina and Hughes 2003).

There are several ways to produce soymilk, including the traditional Chinese and Japanese methods, the Illinois, and Cornell methods. In the traditional Chinese soymilk production the soybeans are first soaked overnight then drained and ground with fresh water. The slurry is then filtered to remove insoluble particulates (okara) and heated (Wilson 1995). In the Japanese processing method the slurry is first heated then pressed through a filter (Toda and others 2007). The heat treatment is an important step because it ensures a safe food product, inactivates lipoxygenase, destroys anti-nutritional factors such as trypsin inhibitors and can extend the shelf-life of the soymilk. However, problems arise with the heat-treated products including the production of cooked flavors, which is a major problem when trying to develop foods containing soy protein (Kwok and Niranjan 1995).

Tofu processing

The most recognized soy food in today market is tofu. The word "tofu" is Japanese, however, its production was believed to have originated in China in 164 BC. The increase in tofu consumption in Western diets has steadily increased in recent years for many of the same reasons as soymilk (Liu and others 2004; Yuan and Chang 2007).

Tofu is a soft or firm, gel-like soybean curd obtained by curdling hot soymilk with a coagulant which has traditionally been a salt ($CaCl_2, CaSO_4$ or Nigari) or an acid (glucono-δ-lactone, GDL). The gelation of soymilk is a two step process: first, the proteins in the soymilk are denatured. This unfolds the protein and exposes the -SH, S-S and hydrophobic amino acid side chains (Liu and others 2004). The coagulant allows the negatively charged residues on the protein to interact with the positively charged coagulant. This results in the aggregation of soy proteins and the formation of a gel. This gel can trap water, lipids, sugars

and other components. Once the gel is formed, the curd is usually pressed to expel the whey and form a solid network. Typically the development of the gel is the most important step in tofu production, but also the hardest to control, as slight changes can have a significant impact on the final product (Hou and others 1997). The final step in tofu production is to package the product in plastic containers or to sell the tofu as an unpackaged item directly to the consumer (Wilson 1995).

Specific varieties of soybeans have been developed specifically for tofu production. These include the Vinton 81, Pioneer 9202 and LS 201 cultivars (Murphy and others 1997). Some reports suggest that soybean varieties with high protein contents produce tofu with high yields and a firm texture (Mujoo and others 2003), while other literature suggests no link between soybean protein content and the final tofu product (Murphy and others 1997).

Several types of tofu exist and are dependent upon the processing method used. With this, different sensory characteristics are obtained, with preferences differing depending on culture and country (Obatolu 2008). Because tofu flavor is quite bland, physical properties such as the texture and moisture are very important to the quality of tofu (Prabhakaran and others 2006). Although texture characteristics differ with tofu type, hardness, springiness, and cohesiveness are important in assessing tofu quality (Guo and Ono 2005). Silken tofu is almost exclusively produced with GDL and has a smoother, finer texture than other tofu. The whey is not removed in silken tofu which results in a product that has higher nutrient levels, but the flavor is compromised due to the whey present (Wilson 1995). Firm tofu is the most popular type in Japan. Typically firm tofu are prepared from soymilk having solid content ranging from 5 to 8%, and are coagulated at 90 to 95 °C (Wilson 1995). Unlike silken tofu, where great care is taken to not disrupt the gel, the curd is broken slightly in firm tofu before

being pressed (Cai and Chang 1997). In dry tofu production, the curd is broken down considerably to remove excess whey. The resulting product is one that is very firm and usually has a moisture content below 76% (Wang and others 1982). The majority of tofu is pasteurized after packaging to prolong its shelf-life. In the production of packed tofu the process takes place in the final package. Cooked soymilk and a coagulant are poured into the final container. The product is heated (90-95 °C) for 40-45 min. Like silken tofu, the whey is not removed and packed tofu is more shelf-stable and less susceptible to microorganisms as post-processing contamination is less likely to occur (Wilson 1995).

The shelf-life of tofu varies greatly depending on the type. Typical ranges include 1-5 days for fresh tofu, 1-3 weeks for pasteurized tofu and up to 2 years for aseptically processed tofu (Wilson 1995). The initial microbial count and the storage temperature largely determine the shelf-life of tofu (Fouad and Hegeman 1993). Processing treatments such as high pressure (400 MPa, 5 °C, 5 min) have also been shown to reduce the microbial population of aerobic mesophiles (Préstamo and others 2000).

Factors affecting soymilk and tofu quality attributes

Of the many different factors affecting the quality of soymilk and tofu, the most important ones include the soybean varieties and storage conditions. Soybean variety has also been reported to affect the soymilk pH, and viscosity along with tofu color and water holding capacity (Khatib and others 2002). Equally important as the variety is the storage and handling of the soybeans, which has been investigated extensively. With an increase in storage time of soybeans, a decrease in the quality of the soybeans occurs. The initial water activity, temperature and relative humidity (RH) all need to be carefully controlled to reduce

the quality degradation (Liu and Chang 2008). After storing soybeans in undesirable conditions (84% RH, 30 °C) tofu that was darker in color was obtained, which is usually disagreeable with consumers, as they prefer a product white in color (Hou and Chang 2004).

Tofu characteristics also depend on the type and concentration of coagulant used. Using a high concentration of coagulant will result in a gel that is compact, hard and has a low yield. On the other hand, using a low concentration of coagulant will not result in a curd at all (Wilson 1995). Nigari and $CaSO_4 \cdot 2H_2O$ are acceptable for making firm tofu but GDL is not (Tsai and others 1981). Using $CaCl_2$ and $MgCl_2$ as the coagulants result in a tofu that is hard, coarse and granular, while using GDL and $CaSO_4$ produces a smooth and uniform curd (deMan and others 1986). When soymilk is coagulated with $CaSO_4$ a high retention of isoflavones was reported, because the coagulation time was slow, resulting in the retention of more isoflavones (Prabhakaran and others 2006). Isoflavones in soy foods are important because they have been reported to have major health benefits. Even when using non-traditional coagulants such as lemon juice and Epsom salt differences were seen in the tofu yield (Obatolu 2008).

Thermal processing of soymilk is an important intermediate step in tofu production because it denatures the proteins but also because it increases the shelf-life of soymilk. This can also inactivate antinutritional factors such as trypsin inhibitors (Liener 1981). However, non-enzymatic browning can occur during the heat treatment causing unwanted color change (Kwok and others 1999). Thermal processing can cause cooked flavors to develop, as well as the loss of some nutrients (Fernandez-Artigas and others 1999). Furthermore, as the heating time increases an increase in the deterioration of color and flavor occurs (Kwok and Niranjan 1995).

Controlling the percent solids in the soymilk is another checkpoint for producing a high quality bean curd. Firm tofu can have lower solids content (5-8%) while soft tofu requires higher total solids content (10-13%) (Wilson 1995). As these factors all play an important role in producing high quality soymilk and tofu, it is important to find the correct combination of technology and processing steps to optimize the final products.

Factor	Quality Parameter Affected	Reference
Soybean variety	Soymilk pH & viscosity; tofu color & water holding capacity	Khatib and others 2002
Soybean storage	Tofu yield, texture & color	Hou and Chang 1998, 2004
Soymilk heat treatment	Soymilk color, cooked flavors, nutrient loss	Kwok and others 1999; Fernandez-Artigas and others 1999
Percent solids in soymilk	Type of tofu produced, texture, yield	Wilson 1995; Hou and Chang 2003
Coagulant type	Isoflavone concentration; tofu yield and color	Prabhakaran and others 2006; Obatolu 2008
Coagulant concentration	Tofu yield and texture	Sun and Breene 1991; Liu and others 2000
Soy protein ratios	Tofu yield and texture	Kohyama and Nishinari 1993

Table 1 – Factors and their effects on the quality of soymilk and tofu

Summary

Although there is a great deal of evidence on the potential detrimental effect that thermal treatment can have on quality and nutritional attributes of soymilk and tofu properties, alternative to thermal treatment has not yet been identified. Furthermore, use of thermal treatment during soymilk and tofu production involves long treatments at high temperatures, which are time consuming and costly. Therefore, finding an alternative processing method that is more efficient, and improves tofu and soymilk quality can benefit the soy industry.

Phosvitin

Structure and composition

Phosvitin is one of the main proteins found in egg yolks, making up ~7% of the yolk protein and ~80% of the protein-bound phosphorus found in the yolk (Stadelman and Cotterill 1977). First isolated in 1949 by Mecham and Olcott, the name "phosvitin" indicates that it has high phosphorus content. With 10% phosphorus, it is indeed a highly phosphorylated protein. Phosvitin contains 6.5% carbohydrates and is obtained from the large precursor protein, vitellogenin, which is produced in the liver of oviparous vertebrates (Taborsky and Mok 1967; Byrne and others 1984).

The amino acid structure of phosvitin is very unique in that it is roughly 50% serine residues, all of which are phosphorylated (Fig. 1; Byrne and others 1984; Clark 1985). The high negative net charge of phosvitin (approximately -179) is due to the phosphorylated state of the serines (Damodaran and Xu 1996). These phosphoserines can be found in lengths of up to 15 residues long, which play a key role in the strong metal chelator property of the protein (Byrne and others 1984; Grizzuti and Perlmann 1975). For example, phosvitin binds 95% of the iron found in the yolk, and to a lesser degree also binds Ca^{2+}, Cu^{3+}, Mn^{2+}, and Co^{2+} (Greegard and others 1964; Grizzuti and Perlmann 1975). Of the 217 amino acid residues, a core region of 99 residues has been identified, with 80 of these being serines grouped together, with arginines, lysines, and asparagines making up the others (van het Schip and others 1987). Some research has suggested that β sheets are the primary component in phosvitin secondary structure; however factors such as pH can affect its secondary structure and shift the percentages of β sheets to α helices and random coils (Renugopalakrishnan and others 1985; Prescott and others 1986). Phosvitin has been

separated into two subunits, α and β, which have molecular weights of roughly 37.5 and 45 kDa and phosphorus contents of 2.97 and 9.20%, respectively (Abe and others 1982). Much work done on phosvitin isolated from hen egg yolk; however, phosvitin has been isolated from fish eggs as well. The phosvitin isolated from fish eggs have many similarities to hen egg yolk phosvitin, but has a lower molecular weight (14.5 and 7.6 kDa) and phosphorus content (Grogan and Taborsky 1986; De Vlaming and others 1980).

AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPNRKKPMDEEENDQVKQA
RNKDASSSSRSSKSSNSSKRSSSKSSNSSKRSSSSSSSSSSSSSSRSSSSSS
SSSSNSSKSSSSSSSKSSSSSSRSRSSSKSSSSSSSSSSSSSSSKSSSSRSSS
SSSKSSSHHSHSHHSGHLNGSSSSSSSSSRSVSHHSHEHHSGHLEDDSSSS
SSSSVLSKIWGRHEIYQ

Figure 1 – Amino acid sequence of phosvitin

Effect of processing treatments on phosvitin structure

Heat treatments are widely used in the food industry to increase shelf-life and ensure the safety of products. Because of its structure, phosvitin is resistant to many processing treatments. After heating phosvitin solutions with a range of different pH values (4-8) for several hours at 100 °C, no precipitate or change in the solution was noted (Mecham and Olcott 1949). After heating phosvitin for 10 min at 100 °C no change in the α and β fragments of phosvitin was noted. Similarly, after a heat treatment at 90 °C for 60 min, no decrease in the iron binding capacity could be detected (Castellani and others 2004).

Changes in pH can have an effect on the structure of phosvitin. This is an important factor as foods can have a wide range of pH values, and can influence the benefit of phosvitin addition. The structure of phosvitin can be altered due to the changes in electrostatic repulsions (Taborsky 1968). A conformational transition of phosvitin was reported between the pH range of 5.5 to 7.0 (Grizzuti and Perlmann 1970). At a neutral pH, circular dichroism

results revealed an irregular structure, which shifts to a more ordered β sheet conformation at an acidic pH level (Taborsky 1970). Castellani and others (2004) reported a change in the iron binding capacity, which was dependent upon the pH and a lower binding capacity for Mg^{2+} and Ca^{2+} when the pH was lowered from 6.5 to 4.5 (Grizzuti and Perlmann 1975).

Enzymatic digestion of phosvitin has also been performed. Upon tryptic digestion of native phosvitin, Goulas and others (1996) reported that 2 peptide fragments could be obtained, however, the core portion of phosvitin remained largely unaltered. It was assumed that the highly negative charge of the protein protects the peptide bonds from tryptic digests, but when the charge was neutralized via iron, peptide formation was not observed. Partial dephosphorylation of phosvitin using an alkaline treatment (0.4 NaOH, 37 °C, 24 h) prior to tryptic digestion seems to favor proteolytic hydrolysis, with formation of <3kDa peptides (Jiang and Mine 2000).

Biological and chemical properties

Some research has been conducted on the biological and chemical properties of native phosvitin, and to a lesser extent on phosvitin peptides. Interest in phosvitin comes at a time when there is an increasing awareness of functional foods. Bioactive peptides can be defined as small fragments of protein that can have positive biological effects after they are digested and released *in vivo* (Megias and others 2004). Bioactive peptides can have a multitude of health benefits ranging from the prevention and treatment of cardiovascular diseases, cancer, and obesity. More than 1,500 bioactive peptides have been identified since 2003, most of which are from milk and soy proteins, and some are from fish and cereal grains (Dziuba and others 2003; Yamamoto and others 2003).

When phosvitin was subjected to a tryptic digest for up to 24 h, an increase in the antioxidant activity was observed (Xu and others 2007). However, when phosvitin was subjected to a combination of pepsin, trypsin and α-chymotrypsin the emulsifying properties were significantly decreased compared to that of the native phosvitin (Sattar Khan and others 1998).

Phosvitin has amphiphilic properties, due to the hydrophilic amino acids found in the core region of the protein (Fig. 2). This suggests that the protein has a high affinity for lipids, and can form excellent emulsions (Kato and others 1987). Additionally, due to the repulsion of the negative phosphorus charges the extended conformation of the protein is observed, which allows oil droplets to become entrapped (Satter Khan and others 1998). The effects of dephosphorylation and protease digestion on the emulsifying activity were studied by Satter Khan and others (1998). After digesting the phosvitin for 24 h with pepsin, trypsin, or α-chymotrypsin the emulsifying activity greatly decreased by more than half of the original value. When the phosvitin was dephosphorylated using phosphatase for up to 10 h, the emulsifying activity decreased as the % of phosphate groups released increased, which further illustrates the importance of the negative phosphorus charges' ability to repel and trap oil droplets.

Figure 2 – Structural diagram of phosvitin displaying hydrophobic and hydrophilic regions

As previously mentioned, 95% of the iron found in the yolk is bound to phosvitin, and is found in the ferric state. The availability of this iron, however, is low because it is not easily released, even when autoclaved (Albright and others 1984). When isolated from egg yolk, 2-3 iron atoms are bound to 1 phosvitin molecule, but phosvitin has the potential to bind up to 60 iron atoms per molecule (Taborsky 1963). Therefore, phosvitin can be considered nutritionally negative (Jiang and Mine 2000). The interaction between iron and phosvitin depends on the method of iron addition. When phosvitin is mixed with a ferric solution the complexes are insoluble due to the intermolecular reaction (McCollum and others 1986). However, when ferrous iron is mixed with phosvitin the ferrous becomes oxidized to ferric and a soluble, intramolecular interaction occurs. The strong iron binding capacity of phosvitin has some positive attributes as it can have antimicrobial properties (Sattar Khan and others 2000).

There is a constant need to find antimicrobial agents that are safe for the food system. Most pathogens are gram negative, and have a lipopolysaccharide (LPS) layer that protects them from many antimicrobial agents. However, the LPS layer is sensitive to chelating agents and, therefore, phosvitin could potentially inhibit these pathogens. When phosvitin was incubated with *E. coli* at 37 °C for 3 h, no antimicrobial effect was observed. An incubation at 50 °C for 40 min was necessary to induce antimicrobial effects, which included an increase of the amount of DNA leakage, indicating that the bacterial cells were irreversibly damaged and that in combination with another processing treatment, such as a heat treatment, phosvitin may have strong bactericidal effects (Satter Khan and others 2000).

Another important factor for maintaining the quality of a food product is limiting the amount of lipid oxidation during processing. Lipid oxidation generates formation of off-

flavors, and leads to the loss of essential fatty acids and protein denaturation (Baker 1985). Chelating agents are often used as antioxidant agents because metal ions, such as Fe^{2+} and Cu^{2+}, catalyze lipid oxidation in food products (Baker 1985). Thus, phosvitin, known for its strong chelating abilities could be a strong, natural antioxidant. Phosvitin has been shown to successfully inhibit both Fe^{2+} and Cu^{2+} catalyzed phospholipid oxidation, and pasteurization treatment did not reduce its antioxidant activity (Baker 1985). Recently it was shown that phosvitin peptides had stronger antioxidant activity in a linoleic acid system when compared to phosvitin, perhaps due to the changes of phosphorus content and the amino acid sequence (Xu and others 2007). However, there is still a lack of understanding of the mechanisms behind phosvitin and its peptide antioxidant activity.

Calcium is an important macronutrient with a Recommended Dietary Allowance of 800-1,200 mg/day. Calcium deficiency results in metabolic bone disease. Moreover, the only source of replenishing calcium in the body is by dietary intake. Because calcium metabolism is a complex process, bone disease treatment is much more rigorous than just adding calcium to the diet. It is widely known that casein peptides, which are highly phosphorylated, can increase the bioavailability and retention of calcium in the body. This is due to the ability of the phosphoserine groups to form soluble complexes with the calcium (Sato and others 1986). Because phosvitin also has many phosphoserine groups the ability of phosvitin peptides to prevent insoluble calcium phosphate was investigated. It was found that phosvitin peptides could increase the solubility of calcium, the amount of phosphate present having a considerable effect on the calcium binding ability (Jiang and Mine 2000; Choi and others 2005). On the other hand, it was reported that undigested phosvitin inhibited calcium

absorption in rats, indicating the need for further studies to better understand the overlaying mechanisms (Ishikawa and others 2007).

Summary

Phosvitin has the potential to be used as a natural source for many biological and chemical functions. However, as several studies have indicated, in its native state, phosvitin is resistant to many processing conditions and can even be nutritionally negative. Therefore, it is necessary to consider novel processing technique, used alone or in combination, to better understand in which processing conditions the structure of phosvitin could be favorably modified. Only a limited amount of data has shown that enzymatic digestion can produce bioactive peptides, and many functions of interest have not been investigated using these peptides.

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High-pressure processing

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Concepts and principles

Today's consumers are demanding foods that taste good, are minimally processed, preservative-free, microbiologically safe and shelf-stable. High-pressure processing (HPP) offers an answer for these demands and is an attractive alternative to the conventional thermal treatment, which can lower the quality attributes of the food during processing. Also known as high hydrostatic pressure (HHP) or ultrahigh pressure (UHP), HPP is a food processing method in which a large amount of pressure, up to 900 MPa (~135,000 psi) is applied to the food product. HPP technology is based on Pascal's Law, which states that pressure is applied instantaneously and uniformly regardless of the shape and size of the food

product (Smelt 1998). This gives HPP an advantage over thermal processing, where size and shape of the food product play an important role in the processing conditions (Hoover and others 1989).

HPP treatment can be applied via batch or semi-continuous system. Batch systems are traditionally used as they can pressurize both liquid and solid food products, while semi-continuous systems can only be used for fluids (Anstine 2003). Single commercial scale equipment can range in size from 35 to nearly 700 L.

In the batch system food that is pre-packaged in a flexible pouch is loaded into vessel and the pressure transmission fluid, usually water, which can be combined with mineral or vegetable oil, is pumped into the vessel, generating pressure. Once the desired pressure level is achieved it can be maintained without expending further energy. After a specific time period, the pressure is released; food is unloaded and can be distributed (Patterson 2005).

During HPP the energy used to generate pressure creates heat in the vessel. This heat is called adiabatic heat. Adiabatic heating results in the homogenous heating of a food product, an advantage over thermal treatments. Adiabatic heating depends on the rate of pressurization, food product composition, pressurization fluid, and initial temperature (de Heij and others 2003). Water at 25 °C has an adiabatic heat rate of approximately 3 °C/100 MPa, whereas soy oil has a rate of approximately 9.1 °C/100 MPa (Matser and others 2004). A temperature decrease of the same extent is observed with the pressure is released. Figure 1 shows a typical pressure and temperature profile for HPP; the rate of pressurization increase is controlled and during this time adiabatic heating occurs. During the holding time there can be a loss of heat through the wall of the high pressure vessel and the pressurization fluid, which usually causes a temperature gradient in the vessel and a decrease of temperature of

the food product, which depends on the dwell time. This heat transfer exchange may result in non-uniform microbial inactivation and quality degradation (Denys and others 2000). After the specified treatment time pressure is released at a rapid rate, and the temperature level decreases as well.
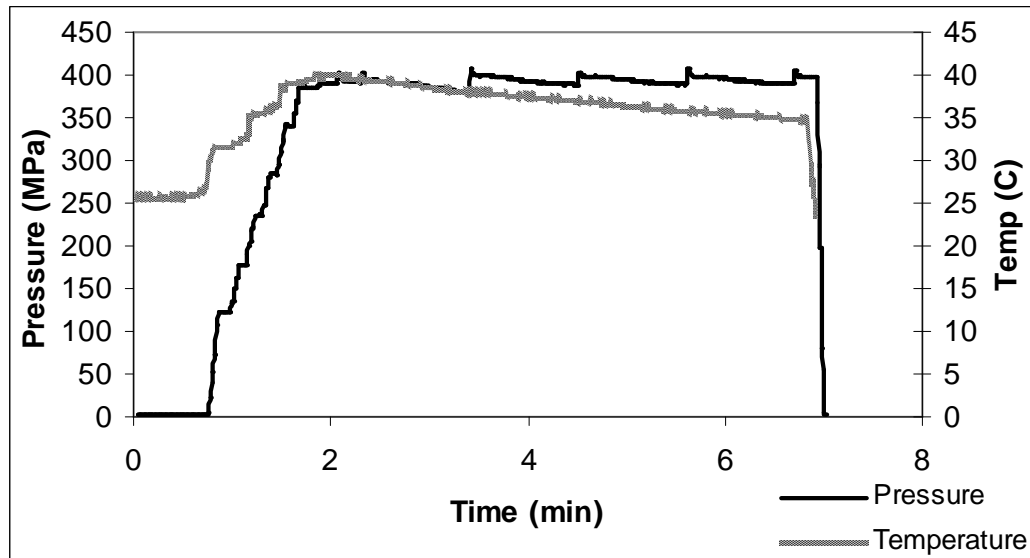

Figure 1 – Typical temperature and pressure profile for a 400 MPa treatment

Brief history and current trends

High-pressure processing first emerged as a food processing technique in 1899, when Hite observed that the shelf-life of milk could be extended by 4 days after a treatment at 600 MPa for 1 h at ambient temperature. A few years later, in 1914, he also observed shelf-life extension of peaches and pears (Hite 1899, 1914). At that time, little thought was given to the idea, and except for a few studies, HPP did not gain considerable interest until the late 1980s (Hayashi 1989). Soon after, in the 1990s the first commercial applications of this technology were seen in Japan with fruit jams (Suzuki 2003). Since then, the number and variety of food products have grown extensively, from guacamole, which was the first high pressure

processed food in the United States, to ready-to-eat meats, salsas, seafood, fruit juices, and

others. Table 2 summarizes the current food companies and products on the market today.

Table 3 summarizes the companies manufacturing laboratory and industrial scale high-

pressure equipment.

Food Sector	Company name	Website	Country	Products
Meat	MapleLodgeFARMS[LTD]	http://www.pressureprotection.ca/	Canada	Sliced deli meats, RTE meats
	Hormel Foods	http://www.hormelfoods.com/	USA	Sliced deli meats, RTE meats
	Perdue Farms Inc	http://www.perdue.com/	USA	Poultry
	Organic Prairie	http://www.organicprairie.coop/	USA	Sliced deli meats
	Espuña	http://www.espuna.es/	Spain	Sliced ham and tapas
	Kraft Foods*	http://www.kraft.com/	USA	Hot dogs
	Tyson*	http://www.tyson.com	USA	Poultry
	Campofrio*	http://www.campofrio.es/	Spain	RTE meats & seafood
Fruits and Vegetables	Fresherized Foods	http://fresherizedfoods.com/	USA	Guacamole, salsas, fruit juices & smoothies
	SimplyFresco	http://www.simplyfresco.com/	USA	Pasta sauces & salsas
	Pressure Fresh Australia	http://www.pressurefresh.com.au/	Australia	Fruits, vegetables & herbs
	Meidi-Ya	http://www.meidi-ya.co.jp/	Japan	Fruit based products
	Donny Boy Fresh Food	http://preshafood.com.au/	Australia	Fruits & juices
	Garden Fresh Salsa	http://www.gardenfreshsalsa.com/	USA	Salsas
	Pampryl	http://www.pampryl.fr/	France	Fruit juices
	Orchard House*	http://www.ohf.co.uk/	UK	Fruit juices
	Leahy Orchards Inc	http://www.applesnax.com/	Canada	Applesauce
	Hannah International Foods*	http://www.hannahfoods.net/	USA	Hummus
Seafood	Motivatit	http://www.motivatit.com/	USA	Oysters
	Nisbet Oyster Co	http://www.goosepoint.com/	USA	Oysters
	Shucks Maine Lobster	http://www.shucksmaine.com/	USA	Fresh raw lobster
	Clearwater Seafoods	http://www.clearwater.ca/	Canada	Frozen raw lobster
Dairy	Fonterra	http://www.fonterra.com/	New Zealand	Yogurt & cultured products

Table 2 – Food companies using HPP technology and applications
*Does not advertise the use of HPP on their website

Food Sector	Company name	Website	Country	Products
Equipment Manufacture	Avure Technologies Inc	http://www.avure.com/	USA	Industrial scale batch & continuous
	Elmhurst Research, Inc	http://www.elmhurstresearch.com/	USA	Ultra high pressure vessels
	Stansted Fluid Power Ltd	http://www.sfp-4-hp.demon.co.uk/	UK	Laboratory, industrial scale & custom
	Engineered Pressure Systems Inc	http://www.epsi-highpressure.com/	Belgium	Laboratory & industrial scale
	Kobelco	http://www.kobelco.co.jp/	Japan	Laboratory & industrial scale
	NC Hyperbaric (NCH)	http://www.nchyperbaric.com/	Spain	Industrial scale equipment
	Resato	http://www.resato.com/	Holland	Complete systems & components
	UHDE High Pressure Technologies	http://www.uhde-hpt.com/	Germany	Industrial scale equipment
Academic	Virginia Tech	http://www.hpp.vt.edu/	USA	Product testing & evaluation
	Ohio State University	http://grad.fst.ohio-state.edu/hpp/	USA	Product testing & evaluation

Table 3 – Companies manufacturing laboratory scale and industrial scale high pressure equipment and academic websites having extensive information regarding high pressure technology

Unlike thermal, chemical, and some irradiation processing technologies, HPP does not, for the most part, affect the color, flavor, or vitamin content in foods. For example, freezing, heat treatment, and drying of herbs results in a significant loss of flavor, which can be problematic as many herbs are added to a food product to improve their flavors. HPP treatment (2 pulses, 85 °C, 700 MPa) of fresh basil resulted in the best retention of flavor compounds when compared to freezing, heat treatment, and drying (Krebbers and others 2002). This is largely due to the fact that pressure affects non-covalent bonds (hydrogen, hydrophobic ionic, and van der Waals), while covalent bonds remain largely unmodified. HPP is also thought to be a "green" technology and requires less energy input than thermal processing.

HPP induces many changes to microorganisms by affecting different components of the cell (Hoover 1989). Although a combination of damages of these constituents causes cell death, it is thought that the cell membrane is the major site of pressure damage (Ulmer and others 2000). Studies have shown ATP leakage from pressure-treated cells (Smelt and others 1994) and the uptake of fluorescent dyes (Benito and others 1999). Cell morphology, genetic makeup and biochemical reactions are also slightly altered. Stationary phase cells are generally more resistant to pressure treatments when compared to exponential-phase cells and usually gram-positives are pressure-resistant compared to gram-negative bacteria (Manas and Mackey 2004; Smelt 1998). In general, pressure levels between 300 and 600 MPa inactivate yeasts and molds, as well as vegetative cells, including many foodborne pathogens (Smelt and others 2001). Much like other processing conditions, spore inactivation is a problem for HPP. Some spores can resist pressures up to 1,000 MPa, with *Clostridium botulinum* spores being among the most pressure resistant (Patterson 1995). One option for

eliminating spores is to use an HPP cycle treatment. An initial low pressure levels (<200 MPa) can initiate spore germination, while a second, higher pressure level would cause death to the germinated spore (Smelt 1998). Other options for eliminating spores are combining a more traditional method, such as a heat treatment with HPP to create a synergistic effect. Pressure in combination with temperatures between 50-70 °C significantly reduced the amount of spores present (Okazaki and others 1996). Very little information is available regarding pressure effects on viruses, although significant variations occur from strain to strain.

Pressure-assisted thermal sterilization (PATS) combines pressure and thermal treatments in order to reduce vegetative and spore-forming bacteria in a shorter time period than the conventional thermal treatment (Matser and others 2004). In early 2009 the U.S. Food and Drug Administration approved the use of pressure-assisted thermal sterilization as a new method of sterilizing mashed potatoes. This method pertains to foods that fall under the low acid canned foods in Title 21, *Code of Federal Regulations*, Parts 108 and 113. Pressure-assisted thermal sterilization has a significant advantage over the typical processing method of these foods as they are typically subjected to very high temperatures, resulting in quality degradation and nutrient loss (Barbosa-Canovas and Juliano 2008).

Impact of high-pressure processing on soy and egg proteins

HPP influences many characteristics of egg products as well as soy foods, and can be very versatile depending on the desired outcome. Liquid eggs are used in a variety of different sectors in the food industry, including bakery and confectionary products, dressings, infant foods and noodles. However, *Salmonella* contamination in egg products has been a

major health concern in recent years. Liquid eggs are especially vulnerable to microbial growth if they are temperature abused because of the high amounts of protein and lipids found in the product (Bari and others 2008). *Salmonella enteritidis* has been reported in 6 to 20% of unpasteurized liquid egg products, but not in pasteurized egg products (Mason 1994; Schroeder and others 2005). However, pasteurization can lead to protein denaturation and coagulation, which can affect the different functional properties of eggs. An HPP pulse treatment of 2 min for 4 cycles at 350 MPa and 50 °C led to a significant reduction in *Salmonella enteritidis* in liquid whole eggs without any protein coagulation, suggesting HPP could be an alternative to thermal treatment in pasteurizing liquid egg products (Bari and others 2008). HPP treatment was shown to have an effect on the foaming properties of egg white proteins; the treatment being both beneficial and detrimental to the foam stability and volume, depending on the pH of the egg white (Van der Plancken and others 2007). By adding 7 to 10% NaCl or sucrose to fresh egg albumin no precipitation or formation of gels was observed even up to treatment levels of 800 MPa (10 min, room temperature), however, foams and gels produced from the treated albumin was less consistent and both stronger and weaker when compared to the untreated samples (Iametti and others 1999). The rheological characteristics of whole liquid eggs (WLE), albumen and yolk after pressurization were investigated by Ahmed and others (2003). Both the WLE and albumen behaved as thixotropic fluids, however, after pressurization at 250 and 200 MPa, the time dependency was significantly reduced. It was also reported that coagulation of the WLE and albumen occurred after pressure treatment at 300 MPa and above (20 °C, 30 min). Ready-to-eat egg products have recently been introduced into the breakfast food market. Commercial sterilization can cause a greenish-grey discoloration of the eggs due to the iron-sulfur

compounds formed, which negatively affects the appearance (Song and Cunningham 1985). When HPP was combined with high temperatures (700 MPa, 98 °C, 5 min) it was found that scrambled egg patties could maintain their color, again suggesting that HPP could be used as an alternative to traditional thermal processing in the egg industry (Juliano and others 2006).

In a recent study, high-pressure processing was applied to alter the structure of phosvitin (Castellani and others 2004). Solution of phosvitin (0.77 mg/ml) was treated at 300 and 600 MPa for 10 min, at an initial temperature of 10 °C. The iron binding capacity of phosvitin was measured as well as changes in the secondary structure, using circular dichroism. After HPP, no changes in the iron binding capacity nor in the circular dichroism profile were noted. The authors concluded that phosvitin is very resistant to elevated pressure, attributing this resistance to its irregular structure, first reported by Taborsky (1970).

The changes made to soy proteins under pressure can also vary widely and generally depend on the environmental conditions. At 400 MPa (20 °C, 15 min) the 7S globulin is mostly unfolded while at pressure levels between 200 and 600 MPa the 11S globulin has a major loss in the protein structure (Molina and others 2001). Treatment from 200 to 600 MPa of soy protein isolates (pH 8.0) increased the protein hydrophobicity, indicating that pressure caused the proteins to unfold and expose their hydrophobic groups. Under pressure, soy proteins can dissociate, and then form new SH residues (Kajiyama and others 1995, Puppo and others 2004). Similarly, Lakshmanan and others (2006) reported a decrease in the total free and surface free sulfhydryl content for soymilk treated at 500 or 600 MPa (25 °C, 10 min). When soymilk was treated with high-pressure at 500 MPa (30 min) formation of a sol was observed, but sol formation was avoided at 400 MPa for 10 min (Zhang and others

2005), indicating that protein changes are pressure and time dependent. Most recently, Zhang and others (2009) reported on the preparation of tofu from untreated soymilk under high pressure (room temp, 200-600 MPa, 10-40 min), noting that the strength of the gel depended on the pressure level applied. As expected, HPP has a positive impact on microbiological attributes of soy foods. For example, the aerobic mesophilic population of a commercially prepared tofu was reduced by 0.31 and 2.38 log units after 5 and 45 min, respectively, at 400 MPa, 5 °C (Préstamo and others 2000). When soymilk was treated at 600 MPa (25 °C, 5 min) and stored for 4 days at 4 °C the total bacterial count decreased from 4.6 to 2.21 log CFU ml^{-1} (Smith and others 2009).

Summary

Many papers focus on the effect of HPP on microbial attributes of food, and not on using this non-traditional food processing to modify protein structure in order to add value to food product. To date, only a few research papers have reported on the production of tofu using HPP, and very little of this research was focused on the quality of the product and how it compares to traditionally made soymilk and tofu. On the other hand, one study has investigated change of phosvitin structure under pressure (Castellani and others 2004), and the treatment was not efficient in modifying its structure. Application of pressure combined with thermal treatment could result in different outcomes and has not yet been explored.

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CHAPTER 3. COMPARISON OF HEAT AND PRESSURE AS PRE-TREATMENT OF SOYMILK AND METHOD OF TOFU PRODUCTION

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Abstract

Effect of pH (6.0 and 6.5) and pre-treatment (none compared to 95 °C, 30 min, and 400 and 600 MPa, 5 min, initial temperature 25 °C) on soymilk stability and particle size was studied. Particle size of the pH 6.5 soymilk was decreased 12-fold after thermal and 400 MPa pre-treatment, while increasing by a factor of 2 at 600 MPa. No correlation was found between particle size and soymilk stability as stability of both thermal and pressure pre-treated (400 and 600 MPa) soymilk was significantly increased. Acidification of the soymilk with addition of glucono-δ-lactone (GDL) at a final concentration of 0.3% (16 mmol/L) followed by a pressure (400 and 600 MPa, 5 min, initial temperature 25 °C) or thermal treatment (85 °C, 30 min), led to soy protein coagulation (i.e., tofu formation) in all instances, except with

thermally pre-treated pH 6.0 soymilk. Hydrolysis of GDL in water was increasing with increase of pressure (200 to 600 MPa) and was 1.3 times faster at 600 MPa than after 30 min at 85 °C. Tofu produced from unpre-treated soymilk, adjusted to pH 6.0, subjected to 600 MPa treatment had an homogeneous structure and was the harder, illustrating that unfolding and coagulation of the proteins occurred simultaneously in these conditions. Benefits of using high-pressure for tofu processing includes shorter processing time (5 min dwell time vs. 30 min thermal treatment) and no requirement of a pressing step, which was necessary with thermal production to obtain a stand-alone tofu.

Introduction

Tofu ranks as one of the most popular soy products in the United States, with eighty-five percent of the population recognizing soy foods as healthy (United Soybean Board 2007). One of the major driving forces behind the increase of soy foods consumption in Western countries is the U.S. Food and Drug Administration approval of the Nutritional Labeling and Educational Act health claim for soy protein in 1999 (Wrick 2003). Tofu is a protein gel that is formed by the coagulation of soymilk proteins. The proteins are denaturated by a thermal treatment, exposing the negatively charged hydrophobic residues, which favor interactions with the positively charged coagulant. The addition of the coagulant therefore neutralizes the protein charge and induces aggregation and coagulation (Kohyama and Nishinari 1993; Kohyama and others 1995). Addition of coagulant to heated soymilk is usually done under thermal treatment and followed by a pressing step to obtain tofu with desirable texture. Glucono-δ-lactone (GDL) is a compound that decomposes gradually in aqueous media and has been used for the production of silken tofu, a soft type of tofu. For

production of silken tofu, pasteurized pre-cooked soymilk mixed with GDL is directly filled into the retail containers and heated up to 60 min at 80-90°C (Tseng and Xiong 2009).

Soymilk coagulation involves complex interactions among soy proteins, particularly glycinin (11S) and β-conglycinin (7S), coagulants, and non-protein components (Hou and Chang 2004). Parameters influencing the quality of tofu include nature of the coagulant and its concentration, and important factors affecting tofu quality are its moisture content, yield and texture (Cai and Chang 1997; Hou and others 1997).

High-pressure processing (HPP) is a non-traditional food preservation method that has generated considerable interest in the last two decades because of its ability to preserve foods while maintaining their fresh-like qualities. Heat primarily affects covalently bonded networks whereas HPP causes the disruption/formation of hydrophobic and ionic bonds but maintains covalent bonds. As a result flavor compounds and vitamins are pressure-resistant (Farr 1990; Molina and others 2002). Both 7S and 11S and 7S-11S interactions are modified by HPP (Molina and others 2001). Environmental conditions such as pH and protein concentration, greatly affect the degree of pressure-induced modifications including the extent of changes in surface hydrophobicity and soluble aggregate formation (Puppo and others 2004).

Use of HPP to increase shelf-life of tofu has been studied by Préstamo and others (2000). These authors reported a reduction of the aerobic mesophilic population by 0.31 log unit and 2.38 log units after treatment at 400 MPa, 5 °C for 5 and 45 min, respectively. HPP, because of its effect on protein structure, could also be used to replace the thermal treatment(s) applied during the tofu making process, which is required for adequate protein unfolding before coagulant addition. Kajiyama and others (1995) showed that pre-treatment

of soymilk with pressure can led to thermal-induced tofu with different hardness while Zhang and others (2005) and Saowapark and others (2008) investigated the potential of obtaining a tofu under pressure. While these studies reported some of the characteristics of the tofu obtained under pressure, the mechanisms involved during tofu making under pressure and key properties of the tofu produced, such as texture, yield and moisture content (Prabhakaran and others 2006; Hou and Chang 2004), have yet to be established. In addition, impact of soymilk characteristics, such as pH and native state of the proteins, i.e., pre-treatment applied to the soymilk, needs to be determined.

This study first compares how soymilk pH, and thermal and high-pressure pre-treatment affect soymilk stability and particle size. Secondly, pressure- and thermal-treatment were applied to coagulant-added untreated, heat-treated and pressure-treated soymilk. Quality attributes of the tofu, including yield of tofu, moisture content, crude protein content, protein recovery, texture and water holding capacity, were determined. Some correlations between microscopic observations of tofu and tofu attributes were established. Effect of pressure level on hydrolysis rate of GDL was also investigated.

Materials and Methods

Soymilk preparation

Soybeans (*Glycine max)* of Vinton 81 cultivar (Pattison Bros., Fayette, IA, USA) were rinsed twice with distilled water and soaked overnight at room temperature in an excess of distilled water. Hydrated soybeans were weighed to determine water uptake and ground with enough water to reach a dry soybean-to-water ratio of 1:6. Grinding was done using a Waring heavy-duty laboratory blender (Waring Commercial, Torrington, CT, USA) on

medium speed for 1 min. The slurry was pressed through 2 layers cheesecloth to separate the okara from the soymilk. The degree Brix was measured with a refractometer (Atago, Tokyo, Japan) and adjusted to a value of 8.0 ± 0.2. The pH of soymilk was measured and adjusted using 2N HCl (SA54-4, Fisher Scientific, Pittsburg, PA, USA) to 6.0 or 6.5 ± 0.2, the latter being close to the soymilk natural pH.

Soymilk pre-treatment

Thermal treatment. Soymilk was packaged with minimum air headspace in polyester pouches (SealPaks, KAPAK Corporation, Minneapolis, MN, USA) and heated at 95 °C for 30 min in a 2.5 L volumetric flask filled with water (Fig. 1). Immediately after treatment, the samples were placed into an ice-water bath until a temperature of 25 °C was reached. The heating and cooling rates were 3.1 °C/min and 11.5 °C/min, respectively.

High-pressure treatment. High-pressure treatment was conducted with a Food-Lab 900 High-Pressure Food Processor (Stansted Fluid Power Ltd., Essex, UK). The rates of pressurization and depressurization were 260 and 500 MPa/min, respectively. A 1:1 mixture of distilled water and 1,2 propanediol 95-100% vol (57556, Global Water Technology Koilguard, Oakbrook Terrace, IL, USA) was used as pressure transmitting fluid. The temperature increase of the pressurization fluid due to adiabatic heating was approximately 3 °C/100 MPa. The pressure and temperature were recorded and continuously monitored during the entire period using Stansted fluid power FPG55000 RAP system and Scan 1000 Supervisory Control and Data Acquisition system (Hexatec, UK). Pressure levels for the high-pressure pre-treatment were 400 and 600 MPa at an initial temperature (IT) of 25 °C for a dwell time of 5 min.

Tofu production

Thermal treatment. Aliquots of untreated and pre-treated soymilks were poured into a flask, and glucono-δ-lactone (GDL, $C_6H_{10}O_6$, G4750-100G, Sigma-Aldrich Corp., St. Louis, MO, USA) was added at a final concentration of 0.3% w/v of soymilk (Karim and others 1999; Guo and Ono 2005; Toda and others 2007). The mixture was stirred for 1 min with a spatula to ensure uniform distribution of GDL. Forty mL of the soymilk was transferred into a 50 mL centrifuge tube which was tightly sealed and incubated in a water bath at 85 °C for 30 min (Karim and others 1999). The tofu was then immediately cooled down by immersion into an ice-water bath to a temperature of 25 °C. The samples were then centrifuged at 3,000 *x* g for 15 min at 25 °C (Rotor JA 2550, Beckman Coulter Avanti [TM] J-20XPI centrifuge, Fullerton, CA, USA).

High-pressure treatment. GDL was added to the soymilk as described above and 40 mL aliquot was loaded into cellulose casing (26 mm in diameter, 650046P, Viscofan USA Inc, Montgomery, AL, USA). Tightly tied samples were placed in polyester pouches filled with water, with minimum air headspace, and pressurized at 400 MPa and 600 MPa, 5 min, at an IT of 25 °C. After both treatments, the samples were stored at 4 °C until further analysis.

Soymilk characterization

Particle size analysis. The particle size analysis was done by laser diffraction using a Mastersizer 2000 particle size analyzer (Malvern Instruments Ltd, Worcestershire, UK) to determine particle size distribution and surface area mean diameter $d_{3,2} = \left(\sum n_i d_i^3 / \sum n_i d_i^2 \right)$,

where n_i is the number of particles of diameter d_i. The refractive index of the soymilk was 1.46, while the refractive index of the dispersant (water) was 1.33.

Particle stability. Aliquot of 40 mL of soymilk was centrifuged at 3,000 x g for 15 min, 25 °C. The stability index (%) was determined as the weight of the precipitate divided by the initial weight of soymilk multiplied by 100.

Tofu characterization

GDL hydrolysis. Hydrolysis of GDL was measured using the method of Schwertfeger and Buchheim (1999). Briefly, GDL was dissolved in DI water at a concentration of 160 mmol/L or 1 mol/L then added to either deionized water or pre-treated soymilk at a final concentration of 0.3% (16 mmol/L) or 100 mmol/L in water. Samples were stirred for exactly 1 min and then the pH was immediately taken. Thermal or high pressure treatments were performed as previously described (30 min, 85 °C or 200, 400, 500, and 600 MPa, 5 min, IT 25 °C). pH measurements were taken with a portable pH probe equipped with a glass electrode (Thermo Fisher Scientific, Orion 3 Star, Waltham, MA, USA) for up to 90 min. Initial pH values for deionized water and soymilks were 4.96, 6.0 and 6.5, respectively. Time 0 was considered the pH measurement immediately after addition of GDL.

Tofu yield. The yield of tofu was based on the solid content of the product and calculated as weight of tofu (as is) divided by a specified amount of soymilk (as is) multiplied by 100 (Prabhakaran and others 2006).

Moisture content, protein recovery and crude protein content. Moisture content of the tofu was determined by using the AACC method 44-15A. Protein recovery (%) was calculated as the protein content in tofu (g) divided by the protein content in original

soybeans (g) multiplied by 100. Crude protein content of soymilk and tofu was determined with the Dumas method by using a rapid N III Nitrogen Analyzer Vario MAX CN from Elementar Americas, Inc. (Mt. Laurel, NJ, USA) using a factor of 6.25 to convert nitrogen to crude protein.

Water holding capacity. The water holding capacity (WHC) was determined using the method described by Sawoapark and others (2007) with slight modifications. Tofu samples were cut into rectangular strips (approximately 15 mm x 5 mm) and placed into microcentrifuge tubes adapted with a 0.45 µm filter (Millipore Ultrafree-CL, Fisher Scientific). Samples were centrifuged for 10 min, at 20 °C at 4,000 x g. WHC was calculated as percent water expelled per gram of original sample.

Texture profile analysis. Refrigerated tofu was left 1 hour at ambient temperature prior to measurement. Tofu samples were cut to a height of 2 cm. Textural properties of tofu were evaluated with a TA.XT2 Texture Analyser (Stable Micro Systems, New York, NY, USA) fitted with a 5 kg load cell. Samples were compressed twice, to simulate a 2 bite cycle, with a cylindrical probe (TA acrylic, 36 mm in diameter, Texture Technologies, Scarsdale, NY, USA) using 50% penetration for the samples. The compression speed was set to 1.2 mm/s (Guo and Ono 2005). Parameters measured included hardness, gumminess, springiness and cohesiveness, and were calculated according to Bourne (1982).

Scanning electron microscopy. Tofu samples were prepared as described previously and were sliced into small cubes (approximately 5 mm x 5 mm x 5 mm) and fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 24 h at 4 °C. Samples were rinsed in deionized water and post-fixed in 2% aqueous osmium tetroxide followed by dehydration in a graded ethanol series up to 100% ultra-pure ethanol and dried

using a Denton DCP-2 critical point dryer (Denton Vacuum, LLC, Moorestown, NJ, USA). After drying, the samples were placed onto adhesive coated aluminum stubs, sputter coated (Denton Desk II sputter coater, Denton Vacuum) with palladium/gold alloy and imaged using a JEOL 5800, LV SEM (Japan Electron Optics Laboratory, Peabody, MA, USA) at 10kV with a SIS ADDA II for digital image capture (Olympus Soft Imaging Systems Inc., Lakewood, CO, USA).

Statistical analysis

Each experimental design was randomly performed in triplicate. The data were analyzed by analysis of variance (ANOVA) and general linear model (GLM). Least significant differences (LSD) were calculated at $P < 0.05$ to compare treatment means by using the SAS system (version 9.1, SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Soymilk characterization

Soymilk appearance. The visual appearance of the soymilk after pre-treatment was dependent upon the pH and pre-treatment applied. A sol can be defined as a flowing fluid, whereas gels cannot flow on an experimental time scale (Jeong and others 2002). The appearance of the pH 6.5 samples was similar to the untreated soymilk regardless of the pre-treatment applied. The pH 6.0 soymilk thermal-treated and treated at 400 and 600 MPa (5 min) had a sol-like appearance. A sol formation was previously observed after treatment of a pH 6.0 soymilk at 500 and 600 MPa, 25 °C, for 10 min (Lakshmanan and others 2006) and a pH 6.5 soymilk after treatment at 500 MPa for 30 min (Kajiyama and others 1995), illustrating that different pH, pressure, dwell time combinations could led to different

changes in soymilk appearance. Our results indicated that a pressure of 400 MPa applied for 5 min to soymilk (pH 6.0) was enough to promote a sol formation. Zhang and others (2005) reported no change in the appearance of soymilk treated at 400 MPa for 10 min, which could be attributed to the pH of the soymilk used in their study, which was not reported. Changes in the protein conformation after pressure treatment was illustrated by the decrease in surface hydrophobicity and sulfhydryl content of the soymilk proteins treated at 500 and 600 MPa, 25 °C, 10 min (Lakshmanan and others 2006) and likely occurred for our samples.

Particle size analysis. Due to the sol formation of the pH 6.0 pre-treated soymilks, particle size analysis was only performed on the pH 6.5 soymilks. The untreated and pressure-treated soymilk at 600 MPa both displayed a monomodal distribution with a peak at 8.7 and 19.9 µm, respectively (Fig. 2 and 3). This shift in the maximum particle size of the pressurized soymilk suggested an aggregation and/or flocculation of the particles during pressure treatment at 600 MPa. The thermal-treated and pressure-treated at 400 MPa soymilk had similar bimodal particle distribution, with peaks around 0.5 µm and 200 µm, the 400 MPa soymilk also having a shoulder at around 5 µm (Fig. 2). Similarly, untreated skim milk and skim milk treated at 500 MPa (30 min, 20 °C) displayed monomodal curve while a bimodal curve was observed after treatment at 300 MPa. This change was attributed to both disintegration and aggregation of the casein micelles (Anema 2008). After thermal treatment and 400 MPa treatment, the d (3,2) value of the soymilk decreased to 0.5 µm when compared to the untreated soymilk (Fig. 3) which suggested that these treatments dissociated some aggregates that were present in the untreated soymilk (Nik and others 2008). Soymilk particle size could therefore be increased or decreased, depending on pressure level, which agreed

with Anema (2008) observing a decrease in particle size in casein micelles after

pressurization at 200 MPa and an increase in size at 300 MPa.

Particle stability. pH was the only factor having a significant impact on the stability

of the soymilk (Table 1, $P < 0.05$), though the interactions between pH and pre-treatment had

a P-value of 0.088. Untreated soymilks had the same stability regardless of the pH (Table 2),

illustrating that this change of pH in the control did not led to any precipitation/coagulation

of the proteins. Heat- and pressure-treated pH 6.5 soymilks were more stable than soymilk at

pH 6.0, with an average difference of more than 26%. At pH 6.0, the stability of the soymilk

decreased from 13% (untreated soymilk) to 32-34%, regardless of the pre-treatment type.

This was expected as sol formation was observed for these soymilks. An increase in the

amount (g as is) of precipitated materials could be due to an increase of the solids that

sedimented and/or increased of interactions between compounds such as water and/or oil and

the precipitated materials (Nik and others 2008). Denaturation of the proteins likely occurred

after the thermal treatment (95 °C for 30 min) and HPP treatment (400 and 600 MPa, 5 min)

that were applied (Molina and others 2001; Tang 2007), opening possibilities for formation

of new interactions between soymilk compounds, and possibly decrease their solubility,

leading to formation of insoluble aggregates.

Tofu characterization

When formation of a sol was obtained after application of the pre-treatment, samples

were manually stirred before addition of the coagulant.

Choice of tofu production method. As indicated in Fig. 1, the thermal-produced

tofu was subjected to a centrifugation step. This step was applied to mimic the pressing step

often applied in industry to obtain a firm tofu. Also, without this centrifugation step the tofu

texture was too loose and the tofu could not be characterized; in other words, the centrifugation step was necessary to obtain a stand-alone tofu. In contrast, when tofu was obtained under pressure, it formed a stand-alone gel without need of any centrifugation step; when it was submitted to one, a very firm tofu was obtained, which could not be analyzed. It was decided to compare the tofu properties regardless of this difference in the processing step, as from an industry point-of-view comparison of the properties of a tofu prepared from a conventional procedure (thermal pre-treatment, tofu making with thermal treatment and pressing step) to a tofu obtained under pressure were of interest.

Effect of treatment conditions on tofu production. Traditionally, tofu is made by first heating soymilk to denature proteins and then reheated with the addition of salt or acid to coagulate the soymilk and form a gel (Yamauchi and others 1991). In our study, not every combination of pH, pre-treatment and method of production resulted in tofu formation. Specifically, when pH 6.0 soymilk was thermally pre-treated and subjected to pressure, small agglomerations of proteins occurred. However, complete coagulation with formation of a tofu curd was not observed (Fig. 4, A). The soymilk used did have sol formation, which probably prevented the proteins from forming a network. However, when the pH 6.0 soymilk formed a sol under pressure, a curd was still formed. In all other conditions, a tofu was obtained. When obtained under pressure, the tofu from pressure-treated soymilk had the best appearance with a smooth and uniform surface, regardless of the pressure level applied during the pre-treatment and method of production (Fig. 4, B). In addition, these tofu fully retained their shape upon removal of the cellulose casing. The formation of tofu under pressure from a untreated soymilk indicated that under pressure protein denaturation and formation of a network that lead to the formation of a stand-alone tofu in one-step (no

pressing step applied) could be done simultaneously and in a short time (5 min dwell time). Therefore, pre-denaturation of the protein before coagulant addition was not a prerequisite for tofu making under pressure. It is generally admitted that thermal denaturation of proteins is performed in order to inactive trypsin inhibitors, unfold the proteins and expose hydrophobic residues, and –SH and S-S bonds (Kwok and Niranjan 1995).

GDL hydrolysis. GDL is an acid precursor, and when added to soymilk for tofu production, a slow increase of temperature (80-90 °C for 40 to 60 min) is necessary to promote protein gelation with minimal syneresis. Schwertfeger and Buchheim (1999) reported an increase of GDL hydrolysis when pressure was increased from 50 to 200 MPa, which was accelerated with increase of its concentration from 100 and 200 mmol/L. When GDL was solubilized into water at a concentration used for tofu production (16 mmol/L), an increase of pressure from 200 to 600 MPa increased its hydrolysis rate almost 2-fold after 90 min (Fig. 5A). Similar to results reported by Schwertfeger and Buchheim (1999), when a higher concentration was used (100 mmol/L), a similar trend was seen, but the pH drop was much greater, with a final value of 2.56 (16 mmol/L) and 2.29 (100 mmol/L) after treatment at 600 MPa (Fig. 5B). This indicated that the rate of hydrolysis was dependent upon concentration. When added to soymilk, the pH shift with increase of pressure was less pronounced, ~1.3-fold (Fig. 6A and B. This less evident shift was probably due to the buffering capacity of the soymilk (Schwertfeger and Buchheim (1999). In both the water and soymilk samples, regardless of coagulant concentration and pH, the drop in pH for the thermal-treated products was less or not significantly different from the treatments at 200 MPa. This trend was seen for the pre-treated soymilks, which did not have a significant impact on the pH changes (Table 1). The formation of a stand-alone tofu was only observed

at a pressure level of 400 MPa or higher, indicating that while there was a pH drop at 200 MPa, the proteins did not denature enough to form a gel.

Tofu yield. The pH and soymilk pre-treatment had a significant impact on tofu yield as well as all interactions, except pH x method of tofu production (Table 1). With the use of pH 6.5 soymilk for the production of tofu with thermal and pressure treatment higher yields were obtained than with pH 6.0 soymilk, except for untreated and thermal pre-treated soymilk with a thermal tofu production treatment where the yields were similar regardless of the pH. This increase could be attributed to an increase of the surface hydrophobicity of the soymilk proteins after HPP treatment (Lakshmanan and others 2006). When soymilk was pre-treated at both pressures and produced with a thermal treatment, the tofu yields were improved by about 46 and 56%, at pH 6.0 and pH 6.5, respectively, as compared to the untreated and thermal-treated soymilk (yield of about 35%; Table 3). When tofu was produced under 400 MPa, highest yields were obtained with pH 6.5 soymilk that was heated or pressurized at 600 MPa (~74%). The highest yield was 82% obtained with pH 6.5 thermal-treated soymilk under 600 MPa. Prabhakaran and others (2006) reported tofu yields in the range of 38.55-49.25%, which were slightly lower than the values reported in our study; however, these differences can be attributed to differences in processing conditions. Huppertz and others (2004) reported an increase in cheese curd yield after pre-treatment of raw whole milk at 400 and 600 MPa (IT 20 °C, 5 min) of 4.3 and 12.9%, respectively, attributing the increase to incorporation of denatured whey proteins into the curd and changes in the moisture content of the curd.

Moisture content, protein recovery and crude protein content. The moisture content (MC) of the tofu varied from 81.2% to 89.3% (Table 4) and a significant correlation

was found between the pre-treatment and MC (Table 1). These values were comparable with the one of Karim and others (1999), who reported a MC of 81.3% for a traditionally made tofu. Martín-González and others (2007) observed that pre-treating whole milk at 483 MPa, 30 °C, before cheese making increased the MC (40.16%) when compared to raw and pasteurized milk (35.26 and 35.96%). In our case, there was also an increase in the MC after pre-treating the soymilk with pressure (400 and 600 MPa) when producing tofu in the traditional method. Furthermore, the moisture content of the pressure-produced tofu was in the 86.1-89.3% range, with the exception of the ones obtained under 600 MPa from untreated and pressure pre-treated (400 MPa) pH 6.0 soymilk, which were slightly lower. The increased moisture content could be attributed to the formation of a finer network structure as observed by Needs and others (2000) for cheese curds after pressure treatment of the milk (600 MPa, IT 20 °C, 15 min).

All parameters tested had a significant impact on protein recovery (PR) except pH (Table 1). The PR of thermal-produced tofu ranged from 53.7-70.8% with the thermal pre-treatment soymilk giving the tofu with the highest PR value (Table 4). The PR of the pressure-produced tofu changed depending on the pressure level. However in both cases, the tofu made with thermal pre-treated soymilk resulted in the lowest values (52.8 and 54.3%, respectively).

Pre-treatment and method of tofu production both had a significant impact on the tofu crude protein content (CPC; Table 1). With the conventional tofu method production, i.e., heat pre-treatment followed by thermal production, a CPC of about 11% was obtained, which was comparable with the study of Karim and others (1999) who reported a CPC of 9.41% (as is). With the thermal tofu production, the pressure pre-treatment significantly decreased the

CPC of the tofu to ~8%. When tofu was produced at 400 MPa, use of untreated pH 6.0 soymilk gave the tofu with the highest CPC (~10%), and the tofu obtained with thermal-pre-treated soymilk had the lowest values (6.5%), whereas tofu prepared from pressure pre-treated soymilks had values ranging from 7 to 9%. Similar trend was obtained when tofu was produced at 600 MPa, illustrating that pressure level for tofu production had no impact on CPC. Overall, a decrease in protein content was concomitant with an increase of moisture content.

Water holding capacity. Both the pH of the soymilk and the method of tofu production had a significant impact on the WHC (Table 1). A lower WHC illustrates a better water retention of the tofu and was overall obtained for the pH 6.5 soymilk, except for the tofu obtained with thermal treatment from heat-treated soymilk, which could be due to the high variability of the results for these samples. The average difference in WHC of tofu prepared from pH 6.0 and 6.5 soymilks was 33% and 22%, for thermal and pressure tofu production, respectively (Table 5). Our results therefore illustrated that a small change in soymilk pH has a large impact on the WHC, which could be attributed to changes in the tofu microstructure, as observed for soy protein gels by Renkema and others (2000). Saowapark and others (2008) reported that tofu produced under pressure (400 MPa, 10 min, room temperature) released approximately 23% more water than thermal (70 °C, 60 min) produced tofu, due to the "loose" texture of the tofu curd. In our study, the method of tofu production also had a significant impact on the WHC of the tofu. For instance, the pressure-produced tofu from pH 6.5 400 MPa pre-treated soymilk released ~15% more water than the thermal-produced tofu. This loss in the ability of the tofu in retaining water was likely due to several

changes at the protein level and could involved incomplete unfolding of the samples (Molina and others 2002).

Texture profile analysis. The hardness, gumminess, springiness and cohesiveness of tofu are shown in Fig. 7. Adhesiveness and chewiness were not reported as they are not considered significant factors in tofu products (Yuan 2007). All factors had a significant effect on the texture of tofu except for the method of tofu production on the springiness and cohesiveness (Table 1). The harder and gummier tofu obtained was from the pressure-produced tofu, with the highest values coming from untreated pH 6.0 soymilk with pressure production at 600 MPa. Increasing the pressure level from 400 to 600 MPa for tofu production, increased the hardness and gumminess of the tofu prepared from pH 6.0 soymilks. Molina and others (2002) also reported an increase in hardness as pressure level increased of 7S and 11S soy protein pressure-induced gels. Studies looking at the effects of pH and heat treatment on gel hardness of soy protein isolate showed that gels formed at low pH (3.8) were harder than gels produced at neutral pH (7.6) (Renkema and others 2000). This trend was also observed in our study for the pressure-produced tofu, but not for the thermal-produced tofu, except for in the case of the tofu produced from untreated, pH 6.0 soymilk. The tofu made with a thermal treatment from pressure pre-treated pH 6.5 soymilk at 600 MPa was the springiest, indicating that it was more elastic and thus more difficult to eat (Fig. 7; Cai and Chang 1997; Prabhakaran and others 2006). Cohesiveness is directly related to the amount of work required to break internal bonds; therefore, a tofu that has a higher cohesiveness value has greater internal bonding (Cai and Chang 1997). The tofu obtained from thermal-produced, untreated soymilk (both pHs) was the least cohesive, while the

pressure-produced (both pressure levels), untreated, pH 6.0 thermal pre-treated soymilk was
the most cohesive, 0.2 vs. 0.7, respectively.

Sulfhydryl-disulfide exchange plays a key role in the forming a three dimensional
structure that gives rigidity to tofu (Saio 1981) and are more than likely involved in the
texture of the tofu obtained in our study. Kajiyama and others (1995) observed a decrease in
free sulfhydryl residues in soy proteins after pressure treatment, but noted that when
dithionitrobenzoic acid was added prior to pressurization, the soymilk had a higher SH
content. This suggests that under pressure, soy proteins dissociate, and create new SH
residues. The trends seen in the gumminess results were very similar to the hardness results.
Overall, method of tofu production did not influence the cohesiveness and springiness of the
tofu and instead soymilk pH and pre-treatment played a larger role in the results. The results
from texture analysis indicate that many different tofu products can be produced depending
on the combination of pH, pre-treatment of the soymilk and method of production. HPP
opens the possibility of producing a tofu with novel textural parameters.

Scanning electron microscopy. Among the 22 tofu that were obtained, scanning
electron microscopy was performed on the ones produced from pH 6.5 soymilks (untreated
and pre-treated) with a thermal and 600 MPa production and on the one produced from pH
6.0 soymilk pre-treated and produced at 600 MPa (total of 9 samples). pH significantly
affected the appearance of the 600 MPa-produced tofu prepared from the 400 MPa-pre-
treated soymilks (Fig. 8, A1 and A2). Tofu produced from the pH 6.0 soymilk had a porous
microstructure, with an open network and sponge-like appearance (A1), while with the pH
6.5 soymilk, the tofu was more compact and the individual particles were smaller and
rounder (A2). These observations could explain the WHC results, where the network formed

with pH 6.0 retained less water than the one obtained with pH 6.5 soymilk. When produced under 600 MPa, untreated soymilk resulted in a tofu with bigger particle size (B1) than the ones obtained with pressure (A2 and B3) and thermal pre-treated soymilks (B2), with the one obtained from thermal pre-treated soymilk being the smallest (B2). There was some similarity in the appearance of the tofu obtained under 600 MPa from heat-treated soymilk (B2) and 600 MPa-treated soymilk (A2), suggesting that when the proteins were denatured by the pre-treatment, the resulting microstructure of the tofu were independent on how the denaturation occurred. Similarly, with thermal tofu production, appearance of the tofu were the same regardless of the method of denaturation of the proteins, i.e., 600 MPa (C1) and heat-treatment (C2). Very little interactions between particles occurred when the tofu underwent thermal pre-treatment (B2). This could be an explanation for the change in cohesiveness and thus the lack of tofu formed when thermal pre-treatment and pressure production (600 MPa, IT 25 °C, 5 min) were combined (Fig. 4, A). Production method of tofu had an effect on the microstructure. With pH 6.5 untreated soymilk (native proteins), pressure-produced tofu displayed larger individual particles and more aggregation within the structure (B1), whereas the thermal-produced tofu had smaller particles and more empty space between the protein networks (C3). Similarly Saowapark and others (2008) showed significant difference in structure of tofu prepared from untreated soymilk, as observed by confocal microscopy, between pressure-induced tofu (400 MPa, 10 min, IT 20°C) and thermal-induced tofu. With denaturated soymilk proteins (600 MPa or thermal treatment), the method of production (thermal treatment vs. 600 MPa) had no significant impact on the tofu microstructure (A2 vs. C2). Interestingly, effect of the native state of the proteins (native or denaturated) was important when tofu was produced under 600 MPa (B1 and B2) leading to

different microstructure, whereas with thermal treatment, the tofu had very similar microstructure (C2 and C3).

Conclusions

Soymilk pH, state of the proteins and method of production had a significant impact on coagulation mechanisms occurring after GDL addition, and significantly impact the quality attributes of the resulting tofu. HPP offers processors the opportunity to produce soymilk and tofu with many superior qualities, including the possibility to obtain a tofu under pressure from an untreated soymilk without need of a pressing step. This would eliminate two steps in traditional commercial tofu production, greatly simplifying the process, and should appeal to the interest of soy food companies. When compared to production of silken tofu production, the pre-denaturation of the proteins can be avoided, and due to the increased hydrolysis rate of GDL under pressure, processing time is significantly reduced compared to a thermal treatment. However, under similar processing conditions (500 MPa, 20 °C, 5 min) trypsin inhibitors of soymilk were not inactivated (van der Ven and others 2005). While the presence of coagulant that we added for the tofu production might slightly modified the effect of pressure on trypsin inhibitors, by decreasing the pH, it is more likely that combination of high pressure and temperature will be necessary to address this issue and is currently under investigation. A combination of pressure and thermal treatment will likely also be a benefit as it can lead to an increase in the shelf-life and food safety of the final products.

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Table 1 – *P*-values of soymilk and tofu parameters

	Soymilk		Tofu										
	Particle size	Stability index	Yield and composition				WHC[f]	Texture characteristics				GDL hydrolysis
			yield	MC[c]	PR[d]	CPC[e]		H[g]	G[h]	S[i]	C[j]	
pH	ND	0.0027	<0.0001	0.5360	0.8293	0.1409	<0.0001	0.0394	0.0380	0.0471	0.0462	0.0034
PS[a]	0.0002	0.2650	0.0046	<0.0001	0.0004	<0.0001	0.3669	0.0090	0.0087	0.0422	0.0473	0.7850
TP[b]	ND	ND	0.7382	0.0535	<0.0001	0.0351	0.0285	0.0016	0.0229	0.0655	0.0790	0.0023
pH x PS	<0.0001	0.0879	<0.0001	0.0016	0.0015	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0009
pH x TP	ND	ND	0.3817	<0.0001	<0.0001	0.0277	0.0013	<0.0001	<0.0001	<0.0001	<0.0001	0.0013
PS x TP	ND	ND	0.0589	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0078
pH x PS x TP	ND	ND	0.0005	0.0018	<0.0001	0.0040	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Significance was determined at *P* < 0.05
[a] Pre-treatment of soymilk (PS)
[b] Method of tofu production (TP)
[c] Moisture content
[d] Protein recovery
[e] Crude protein content
[f] Water holding capacity
[g] Hardness
[h] Gumminess
[i] Springiness
[j] Cohesiveness
ND: Not determined

Table 2 – Stability index of soymilk

Samples	pH	Stability index (%)
US[a]	6.0	13.4 ± 1.4^c
	6.5	12.0 ± 1.1^c
TS[b]	6.0	34.0 ± 3.8^d
	6.5	7.4 ± 1.1^b
PS400[c]	6.0	31.6 ± 1.4^d
	6.5	5.0 ± 0.2^a
PS600[d]	6.0	32.6 ± 0.9^d
	6.5	5.4 ± 0.7^{ab}

Values sharing the same superscript are not significantly different at $P < 0.05$

[a] Untreated soymilk
[b] Thermal-treated soymilk (30 min, 95 ℃)
[c] Pressurized soymilk at 400 MPa, 5 min, IT 25 ℃
[d] Pressurized soymilk at 600 MPa, 5 min, IT 25 ℃

Table 3 – Yield of tofu (%)

Samples	pH	Thermal treatment	Pressure treatment (MPa) 400	600
	6.0	35.7 ± 0.7^{ad}	57.9 ± 4.3^{d}	55.1 ± 4.2^{b}
US[a]	6.5	34.3 ± 1.1^{a}	61.0 ± 4.8^{c}	62.0 ± 6.2^{c}
	6.0	35.9 ± 2.7^{ad}	ND	ND
TS[b]	6.5	38.8 ± 4.4^{d}	74.9 ± 8.4^{a}	82.5 ± 6.0^{e}
	6.0	46.4 ± 2.0^{c}	54.9 ± 2.5^{e}	48.6 ± 6.2^{a}
PS400[c]	6.5	54.8 ± 2.8^{b}	59.7 ± 6.5^{cd}	73.5 ± 1.8^{d}
	6.0	47.1 ± 1.4^{c}	56.4 ± 4.7^{e}	58.0 ± 5.5^{bc}
PS600[d]	6.5	56.5 ± 5.5^{b}	72.9 ± 3.6^{a}	70.8 ± 4.7^{d}

Values in column sharing the same superscript are not significantly different at $P < 0.05$
[a] Untreated soymilk
[b] Thermal treated soymilk (30 min, 95 ℃)
[c] Pressurized soymilk at 400 MPa, 5 min, IT 25 ℃
[d] Pressurized soymilk at 600 MPa, 5 min, IT 25 ℃
ND: Not determined because of the absence of tofu curd

Table 4 – Protein recovery (%), moisture content (%) and crude protein content (%) of tofu

		Tofu production								
					Pressure treatment (MPa)					
		Thermal treatment			400			600		
Samples	pH	PR[e]	M[f]	CPC[g]	PR[e]	M[f]	CPC[g]	PR[e]	M[f]	CPC[g]
	6.0	63.1 ± 16.1[a]	81.7 ± 2.8[a]	11.5 ± 0.1[ac]	65.9 ± 10.1[d]	86.4 ± 2.8[b]	10.0 ± 1.0[a]	81.7 ± 8.8[d]	84.1 ± 1.3[ab]	10.6 ± 0.9[ef]
US[a]	6.5	61.7 ± 15.5[b]	81.8 ± 3.1[a]	12.0 ± 0.6[a]	62.8 ± 5.4[cd]	88.1 ± 2.8[c]	8.3 ± 0.8[d]	64.9 ± 9.7[b]	86.4 ± 0.8[cd]	9.0 ± 1.1[d]
	6.0	57.4 ± 11.3[a]	81.2 ± 0.9[a]	11.5 ± 0.6[ac]	ND	ND	ND	ND	ND	ND
TS[b]	6.5	70.8 ± 15.9[c]	81.4 ± 1.2[a]	10.5 ± 0.4[c]	52.8 ± 12.8[a]	88.6 ± 1.8[c]	6.5 ± 1.1[e]	54.3 ± 11.2[a]	89.2 ± 1.6[e]	6.1 ± 1.1[a]
	6.0	54.1 ± 7.2[ab]	84.7 ± 0.6[b]	8.5 ± 0.0[b]	60.5 ± 3.9[c]	86.6 ± 1.6[b]	8.9 ± 0.8[b]	73.1 ± 10.3[c]	83.4 ± 1.2[a]	10.8 ± 2.8[f]
PS400[c]	6.5	61.7 ± 13.2[b]	87.1 ± 1.7[c]	7.7 ± 0.5[b]	59.0 ± 8.3[bc]	86.5 ± 2.3[b]	8.2 ± 1.2[d]	61.6 ± 14.2[a]	85.9 ± 3.1[bc]	8.6 ± 2.5[cd]
	6.0	53.7 ± 7.1[a]	84.7 ± 0.7[b]	8.4 ± 0.2[b]	59.6 ± 6.3[bc]	86.5 ± 2.2[b]	8.6 ± 0.7[bd]	63.2 ± 10.5[b]	86.1 ± 2.3[c]	9.5 ± 1.3[de]
PS600[d]	6.5	60.8 ± 11.6[a]	87.0 ± 1.7[c]	7.5 ± 0.8[b]	64.2 ± 5.1[d]	89.3 ± 1.2[a]	7.1 ± 1.0[c]	66.7 ± 3.3[b]	88.6 ± 1.8[e]	7.3 ± 0.8[ab]

Values in a column sharing the same superscript are not significantly different at $P < 0.05$

[a] Untreated soymilk
[b] Thermal treated soymilk (30 min, 95℃)
[c] Pressurized soymilk at 400 MPa, 5 min, 25 ℃
[d] Pressurized soymilk at 600 MPa, 5 min, 25 ℃
[e] PR: Protein recovery (%) was calculated as the percentage of protein in the original soybeans recovered in the tofu (dry basis)
[f] M: Moisture content (%)
[g] CPC: Crude protein content (as is, %)
ND: Not determined because of the absence of tofu curd

Table 5 – Water holding capacity of tofu

Samples	pH	Thermal treatment	Pressure treatment (MPa)	
			400	**600**
	6.0	43.9 ± 13.1^{bcd}	66.7 ± 4.5^{a}	64.1 ± 11.1^{a}
US[a]	6.5	22.5 ± 5.6^{g}	44.4 ± 3.7^{b}	62.0 ± 6.2^{b}
	6.0	32.8 ± 15.7^{cde}	ND	ND
TS[b]	6.5	23.3 ± 11.2^{efg}	44.7 ± 14.5^{bc}	32.2 ± 11.5^{def}
	6.0	68.5 ± 4.5^{a}	67.8 ± 5.0^{a}	68.2 ± 2.1^{a}
PS 400[c]	6.5	23.8 ± 6.4^{fg}	43.9 ± 4.2^{bc}	36.1 ± 7.9^{bcde}
	6.0	68.5 ± 3.8^{a}	55.2 ± 8.5^{a}	67.8 ± 2.0^{a}
PS 600[d]	6.5	19.4 ± 10.3^{fg}	38.5 ± 11.0^{bcde}	44.5 ± 11.3^{bcd}

Values in column sharing the same superscript are not significantly different at $P < 0.05$

[a] Untreated soymilk
[b] Thermal treated soymilk (30 min, 95 °C)
[c] Pressurized soymilk at 400 MPa, IT 25 °C, 5 min
[d] Pressurized soymilk at 600 MPa, IT 25 °C, 5 min
ND: Not determined because of the absence of tofu curd

Figure 1 – Schematic diagram of soymilk pre-treatment and tofu production
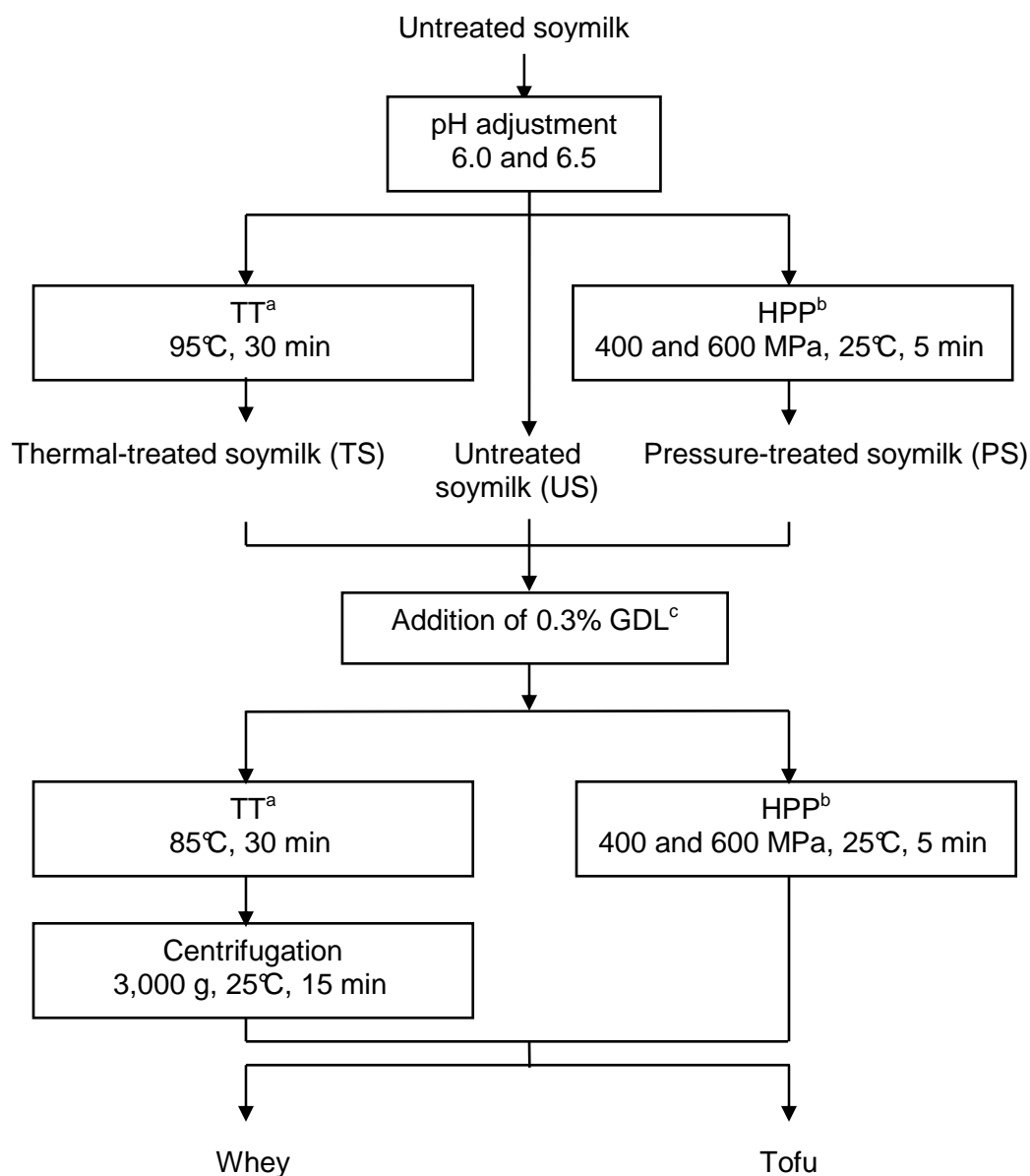
[a] TT: Thermal treatment
[b] HPP: High-pressure processing (temperature reported is the initial temperature (IT) of treatment)
[c] GDL: Glucono-δ-lactone

Figure 2 – Particle size distribution of untreated, pressurized, and thermal-treated pH 6.5 soymilk
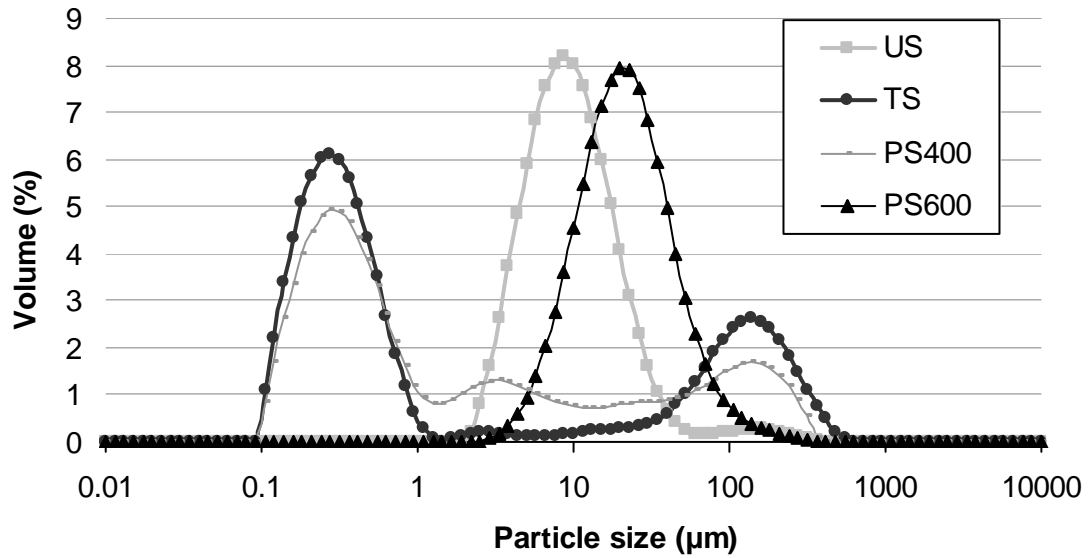
US: Untreated soymilk
TS: Thermal-treated soymilk (95 ℃, 30 min)
PS400: Pressurized soymilk at 400 MPa, 5 min, IT 25 ℃
PS600: Pressurized soymilk at 600 MPa, 5 min, IT 25 ℃

Figure 3 – Particle size diameter d $_{(3, 2)}$ of untreated, pressurized, and thermal-treated pH 6.5 soymilk
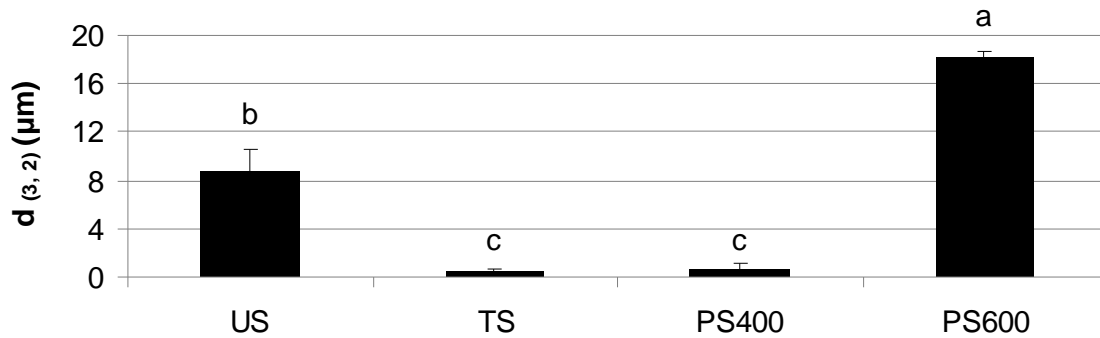
Columns sharing same letter are not significantly different ($P < 0.05$)
US: Untreated soymilk
TS: Thermal-treated soymilk (95 ℃, 30 min)
PS400: Pressurized soymilk at 400 MPa, 5 min, IT 25 ℃
PS600: Pressurized soymilk at 600 MPa, 5 min, IT 25 ℃

Figure 4 – Appearance of tofu made under pressure with pH 6.0 soymilk submitted to (A) thermal pre-treatment and (B) pressure pre-treatment
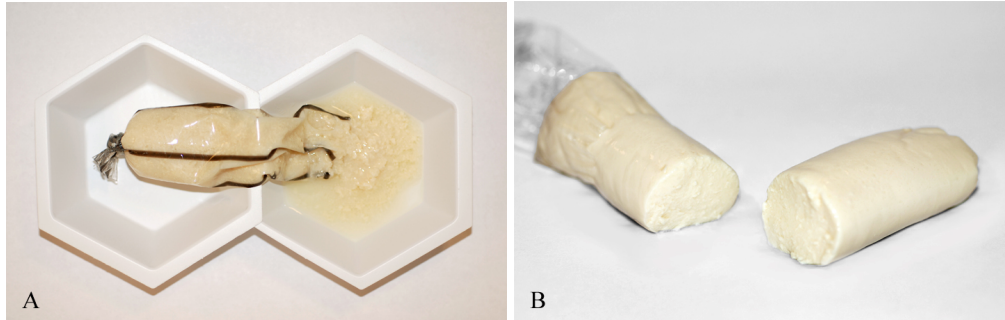
Tofu production: 600 MPa, 5 min, IT 25 ℃; thermal p re-treatment: 30 min, 95 ℃; pressure pre-treatment: 600 MPa, IT 25 ℃, 5 min

Figure 5 – Effect of thermal and pressure treatments on the change in pH of a GDL in water solution at (A) 16 mmol/L or (B) 100 mmol/L concentrations
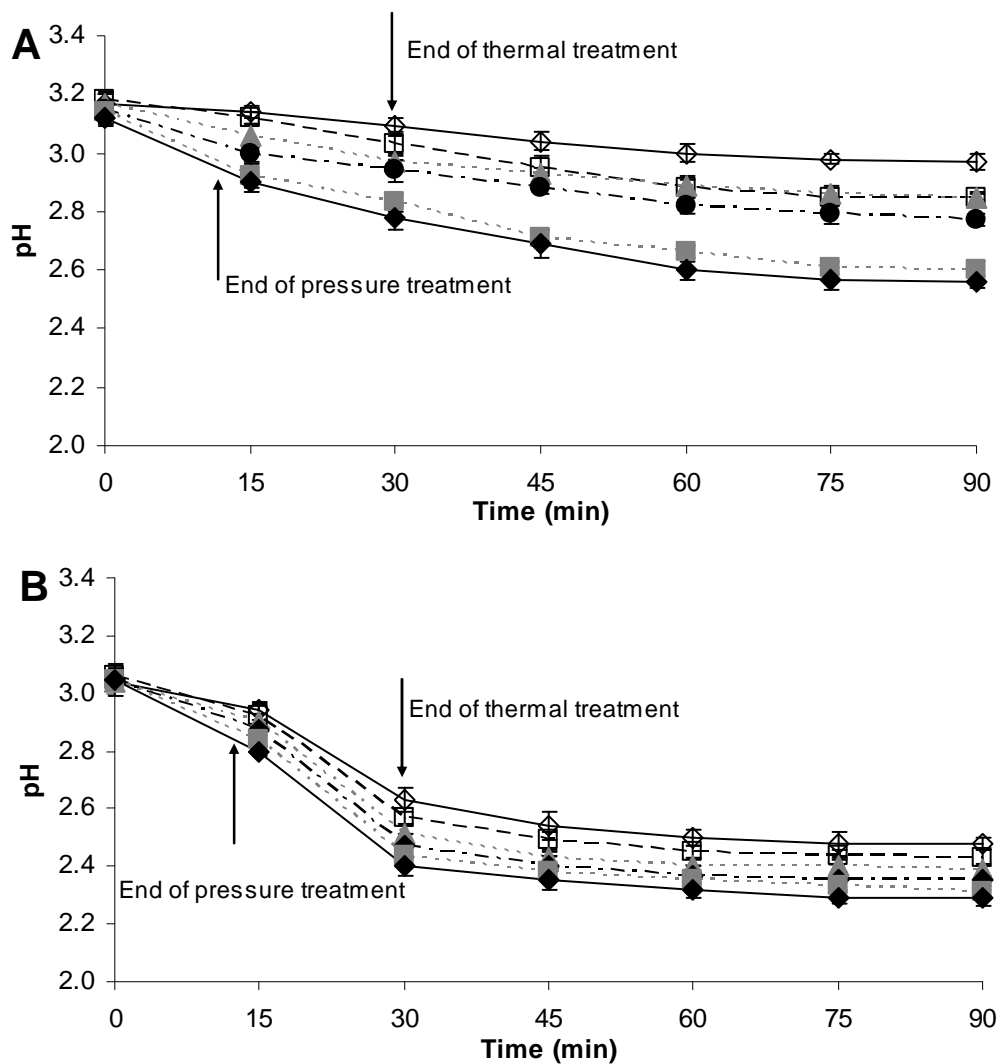
◇: Control (25 ℃, no treatment)

□: Thermal treatment (30 min, 85 ℃)

▲: 200 MPa (5 min, IT 25 ℃)

●: 400 MPa (5 min, IT 25 ℃)

■: 500 MPa (5 min, IT 25 ℃)

♦: 600 MPa (5 min, IT 25 ℃)

Figure 6 – Effect of thermal and pressure treatments on the change in pH of a GDL (16 mmol/L) in untreated soymilk solution at (A) pH 6.0 or (B) 6.5
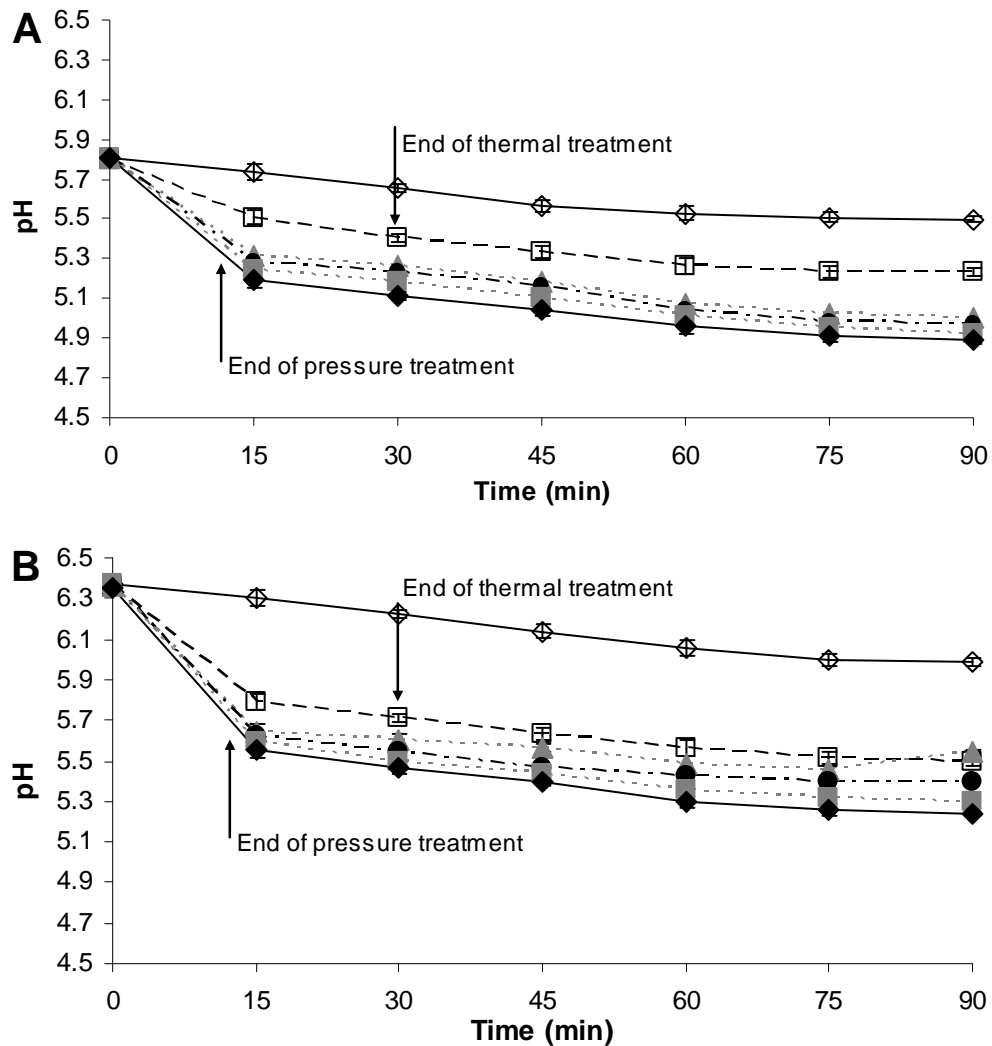
◇: Control (25 ℃, no treatment)

□: Thermal treatment (30 min, 85 ℃)

▲: 200 MPa (5 min, IT 25 ℃)

●: 400 MPa (5 min, IT 25 ℃)

■: 500 MPa (5 min, IT 25 ℃)

♦: 600 MPa (5 min, IT 25 ℃)

Figure 7 – Texture profiles of tofu produced from thermal treatment, and pressure treatment at 400 MPa and 600 MPa
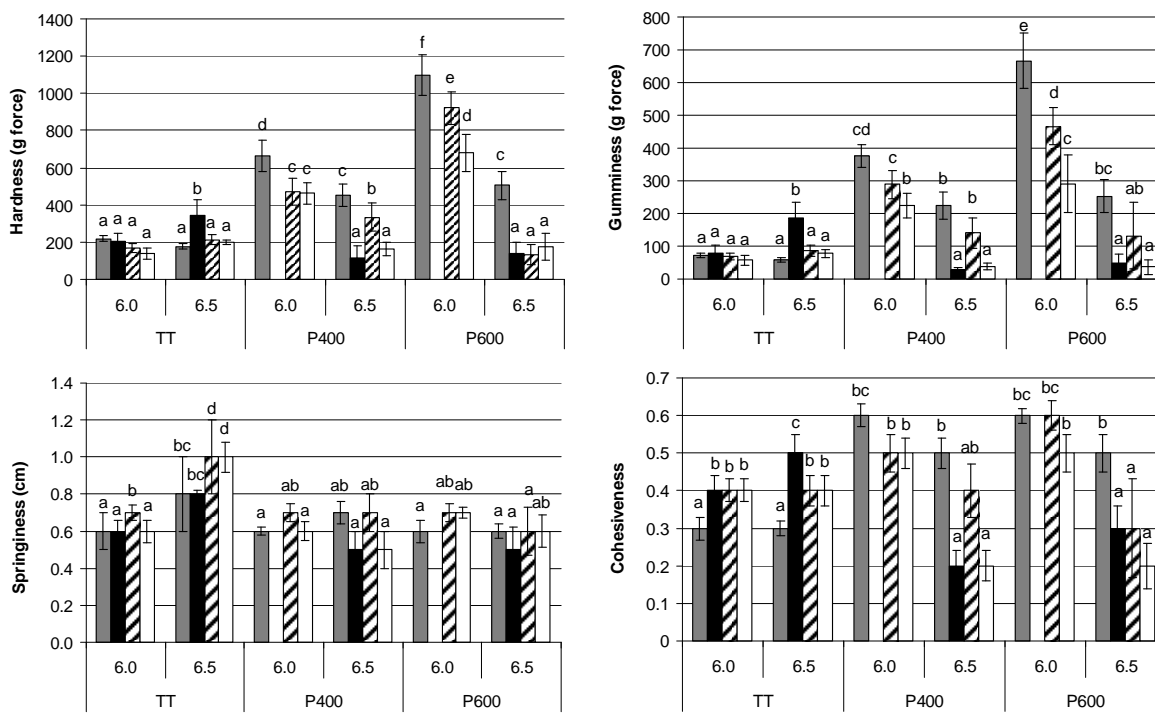
Column of same production method (thermal or pressure) bearing same letter are not significantly different at *P* < 0.05

TT: Thermal-produced tofu (85℃, 30 min)

P400: Pressure-produced tofu at 400 MPa, 5 min, IT 25 ℃

P600: Pressure-produced tofu at 600 MPa, 5 min, IT 25 ℃

■: Untreated soymilk

■: Thermal-treated soymilk (95℃, 30 min)

▨: Pressurized soymilk at 400 MPa, 5 min, IT 25 ℃

□: Pressurized soymilk at 600 MPa, 5 min, IT 25 ℃

Figure 8 – Effect of soymilk pH, pre-treatment, and type of production on microstructure of tofu
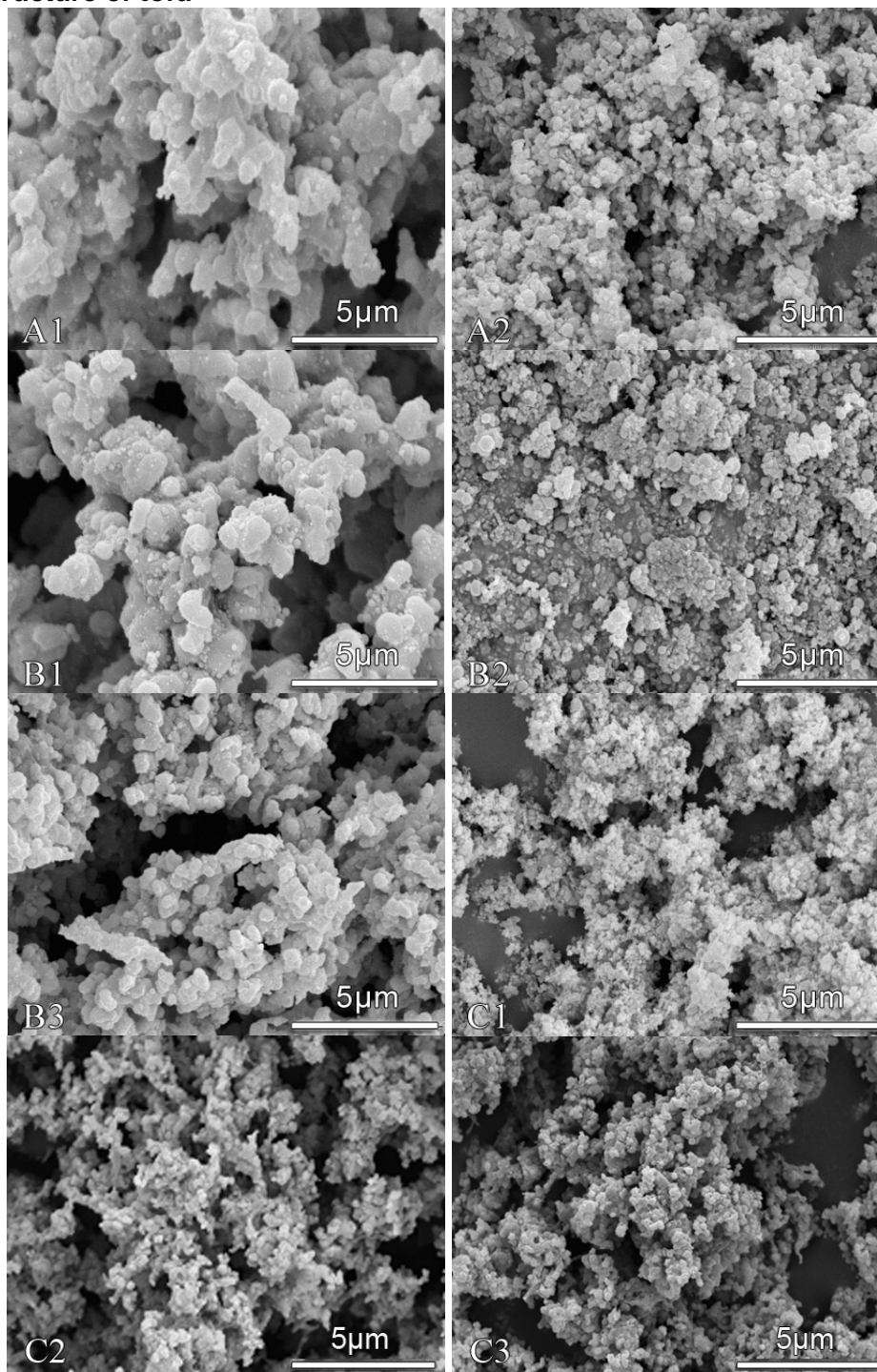
Tofu obtained under pressure (600 MPa) from pressurized soymilk (600 MPa) at (A1) pH 6.0 and (A2) pH 6.5; Tofu obtained under pressure (600 MPa) from pH 6.5 soymilk that was (B1) untreated, (B2) thermal-treated, and (B3) pressure pre-treated (400 MPa); Tofu obtained from pH 6.5 soymilk by thermal treatment that was (C1) pressure pre-treated (600 MPa), (C2) by thermal-treated, and (C3) untreated

CHAPTER 4. IMPACT OF ENZYMATIC AND NON-TRADITIONAL PROCESSING FOR PRODUCTION OF BIOACTIVE PEPTIDES FROM EGG PHOSVITIN

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Abstract

Phosvitin was subjected to high-pressure processing applied at an initial temperature of 65 °C, and to enzymatic dephosphorylation, prior to protease digestion in conditions that mimic *in vivo* gastrointestinal digestion. Phosvitin was resistant to treatment at 600 MPa for up to 30 min. Decrease of pH from 7.0 to 2.3 increased the α helices and β sheets by 8.1 and 22.6%, respectively. Treatment of phosvitin with phosphatase increased the degree of dephosphorylation from 24.3 to 62.8%, after 2 and 18 h, respectively. While no peptides were obtained with the proteolytic treatment of untreated and pressurized phosvitin, dephosphorylation of phosvitin prior to enzyme digestion promoted the formation of three

major peptides with 29, 27 and 21 kDa molecular weights. Percent of dephosphorylation had no impact on the extent of proteolysis. The ACE inhibition and antioxidant activity after 18h dephosphorylation followed by protease treatment, increased by 48 and 62%, respectively.

Introduction

Egg yolk phosvitin is a highly phosphorylated protein that represents about 7% of the proteins found in the egg yolk (Stadelman and Cotterill 1977). Phosvitin has two subunits, α and β which differ in molecular weight (37.5 and 45 kDa, respectively) and phosphorus content (2.97 and 9.20%, respectively; Abe 1982). Phosvitin is rich in serine residues, giving it a highly negative charge, most of which are phosphorylated. These phosphoserines account for ~50% of the total amino acid residues (Belhomme 2007). Due to its high phosphorus content, phosvitin acts as a strong metal chelator, with 95% of the iron found in the egg yolk bound to phosvitin in a stable complex (Choi 2005). When isolated from egg yolk, phosvitin contains 2 to 3 iron atoms per molecule. Phosvitin, however, has the potential to bind up to 60 iron atoms per molecule *in vivo*, these free binding sites being saturated with other cations such as calcium and magnesium. (Tarborsky 1963; 1983). This property could be used to scavenge iron from microorganisms, which could give phosvitin the potential to be a natural antimicrobial agent. However, native phosvitin is usually considered to be nutritionally negative, because bound iron is not easily released, and therefore not accessible to human metabolism (Jiang and Mine 2000).

Bioactive peptides are defined as protein fragments that exert biological effects once they are digested *in vivo* (Megias and others 2004). There has been an increasing demand for natural sources of bioactive peptides in recent years. Bioactive peptides can provide health

benefits such as antioxidant and antihypertensive activity and also have the possibility to break into the nutraceutical and cosmetics industry. As of 2003, more than 1,500 bioactive peptides have been identified (Dziuba and others 2003). Many of these peptides have been identified in milk and soy proteins and to a lesser degree in fish and cereal grains (Yamamoto and others 2003). As of today, little investigation has been made on identifying bioactive peptides obtained from eggs. Phosvitin could be a good candidate as a bioactive protein. Indeed highly phosphorylated portions of casein proteins increase bioavailability of calcium in the body and help in calcium retention (Jiang and Mine 2000). Providing further insight into egg-derived bioactive peptides would provide an alternative to soy and milk peptides, as both of these are considered to be highly allergenic foods.

The major challenge in obtaining peptides from phosvitin is its highly stable structure, due to the protection of the phosphoserine groups, the negative charge on the residue interfering with suitable enzyme-substrate complexes. Because of its structure, phosvitin is also highly resistant to processing treatments including autoclaving (Albright 1984), proteolytic action (Goulas 1996; Jiang and Mine 2000), and high-pressure treatment applied at 10 °C (Castellani 2004), but pH changes have been shown to alter the conformation of phosvitin (Grizzuti and Perlmann 1970). Our objectives were to assess structural changes of the phosvitin after high-pressure processing in combination with a thermal treatment, identify if enzymatic dephosphorylation would affect protease digestion and characterize peptides for their biological (ACE inhibition) and chemical (antioxidant) activity.

Materials and Methods

Phosvitin preparation. Phosvitin samples were kindly provided by Dr. Ahn from the Department of Animal Science, Iowa State University. The sample was obtained as freeze-dried powder and was approximately 80% protein (as is), as determined by the Dumas method, with a protein conversion factor of 6.25. The sample was stored at -20 °C. Samples were diluted with distilled water to the appropriate dilution depending on which measurement was being performed and the pH of the solution was adjusted to 2.3, 7.0 or 11.0 with 2N HCl or 2N NaOH (SA54-4 and A669-500, Fischer Scientific, Pittsburg, PA, USA). The natural pH of the phosvitin solution had a value of ~3.80.

High-pressure treatment. High-pressure treatment was done with a Food-Lab 900 High-Pressure Food Processor (Stansted Fluid Power Ltd., Essex, UK). The rates of pressurization and depressurization were 260 and 500 MPa/min, respectively. A 1:1 mixture of distilled water and 1,2 propanediol 95-100% vol (57556, Global Water Technology Koilguard, Oakbrook Terrace, IL, USA) was used as pressure transmitting fluid. The pressure and temperature were recorded and continuously monitored during the entire period using Stansted fluid power FPG55000 RAP system and Scan 1000 Supervisory Control and Data Acquisition system (Hexatec, UK). Twenty mL solutions of phosvitin were packaged with minimum air headspace in polyester pouches (SealPaks, KAPAK Corporation, Minneapolis, MN, USA). The pressure level for the high-pressure treatment was 600 MPa at an initial temperature (IT) of 65 °C with the dwell time varying between 0 and 30 minutes. Prior to pressure treatment samples were allowed to sit in the holding container within the equipment for 5 min in order for the sample to come up to temperature (65 °C). Control samples were loaded into the pressurization vessel and allowed to sit for 7.5 min. This time

includes the 5 min temperature equilibrium time, as well as 2.5 additional min to include the come up and down time of pressurized samples. The initial temperature of the sample was 65 °C and reached 83 °C when the pressure was reached, because of the temperature increase of the pressurization fluid due to adiabatic heating which was approximately 3 °C/100 MPa.

Circular dichroism. Phosvitin samples were freshly prepared at a concentration of 0.5 mg/mL, based on dry powder weight. Circular dichrosim (CD) measurements were performed on a Jasco J-710 spectropolarimeter at room temperature, in 0.1 cm path cell length. All measurements were taken within 4 h of HPP treatments or pH change. Data was recorded in a wavelength range of 190-250 nm and CD analysis was done using the JFit program (Hugonin and others 2008) to determine the percent alpha helix, beta sheet, and random coil (Altschuler and others 1997).

High performance size exclusion chromotography (HPSEC). Phosvitin samples were freshly prepared at a concentration of 20 mg/mL, based on dry powder weight. Size exclusion chromatography was performed on a Galaxie controlled Varian HPLC system including a Prostar 410 Autosampler, Prostar 210 Solvent Delivery Module, and Prostar 325 UV/vis Detector. Proteins were separated on a Biorad Bio-sil 400-5 column (300 x 7.8 mm) with a Biorad Bio-sil 400 Guard, 80 x 7.8 mm guard column upstream. The mobile phase used was 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, and 0.15 M NaCl (pH 6.8) in 18 MilliQ water at a flow rate of 1.0 mL/min and ambient temperature at a concentration of 2 mg/mL. Sample (50 µL) was injected and the absorbencies were followed at 280 and 215 nm.

Phosphatase treatment. Phosvitin samples were treated with phosphatase (P3752, Sigma-Aldrich Corp., St. Louis, MO, USA) following a modified method of Satter Khan and others (1998). To 20 mL of a 5% phosvitin solution, based on dry powder weight, prepared

in distilled water, 500 µg of phosphatase was added directly to the solution, which was heated to 37 °C prior to addition. This mixture was vortex mixed for 1 min and the incubation was done at 37 °C for up to 18 h at pH 4.8 in a shaking water bath. The pH of the solution was monitored and maintained at 4.8 using 2 N NaOH. During the 18 h of incubation, 5 mL of the sample was taken periodically for further analysis. The phosphatase in the 5 mL aliquot was inactivated by heating in a 95 °C water bath for 10 min, while the remaining 15 mL was subjected to pepsin and pancreatin digestions.

Protease digestion. Phosvitin samples were subjected to protease digestion using pepsin (P6887, Sigma-Aldrich Corp.) and pancreatin (P7545, Sigma-Aldrich Corp.) to mimic *in vivo* gut digestion following the method of Majumder and Wu (2009) with slight modifications. The pH was lowered to 2.3 by adding 2N HCl and after stabilization of the pH, the sample was digested with pepsin (4% w/w of protein) for 3 h at 37 °C in a shaking water bath. Five mL of the sample was then removed and heated in a 95 °C water bath for 10 min to inactivate the pepsin. The remaining sample was raised to pH 7.5 using 2 N NaOH and digested with pancreatin (2% w/w of protein) for an additional 3 h at 37 °C. A 3 h time period for each treatment was chosen to mimic *in vivo* digestion conditions (Majumder and Wu 2009). In both series of digestions the pH was monitored throughout the 3 h time period and adjusted with either 2 N HCl or 2 N NaOH. The pancreatin digestion was terminated by incubating the sample in a 95 °C water bath for 10 min. Further analysis was complete without an additional centrifugation step to mimic gut conditions.

Determination of phosphorus content. The percent of dephosphorylation was measured following the method of Fiske and Subbarow (1925) with slight modifications. Briefly, 3.75 g of sodium molybdate (243655, Sigma-Aldrich Corp.) was dissolved in 37.5

mL distilled water and 37.5 mL of concentrated (36 N) H_2SO_4 was slowly added. The mixture was kept in an ice water bath and once the solution reached a temperature of 10 °C, 50 mL of 70% perchloric acid was added. Fiske-Subbarow reducer (sodium 1-napthol-2-amino-4-sulfonate, $NaHSO_3$, and Na_2SO_3) (46345, Sigma-Aldrich Corp.) was diluted to 1 mg of substance in 6.3 mL of distilled water and stored in complete darkness at room temperature. Thirty µL of digested phosvitin sample was added to 2.5 mL of the sodium molybdate solution, 37.5 mL distilled water, and 0.5 mL Fiske-Subbarow reducer. The solution was mixed with a vortex for 1 min. Color was allowed to develop for 30 min at room temperature prior to reading at 660 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Commercially prepared phosvitin was used as a comparison against our native phosvitin (P1253, Sigma-Aldrich Corp.). SDS-PAGE of digested samples was done according to a modified method of Laemmli (1970). After inactivation of the enzymes the pH was adjusted back to 6.8 using 2N HCl or 2N NaOH. Digested phosvitin samples were diluted to a final concentration of 0.5 mg/ml with a sample buffer containing 0.5 M Tris-HCl, urea, glycerol, 0.4% bromophenol blue, 10% SDS and 1 M DDT. Fifteen µg of phosvitin protein and 10 µL of a commercial molecular weight marker covering the molecular weight range of 6,500 to 200,000 Da (S8445, Sigma-Aldrich Corp.) were loaded on a 15% Tris-HCl gel (161-1175, Bio-Rad Laboratories, Hercules, CA). The gels were run at 200 V and stained for 1 h using 0.25% coomaisse blue, 10% acetic acid, 50% methanol and aluminum nitrate, and destained using a solution of 25% methanol and 5% acetic acid. The gels were stored in a 25% ammonium sulfate solution at 25 °C.

Angiotensin converting enzyme inhibitory activity. ACE inhibitory activity was determined using the method of Cushman and Cheung (1971) with some modifications (Miguel and others 2006). Briefly, 40 µL of a 1.25 mg/mL solution of phosvitin, based on dry powder weight, were incubated with 100 µl borate buffer (pH 8.3), containing 0.3 M NaCl and 5 mM hippuryl-his-leu solution (H1635, Sigma-Aldrich Corp.) and 20 µL (2 mU) ACE (A6778, Sigma-Aldrich Corp.) at 37 °C for 30 min in a shaking water bath. Following incubation the reaction was stopped by the addition of 150 µL of 1 M HCl. One mL of ethyl acetate was added and mixed with a vortex for 30 sec to extract hippuric acid. The solution was centrifuged (1,500 g, 10 min) and a 750 µL aliquot of the organic phase was transferred to a tube and evaporated in a hot air oven at 120 °C for 30 min. The residue was dissolved in 800 µL of distilled water and the absorbance was measured at 228 nm. Captopril, a known ACE inhibitor (50% inhibitory concentration (IC_{50}), 6 µM) was used as a reference ACE inhibitory material, at a concentration of 0.5 µg/mL (C4042, Sigma-Aldrich Corp.).

Antioxidant activity. The antioxidant activity of the digested phosvitin samples was measured using the oxygen radical absorbance capacity (ORAC) assay according to Zulueta and others (2009). This assay utilizes the hydrogen atom transfer reaction, using 2,2′-Azobis(2-methylpropionamidine) (AAPH) as the free radical source and fluorescein as the target source. Briefly, a stock solution of fluorescein (46960, Sigma-Aldrich Corp.) was prepared by dissolving 44 mg of fluorescein in 100 mL of phosphate buffer (75 mM, pH 7.0). This solution was stored in complete darkness at 4 °C. A final working solution of 0.078 μM was freshly prepared by diluting 0.167 mL of the stock solution in 25 mL of phosphate buffer. AAPH (440914, Sigma-Aldrich Corp.) was prepared fresh on the day of the experiment by dissolving 600 mg of AAHP in 10 mL of phosphate buffer (221 µM). The

reference standard was a freshly prepared 20 µM solution of 6-Hydroxy-2,5,7,8-
tetramethylchromane-2-carboxylic acid (trolox) (238813, Sigma-Aldrich Corp.).

The fluorescein consumption during incubation with AAPH was measured by the
decrease in sample fluorescence and was carried out on a Turner fluorometer, model 112
(Turner Designs, Sunnyvale, CA, USA) with an excitation wavelength of 485 nm and an
emission wavelength of 535 nm. Two and a half mL of fluorescein, 2.5 mL of the digested
phosvitin samples, blank (phosphate buffer) or standard (trolox) and 1.25 mL of AAPH were
added and the sample was vortexed for 30 s. The reaction was carried out at 37 °C. The
fluorescence was measured immediately after the addition of the AAPH and then every 5 min
until the relative fluorescence intensity was less than 5% of the initial fluorescence reading.

The ORAC values were expressed as the µM trolox equivalents and were calculated
using the formula:

$$\text{ORAC value} = \frac{C_{Trolox} \cdot (AUC_{Sample} - AUC_{Blank})}{(AUC_{Trolox} - AUC_{Blank})}$$

C_{Trolox} is the concentration of trolox (20 µM), and AUC is the area under the fluorescence
decay curve for the phosvitin sample, blank, and trolox, respectively. This was calculated in a
Microsoft Excel spreadsheet (Microsoft, Redmond, WA, USA) using the formula:

$$AUC = (0.5 + f_5/f_0 + f_{10}/f_0 + \ldots + f_{n+5}/f_0) * 5$$

F_0 is the initial fluorescence and f_n is fluorescence at time n.

Statistical analysis. Each experimental design was randomly performed in triplicate.
Results are reported as mean values of three determinations ± standard deviation (SD). The
data was analyzed by analysis of variance (ANOVA). Least significant differences (LSD)

were calculated at $P < 0.05$ to compare treatment means by using the SAS system (version 9.1, SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Effect of pH and high-pressure treatment on phosvitin structure. Secondary structure of phosvitin was dependent on pH value (Fig. 1). In acidic environment, phosvitin has a high level of α helices and β sheets and in a basic environment a more irregular structure is observed (Table 1). Our observations agree with Taborsky (1970) observing the β sheet structure of phosvitin at an acidic pH and an irregular structure at a neutral pH. The conformational transition of phosvitin to a β sheet structure at an acidic pH can be attributed to the neutralization of the negative charges of the serine groups (Grizzuti and Perlmann 1970). After HPP treatment at 2.3 a slight decrease of 5.68% in the amount of α helix and 8.39% of β sheet occurred while the random coils increased by 6.14% after pressurization for 30 min compared to the control. Interestingly, after HPP treatment the greatest amount of change was observed, even though this sample was the most stable, with the highest α helices and β sheet values. At pH 7.0 and 11.0 there was no significant change in the secondary structure after 30 min of pressurization compared to the control (Table 1). The high-pressure profiles obtained from the treatments were very similar to thermal-treated profiles from Castellani and others (2004), who reported unordered structures and no change after two different heat treatments (50 and 90 °C). The authors also reported no change in the circular dichroism profile of phosvitin treated at 10 °C, up to 600 MPa for 10 min at pH 6.5.

High performance size exclusion chromatography (HPSEC) is a widely used technique to determine the molecular weight of proteins and polysaccharides and

aggregations among these molecules (Lucey and others 2000). Overall, pH adjustment did not result in major changes of the HPSEC elution profile of phosvitin (Fig 2A). After HPP treatment at pH 7.0, small differences in the elution profiles of the phosvitin samples was observed (Fig. 2B). A slight decrease in the intensity of the peaks at 11, 12, and 14 min was observed for the phosvitin treated for 30 min, indicating that over the prolonged HPP treatment some structural changes were occurring, resulting in the decreased intensity of the peaks. Similar results were obtained with the pH 2.3 and 11.0 HPP treated phosvitin samples.

The elution profiles of the phosvitin dephosphorylated for 2 and 18 h were different when compared to the control. The third peak disappeared, and the elution time of the peaks shifted by ~ 1 min (Fig. 3A). The change in the molecular weight was most likely due to the removal of the phosphate groups. This shift also occurred in the samples that were dephosphorylated then treated with pepsin (Fig. 3B). There was total disappearance of the peak at 8 min after 18 h of dephosphorylation, as well as the peak at 14.5 min. Treating the samples with phosphatase for 18 h then pepsin completely dissociated the peak. Similarly the intensity of the peaks at 13 and 16 min decreased with an increased dephosphorylation time. The largest changed observed came after the samples were dephosphorylated then treated with pepsin and pancreatin, compared to just a protease treatment (Fig. 3C). The pepsin and pancreatin digested phosvitin displayed 3 peaks at 8, 13, and 15 min. After dephosphorylating, then digesting the samples 4 large peaks were seen at 15, 16, 18, and 20 min. This indicates that phosphatase treatment played a significant role in the formation of phosvitin peptides, and produced significantly smaller peptides compared to just a protease treatment. Dephosphorylation of the samples between 2 and 18 h did not result in significant changes in the elution profiles for any of the results shown (data not shown).

Phosphatase treatment. After dephosphorylation of phosvitin for 2, 4, 12, and 18 h, the percent of phosphate released increased from 24% to 28, 51 and 62%, respectively (Fig. 4). Sattar Khan and others (1998) reported similar findings, with 21, 32, and 48% of the original phosphate removed after 1, 5, and 10 h when using phosphatase to dephosphorylate. This data illustrates that the phosphate is removed at a somewhat constant rate over the incubation time. Different finding were observed with chemical dephosphorylation treatment. When dephosphorylation of phosvitin was performed with NaOH, an increase of the phosphate released was followed by a decrease in the phosphate released between 1 to 3 h of incubation, followed by a plateau (Jiang and Mine 2000). The percent released was dependent upon the concentration of NaOH used in the process. Although the alkaline treatment removed more phosphates than the phosphatase treatment, this may have a negative impact as it can leave the phosvitin very susceptible to enzymatic digestion creating peptides with limited functionality.

SDS-PAGE. As expected, two large fragments between 29 and 36 kDa were obtained for untreated phosvitin. Small fragments <14.2 kDa were seen in our samples that were not observed in the Sigma standard (Fig. 5, lane 3 vs. lane 2). This discrepancy could be explained to procedure differences used to purify phosvitin between the commercial sample and our sample. High-pressure treatment had no effect on the phosvitin peptide profile, regardless of the pressure dwell time (0, 2 or 30 min) (lanes 4-6). This result was expected as pressure has no effect of primary structure of protein (Castellani and others 2004). Similarly, dephosphorylation of phosvitin, regardless of its extent, did not alter its peptide profile (Fig. 6, lanes 7-11). The removal of the phosphate group as dephosphorylation time increased could, therefore, not be illustrated by changes in SDS PAGE profile, certainly due to the

slight change in the MW of the dephoshorylated phosvitin. After pepsin hydrolysis of native phosvitin, the two major bands were replaced with peptides of ~34, 29, 27 and 21 kDa (Fig. 6, lane 3; I, II, III, IV). When the phosvitin was dephosphorylated prior to pepsin treatment band I completely disappeared. Band II and III were similar, while bands IV and V were less pronounced. Dephosphorylation time did not appear to have an impact on the bands (lanes 4-6). When phosvitin was digested with pepsin followed by pancreatin without prior dephosphorylation treatment, three distinct peptides were observed at 24, 20, and 16 kDa (lane 7; VI, VII, VIII). After dephosphorylation, it appears that new peptides were present, with slightly lower molecular weights (23, 19, and 14 kDa; lanes 8-10). This result illustrated that dephosphorylation of phosvitin before proteolytic digestion increase accessibility of the enzyme to the proteins, leading to the formation of smaller peptides. Again dephosphorylation time did not influence the results. Jiang and Mine (2000) reported that after alkaline dephosphorylation and tryptic digestion only phosvitin peptides of <3 kDa in size were observed. However, up to 96% of the phosphates were released from the phosvitin after this chemical treatment, whereas our samples lost a maximum of 62% phosphate groups after 18 h of treatment. This supports the hypothesis that removing most of all of the phosphate groups will leave the phosvitin particularly vulnerable to protease attack. Therefore, in the current study, we may not have observed such a dramatic production of peptides due to the protection of the phosphate groups. Interestingly, between the 24-62% phosphates removed, no difference was seen in the peptides, suggesting that almost completely stripping the phosvitin of phosphates must occur to fully digest phosvitin.

ACE Inhibitory Activity. The native phosvitin (control) has little ACE inhibitory activity (3.4%, Table 2). While ACE inhibitory activity of purified phosvitin has not been

previously reported in the literature, similarly low ACE inhibitory activity was reported for boiled egg yolk (Majumder and Wu, 2009). Once the native phosvitin was digested with pepsin, and pepsin followed by pancreatin, the percent of inhibition increased to 20.7 and 34.1%, respectively, suggesting that in the condition mimicking *in vivo* digestion, formation of peptides with higher inhibiting ACE activity can be obtained. Dephosphorylation of phosvitin for 2 h increased the ACE inhibitory activity by 5.6%, compared to the control, while increasing the dephosphorylation incubation time did not have further advantage. Similarly, the 2 h dephosphorylation treatment prior to pepsin and pepsin followed by pancreatin digestion, increased the ACE inhibitory activity of the peptides formed, but increasing the dephosphorylation time had no further effect. However, although improvement in ACE inhibitory activity was obtained after digestion of dephosphorylated phosvitin, the concentration needed to obtain 50% inhibition remains relatively high (1.25 mg/mL), and therefore phosvitin peptides would be considered a poor ACE inhibitor. For comparison, when milk whey was digested in a similar fashion to the phosvitin samples (4 h pepsin treatment, 37 °C, followed by 4 h pancreatin treatment, 37 °C) a 44% decrease in the amount necessary to obtain an IC_{50} value was observed after pepsin digestion. After pancreatin digestion a decrease in the ACE inhibition activity was observed, however, these values were still better than the control. (Tsai and others 2008). The authors concluded that while the ACE inhibitory peptides are released by pepsin digestion, they could be maintained in the active form even after pancreatin digestion. In the current study, a further increase in the ACE inhibitory activity was seen after pancreatin digestion. We can hypothesize that the intense conditions of the small intestine is necessary to obtain maximum inhibition activity of the stable phosvitin. Majumder and Wu (2009) studied the effects of cooking methods

(boiling and frying) as well as enzymatic digestion (pepsin and pancreatin) on the ACE inhibitory activity of egg white, egg yolk, and whole egg. While the egg yolk IC_{50} values were lower than the egg white and whole egg values, a protein concentration of 1.118 mg/ml was reported for pepsin digested boiled egg yolk. Digesting the sample further with pancreatin lowered the amount necessary to obtain an IC_{50} value, which was in agreement with our results, and further supports the idea of maximum inhibition obtained when combining pepsin and pancreatin.

Antioxidant activity. Pepsin digestion of the native phosvitin had no effect on the protein antioxidant activity (Table 3). This observation was unexpected as changes in the peptide profile and ACE inhibitory activity were obtained. However, when the native phosvitin was treated with combination of pepsin and pancreatin, a slight but significant difference was obtained, i.e., 1.84 vs. 1.95. Dephosphorylation prior to pepsin and pancreatin treatment significantly increased the antioxidant activity, when compared to the native digested samples, with an average increase of 0.5 and 0.73 units. As seen with the ACE inhibitory activity results obtained, dephosphorylation time did not have an effect on the antioxidant activity. After dephosphorylation, significant differences were seen between the pepsin and the pepsin + pancreatin digestions. These differences were not seen in the native phosvitin digestions. Previous work has reported that native phosvitin has strong antioxidant capacity (Baker 1985; Lu and Baker 1986; Xu and others 2007). Xu and others (2007) measured the antioxidant activity of native and phosvitin peptides derived from tryptic digest in a linoleic acid system. Similar to our results, the authors reported an increase in the antioxidant activity when alkaline dephosphorylated phosvitin peptides were used in comparison to native phosvitin, although percent dephosphorylation was not reported.

Interestingly, the phosphorus groups have strong iron chelating abilities, and were previously suggested to be the reason for the antioxidant activity. However, because these were removed and the activity increased, the amino acid sequence and the peptide sequence may also play a role in the antioxidant activity (Xu and others 2007).

Conclusions

High-pressure processing coupled with a thermal treatment does not alter the overall structure of phosvitin, further supporting the idea that phosvitin is very resistant to physical changes. Phosvitin is more susceptible to chemical changes such as pH adjustments with shifts in the percent of α helices and β sheets at pH 2.3. Phosphatase treatment resulted in the dephosphorylation of phosvitin, and pepsin and pancreatin digestion produced small bioactive peptides. While these peptides did not largely differ in their molecular weights compared to native digested phosvitin, significant improvements were seen in their ACE inhibition. Extent of phosvitin dephosphorylation did not affect these activities. Therefore, a 2 h dephosphoryation time prior to proteolytic digestion is sufficient to produce maximum results. Treating dephosphorylated phosvitin with high-pressure may result in a very different outcome, and is currently under progress.

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antioxidant capacity of food products. Analy Methods 114:310-6.

Table 1 – Effect of pH and dwell time at 600 MPa on phosvitin structure

pH	Dwell time (min)	α helix	β sheet	random coil
2.3	Control	23.99 ± 2.41^c	25.62 ± 2.92^d	58.32 ± 4.01^a
	0	23.85 ± 3.15^c	24.98 ± 3.47^d	59.45 ± 4.60^a
	2	19.32 ± 2.57^{bc}	21.47 ± 5.70^d	54.97 ± 3.85^a
	30	18.31 ± 3.57^b	17.23 ± 4.68^c	64.46 ± 4.46^b
7.0	Control	15.88 ± 4.59^{ab}	3.00 ± 0.89^a	82.11 ± 4.07^c
	0	16.00 ± 4.17^b	3.23 ± 0.67^a	80.76 ± 3.74^c
	2	17.24 ± 4.16^b	5.82 ± 0.94^b	76.94 ± 5.02^c
	30	18.74 ± 4.42^b	4.82 ± 0.54^{ab}	76.43 ± 3.86^c
11.0	Control	12.25 ± 2.91^a	0.00 ± 0.00^a	87.03 ± 4.19^d
	0	13.84 ± 3.44^a	0.00 ± 0.00^a	86.16 ± 3.44^d
	2	15.92 ± 3.58^{ab}	0.00 ± 0.00^a	84.01 ± 4.58^d
	30	11.51 ± 5.00^a	3.71 ± 0.49^a	84.79 ± 3.77^d

Values sharing the same superscript in the same column are not significantly different at $P < 0.05$.

Table 2 – Percentage of ACE inhibitory activity of phosvitin and phosvitin peptides as function of dephosphorylation and protease

Protease addition	Phosphatase treatment (h)				
	0	2	4	12	18
Control	3.44 ± 1.44^a	9.09 ± 0.40^b	10.61 ± 0.47^b	12.28 ± 0.42^b	13.23 ± 0.22^b
PE	20.71 ± 1.39^c	35.88 ± 1.08^d	34.81 ± 1.72^d	35.52 ± 0.54^d	32.67 ± 3.04^d
PE + PA	34.10 ± 2.85^e	46.23 ± 0.65^f	44.72 ± 0.74^f	47.72 ± 1.49^f	52.14 ± 0.29^f

Values sharing the same superscript are not significantly different at $P < 0.05$
PE: Pepsin (4% w/w of protein; 3 h, 37 ℃)
PA: Pancreatin (2% w/w of protein; following pepsin digestion 3 h, 37 ℃)
Values are expressed as % ACE inhibition at 1.25 mg/mL phosvitin solution

Table 3 – ORAC values of phosvitin and phosvitin peptides as function of dephosphorylation and protease

Protease addition	Phosphatase treatment (h)				
	0	**2**	**4**	**12**	**18**
Control	1.84 ± 0.03^{a}	2.12 ± 0.01^{c}	2.14 ± 0.01^{c}	2.17 ± 0.01^{c}	2.19 ± 0.01^{c}
PE	1.91 ± 0.01^{ab}	2.35 ± 0.01^{d}	2.53 ± 0.26^{d}	2.37 ± 0.11^{d}	2.40 ± 0.08^{d}
PE + PA	1.95 ± 0.01^{b}	2.63 ± 0.03^{e}	2.69 ± 0.05^{e}	2.69 ± 0.06^{e}	2.72 ± 0.03^{e}

Values sharing the same superscript are not significantly different at $P < 0.05$
PE: Pepsin (4% w/w of protein; 3 h, 37 ℃)
PA: Pancreatin (2% w/w of protein; following pepsin digestion 3 h, 37 ℃)
Values are expressed as micromoles of Trolox equivalent per mg of phosvitin protein

Figure 1 – Circular dichroism profile of phosvitin as function of pH
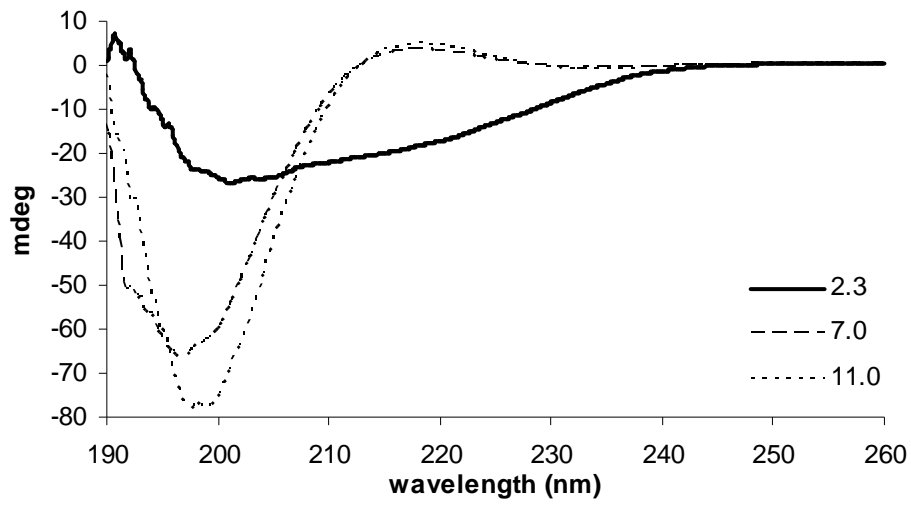
Figure 2 – Elution profile of phosvitin as a function of (A) pH and (B) dwell time (min) at 600 MPa
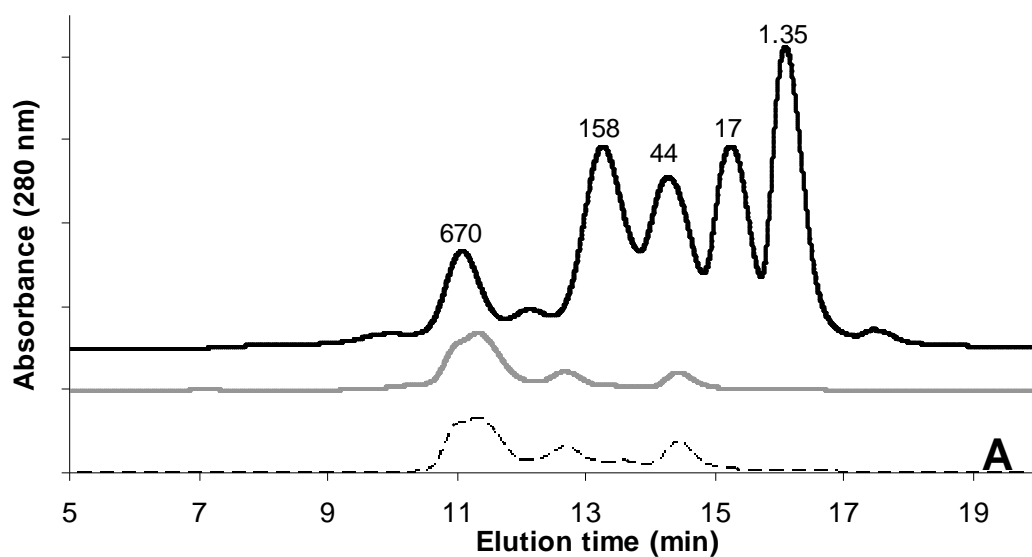
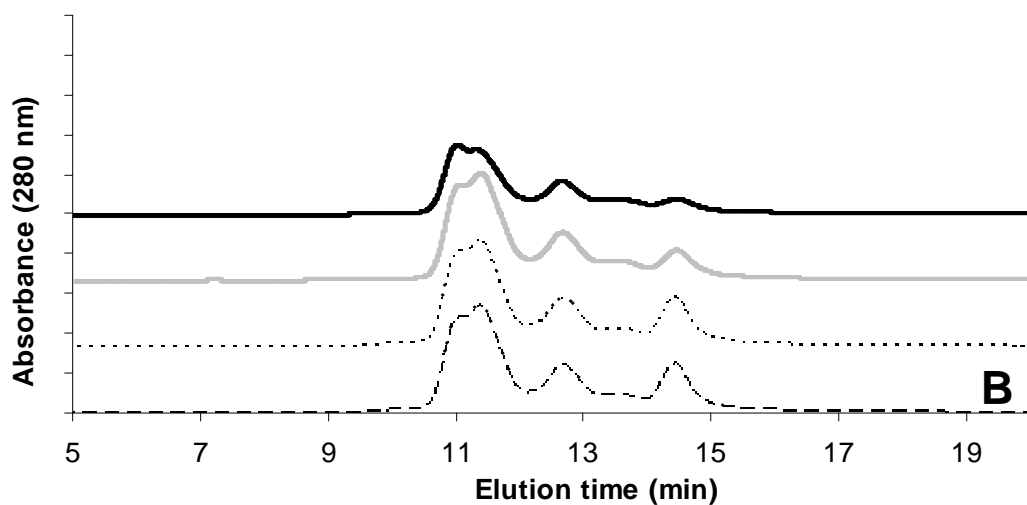_ _ _ _ : pH 2.3; .... : pH 7.0;_____ : pH 11.0; _____ : Molecular weight marker (kDa)
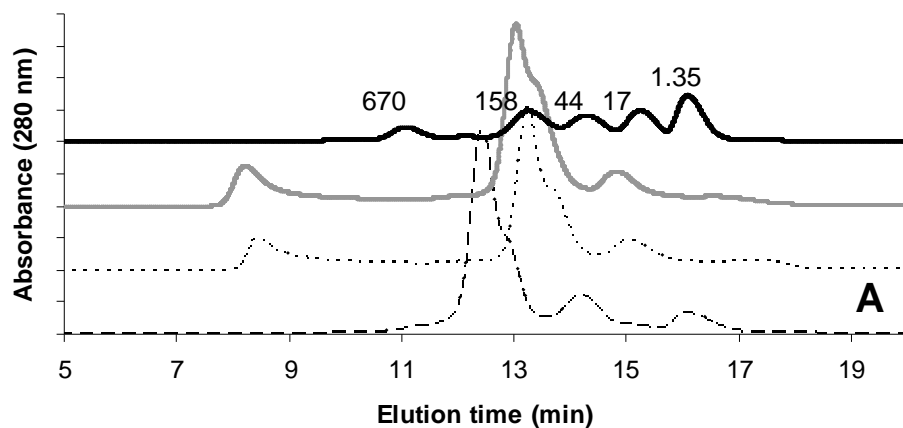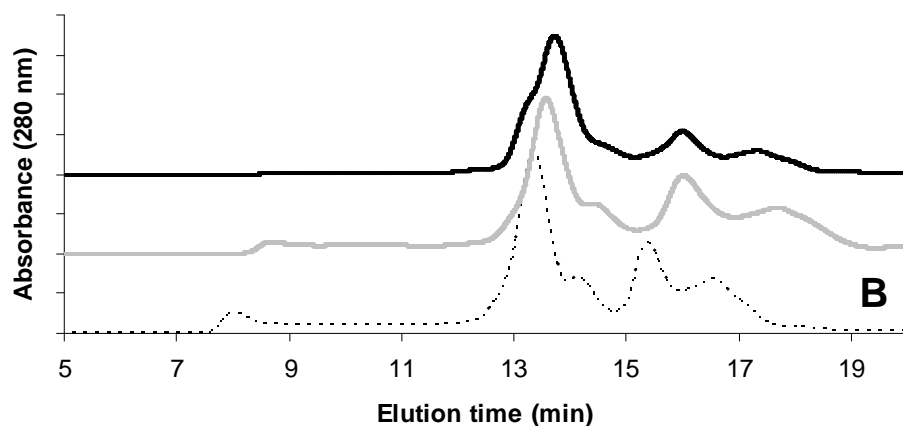

Figure 2B was obtained with a solution of phosvitin adjusted at pH 7.0

_ _ _ _:Control; ....:0 min; _____:2 min; _____ : 30 min

Figure 3 – Elution profile of phosvitin as a function of (A) dephosphorylation time (h), (B) pepsin treatment combined with dephosphorylation and (C) pepsin + pancreatin combined with dephosphorylation
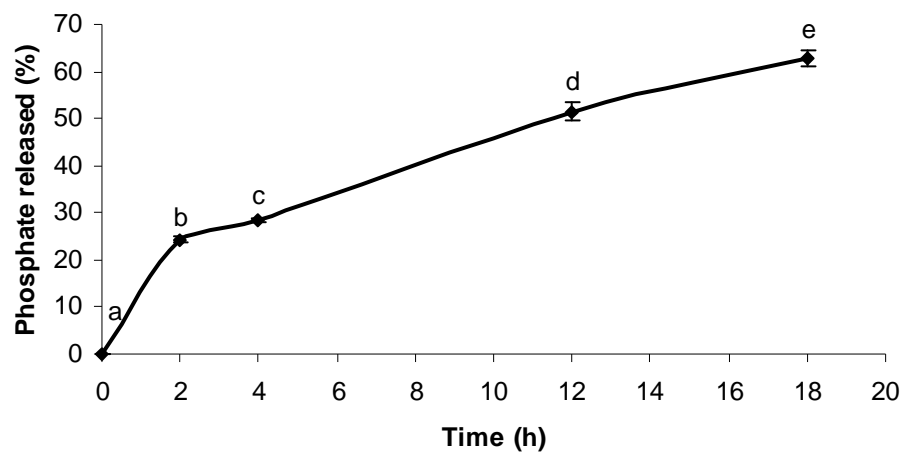

- - - :Control; ....:2h; ——— :18 h; ——— : Molecular weight marker (kDa)

..........:Pepsin; ——— :2h + pepsin; ——— :18 h + pepsin
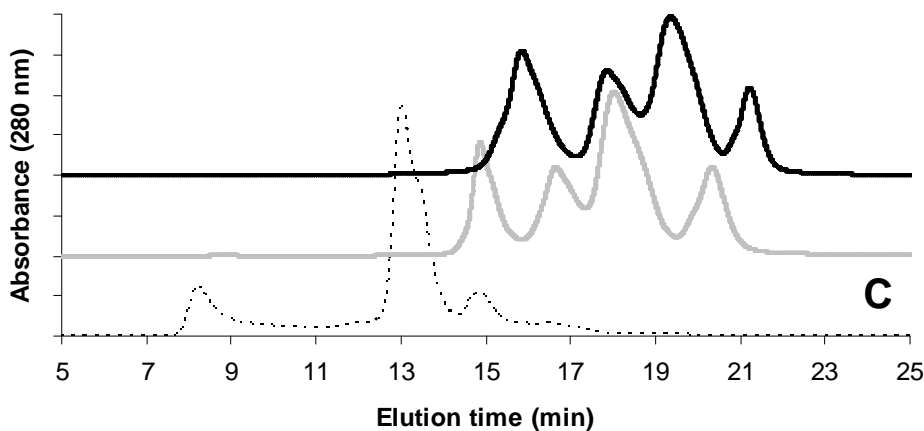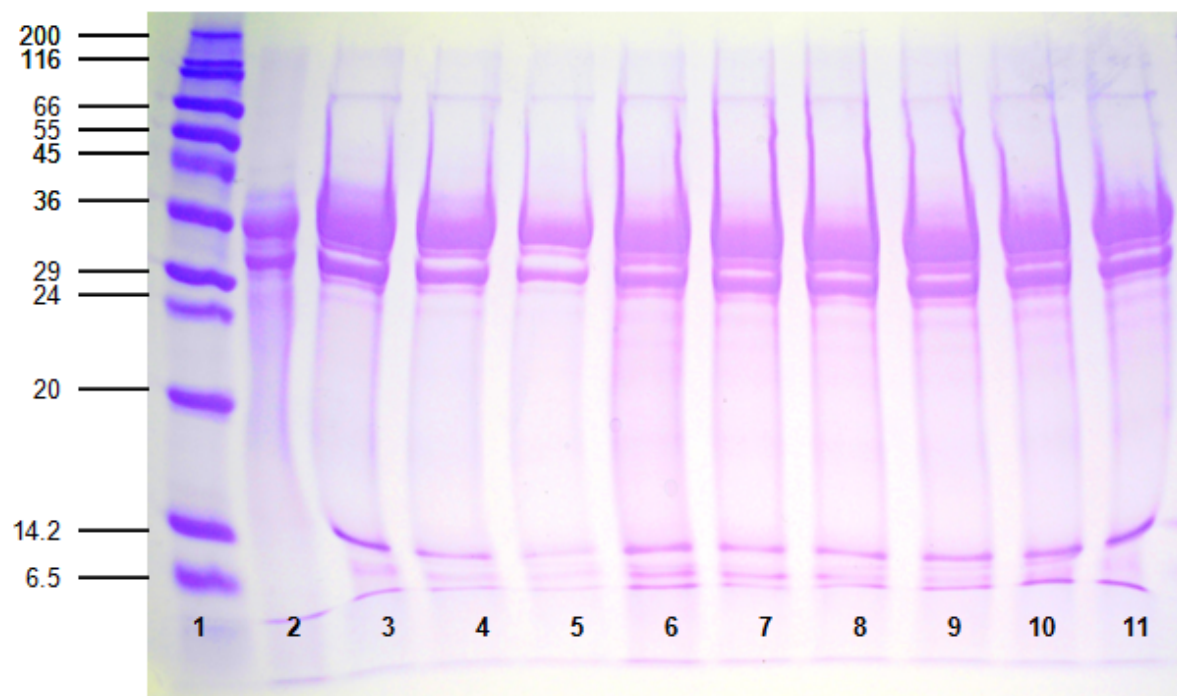
..........:Pepsin + pancreatin; ——— :2h + pepsin + pancreatin; ——— :18 h + pepsin + pancreatin

Figure 4 – Timeline of dephosphorylation of phosvitin

Values sharing the same superscript are not significantly different at $P < 0.05$

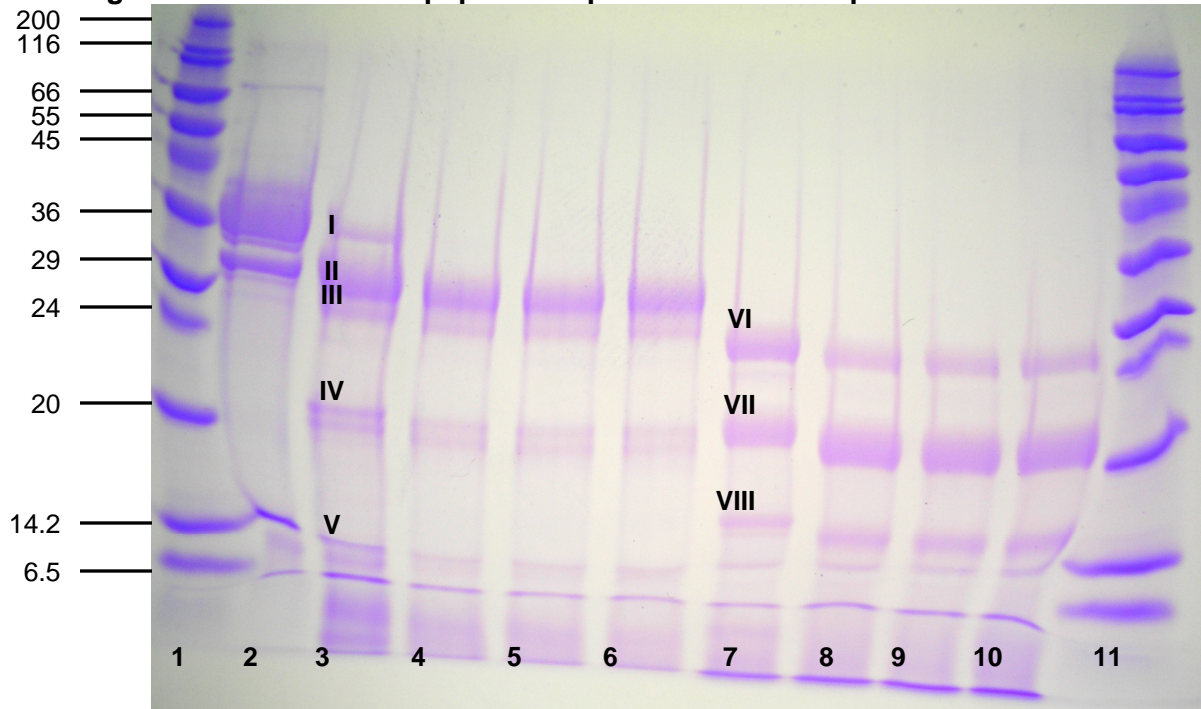**Figure 5 – SDS-PAGE of pressure-treated and dephosphorylated phosvitin**

lane 1: molecular marker
lane 2: native phosvitin (Sigma)
lane 3: native phosvitin (our sample)
lane 4-6: HPP (600 MPa, 65 ℃): 0, 2, 30 min, respe ctively
lane 7-11: dephosphorylation: 1, 2, 4, 12, 18 h, respectively

Figure 6 – SDS-PAGE of pepsin and pancreatin treated phosvitin

lane 1: molecular maker
lane 2: native phosvitin
lane 3: native phosvitin digested with pepsin
lane 4-6: pepsin digested dephosphorylated phosvitin: 1, 12, 18 h, respectively
lane 7: pepsin + pancreatin digested native phosvitin
lane 8-10: pepsin + pancreatin digested dephosphorylated phosvitin: 1, 12, 18 h, respectively
lane 11: molecular marker

CHAPTER 5. GENERAL CONCLUSIONS

In the first study, the quality attributes of soymilk and tofu pre-treated and produced, respectively, with HPP or thermal treatment were investigated. The three parameters tested (soymilk pH, pre-treatment and method of tofu production) had a significant impact on the quality of the final products. Even though the pH change was slight (0.5 unit), it had a major impact on the stability of the soymilk and the water holding capacity (WHC) of the tofu, with an average WHC increase of 11% for the pH 6.5 soymilk compared to pH 6.0 soymilk. Microstructure of tofu was pH-dependent, and changes in the tofu three-dimensional network impacted the WHC results. Tofu was not produced under high-pressure when pH 6.0, thermally pre-treated soymilk was used. However untreated soymilk was able to form a curd under high-pressure. This result suggested that under high-pressure, denaturation of the proteins and formation of a gel is occurring concurrently. Hydrolysis of GDL in both water and soymilk occurred at a faster rate using high-pressure than thermal treatment. As the pressure level increased from 200 to 600 MPa, the rate of GDL hydrolysis increased, which suggests that processing time could be reduced at higher pressure. An improvement in certain quality attributes, including the tofu yield, was observed when using pressure to produce tofu, with the increase being more evident as the pressure level increased. The gumminess and hardness of the pressure-produced tofu regardless of the pH and pre-treatment, except in the case of the 400 MPa pre-treated soymilk, increased as the pressure level increased, while the springiness decreased. The pressure-produced tofu was less elastic, making it easier to chew. This study also identified that HPP could be a quicker method of producing tofu, as some of the time consuming pre-treatment steps could be eliminated. This constitutes an important advantage of HPP vs. thermal treatment, which would provide a financial benefit

to soy food producers. While this study provides insight to many quality parameters that are important to the soymilk and tofu industry, future work is necessary to make this a viable process. Microbial analysis should be performed to determine if the conditions used are effective in lowering bacterial counts. Also, even though nutrients are better retained through HPP some antinutritional factors, such as trypsin inhibitors are also retained. Using pressure-assisted thermal processing may provide a balance between a high quality product, while eliminating negative factors such as trypsin inhibitors.

In the second study it was shown that, unlike soy proteins, phosvitin is pressure resistant, even at a high temperature. Indeed, high pressure treatment coupled with thermal treatment failed to change the phosvitin structure. pH did not result in a change in the elution profile either, signifying a change in the molecular weights did not occur. However, shifts were seen in the percent of α helices, β sheets and random coils after pH adjustment. Specifically, when pH was adjusted from 7.0 to 2.3, the amount of α helices and β sheets increased by 8.1 and 22.6%, respectively, while the random coils decreased by 21.3%. Peptides were produced after enzymatic treatment simulating gastrointestinal digestion. Differences were seen in the intensity and the size of the peptides obtained from dephosphorylated and native phosvitin. Peptides obtained from native and dephosphorylated phosvitin had higher antioxidant activity and ACE inhibition, as compared to untreated phosvitin. However, dephosphorylation prior to proteolytic digestion was more efficient in producing peptides with improved antioxydant and ACE inhibition activities.

Because HPP did not alter the structure of native phosvitin, adding phosvitin prior to HPP treatment may be an effective way of increasing food product shelf-life and reducing the risk of pathogenic outbreaks. Future research could also include anti-microbial activity of the

bioactive peptides, as well as other functionality properties such as emulsifying abilities. Isolating the peptides to determine what effects each has on the functionality of phosvitin, as well as determining the amino acid structure of these peptides, will help to better understanding the mechanisms of action behind the antioxidant and ACE inhibitory activity.

Overall, the soy study and egg phosvitin study demonstrate the opportunity and limitation provided by high-pressure processing, respectively.

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