APPLICATION OF NON-THERMAL AND THERMAL TREATMENTS TO REDUCE SOYBEAN ALLERGENS

By

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To my parents and my lovely husband……..

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CMVS	Color Machine Vision System
ELISA	Enzyme Linked Immuno Sorbent Assay
FAO	Food and Drug Administration
GM	Genetic Modified
HRP	Horseradish Peroxidase
PL	Pulsed Light
PU	Power Ultrasound
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

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Soybeans are considered one of the eight most allergenic foods. Thus, it is necessary to find approaches to reduce the allergenicity of soybean. Non-thermal treatments including pulsed light (PL) and power ultrasound (PU) are novel technologies introduced recently in food processing, but applications of non-thermal treatments to reduce soybean allergens are limited. In this study, the objectives were to reduce the allergenicity of soybean extracts and soymilk by application of PL and PU. Immunological methods, such as western blot and enzyme-linked immune assay (ELISA), were conducted to investigate the IgE binding to soybean allergens. The results showed that there was a significant reduction after treatment of soymilk samples with PL for 0.5, 1, 2, 4, and 6 min compared to heated and untreated samples. It also was observed that the temperature of the samples increased with increasing treatment time.

Power ultrasound was applied to reduce the allergenicity of soybean extract and soymilk. The results demonstrated an obvious reduction after different treatment times with a notable disappearance of allergens after 15 min. However, there was a difference in the allergen reduction for soybean extract and soymilk due to the release of the major

allergen P34 during treatment. The increase in temperature was an interesting phenomenon observed during the PU treatment. Thermal treatments were conducted to compare their effects with those of PU in soymilk samples. The results showed no reduction in bands of glycinin subunits and P34, which are the major soybean allergens. Additionally, the physicochemical properties of soybean extract and soymilk samples treated with PU were examined. Degradation of lightness (L*) was observed with increasing time of treatment. Temperature and viscosity of the samples significantly increased (p<0.05) in all treatments, whereas the pH of the samples decreased with increasing time of treatment.

Transgenic soybeans are found widely in the market, and there is a public fear that transgenic soybeans may increase the allergenicity of soybean products. Hence, the protein profiles and allergenicity of transgenic and non-transgenic soybeans were compared in this study. The results showed that there was no difference between non-transgenic and transgenic soybeans. In addition, thermal treatments, such as heating, steaming, and roasting, which are commonly applied in food processing, produced similar allergens in both cultivars, and no neoallergens were detected in transgenic soybeans

CHAPTER 1
INTRODUCTION

Soybean (*Glycine max*) is an ancient legume, its use is widespread in Southeast Asia, and it is relatively inexpensive compared to other protein sources. Soybean meal is fed to livestock, because it contains high-quality protein. Furthermore, it is used widely for human and animal consumption. The United States are one of the largest soybean producers in the world, and attention to this crop has increased because of its low cost, nutritional quality, and health benefits (Stacy 2008). Americans consume approximately one to three grams daily in soybean products, such as soy drinks, energy bars, and “soy burgers” (Omani et al. 2005). Soybean has gained more attention after the U.S. Food and Drug Administration (FDA) approved labeling of foods containing soy protein as protective against coronary heart disease. In addition, the American Heart Association (AHA) Nutrition Committee approved a scientific advisory on soy protein and cardiovascular disease (Sacks et al. 2006). Furthermore, soybeans are rich in isoflavones, such as genistin and daidzein, which are claimed to prevent prostate and breast cancers (Liggins et al. 2000). However, there is a major issue related to their potential allergenicity. Soybean is considered one of the “big eight” most allergenic foods.

Food allergies are receiving more attention, especially in the United States, because this country is the leading producer of soybean. Most food products are cooked for safety reasons. Thermal treatments can enhance flavor, texture, and other qualities that improve satiability. Thermal treatments also may cause major changes in the potential allergenicity of the food product. However, one cannot predict if the thermal

treatment decreases or increases the allergenicity of food, because potential effects are governed by molecular properties of food allergens (Davis et al. 2001).

In the last few years, non-thermal treatments have been introduced in the food industry, because they have negligible effects on food quality. The following non-thermal treatments have been used recently for food allergen control: pulsed ultraviolet light, pulsed electric fields, power ultrasound, and gamma irradiation (Johnson et al. 2010; Lin et al. 2011; Yang et al. 2011). However, publications on the application of non-thermal treatments to eliminate the allergenicity of soybean products are limited.

Most of the soybeans produced in the United States are genetically modified, and there is a public fear that genetic modification may introduce a new protein or allergen into the modified cultivar (Panda 2012). Moreover, there is little information on the behavior of allergens during food processing, and it is not clear whether new allergens are introduced or whether allergenicity changes during food processing.

Justification of the Study

Soybean has several benefits because of the high content of proteins, lipids, and isoflavones. The consumption of soybeans in the United States is increasing because of the soybean's nutritional benefits and the claim that it reduces cancer in both women and men. However, soybean proteins have been shown to cause allergies, and recently, 38 allergens have been identified in soybean. During food processing, the seeds are subjected to thermal treatment for quality and safety purposes, but thermal treatment also may increase or reduce the allergenicity of proteins. Non-thermal treatments have been used in food processing to retain nutrient content and flavors. Some non-thermal treatments have been found to reduce the allergens in peanut, shrimp, soybean, and almond. The scarcity of articles related to the application of

pulsed light and power ultrasound in order to reduce allergens in soybean extract and soymilk is the reason for this study. More specifically, no publications exist that address the reduction of allergens in soybean by ultrasound treatment. It is hypothesized that power ultrasound and pulsed light will lead to a reduced allergenicity of soybeans. Furthermore, most soybeans in the market can be considered genetically modified, and there is little data published on the behavior of genetically modified soybeans during food processing, specifically during thermal treatments.

Overall Objectives

The overall objective of this study was to reduce the allergens in soybean by application of the following non-thermal treatments: pulsed light and power ultrasound. In addition, the study compared the effect of thermal treatments on allergenicity of non-transgenic and transgenic soybean.

The specific objectives included the following: 1) to study the effect of pulsed light on soymilk allergens; 2) to investigate the effect of power ultrasound on the allergenicity of soybean extracts; 3) to test the effect of power ultrasound on soymilk and compare it with that of thermal treatment; 4) to examine the effect of power ultrasound on the physicochemical properties, which include viscosity, color, pH, and temperature, of soybean extracts and soymilk; and 5) to elucidate the effect of thermal treatments (heating, roasting, and steaming) on the allergenicity of non-transgenic and transgenic soybean extracts.

CHAPTER 2
LITERATURE REVIEW

Food Allergy

While eating is essential to sustaining life and is an enjoyable hobby for some people, consuming certain foods can be life threatening for individuals with food allergies. Consequently, individuals with food allergies must avoid consuming certain foods or ingredients that can cause allergic reactions.

Food allergies are defined as adverse immune-mediated reactions that can occur in individuals who eat or drink certain kinds of food. A food allergy is an abnormal immunological reaction to food allergens, such as peanuts or milk. There are several types of reactions that often are considered a response to food allergens; those include immunoglobulin E (IgE) mediated and non-IgE-mediated primary immunological sensitivities, secondary sensitivities, and non-immunological food intolerances. True food allergies are divided into two types based on the response to food allergens: IgE-mediated (immediate hypersensitivity reactions) and non-IgE–cell-mediated (delayed hypersensitivity reactions). In immediate hypersensitivity reactions, symptoms develop within minutes to an hour after ingestion of food allergens. In delayed hypersensitivity reactions, symptoms take 24 hours or longer to develop (Koppelman and Hefle 2004; Taylor and Hefle 2001).

Food allergens can be defined as foreign proteins with small macromolecules to which IgE binds. They are usually water soluble and have low molecular weights of 10-70 kDa. Only a few types of natural food proteins can be considered food allergens based on the ability to bind to IgE antibodies and produce allergic reactions. Food proteins designated to the most common food allergies were labeled as “The Big Eight”

and include milk, peanuts, soybeans, shrimp, and almonds (Koppelman and Hefle 2006; Yada 2004). There is a small fragment in the allergen's structure that plays a certain role in the immunological reaction and is known as epitope. The epitope is composed of five to seven amino acids or three to four sugar residues, which can cause IgE-mediated allergic responses (Yada 2004; Yang et al. 2011). The epitope structure can be a sequence of amino acids known as a liner epitope. Also, it can be found as a three-dimensional structure that is called a conformational epitope. To change the activity of proteins, it is necessary to restructure the epitopes by distributing the IgE-binding epitopes (Tanab 2007). In the case of the liner epitopes, it is a challenge to change the reactivity, but genetic modification of amino acid sequences can lead to the altering of the liner epitope structure. The conformation structure of epitopes can be altered by denaturation and/or aggregation, which lead to cross linking (Shriver and Yang 2011; Yada 2004).

The mechanism of IgE-mediated allergic reactions is illustrated in Figure 2-1. The first phase of the sensation occurs when allergens are exposed and stimulate production of specific IgE antibodies. During the circulation of the blood, the specific IgE attaches to the mast cell in various basophils and tissues, which contain active chemical compounds that mediate the allergic reactions. On the surface of the sensitized cell, two specific IgE antibodies can stimulate and release allergen response mediators, such as histamine, which is responsible for several allergy symptoms (Taylor and Hefle 2001). There are many methods that have been used to detect food allergens based on IgE binding to allergens, and they are used for qualifying or quantifying food allergens. For example, enzyme-linked immunosorbent assay (ELISA), western blot, and radio-

allergosorbent tests (RAST) are tests that assist in confirming the presence allergens. Also, there are in vivo tests, such as a skin prick test or an oral food challenge (Koppelman and Hefle 2006).

As mentioned before, soybeans are one of “The Big Eight” allergens. Soybean allergies have become important since soybeans are consumed widely and found as ingredients in several food products. Just a small amount of soybeans can trigger severe symptoms in individuals with soybean allergies.

Soybean Overview

Soybean (*Glycine max*) is a legume that is used widely in Asian cuisine. It is used to prepare both fermented and non-fermented foods. The main advantage of the soybean is the nutritional value for human health in the form of energy and protein. Soybean is considered one of the most important sources of protein and oil. Although the amount of protein content in soybean is high (38%), the quality of protein must also be considered. Soybeans contain all essential amino acids required by the human body (Lambein 2005). The United States, Brazil, and Argentina are the predominant soybean-producing countries. The conservative market for this crop is estimated at $48.6 billion; the market is $18.7 billion in the United States alone. The demand for soybeans is strong, and it continues to grow because of the application of soybeans in a wide range of soy foods, soybean oil, and vegetable protein substitutes for meat and dairy products.

The origin of the soybean is China. Samuel Bowen introduced the soybean to North America in 1765 to produce soy sauce. Benjamin Franklin also contributed to the spread of soybean in the United States, but his interests were only for forage and ground cover. In 1915, in North Carolina, soybeans were first crushed to extract the oil

from the seeds. The discovery of oil extraction from soybeans led to additional efforts by the United States Department of Agriculture (USDA) to establish a new program related to soybean breeding in cooperation with universities. This development led the United States to be the leader in soybean production (Wilson 2008).

Soybean Allergens

Although soybean protein has excellent nutritional value and health benefits, the soybean ranks among the top eight significant food allergens. Soybean allergy is the most common allergy among infants and children and affects 1-6% of infants (Tryphonas et al. 2003). The allergy symptoms include skin, respiratory, and gastrointestinal reactions. Three main types of soy allergenic reactions are common. The first type of IgE-mediated reactions can produce cutaneous, respiratory, and gastrointestinal symptoms. The second type consists of non-IgE-mediated reactions, including soy-induced enterocolitis that can always be outgrown; symptoms of this reaction often include vomiting, fever, and diarrhea. The third type, and least common reaction, is anaphylaxis, which affects 10.8/100,000 persons in the United States (Wilson et al. 2005).

Soybean allergy has become a challenge for both the consumers and the food industry. The only way to circumvent the soybean allergen is to avoid foods that contain soybean or derived soybean protein. In the Unites States, the Food Allergen Labeling and Consumer Protection Act (FALCPA) amended the Federal Food, Drug, and Cosmetic Act (FFDCA) and required food producers to clearly label a product that contains a “major food allergen” (US FDA 2007). Currently, a total of 38 soybean allergens have been identified and listed in the allergen online database (FARRP 2008).

The protein in soybean seeds is comprised of two major fractions (β-conglycinin and glycinin), which represent 70-80% of the total protein composition. Several soybean allergens, such as β- conglycinin, glycinin, and *Gly m* Bd 30 K, have been identified and listed as major allergens (Baur et al. 1996).

β- Conglycinin

The β-conglycinin, also called 7S, is considered one of the major allergens in soybeans, and several researchers studied the allergenicity of β-conglycinin extensively. It is a glycoprotein and a trimer that includes the three unique subunits α, α′, and β with molecular weights of 72, 68, and 52 kDa, respectively. The amino acid sequence of the β subunit does not contain cysteine residues (-SS-), whereas α and α′ subunits each possess one cysteine residue (-SH) near the N-terminal (Yada 2004). It is documented that IgE binds to all three subunits, which have been labeled as major allergens (Burks et al. 1988). Ogawa et al. (2000) reported that almost 25% of Japanese are sensitive to α and β subunits of β-conglycinin. In addition, Krishnan et al. (2009) confirmed that all β-conglycinin subunits induced allergic reactions in Brown Norway rats.

Glycinin

Glycinin, also known as 11S, is a hexamer with five subunits that are composed of basic (~20 kDa) and acidic (~35 kDa) polypeptides that are linked together by disulfide bonds (Yada 2004). Glycinin is known to form a harder gel and be more turbid compared to β-conglycinin. Also, glycinin is more stable thermally because of the large amount of SH groups and the higher hydrophobicity compared to β-conglycinin. L'hocine and Boye (2007) reported that IgE binds to all glycinin subunits, and glycinin is considered a major allergen in soybeans.

P34

P34 and *Gly m* Bd 30 K are considered the same allergen because the N-terminal amino acid sequence and the amino acid composition of *Gly m* Bd 30 K and P34 are identical (Wilson et al. 2008; Helm et al. 2000). P34 is a monomeric and insoluble glycoprotein containing 257 amino acid residues linked by disulfide bonds in the 7S globulin protein fraction, and it probably has an important role in protein folding. IgE binding assays with immunoglobulins from soybean-sensitive individuals showed that 65% of the total allergic response was related to *Gly m* Bd 30 K, which is known as the strongest immunodominant allergenic protein (Wilson 2008). In a previous study, P34 was reported to contain a minimum of 12 distinct liner epitopes rich with aspartic, histidine, lysine, and glutamic acids. It was recognized as heat resistant and is hard to be modified because of the complex structure and number of the epitopes in P34 (Wilson et al. 2005). Fermentation is an effective procedure to reduce the allergenicity of P34, because this process hydrolyzes proteins into small peptides. The fermented soybean products (miso, tempeh, and mold-hydrolyzed soy sauce) showed a significant reduction in allergenicity compared to non-fermented soybeans with less content of P34 (Herian et al. 1993).

Kunitz Trypsin Inhibitor

Kunitz trypsin inhibitor is a minor allergen (~21.5 kDa) with two disulfide bonds per mole of protein and known to induce anaphylaxis. It is unstable thermally due to the low amount of disulfide linkages. Heat processing of soybean products can inactivate the Kunitz trypsin inhibitor, and soybean products for infants were found to have lower levels of Kunitz trypsin inhibitor compared to raw soybean (Friedman and Brandon 2001; Vorlova 2011).

Other Soybean Allergens

Profilins (14 kDa) are heat label proteins, and heating, enzymatic hydrolysis, and fermentation can reduce the antibody binding capacity of profiling (Amnuaycheewa and Mejia 2010). There are other less known allergens with low molecular weights, such as Gly m1 (7 kDa) and Gly m 2 (8 kDa). In some cases, these allergens play a significant role in inducing the allergy (Vorlova 2011).

Approaches to Changing the Allergenicity of Soybeans

For individuals with food allergies, strict avoidance of the allergenic proteins is necessary to avoid allergenic reactions. This can be a challenge for individuals who suffer from food allergies, especially peanut and soybean, because there is a large quantity of products in the market not labeled to contain these commodities (L'hocine and Boye 2007). Past researchers reported several ways to change the allergenicity of food allergens, and for soybean allergens, either an increase or decrease in allergenicity during processing could occur. The following sections give examples.

Thermal Treatment

Thermal treatment is intended to enhance the safety aspect of foods; however, physicochemical properties, especially flavor and color, are affected by this treatment. Thermal treatment also can reduce the allergenic potential of some foods. The principle of an allergenic reduction is based on the alteration of the protein structure. Heating can cause protein denaturation, introduction of new intra-molecular or inter-molecular bonds, aggregation, cleavage of disulfide bonds, and other conformational modifications (Wal 2003). Thermal treatment can stretch a few amino acids along the primary structure, and when the temperature increases, the extent of the structure can become

larger. Even some of the protein tertiary structure can be altered by low temperatures, such as 50 °C (Davis and Williams 1998).

In the food industry, dry roasting is used widely to make roasted soybean products. The seed is roasted approximately 30 min, until it becomes brown. The main purposes of roasting are to inactivate undesirable bacteria, to improve the quality of protein, and to inactivate the beany flavor. In thermal processing of soybeans, there are important factors that should be considered, and they include physiological and nutritional aspects, functional properties, and allergenic potential. It is known that thermal treatments may have an impact on the potential allergenicity of proteins. Commercial soy-based foodstuffs, which include beverages, snacks, cow's milk supplemented with soy isoflavones, and biscuits, were investigated to study the relation between food processing and allergenicity. The data obtained by RP-HPLC and SDS-PAGE indicated that the 7S and 11S soybean allergens changed due to thermal processing applied to produce instant powdered soymilk, soy snacks, and biscuits (Benavent et al. 2008). In some cases, thermal treatment can decrease the allergenicity. Heat treatment, for example, can cause a reduction of Kunitz trypsin inhibitor, which is considered a soybean allergen (Breiteneder and Ebner 2000). However, other allergens that are stable thermally during processing were not modified easily. For example, in P34, because of the complex structure of epitopes, heating alone cannot reduce the allergen. Application of more than just chemical and heat treatments may help to reduce or denature an allergen. Yamanishi et al. (1995) reported that IgE binding activity of P34 was enhanced when autoclave heat treatment was used. Similarly, their results showed that IgE binding activity significantly increased after

retorting at a temperature of 121.1 °C. Wilson et al. (2008) documented that P34 allergenicity increased after 5 min of boiling but sharply decreased after 60 min of boiling.

There are no general rules about the effect of thermal treatment on allergens. Some food allergens are heat stable, whereas others are unstable during food processing. Also, it is important to note that the change in food allergenicity is based on the type of processing as well as other factors. For example, it was observed that P34 was not found in texturized soy protein after extrusion. Although the thermal energy was low for texturizing, P34 could not be found in the product. Hence, it can be assumed that there are other factors responsible for the disappearance of the allergen in a texturized soy protein after extrusion (Franck et al. 2002).

Pulsed Light (PL)

Pulsed Light is an emerging non-thermal technology and was used for the first time to inactivate a wide range of microorganisms. This technology is known in the scientific literature as pulsed light (PL), pulsed ultraviolet light (PUV), high intensity broad-spectra pulsed light, or pulsed white light (Gomez-Lopez et al. 2007). Pulsed light contains an intense broad spectrum, rich in approximately 54% UV-C, 25% visible light, and 20% infrared light (Yang et al. 2011). Generally, a PL system used during processing contains a power unit, one or more xenon lamp units, and a capacitor in which high-voltage electrical energy is accumulated. During the PL process, energy that is accumulated in a high-power capacitor is released in short bursts (nanoseconds to milliseconds) of light producing several high-energy flashes per second. This technique delivers during the pulse a spectrum 20,000 times more intense than sunlight at the earth's surface (Barbosa-Gustavo et al. 2000). The intensity of these flashes has been

used in the food industry in pulsed or continuous modes. There are two types of PL systems: the batch system and the continuous system. The batch system is used by the University of Florida, and the time, distance, and pulse frequency (1-20 pulses/s) can be adjusted for single samples or groups of samples (Shriver and Yang 2011). Pulsed light was originally performed to decontaminate surfaces by inactivation of microorganisms. The efficiency of PL in inactivation of viruses, molds, and bacteria is well studied and reviewed. Krishnamurthy et al. (2004) documented that PL applied for 5 s was effective to reduce 7 to 8 log CFU/mL of *Staphyloccocus aureus* on suspended and agar-seeded cells without an increase in temperature. The *Aspergillus niger* spore was inactivated by 4.8 log cycles when treated with 5 pulsed-light flashes at 1 J/cm^2 (Wekhof et al. 2001). The mechanism by which PL inactivates microorganisms was explained by photothermal or photochemical effects; both effects can co-occur and lead to the lethal action on microbes. Most of the studies explained the mechanisms of PL based on continuous-wave ultraviolet light (CW UV), in which the photothermal or/and photochemical effects produce structural changes in the DNA of viruses, bacteria, and other pathogens, which prevent cells from replicating.

It is believed that PL treatment has photophysical, photothermal, and photochemical effects in food products. The PL consists of intense broad photonic spectra emitted within a number of nanoseconds from ionized inert gas under high voltage (Chung et al. 2008; Krishnamurthy et al. 2009; Yang et al. 2010; Shriver et al. 2011). Fiedorowicz et al. (2001) reported that prolonged treatment with UV light could affect peroxidation of unsaturated fatty acids, formation of insoluble complexes in food,

crosslinking of carbohydrate, depolymerization of starch, crosslinking of protein, and fragmentation of protein.

UV light and PUV (and PL) are already approved by the FDA to reduce pathogens in different types of food products. In the last few years, PL has been applied to reduce allergens in peanut, soybean, and shrimp. Specifically, the soybean study by Yang et al. (2010) showed that PL was effective in reducing soybean allergens when applied for up to 6 min with 3 pulses per second. The results, which were obtained by the SDS-PAGE method, showed a significant reduction in glycinin (14-34 kDa) and β-conglycinin (50 kDa). In contrast, soybean proteins of higher molecular weights (45-75 kDa) were affected only slightly. The ELISA results showed a notable reduction in IgE binding after 6 min of PL treatment. In another study, peanut protein extracts and liquid peanut butter were treated by PL, and the results demonstrated that there was a reduction in the IgE binding up to 6- to 7-fold compared to the control (Chung et al. 2008). Shriver et al. (2010) treated shrimp protein extracts with PL for 4 min and reported a notable reduction in tropomyosin, which is a major shrimp allergen. A slight allergen reduction occurred when the samples were boiled to 100 °C. The authors attributed the shrimp allergen reduction by PL treatment to conformational changes of tropomyosin. Anugu et al. (2009) found that PL reduced the allergen levels in milk, and the milk allergens casein and whey were undetectable by SDS-PAGE after treatment of the sample by PL for 150 s. ELISA results illustrated that IgE binding was reduced 7.7-fold for whey protein and 7.4-fold for α-casein. Further studies on egg protein extracts showed that PL was effective in reducing egg allergens as determined by SDS-PAGE.

All IgE binding to major allergens was undetectable after 2 min of PL treatment (Anugu et al. 2010).

Power Ultrasound

The application of ultrasound in food processing started in the years preceding the Second World War. In recent years, the majority of the applications were aimed at quality assessment. For example, ultrasound technology has been used to detect the foreign bodies in food. There are two types of ultrasound that can be used in food processing: 1) low-frequency, high-intensity, or power ultrasound (PU) in the kHz range and 2) high-frequency, low-intensity diagnostic ultrasound in the MHz range. Power ultrasound uses mechanical waves within frequencies of 20-100 kHz, and the high-energy waves promote formation of sonication bubbles in foods that undergo compression and intermittently rarefaction. At the end, the bubbles collapse at critical sizes under pressure of up to 1,000 atm and at a temperature of 5,000 K (Feng et al. 2009).

The early applications of ultrasound were for emulsification. The emulsions formed by ultrasound are more stable than emulsions produced conventionally (Mason et al. 1996). Recent applications of ultrasound have been used to reduce the chemical (lye) peeling in tomato (Rock et al. 2012), to increase the extractability of protein for allergen detection (Albillos et al. 2011), to remove surface compounds and extend the shelf life of potato chips (Wambura and Yang 2009), and to reduce the allergens in shrimp (Lie et al. 2006). Recently, researchers have paid more attention to the reduction of allergens by non-thermal treatments. However, few publications can be found in the literature on this area, and none has applied PU for soybean allergen reduction. Lie et al. (2006) used PU to alter the structure of shrimp proteins and studied the effect on the

IgE binding capacity of Pen 1 (tropomyosin), which is a major allergen in shrimp. The samples were treated with PU for 30-180 min. Extracts of treated samples were analyzed by ELISA with pooled serum from patients with shrimp allergy, by polyclonal anti-allergen antibodies, and by immunoblotting after SDS-PAGE. The results showed that PU could reduce the allergenicity of shrimp. Li et al. (2011) studied the effect of PU on the allergenicity and texture properties of shrimp. The raw and boiled shrimp were treated with PU (30 kHz, 800 W) at 0 and 50 °C for 2, 8, 10, and 30 min. The results indicated that PU had a significant effect on the allergenicity of the boiled shrimp when compared with raw shrimp. The data also indicated that PU could be used to reduce the allergens in shrimp without changes in texture.

Genetically-Modified Soybean

Genetically modified plants have become more important because of the modifications that improve yield under environmental challenges. This technology was considered revolutionary in the early stages of development and application. It is a powerful tool that comprises a set of modern biological techniques to manipulate an organism's genetic endowment or introduce a specific gene (Batista and Oliveira 2008). Genetically modified plant or food is the common term in the media and in some of the scientific publications. To be precise, transgenic plant/food should be the scientific term that refers to this technology. Flavr Savr tomato, produced by the Calgene Company, was the first genetically modified food approved for human consumption. The product was brought to a limited market in 1994 in the United States after approval by the FDA to ensure there was no evidence for changes in the nutritional value or health risks (Smith et al. 1990; Talyer 1997).

The History of Genetic-Modification Soybean

The first trait to be introduced in soybean by means of genetic engineering was herbicide resistance to glyphosate and glufosinate. The plant acquires glyphosate resistance through the action of a genetically introduced enzyme of bacterial origin (Thompson et al. 1987). Glyphosate (N-phosphonomethyl glycine) inhibits the 5-enylpyruvylshikimate-3-phosphate synthase (EPSPS) of plants and certain bacteria. This enzyme initiates a key step in the synthesis pathway of aromatic acids, hormones, plant metabolites, lignins, and other phenolic compounds (Dill 2005). Glufosinate (DL-homoalanin-4-yl methylphosphinic acid) inhibits glutamine synthase, which is required for the conversion of L-glutamic acid to L-glutamine in the presence of ammonia. If this pathway is blocked in plants, the result is toxic ammonia levels and cell death (Duke 1990). Monsanto and Bayer Crop Science have commercially developed and launched genetically modified glyphosate and glufosinate resistant soybean lines, respectively, both of which have been approved for human and animal consumption. Since its first commercial cultivation in the United States in 1996, Roundup Ready soy developed by Monsanto is still the principal biotech crop at global scale. The total herbicide resistant genetically modified (GM) soybean area is cultivated on 73.3 million hectares, representing 50% of the total area of GM crops in the top-ten biotech crops. Countries that are growing GM soy are the United States, Brazil, Argentina, Paraguay, Uruguay, Bolivia, Mexico, Chile, and Costa Rica. In Paraguay and Bolivia, GM soy accounts for the total area of GM crops (James 2010).

Herbicide tolerance is the only GM soybean trait that has been commercialized to date. Only three different lines of genetic modification can be found worldwide: Roundup Ready soy, Roundup Ready 2 soy, and Liberty Link soy. Roundup Ready soy and

Roundup Ready 2 soy (Monsanto Company) are both resistant to glyphosate, and Liberty Link soy (Bayer Crop Science) is resistant to glufosinate. Eleven additional GM soybean lines have received regulatory approval in at least one country and are ready for commercialization. Among these are the first GM soybeans modified to increase the value for industry by having an increased oleic acid content, and they were expected to be ready for global use by 2012 (Plenish and Dupont). Three other lines (Optimum GAT, Cultivance, Bt Roundup Ready 2 Yield) also are expected to be commercially launched within the next two to five years (Dupont, BASF Plant Science, and Monsanto Company). Other GM soybean lines like 260-05 (Dupont), W62, W98, GU262 (Bayer Crop Science) already have obtained regulatory approval but have not yet been commercialized (James 2010; Stein and Rodriguez-Cerezo 2009).

Genetic Modified Soybean and Food Allergies

As a biological method, genetic modification has gained much attention despite its controversial nature. It prevents the translation of selected allergens using posttranscriptional gene silencing or co-suppression (Shewry et al. 2001). Herman et al. (2003) used transgene-induced gene silencing to prevent the accumulation of *Gly m* Bd 30 K (or P34) protein in soybean seeds. It was found the *Gly m* Bd 30 K-silenced plants and their seeds lacked any compositional, developmental, structural, or ultrastructural phenotypic differences when compared with control plants. The silencing of *Gly m* Bd 30 K almost completely inhibited IgE binding. Experimental evidences by electron microscopic–immunocytochemical assays, two-dimensional protein analysis, and tandem mass spectrometric identification all indicated no significant differences between the modified soybean and the control with respect to structural morphology and protein composition. Furthermore, comparative testing showed that no new

allergens were formed during silencing. Genetic modification is still under scrutiny regarding the stability of hypoallergenic foods produced by it; therefore, food safety risks exist with foods genetically modified for allergen silencing. Also, the allergen removal process may entail the alteration of the foods' functional and physical properties (Shewry et al. 2001), because many allergenic proteins are parts of plant development and metabolism. Genetic modification of IgE epitopes instead of the protein fraction may be a better option to reduce allergens. The combination of physical (novel technologies), chemical, and biological techniques may be an effective approach to maximize the alleviation of allergenicity and minimize the change in functionality.

Advantages and Disadvantages

The main purpose of genetically modified crops was to ensure that food would be secure in the future, especially in developing countries where there is a lack of adequate food supply for the coming generations. One of the common purposes of genetic modification of crops is to protect against pests, and this leads to improved yields and reduces the use of chemical pesticides. A good example of GM crops produced by Monsanto is Bt cotton, which was designed to protect against key Lepidopteran insect pests. It works by controlling a protein (Cry 1Ac) that is derived from a naturally found soil bacterium, *Bacillus thuringiensis* subsp (Betz et al. 2000). GM crops designed to be resistant to herbicide are very powerful in helping to reduce the amount of herbicide used and prevent damage to the environment. Monsanto also created the herbicide known as glyphosate or Roundup, and this company has a genetically modified strain of soybean that is not affected by glyphosate (Byrne 2004).

It is important to note that great attention has been given to improve and change the nutritional profiles of foods that contribute to positive health benefits, which include increasing β-carotene, flavonoids, calcium, and iron availability (Lemaux 2008).

Despite the advantages of genetically modified crops, the general public remains largely unaware of the specifics of genetic modification as a new technology. However, two main areas of concern have emerged. First, there is concern about the risk to the environment, and second, there is concern in regard to human health. In the last few years, researchers have focused on the toxicity of GM foods. Seralini et al. (2007) studied the toxicity of transgenic corn MON 863 under the responsibility of Monsanto Company, and the data indicated that after animals' consumption of MON 863 for 90 days, there was a 3.3% decrease in weight for males and 3.7% increase for females. In addition, signs of hepatorenal toxicity were noticed, and the triglyceride count increased by 24-40% in females. Based on the data, GM corn MON 863 was not safe to consume. However, regulatory agencies and researchers did not agree with the results, and there was an argument regarding the interpretation and statistical analysis of data (http://www.ensser.org/democratising-science-decision-making/ensser-comments-on-seralini-study/).

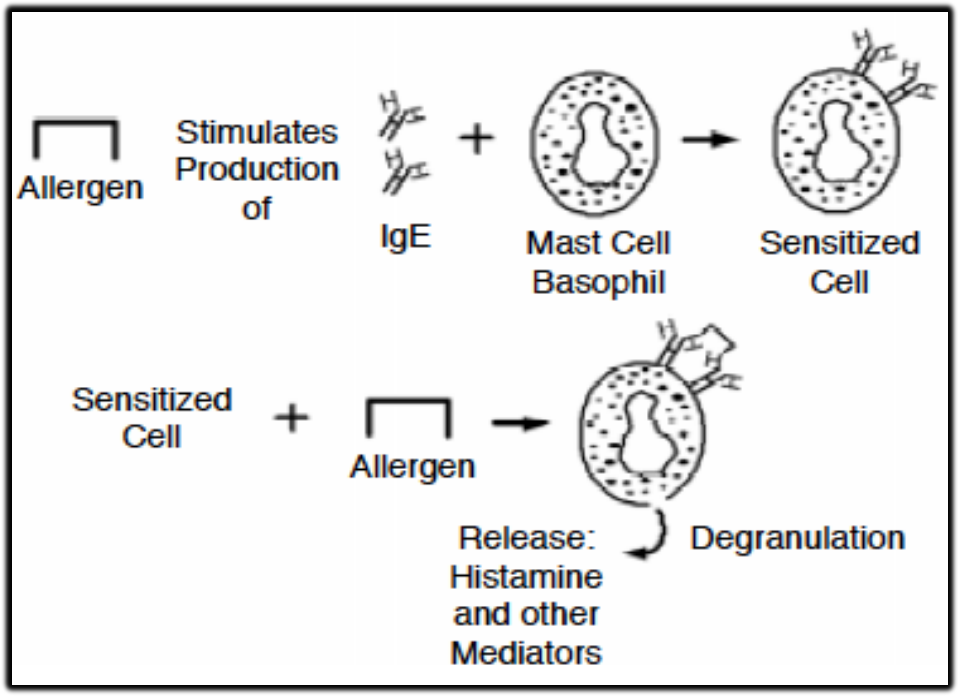

Figure 2-1. Mechanism of IgE-mediated allergic reaction (Taylor and Hefle 2001).

CHAPTER 3
THE EFFECT OF PULSED LIGHT ON ALLERGIC POTENCY OF SOYMILK

Background

Soymilk is considered a high-quality protein source in Eastern countries. It is defined as the aqueous extract of soybeans and has a composition and physical appearance similar to dairy milk. Soymilk, a turbid colloidal dispersion, is prepared by heating, swelling, and grinding soybeans (Giri and Mangaraj 2012). It contains a high quantity of protein (2-4%), carbohydrate (2%), lipid (2%), and total solids (8-10%) similar to the original composition of the soybeans and of the soy pulp (okara) that is obtained after separation from the slurry. The main storage proteins 7S (β-conglycinin) and 11S (glycinin) constitute about 30 and 40%, respectively, of the total soybean proteins (Nik et al. 2008).

Although several health benefits are attributed to soymilk and soybean products, there is a major issue related to soybean allergens. Soybean is considered as one of the most allergenic foods, which include wheat, crustacea, eggs, fish, peanut, milk, tree nuts, and soybeans (Tayler and Hefle 2001). The threshold level to trigger an allergic reaction is very low at about 0.0013 to 500 mg of soy protein (Song et al. 2008).

Thermal treatment during soymilk preparation is a step that facilitates food safety, extension of shelf life, and inactivation of undesirable biological compounds, such as lipoxygenase and trypsin inhibitors (Lakshmanan et al. 2006). Thermal treatment provides several quality benefits, such as enhanced flavor and texture; however, it also causes major changes in allergenicity. Thermal treatment of legumes may eliminate or enhance their allergenicity. It was reported that microwaving as well as boiling for 120 min could eliminate the immunoreactivity of proteins in soybean, whereas

thermal treatment at various times and temperatures did not alter the immunoreactivity of soybean and peanut protein extracts (Burks et al. 1992).

Non-thermal treatment has negligible effects on potential properties of food quality and thus is seen as a favorable technology in food processing. Recently, several non-thermal treatments have been used for food allergen control; these include pulsed light (PL), high-intensity ultrasound, and gamma irradiation. A good example of non-thermal treatment is PL, which is an emerging technology that has evolved very quickly in food processing to minimize the use of thermal processing.

During the past few years, it has been reported that PL reduced the allergenicity of peanut, soybean, almond, and shrimp. For soybean, Yang et al. (2010) showed that PL was effective at reducing soybean allergens (glycinin and β-conglycinin) for treatments up to 6 min with 3 pulses per second. There is little literature published on applying non-thermal treatments, specifically PL, to reduce allergens in soymilk. Therefore, this study was conducted.

Material and Methods

Materials

Certified organic soybean seeds were purchased from wheatgrass kits. Low fat soymilk (West soy), Tris buffered saline (TBS) 0.05% (v/v), 96-well microtiter plates, defatted cotton sheet, Tween 20 (Sigma Chemical Co., St. Louis, MO, USA), and goat anti-human IgE conjugated to horseradish peroxidase (HRP) **(**Invitrogen, Carlsbad, CA, USA) were used. Furthermore, Tris-glycine gels (4-10%), gel electrophoresis apparatus, Coomassie Plus (Bradford) Protein Assay **(**Pierce Chemical Company, Rockford, IL, USA), bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA), GelCode Blue Stain Reagent, nonfat dry milk (local grocery store), *o*-phenylenediamine

dihydrochloride (OPD) and SuperSignal West Pico Chemiluminescent (ECL) substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) were obtained. Plasma from 4 donors with documented soybean allergy and negative soybean allergy (Plasma Lab International, Everett, WA, USA) were acquired for testing.

Preparation of Soymilk

Soymilk was prepared according to the methods described by Shun-Tang et al. (1997) with slight modification. Soybeans were soaked in deionized water overnight at 4 °C. The swollen beans were ground in deionized water (w/v 1:7) and blended using a kitchen aid blender (St. Joseph, Michigan, USA). The homogenate was filtered using a defatted cotton sheet. The resulting raw soymilk was heated in a water bath at 80 °C for 10 min and then quickly cooled to approximately 24 °C. The soymilk was divided in small tubes and kept at -20 °C until used for further experimentation.

PL Treatment of Soymilk Samples

Pulsed light was operated at 3 pulses per second using a Xenon LH 840 LMPHSG PL sterilizer (Xenon Corp., Woburn, MA, USA) (Figure 3-1). Samples were treated at 11 cm from the light source for 0, 0.5, 1, 2, 4, and 6 min (three replications each). The temperature (°C) of the samples was measured in the untreated and treated samples with an infrared thermometer (Omega OS423-LS, Omega Engineering, Inc, Stanford, CT, USA), recorded before and after treatment, and reported as an average of the triplicate samples. In addition, each sample was weighed before and after treatment to determine percent water loss.

Protein Concentration

Untreated and treated samples were subjected to protein determination using the Bradford assay. Ten mL of each sample (treated and untreated) were added to 300 mL

Bio-Rad Bradford reagent. The absorbance of the mixture was read at 595 nm in a Spectramax 340^{384} (Molecular Devices, Sunnyvale, CA, USA) and quantified using bovine serum albumin as standard.

Determination of IgE Binding to Soybean Samples with Indirect ELISA

Polystyrene 96-well plates were coated with untreated and treated samples (diluted to a protein content of 20 µg/mL) at 100 µL per well in triplicates and incubated overnight at 4 °C. The plates were washed with TBS/0.05% Tween 20 (TBST) and blocked with Starting Block blocking buffer (200 µL per well) at room temperature for 2 h and then washed 3 times with TBST. Pooled human plasma (cap no 24.8 KU/L) from patients allergic to soybean was diluted in phosphate buffered saline (PBS) (1:10), and 100 µL were added to each well. Plasma from patients with negative soybean allergy served as a negative control. The plate was incubated for 1 h at room temperature and then washed with TBST. Secondary antibody, anti-human IgE HRPO conjugated, was diluted in PBS (1:1000), added to each well (100 µL per well), and incubated for 1 h at room temperature. After incubation, the wells were washed and then treated with 100 µL of substrate peroxide buffer containing OPD substrate (0.5 mg/mL). The absorbance was measured at 450 nm using a Spectramax 340^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA, USA)

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The samples were analyzed by SDS-PAGE according to the protocol described by Chung et al. (2008). After PL treatment, samples with equal amounts of protein in Laemmli sample buffer containing 0.05% 2-mercaptoethanol were heated in a water bath (100 °C) for 10 min. The samples were loaded into (4-10%) Tris-glycine precast gels (Bio-Rad Laboratories, Hercules CA, USA) and subjected to electrophoresis for 1.5

h at 150 V. The gels were stained with GelCode Blue solution, de-stained with water, and scanned using a Canon scanner (Canon Inc., Sunnyvale, CA, USA).

Western Blot Analysis

After separation of the protein by SDS-PAGE, unstained gels were washed with distilled water and then transferred onto an Immun-Blot PVDF membrane at 15 V for 30 min. After the transfer was completed, the membrane was washed with TBST, and the unbound membrane was blocked using 5% nonfat dry milk in 0.01% Tween 20 in TBS buffer for 1 h. The membrane was washed with TBST 3 times and incubated with a pooled patient plasma (cap no 24.8 KU/L), which was diluted in 5% nonfat dry milk in TBS buffer (1:6), for 4 h at room temperature with gentle shaking. After 3 washes with TBST buffer, the membrane was incubated at room temperature for 1 h with a goat anti-human IgE HRP (diluted in TBST at 1:1,000). The membrane was washed again 3 times with TBST for 5 min. Super Signal West Pico Chemiluminescent substrate was used for detection

Statistical Analysis

Statistical analysis was conducted using a one-way analysis of variance (ANOVA) with SAS version 9.0 (Cary, NC, USA) and Tukey's comparison of means. Differences were considered at $\alpha=0.05$. All experiments were conducted in triplicate.

Results and Discussion

Effect of PL on Sample Temperature

Although PL is a non-thermal treatment, heat can be generated during treatment as a result of absorbance of the high energy by the food components. Table 3-1 shows the change in water loss and temperature of soymilk after exposure to PL. All samples showed an increase in temperature over treatment time, and the temperature rose up to

117 °C after 6 min of treatment. Also, a decrease in samples weights by approximately 1-48% after 1 to 6 min of PL treatment was observed. Longer treatment times (8, 11 min) led to reduced sample size and sample burning (data not shown). A similar increase in temperature up to 115 °C was observed by Li et al. (2011) after they treated almond extract for 7 min. The results are in agreement with Chung et al. (2008) and Yang et al. (2010), who reported an increase in sample temperature and decrease in sample weights after PL treatment. Based on the literature and the observations made during the herein described experiments, the following parameters should be considered during evaluation of the results: 1) The temperature was not recorded during the treatment, and the temperature measurement after the treatment was delayed for a few seconds. Li et al. (2011) emphasized that such a delay could affect accuracy of measurements. 2) The distance of the samples from the lamp could change the temperature. For instance, a long exposure and close distance to the lamp caused higher temperatures compared to shorter exposure and farther distance (Wambura and Verghese 2011). 3) In our experiments, we used a sample size of 10 mL. Sample size could affect the penetration of PL, because the intensity of PL in a sample diminishes with increasing distance from the surface (i.e., the larger the sample size, the lower the total intensity of penetration). 4) The number of pulses could change the temperature sensitivity of the sample. Gelling temperature of egg white, for example, was decreased by progressive increases in the number of pulses (Manzocco et al. 2013).

SDS-PAGE of PL-Treated Soymilk Samples

Figure 3-2 shows the SDS-PAGE profile of the untreated and treated soymilk samples. The electrophoretogram shows protein bands ranging from 8-140 kDa. The two major bands observed in the soymilk samples corresponded to the known soybean

proteins 7S (β-conglycinin, 50-72 kDa) and 11S (glycinin, 12-34 kDa). This result is in agreement with Shun-Tang et al. (1997), who reported that unheated soymilk was comprised of 70% glycinin and 30% β-conglycinin. The SDS-PAGE pattern of untreated soymilk samples (lane 2) exhibited high-intensity bands with molecular weights of approximately 8-140 kDa (Figure 3-2). A similar pattern that included 7S and 11S for untreated soybean extract was reported by Yang et al. (2010). Both 7S and 11S are considered major allergens in soybean (Wilson et al. 2005; Vorlova 2011). In the present study, no obvious changes were seen in the protein bands after heat treatment of the samples for 10 min. Similarly, PL treatment for a short duration (0.5, 1, and 2 min) was not efficient to reduce the major allergens glycinin (11S) (50-72 kDa) and ß-conglycinin (7S) (12-34 kDa). Similarly, the major allergens of almond extracts were not affected by short exposure to PL for 0.5 and 1 min (Li et al. 2011). Unlike soymilk and almond allergens, milk and eggs allergens were documented to be reduced after short PL treatment times (Anugu et al. 2009; 2010). The differences in response to treatment time can be explained by the large variety of food matrix properties. For instance, liquid foods are more susceptible to PL because they allow higher penetration compared to solid foods (Cullen et al. 2004). Exposure to PL for a longer time (4 or 6 min) with an additional step of homogenizing the samples each minute to increase the penetration of PL resulted in an obvious reduction of major protein bands with molecular weights between 55 and 140 kDa (Figure 3-2). According to Yang et al. (2010), PL treatment (4 and 6 min) decreased the level of glycinin and ß-conglycinin in soybean extract, potentially because of increasing temperature causing protein aggregation. In the case of milk protein, PL treatment caused changes in milk protein spectra as a result of

protein aggregation by disulfide bonds without significant changes in conformation of protein structure and amino acid composition (Elmnasser et al. 2008). Recently, Manzocco et al. (2013) showed that PL treatment caused modified egg white protein spectra by aggregation phenomena and protein backbone cleavage, and different egg white proteins differed in their response to PL. A novel band was observed after exposure of white egg protein to PL and was assumed to be a result of protein aggregation (Manzocco et al. 2013). No changes in gel strength and viscosity were reported.

Western Blot

To evaluate the immunoreactivity of untreated and treated soymilk samples, electrophoretically separated extracts were examined for their allergenicity with pooled human sera from patients with soybean allergy. Figure 3-3 shows the western blot of the soymilk samples. Here, the untreated soymilk sample (Lane 2) showed high-intensity bands that corresponded to 7S subunits with approximately 50-90 kDa and 11S subunits with 15-37 kDa. A similar pattern with soybean samples was observed by Song et al. (2008). Heat treatment led to a decrease in the immunoreactivity of proteins (Lane 3, Figure 3-3). Reduction of the major allergens (7S and 11S subunits) was observed mainly in the range of 15-75 kDa. Food processing technologies, such as fermenting and heating, were documented to modify the allergenicity of food protein, and our results agree with Frank et al. (2002), who reported that a 30 kDa protein (Glym Bd 30), which is a major soybean allergen, was absent in texturized soy protein and caused only weak allergenicity in Sojasun soymilk. In contrast, heat treatment did not significantly change the IgE- or IgG-specific binding to soybean protein when the

samples were subjected to room temperature for 1 h, 37 °C for 1 h, 56 °C for 1 h, 100 °C for 5 min, and 100 °C for 1 h (Burks et al. 1992).

It has been reported in the literature that thermal processing may affect the liner structure or conformation of epitopes (Yang et al. 2011). Heat treatments that are used mainly in food processing can alter the structure and reduce or increase the immunoreactivity of allergens. Heat causes protein denaturation and thus reduces the inhibitory capacity of soybean proteases, such as Kunitz trypsin, which is considered a minor allergen. However, the effect of thermal processing cannot be predicted because the mechanism of allergen reduction is based on both the chemical properties and the structure of allergens (Mondoulet et al. 2005). A significant reduction in soybean allergens was observed after treatment of soymilk samples with PL for 0.5, 1, and 2 min (Figure 3-3). Anugu et al. (2009) showed the same reduction of protein allergenicity in milk after they treated the samples with PL for 150 s. Furthermore, Anugu et al. (2010) found that PL was effective in reducing egg allergens and that the major allergens were undetectable after 2 min of PL treatment. It also has been reported that PL was effective in reducing the allergenicity of peanut (Chung et al. 2008; Yang et al. 2011), shrimp extract (Shriver et al. 2010), and wheat extract (Nooji et al. 2010).

In the present study, PL treatment of soymilk samples for 4 and 6 min in combination with homogenization of each sample every minute resulted in a slight increase in 7S and 11S subunits (Figure 3-3). Moreover, 6 min of treatment led to a decrease in small molecular 11S subunits (~15 kDa). During the treatment, it was observed that PL caused a browning layer on the surface of the samples; therefore,

samples were homogenized each minute to increase the penetration of PL and prevent the formation of browning layers.

The mechanisms by which PL modifies the structure of allergens could include photophysical, photothermal, and photochemical effects as well as a combination thereof. Several researchers have suggested that microbial inactivation, for example, is based on photochemical effects (Gómez-López et al. 2007). The question how PL reduces the allergenicity of proteins has not been answered yet. However, most of the authors suggested that the observed increase in temperature over the time of treatment may indicate a photothermal effect of PL. The prolonged exposure to PL can cause an increase in temperature and a decrease in the moisture content of the sample (Yang et al. 2011). In our experiments, the temperature of samples increased to 117 °C during PL, and photothermal effects may have caused protein modification and alteration of the conformation or liner structure of epitopes so that the allergens were not recognized by IgE antibodies. Wall (2003) demonstrated that heat treatments could disorganize protein structure and thus cause protein denaturation and formation of aggregates. In general, thermal processing can unfold the secondary structure of proteins (at 55-70 °C), cause cleavage and rearrangement of disulfide bonds (at 70-80 °C), and induce the formation of aggregates (at 90-100 °C).

Results from the presented experiments showed that PL was more effective in reducing β-conglycinin than glycinin in soymilk. This may be explained by the large number of SH groups in glycinin, which prevent unfolding of the protein (Yada 2012). More energy may be required to change the protein structure of glycinin and dissociate the disulfide bonds.

Indirect ELISA

To confirm the results of western blot analysis, ELISA was conducted on pooled plasma from individuals allergic to soybean and from individuals with no allergy to soybean (negative control). IgE binding to the untreated soymilk exhibited significantly (p<0.05) higher immunoreactivity with test plasma compared to the negative control (Figure 3-4). Heat-treated samples showed a significant (p<0.05) increase in allergenicity against test plasma compared to untreated soymilk samples. In addition, ELISA results showed that treatment of soymilk with PL for 0.5, 1, 2, and 4 min did not reduce the allergenicity compared with untreated soymilk. In contrast, samples treated with PL for 6 min showed a significant reduction in allergenicity compared to untreated soymilk samples. These results differed from those obtained with western blot analysis, which showed a noticeable reduction of allergenicity after short treatment times (0.5, 1, and 2 min). This discrepancy between the results suggests that PL was inefficient to reduce the minor allergens of low molecular weight (<10 kDa), which were not detected by western blot analysis. Wilson (2005) documented that soybean contains low-molecular-weight allergens, such as hull proteins (7-8 kDa). No data that documents the presence of allergens with high molecular weight (>72 kDa) in soybean was found in the literature.

The Effect of PL on Commercial Soymilk

For further investigations, the effect of PL on a commercial soymilk product was tested. Figure 3-5 shows the SDS-PAGE and western blot profiles of the treated and untreated commercial soymilk samples. The SDS-PAGE profile showed similar protein patterns with bands ranging from 8-100 kDa in untreated, heat-treated, and PL-treated commercial soymilk. However, the bands of PL-treated samples had lower intensity

than those of untreated and heat-treated samples. The western blot result showed not only a reduction in β-conglycinin (49-75 kDa) and glycinin (10-25 kDa) but also the presence of two obvious bands that could correspond to P34 (~34 kDa) and glycinin subunits (~20 kDa) in samples after PL-treatment. It is known that certain soybean allergens, including P34, are heat resistant; because of the complex structure of epitopes in P34, heat alone will not denature the protein effectively to reduce the allergenicity (Wilson et al. 2005). The ELISA results showed a significant (p<0.05) increase in IgE binding to the heat-treated and PL-treated samples compared to the untreated samples (Figure 3-6). Binding to the untreated samples was significantly lower (p<0.05) in the negative control (plasma from patients without soybean allergy) than in the positive control (plasma from patients allergic to soybean).

Comparison of the results of western blot analyses showed that a short PL treatment (1 or 2 min) was more effective in reducing allergenic proteins in prepared soymilk than in commercial soymilk. The process of commercial soymilk production includes thermal treatment to assure food safety and extend shelf life of the product and may result in the presence of proteins that are less sensitive to PL treatment. Another possible explanation for the observed differences could be that the variety of soybean cultivar that was used to prepare the soymilk was not the same as that in the commercial soymilk. Finally, the extraction efficiency of our method likely differed from that of commercial soymilk production and may have resulted in a different composition of protein compounds.

Figure 3-1. Pulsed light system (Xenon Corp. Woburn, MA, USA) at the University of Florida. Image courtesy of author.

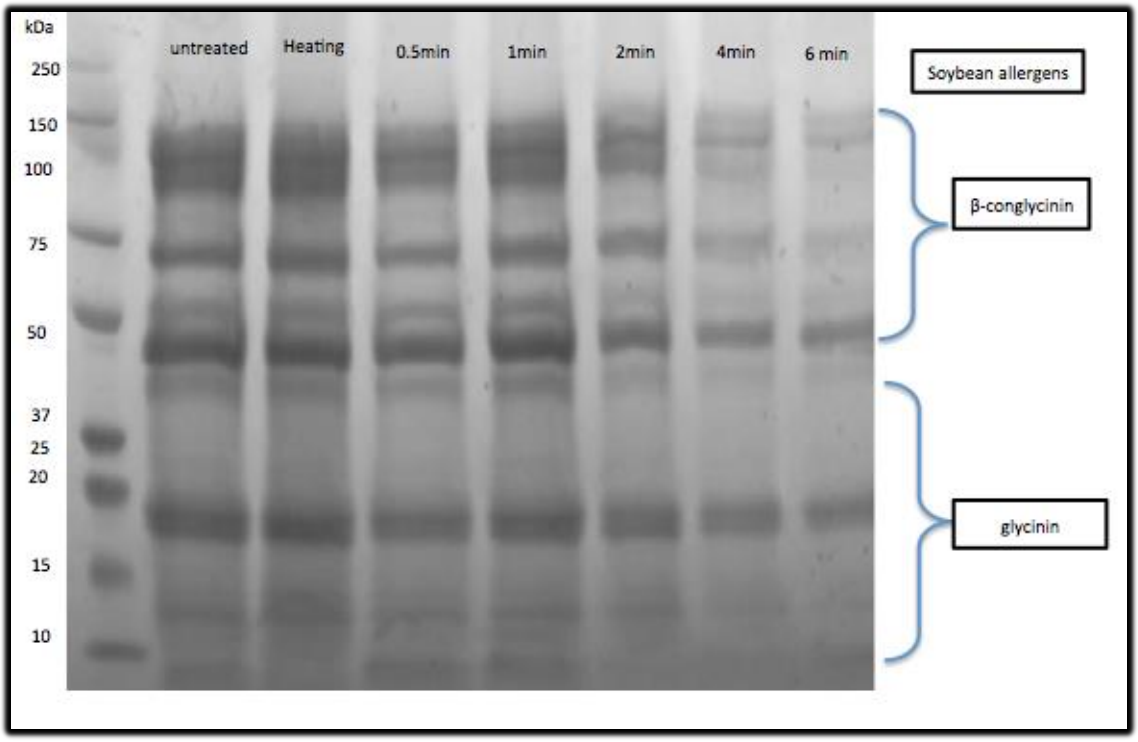

Figure 3-2. SDS-PAGE profile of soymilk samples treated with pulsed light for 0.5, 1, 2, 4, and 6 min, compared to untreated and heated samples. Molecular weights are expressed in kDa**.**

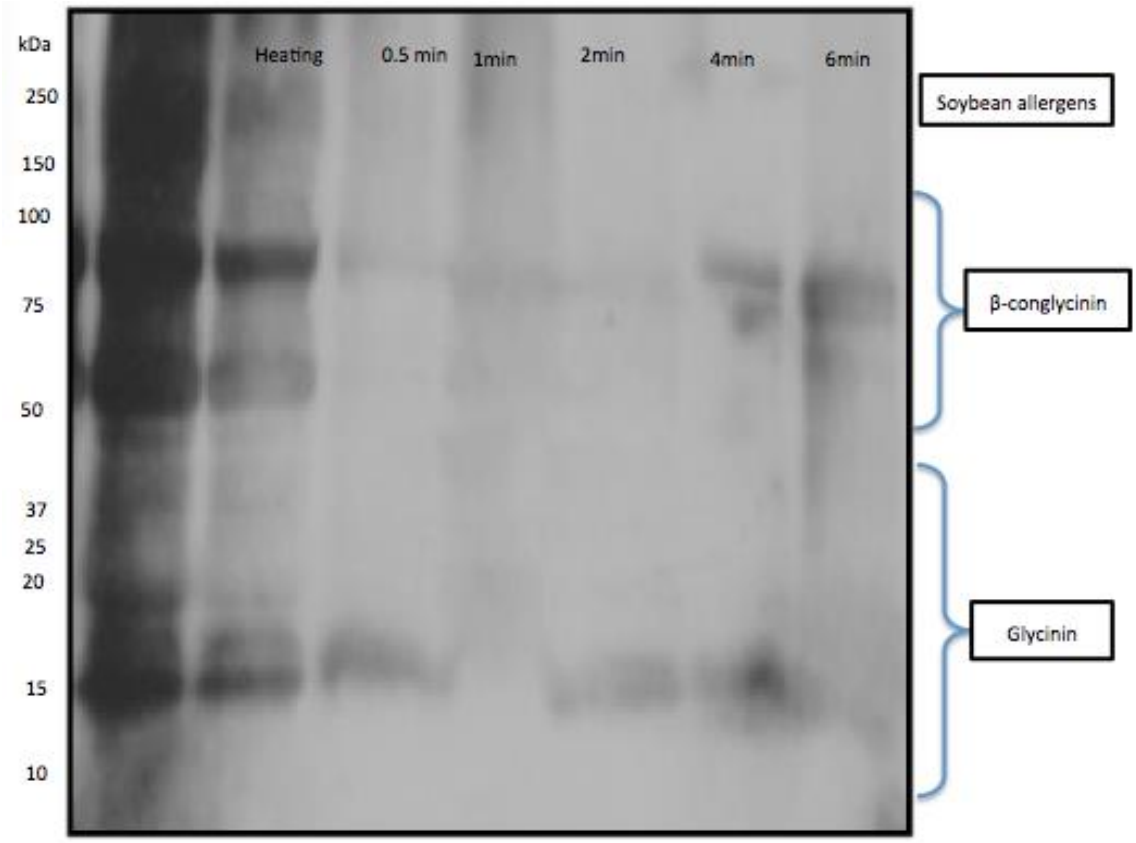

Figure 3-3. Western blot profile of soymilk samples treated with pulsed light for 0.5, 1, 2, 4, and 6 min, compared to untreated and heated samples. Molecular weights are expressed in kDa.

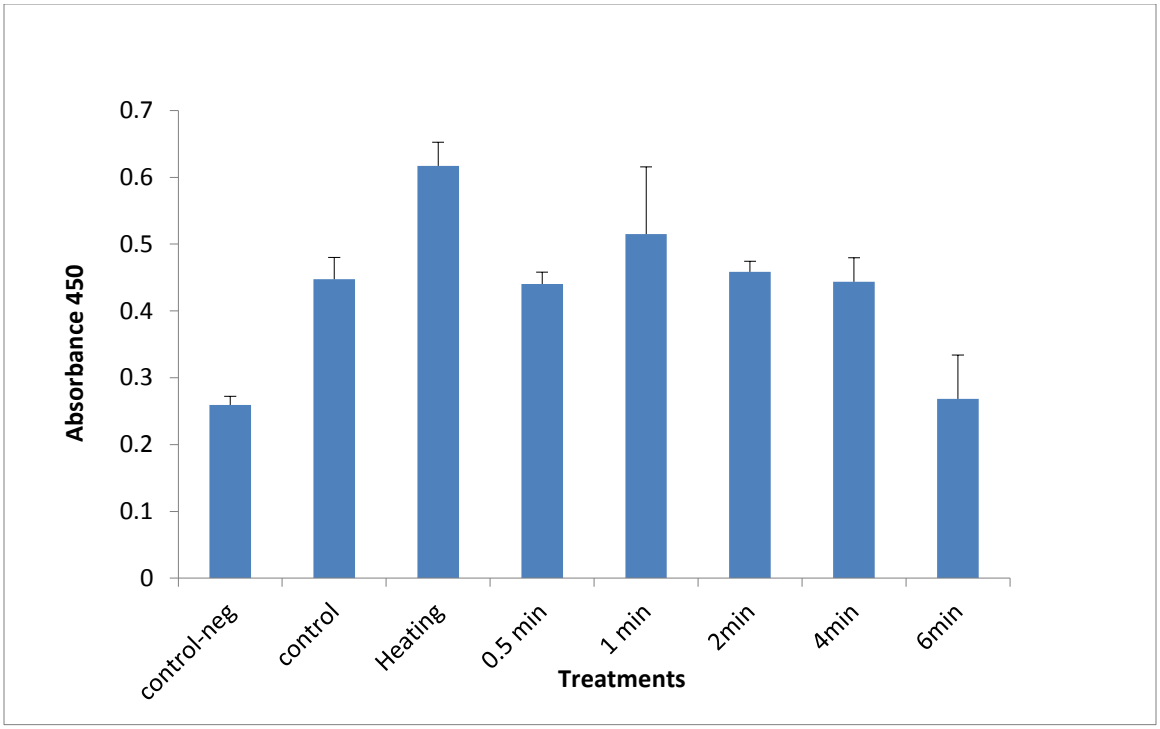

Figure 3-4. Results from indirect ELISA conducted with soymilk samples treated with pulsed light for 0.5, 1, 2, 4, and 6 min, compared to untreated (control) and heat-treated samples. ELISA was performed with human plasma from patients allergic to soybean, and normal plasma (from humans without soybean allergy) was used as a negative control (control-neg). The values show the mean absorbance of three replicates with standard deviation.

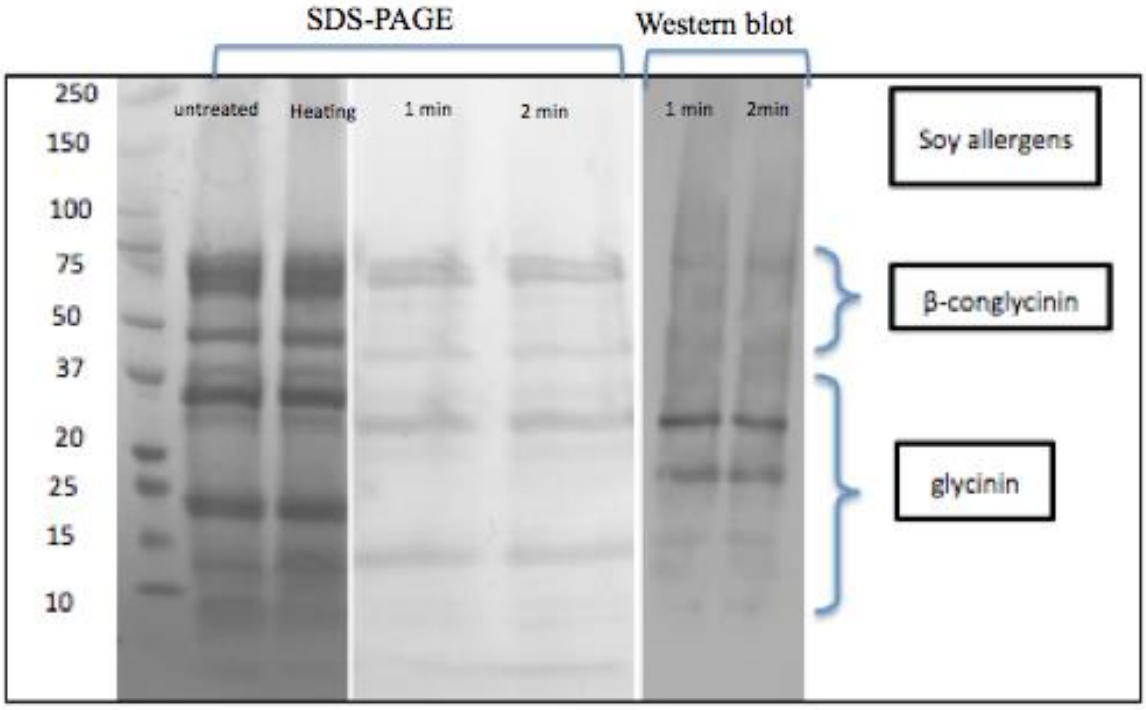

Figure 3-5. SDS-PAGE and western blot profiles of commercial soymilk treated with pulsed light (PL) for 1 and 2 min, compared to untreated and heated samples. Molecular weights are expressed in kDa**.** Lane (1) SDS-PAGE of untreated soymilk. Lane (2) SDS-PAGE of heated soymilk. Lane (3) SDS-PAGE of PL-treated (1 min) soymilk. Lane (4) SDS-PAGE of PL-treated (2 min) soymilk. Lane (5) western blot of PL-treated (1 min) soymilk. Lane (6) western blot of PL-treated (2 min) soymilk.

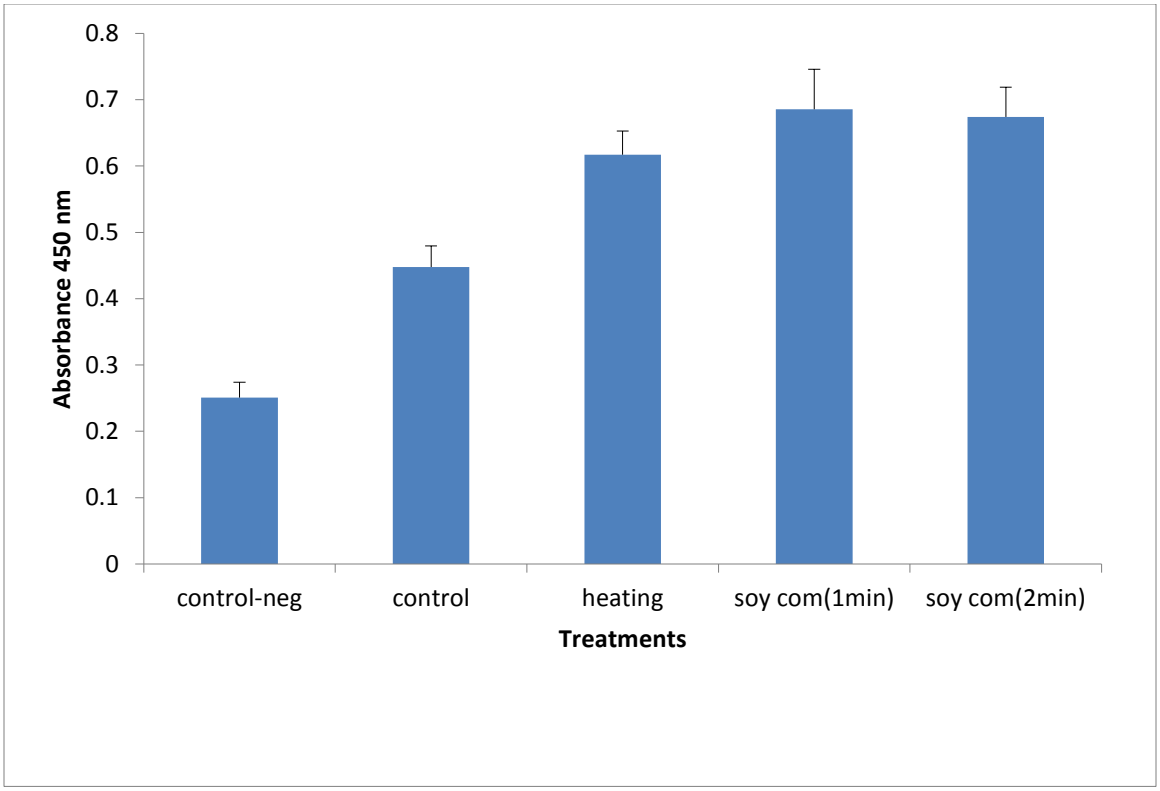

Figure 3-6. Results from indirect ELISA conducted with commercial soymilk samples treated with pulsed light for 1 and 2 min, compared to untreated (control) and heat-treated samples. ELISA was performed with human plasma from patients allergic to soybean, and normal plasma (from humans without soybean allergy) was used as a negative control (control-neg). The values show the mean absorbance of three replicates with standard deviation.

Table 3-1 Change in temperature and water loss of soymilk after PL treatment

Treatment time (min)	Water loss (%)	Initial temperature (°C)	Final temperature (°C)
0.5	1	16	51
1	3	17	55
2	12	17	93
4	31	17	113
6	48	16	117

CHAPTER 4
THE APPLICATION OF POWER ULTRASOUND TO REDUCE THE ALLERGENS OF SOYBEAN EXTRACT

Background

In soybean, 7S (β-conglycinin) and 11S (glycinin) are the main storage proteins and are considered the major allergens (Vorlova 2011). According to Yang et al. (2011), a total of 34 soybean allergens have been identified in the allergen online database. Most of the allergens have been shown to bind to immunoglobulin E (IgE) from sera of soybean-sensitive patients.

Conventionally, thermal treatment is used to eliminate the pathogens in food products to enhance their safety. Thermal treatment also may eliminate the immunoreactivity of most allergens. Although there is a chance to reduce the allergenicity of soybean and soybean products during thermal treatment under certain conditions, there is an equal chance to increase it (Davis et al. 2001). Some of the soybean allergens are heat resistant and can be changed during food processing (Penas et al. 2011). The soybean hull allergens are a good example: Although fresh soybean hulls showed a weak allergenicity, soybean samples induced asthma after exposure to heat under storage and transportation conditions (Davis et al. 2001). In general, it is believed that thermal treatment can reduce some of the allergenicity of some foods (Wale 2003), but there is no general rule because of the variable consequences of heat exposure on allergenicity.

Recently, the following non-thermal treatments have been used for food allergen control: pulsed ultraviolet light, gamma irradiation, high-intensity ultrasound, and high hydrostatic pressure (Li et al. 2006; Yang et al. 2010; Penas et al. 2011).

Power ultrasound (PU) has been applied in food processing to improve mixing, drying, extraction, and homogenization (Li et al. 2004). Recently, PU has been used to reduce the chemical (lye) peeling in tomatoes (Rock et al. 2012), to increase the extractability of protein for allergen detection (Albillos et al. 2011), to remove surface lipids from and extend the shelf life of potato chips (Wambura and Yang 2009), and to reduce allergens in shrimp (Lie et al. 2006). Although a few publications have focused on the reduction of allergens by non-thermal treatments, none has dealt with the effect of PU on soybean allergens.

The main objective of this study was to evaluate the effects of PU treatment on the allergenicity and IgE binding of soybean extracts and to determine the optimal conditions to eliminate the IgE binding.

Materials and Methods

Materials

Certified organic soybean seeds were purchased from wheatgrasskits. Low fat soymilk (West soy), Tris buffered saline (TBS) 0.05% (v/v), 96-well microtiter plates, defatted cotton sheet, Tween 20 (Sigma Chemical Co., St. Louis, MO, USA), and goat anti-human IgE conjugated to horseradish peroxidase (HRP) **(**Invitrogen, Carlsbad, CA, USA) were used. Furthermore, Tris-glycine gels (4-10%), gel electrophoresis apparatus, Coomassie Plus (Bradford) Protein Assay **(**Pierce Chemical Company, Rockford, IL, USA), bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA), GelCode Blue Stain Reagent, nonfat dry milk (local grocery store), *o*-phenylenediamine dihydrochloride (OPD) and SuperSignal West Pico Chemiluminescent (ECL) substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) were obtained. Plasma from 4 donors

with documented soybean allergy and negative soybean allergy (Plasma Lab International, Everett, WA, USA) were acquired for testing.

Sample Preparation

Raw soybeans were ground and defatted in hexane (w/v 1:4). The defatted soybeans (1 g) were stirred with 10 mL 0.01 M phosphate buffered saline (PBS) pH 7.4 for 30 min at room temperature. The untreated and PU-treated samples were centrifuged (Mini centrifuge, Fisher Scientific, USA) at 2,200 *g* for 10 min.

Power Ultrasound Treatment of Soybean Extract

The samples were treated with PU at 20 kHz and 1,500 W (Sonics and Materials Inc., CT, USA). The ultrasonic system consisted of an air-cooled converter (CV 294), fabricated with piezoelectric lead zirconate titanate (PZT) crystals and a standard autoclavable titanium alloy probe (Tl-6Ai-4V, 2.5 cm diameter and 25 cm length (Figure 4-1). Samples were placed in 400 mL aluminum beakers and treated for 2, 4, 6, 8, 10, 12, and 15 min.

Protein Concentration

Untreated and treated samples were subjected to protein determination using the Bradford assay. Ten mL of each sample (treated and untreated) were added to 300 mL Bio-Rad Bradford reagent. The absorbance of the mixture was read at 595 nm in a Spectramax 340^{384} (Molecular Devices, Sunnyvale, CA, USA) and quantified using bovine serum albumin as standard.

Determination of IgE Binding to Soybean Samples with Indirect ELISA

Polystyrene 96-well plates were coated with untreated and treated samples (diluted to a protein content of 20 µg/mL) at 100 µL per well in triplicates and incubated overnight at 4 °C. The plates were washed with TBS/0.05% Tween 20 (TBST) and

blocked with Starting Block blocking buffer (200 µL per well) at room temperature for 2 h and then washed 3 times with TBST. Pooled human plasma (cap no 50 KU/L) from patients allergic to soybean was diluted in phosphate buffered saline (PBS) (1:10), and 100 µL were added to each well. Plasma from patients with negative soybean allergy served as a negative control. The plate was incubated for 1 h at room temperature and then washed with TBST. Secondary antibody, anti-human IgE HRPO conjugated, was diluted in PBS (1:1000), added to each well (100 µL per well), and incubated for 1 h at room temperature. After incubation, the wells were washed and then treated with 100 µL of substrate peroxide buffer containing OPD substrate (0.5 mg/mL). The absorbance was measured at 450 nm using a Spectramax 340[384] spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The samples were analyzed by SDS-PAGE according to the protocol described by Chung et al. (2008). After PU treatment, samples with equal amounts of protein in Laemmli sample buffer containing 0.05% 2-mercaptoethanol were heated in a water bath (100 °C) for 10 min. The samples were loaded into (4-10%) Tris-glycine precast gels (Bio-Rad Laboratories, Hercules CA, USA) and subjected to electrophoresis for 1.5 h at 150 V. The gels were stained with GelCode Blue solution, de-stained with water, and scanned using a Canon scanner (Canon Inc., Sunnyvale, CA, USA).

Western blot analysis

After separation of the protein by SDS-PAGE, unstained gels were washed with distilled water and then transferred onto an Immun-Blot PVDF membrane at 15 V for 30 min. After the transfer was completed, the membrane was washed with TBST, and the unbound membrane was blocked using 5% nonfat dry milk in 0.01% Tween 20 in TBS

buffer for 1 h. The membrane was washed with TBST 3 times and incubated with a pooled patient plasma (cap no 50 KU/L), which was diluted in 5% nonfat dry milk in TBS buffer (1:6), for 4 h at room temperature with gentle shaking. After 3 washes with TBST buffer, the membrane was incubated at room temperature for 1 h with a goat anti-human IgE HRP (diluted in TBST at 1:1,000). The membrane was washed again 3 times with TBST for 5 min. Super Signal West Pico Chemiluminescent substrate was used for detection.

Statistical Analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) with SAS version 9.0 (Cary, NC, USA) and Tukey's comparison of means. Differences are considered at $\alpha=0.05$. All experiments conduct in triplicate.

Results and Discussion

SDS-PAGE profile of untreated and ultrasound treated soybean extract

Figure 4-2 shows the SDS-PAGE profile of the protein extracts for untreated and treated samples. The electrophoretograms of all samples showed bands that ranged from 10-100 kDa. The known major proteins of soybean were observed and included the bands corresponding to 7S (β-conglycinin, 50-72 kDa) and 11S (glycinin, 12-34 kDa). The SDS-PAGE pattern of untreated samples (lane 2) exhibited high-intensity bands with molecular weights of approximately 90, 70, 50, 40, 28, 20 and 17 kDa, which corresponded to glycinin subunits and β-conglycinin subunits. A band possibly corresponding to the trypsin inhibitor (approximately 20 kDa) also was observed. A similar pattern with the proteins 7S and 11S in non-treated soybean extracts was observed previously by Yang et al. 2010. These proteins are considered major and minor allergens in soybean with molecular weights ranging from 7-71 kDa (Wilson et al.

2005; Vorlova 2011). In this study, it was observed that treatment of soybean extract with PU for relatively long times (8, 10, 12, and 15 min) caused a reduction in the major and minor allergens, specifically the β-conglycinin and glycinin subunits. Although PU is considered a non-thermal treatment, high temperatures and pressures are generated during the treatment, and the extreme temperature can modify and precipitate proteins and may explain why the intensity of bands decreased. Yang et al. (2010) reported a reduction in the intensity of soybean extract protein bands after they treated the soybean extract with pulsed ultraviolet light (PUV), which also is considered a non-thermal treatment. Furthermore, Herrero et al. (2009) have documented that heat may contribute to unfolding and aggregating of proteins thus affecting protein solubility.

Western Blot

To evaluate the immunoreactivity of untreated and treated soybean extract samples, electrophoretically separated extracts were tested for their allergenicity with pooled human sera of patients with soybean allergy. Figure 4-3 shows western blots of the soybean extracts. The two major allergens 11S and 7S were detected in the untreated samples (lane 2). A similar pattern for soybean samples was observed previously by Song et al. (2008). After PU treatment of the samples for 2 and 4 min, a slight reduction in the major allergens occurred, as recognized by the lower intensity of the bands (Figure 4-2). A weak intensity of bands with small molecular weight (<10 kDa) was observed (lanes 2 and 3). A new peptide was detected after treatment for 6 min and could represent P34. The presence of P34 after 6 min of PU treatment could be attributed to the inefficient extraction of protein from the untreated soybean, whereas the PU possibly enhanced the extraction of P34. In addition, a significant increase in protein concentration, which was measured by the Bradford assay, was observed after

treatment of the samples with PU (data not shown). Albillos et al. (2011) suggested that ultrasound treatment could improve the extraction efficiency of protein for allergen detection. In another study, it was reported that PU can improve protein extraction from defatted soy flakes and enhance the protein content of soymilk extracted from soybeans (Karki et al. 2010). A remarkable reduction of allergens was observed after 10 min of treatment, whereas 12 and 15 min had no impact on immunoreactivity (Figure 4-2). Possibly, the major and minor allergens were not recognized by the IgE antibody after long treatment with PU. The application of PU may have changed the conformation structure of the proteins and destroyed some epitopes. As a result, there was no interaction between IgE and protein. The results agreed with those of Lie et al. (2006), who studied the effect of PU on the allergenicity of shrimp. Shrimp samples were treated with PU for 30-180 min and analyzed by ELISA and immunoblotting with pooled serum of patients with shrimp allergy, and a linear relationship was observed between treatment time and decrease in allergenicity. However, the treatment time in our study was shorter than that in the previous study. Li et al. (2011) studied the effect of PU on the allergenicity and textural properties of shrimp. The raw and boiled shrimp were treated with PU (30 kHz, 800 W) at 0 and 50 °C for 2, 8, 10, and 30 min. The results indicated that the PU had a significant influence on the allergenicity of the boiled shrimp compared to the raw shrimp. The data indicated that PU could be used to reduce the allergens in shrimp without changes in texture.

When the ultrasound waves propagated through the soybean extracts examined in the present study, cavitation bubbles were generated, and the collapse of transient bubbles may have caused chemical and mechanical changes in the extracts including

changes in protein structure. Another possible explanation for the observed results is that the increase in temperature during the treatment may have modified protein structure, destroyed existing epitopes, and thus led to significant changes in allergenicity. Wal (2003) indicated that significant modification in protein structure could occur during food processing and mainly at high temperature (100-125 °C). In contrast, Herrero et al. (2009) documented that there were no significant differences in secondary structure of isolated soy protein when the protein was heated at 70, 100 and 200 °C. A reduction in soybean allergens was observed after treatment with pulsed ultraviolet light (PUV), which is a non-thermal treatment (Yang et al. 2010). In that study, an increase in temperature up to 85 °C was observed due to the generation of energy, which was converted to heat. The authors suggested that heat might be a possible cause of protein modification that led to the reduction of soybean allergens (Yang et al. 2010). Sonication treatment of β-lactoglobulin caused structural changes in the molecule and thus affected allergenicity of β-lactoglobulin (Vucinic et al. 2012).

Indirect ELISA

To confirm the results of western blot, ELISA was conducted using pooled plasma from patients allergic to soybean. IgE binding to the untreated soybean extract demonstrated significantly ($p<0.05$) higher immunoreactivity compared to the normal plasma from healthy individuals (Figure 4-4). A slight increase in allergenicity was observed after 2 min of treatment, and it is possible that PU enhanced extraction of soybean protein and caused the release of major and minor allergens. A significant ($p<0.05$) reduction in immunoreactivity was achieved after 10 min of PU treatment, and the results were similar to western blot results. Although major and minor allergens

were not observed with the western blot method, ELISA data showed that there was significantly (p<0.05) higher IgE binding to treated soybean extract after 12 and 15 min compared to normal plasma (negative control). The reason could be that minor soybean allergens, such as soybean hydrophobic protein (7.5 kDa), hull protein (7.0 kDa), and defensin (8.0 kDa), were not detected in western blots but could be seen with the ELISA method, which is based on a different concept of immunoreactivity than western blot analysis. These minor allergens also were recognized on immunoblot by serum IgE of patients allergic to soybean (Vorlova 2011). Also, large molecular weight could not be detected with the western blot method.

Figure 4-1. Power ultrasound system at University of Florida. Image courtesy of author

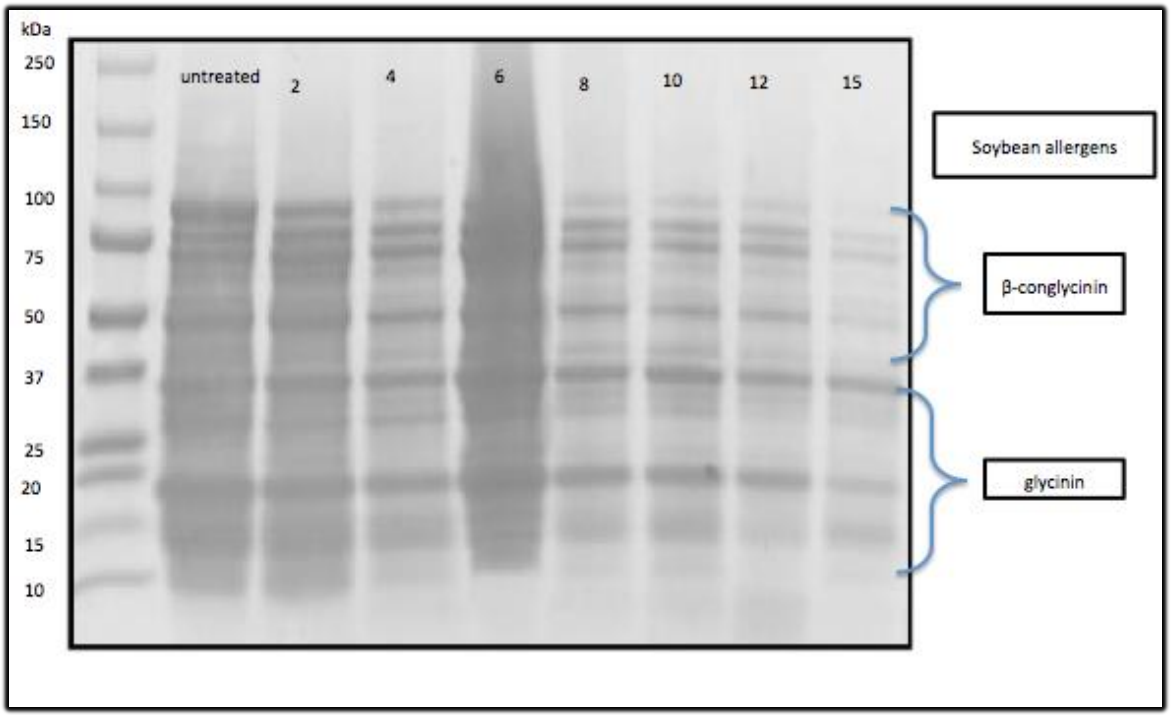

Figure 4-2. SDS-PAGE profile of soybean extracts treated with power ultrasound for 2, 4, 6, 8, 10, 12, and 15 min, compared to untreated extract. Molecular weights are expressed in kDa**.**

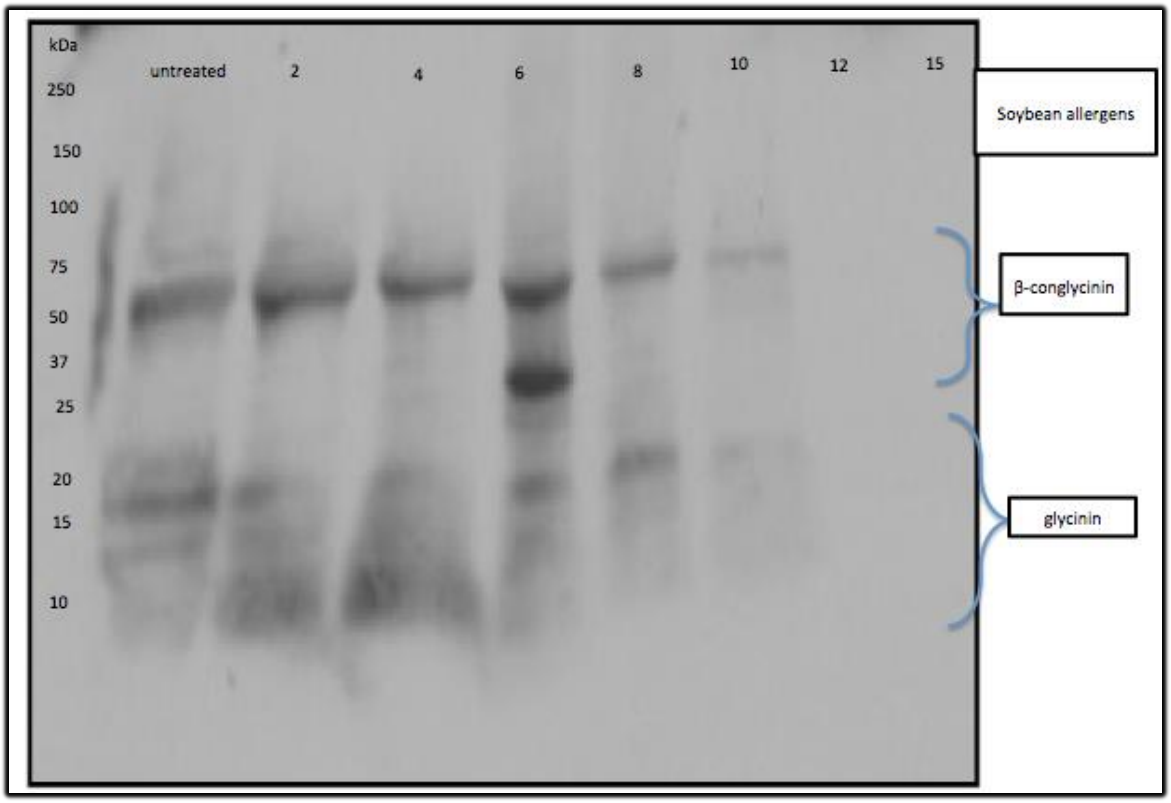

Figure 4-3. Western blot profile of soybean extracts treated with power ultrasound for 2, 4, 6, 8, 10, 12, and 15 min, compared to untreated extract. Molecular weights are expressed in kDa.

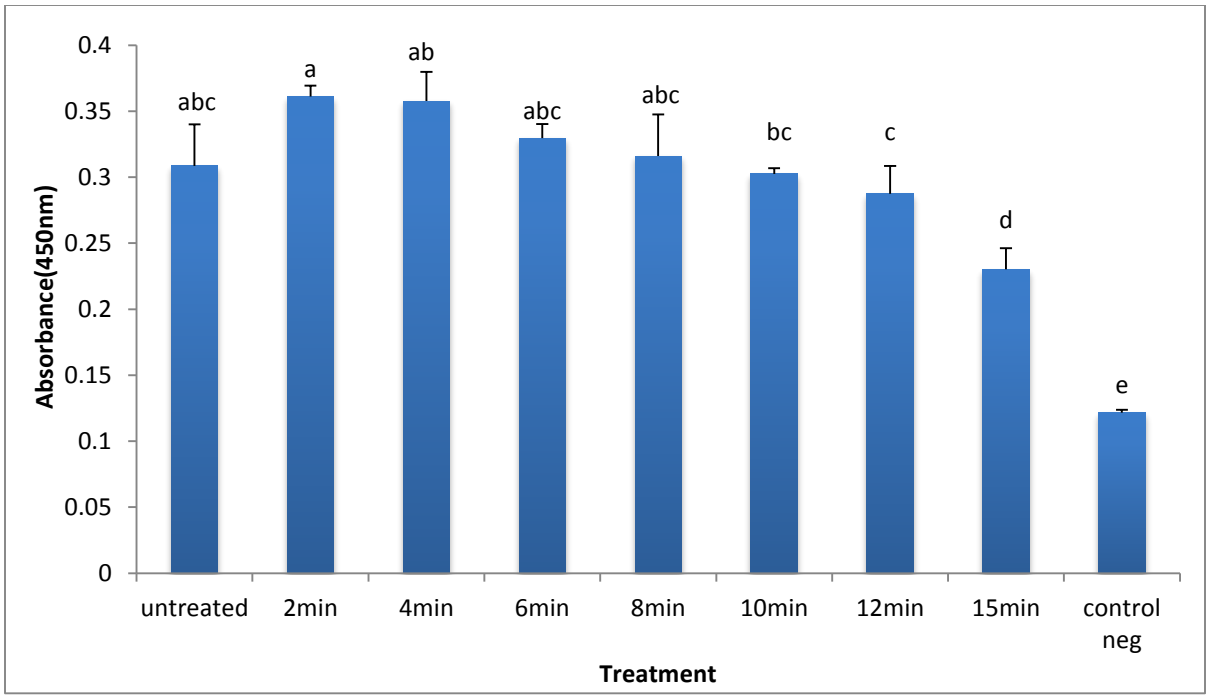

Figure 4-4. Results from indirect ELISA conducted on soybean extracts treated with power ultrasound for 2, 4, 6, 8, 10, 12, and 15 min, compared to untreated samples. ELISA was performed with human plasma from patients allergic to soybean, and normal plasma (from humans without soybean allergy) was used as a negative control (control neg). The values show the mean absorbance of three replicates with standard deviation. Different letters indicate statistically significant differences (ANOVA and Tukey test at $p<0.05$).

CHAPTER 5
THE APPLICATION OF POWER ULTRASOUND AND THERMAL TREATMENTS ON SOYMILK

Background

Power ultrasound (PU) is a growing technology in food processing due to consumers' demand for a high quality of processed food without addition of chemicals. Generally, the application of PU can enhance the extraction of intracellular compounds from plants. In food processing, PU has a wide range of mechanical/physical applications to improve food products that are subjected to various forms of processing, such as cooking, freezing, extraction, and tenderization. The PU is applied at low-frequency ranges of 20-100 kHz with intensity ranges of 10-100 W/cm^2 (Cullen et al. 2004).

Soymilk is widely consumed in Asian countries, and drinking soymilk as a beverage has become more popular throughout the world due to the health benefits of soymilk components. Although soymilk has excellent nutrients crucial for the human body, it contains large amounts of β-conglycinin and glycinin, which are major allergens derived from soybeans (Tezuka et al. 2000). Traditionally, soymilk is subjected to thermal treatment to inactivate spoilage-causing microorganisms and, consequently, to extend the shelf life of the product. Food processing has a significant impact on food allergens by either increasing or decreasing their allergenicity. In the past, many attempts have been made to reduce food allergens by applying non-thermal treatments, such as pulsed light and PU. Moreover, PU was applied to improve the contents of isoflavones in soymilk (Li et al. 2012; Giri and Mangaraj 2012). There are few published research works on reducing allergens by PU; however, none has been published on soymilk.

Therefore, the aim of this study was to investigate the effects of PU on soymilk allergens by performing immune assays including western blot and ELISA and by comparing the results to the effects of thermal treatments.

Material and Methods

Certified organic soybean seeds were purchased from wheatgrasskits. Low fat soymilk (West soy), Tris buffered saline (TBS) 0.05% (v/v), 96-well microtiter plates, defatted cotton sheet, Tween 20 (Sigma Chemical Co., St. Louis, MO, USA), and goat anti-human IgE conjugated to horseradish peroxidase (HRP) (Invitrogen, Carlsbad, CA, USA) were used. Furthermore, Tris-glycine gels (4-10%), gel electrophoresis apparatus, Coomassie Plus (Bradford) Protein Assay (Pierce Chemical Company, Rockford, IL, USA), bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA), GelCode Blue Stain Reagent, nonfat dry milk (local grocery store), o-phenylenediamine dihydrochloride (OPD) and SuperSignal West Pico Chemiluminescent (ECL) substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) were obtained. Plasma from 4 donors with documented soybean allergy and negative soybean allergy (Plasma Lab International, Everett, WA, USA) were acquired for testing.

Preparation of Soymilk

Soymilk was prepared according to Shun-Tang et al. (1997) with slight modification. The soybeans were soaked in deionized water for overnight at 4 °C. The swollen beans were ground by adding deionized water (w/v 7:1) and blended using a kitchen aid blender (St. Joseph, Michigan, USA). The homogenate was filtered using a defatted cotton sheet. The raw soymilk was subjected to PU and thermal treatments.

Power Ultrasound Treatment of Soymilk

The samples were treated with PU (20kHz,1500W) (Sonics and Materials Inc., CT, USA). The ultrasonic system consists of an air-cooled converter (CV 294), fabricated with piezoelectric lead zirconate titanate (PZT) crystals and a standard autoclavable titanium alloy probe (Tl-6Ai-4V,1” diameter and 10 length). Samples were placed in 400 mL aluminum beaker and treated for (2, 4, 6, 8, 10, 12, 15 min).

Thermal Treatments

Soymilk samples were placed in 10 mL plastic tubes. Thermal processing was performed for 10 min at seven different temperatures (40, 50, 60, 70, 80, 90, and 100 °C) in a water bath (DW-Ac 920, D.W. Industries, Seoul, Korea). After the treatments, samples were removed immediately from the water bath and cooled to stop any further denaturation.

Protein Concentration

Untreated and treated samples were subjected to protein determination using the Bradford assay. Ten mL of each sample (treated and untreated) were added to 300 mL Bio-Rad Bradford reagent. The absorbance of the mixture was read at 595 nm in a Spectramax 340^{384} (Molecular Devices, Sunnyvale, CA, USA) and quantified using bovine serum albumin as standard.

Determination of IgE Binding to soybean samples with indirect ELISA

Polystyrene 96-well plates were coated with untreated and treated samples (diluted to a protein content of 20 µg/mL) at 100 µL per well in triplicates and incubated overnight at 4 °C. The plates were washed with TBS/0.05% Tween 20 (TBST) and blocked with Starting Block blocking buffer (200 µL per well) at room temperature for 2 h and then washed 3 times with TBST. Pooled human plasma (cap no 50 KU/L) from

patients allergic to soybean was diluted in phosphate buffered saline (PBS) (1:10), and 100 µL were added to each well. Plasma from patients with negative soybean allergy served as a negative control. The plate was incubated for 1 h at room temperature and then washed with TBST. Secondary antibody, anti-human IgE HRPO conjugated, was diluted in PBS (1:1000), added to each well (100 µL per well), and incubated for 1 h at room temperature. After incubation, the wells were washed and then treated with 100 µL of substrate peroxide buffer containing OPD substrate (0.5 mg/mL). The absorbance was measured at 450 nm using a Spectramax 340^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The samples were analyzed by SDS-PAGE according to the protocol described by Chung et al. (2008). After PU treatment, samples with equal amounts of protein in Laemmli sample buffer containing 0.05% 2-mercaptoethanol were heated in a water bath (100 °C) for 10 min. The samples were loaded into (4-10%) Tris-glycine precast gels (Bio-Rad Laboratories, Hercules CA, USA) and subjected to electrophoresis for 1.5 h at 150 V. The gels were stained with GelCode Blue solution, de-stained with water, and scanned using a Canon scanner (Canon Inc., Sunnyvale, CA, USA).

Western Blot Analysis

After separation of the protein by SDS-PAGE, unstained gels were washed with distilled water and then transferred onto an Immun-Blot PVDF membrane at 15 V for 30 min. After the transfer was completed, the membrane was washed with TBST, and the unbound membrane was blocked using 5% nonfat dry milk in 0.01% Tween 20 in TBS buffer for 1 h. The membrane was washed with TBST 3 times and incubated with a pooled patient plasma (cap no 50 KU/L), which was diluted in 5% nonfat dry milk in TBS

buffer (1:6), for 4 h at room temperature with gentle shaking. After 3 washes with TBST buffer, the membrane was incubated at room temperature for 1 h with a goat anti-human IgE HRP (diluted in TBST at 1:1,000). The membrane was washed again 3 times with TBST for 5 min. Super Signal West Pico Chemiluminescent substrate was used for detection.

Statistical Analysis

Statistical analysis was conducted using one way analysis of variance (ANOVA) with SAS version 9.0 (Cary, NC, USA) and Tukey's comparison of means. Differences are considered at α=0.05. All experiments conduct in triplicate.

Results and Discussion

Figure 5-1 shows the SDS-PAGE profile of the untreated and treated soymilk samples. The electrophoretogram showed protein bands ranging from approximately 18-140 kDa. The SDS-PAGE patterns revealed high-intensity bands of major soybean allergens (β-conglycinin and glycinin). Treatment of soymilk with PU for 2 min did not cause any changes in protein bands, whereas longer treatment times (4 and 6 min) caused a slight reduction in β-conglycinin without any change in glycinin. Treatment of samples for 8 and 10 min increased the band intensities for β-conglycinin and glycinin. Also, a new band was observed after 10 min of treatment, and it was as assumed to be P34 (~34 kDa). The presence of P34 after 10 min could be attributed to its release after PU treatment. Finally, there was a pronounced reduction of band intensities after treatment of the samples for 12 and 15 min.

Western blot was performed to evaluate the immunoreactivity of PU-treated and untreated soymilk (Figure 5-2). The untreated soymilk showed high intensities of bands

corresponding to β-conglycinin and glycinin subunits. There was no obvious reduction in the allergenicity after treatment of the samples for 2 min. Short treatments (4 and 6 min) caused a significant decrease in major allergens of soymilk. In contrast, longer treatments (8 and 10 min) induced an increase in allergens. Major and minor allergens were clearly reduced after treatment of the samples for 12 min and were absent after treatment for 15 min. To confirm the western blot results, ELISA was conducted (Figure 5-3). The results showed that there was no significant difference in IgE binding to untreated and treated soymilk at 2 min. There was a slight decrease in IgE binding to the treated samples at 4, 6, and 8 min. In contrast, treatment of the samples for 10 min caused an increase in IgE binding compared to shorter treatment times. This increase could be related to the new band that was observed in western blot analysis after 10 min and was assumed to be P34. It was reported that ultrasound treatment improved the efficiency of protein extraction and increased the extractability of allergens (Albillos et al. 2011). A significant reduction of immunoreactivity was observed in samples treated for 15 min compared to untreated samples, and this reduction reached a level almost equal to that observed with the normal plasma (negative control). The application of PU to reduce allergenicity is a novel processing idea, and therefore past research is very limited. Lie et al. (2006) studied the effect of PU on the allergenicity of shrimp. Samples were treated with PU for 30-180 min and then analyzed by ELISA and immunoblotting with pooled serum from patients with shrimp allergy. A linear relationship was observed between treatment time and a decrease in allergenicity of shrimp samples. However, comparison of our results with those from the shrimp study is

difficult, because the treatment time applied in our study was less but the system used in our experiment was more powerful.

The findings from SDS-PAGE, western blot, and ELISA demonstrated that PU did change the immunoreactivity of soymilk compared to untreated soymilk. There was a pronounced reduction after both short times (4 and 6 min) and long times (up to 15 min). However, treatment of the samples for 10 min allowed a significant increase in IgE binding.

Another phenomenon that was observed during PU treatment was an increase in the temperature of samples (data not shown). As known from the literature, PU can cause the formation of cavitation bubbles that increase in size during treatment causing an extreme pressure (1,000 atm) and heat (5,000 K) as hot spots. At sufficient power, the size of bubbles increases, and bubbles are distributed throughout the liquid and eventually collapse violently. The cavitation bubbles and other effects of the PU application, which include heat, pressure, and turbulence, can lead to chemical, physical, and mechanical effects on food matrixes (Patist and Bates 2008; Soria and Villamiel 2010; Knorr et al. 2004). In recent studies, PU application was successful in reducing the allergenicity of shrimp by causing tropomyosin sensitization (Li et al. 2006; Li et al 2011). In previous publications, PU was documented to cause destabilization of protein structure, which may affect the epitopes of allergens so that they would not be recognized by IgE antibodies (Garino et al. 2012). However, the mechanisms by which PU could modify epitopes are unknown. Furthermore, no data has been published on the effects of PU on soymilk. A reduction in allergens may be explained by thermal, physical, and chemical changes resulting from the cavitation phenomena. Single or

synergistic changes, such as an increase in temperature in combination with the destruction of bonds between proteins, may contribute to the reduction.

An experiment was conducted to evaluate the impacts of thermal treatments on allergens and compare them to the impact of PU. The thermal treatment experiment was designed based on the average time applied and the temperatures observed during PU treatment. The results of SDS-PAGE of soymilk samples heated at 40, 50, 60, 70, 80, 90, and 100 °C for 10 min are shown in Figure 5-4. The major allergens of soybean, β-conglycinin and glycinin, were observed in all the treatments. This result is in good agreement with that reported by Shun-Tang et al. (1997), where heated soymilk showed a pattern of proteins similar to that observed here. The intensity of bands and the overall distribution pattern of proteins were similar in all treated samples (Figure 5-4).

To evaluate the allergenicity of heat-treated soymilk samples, western blot analysis was conducted (Figure 5-5). The immunoblotting revealed strong reaction against glycinin subunits and weak reaction against β-conglycinin. All the samples exhibited a very similar and stable pattern in the different thermal treatments. The high-intensity bands with approximate molecular weight of 34 kDa were assumed to represent the P34 allergen. It was reported that P34 was heat resistant because of the complex structure of the epitopes, which requires a strong treatment to be modified and to reduce the allergenicity. Interestingly, one study documented that autoclaving enhanced the allergenicity of P34 (Wilson et al. 2005). Thermal treatments can induce denaturation and unfolding of protein and, as a consequence, decrease protein extractability/solubility because of surface exposure of hydrophobic groups and formation of covalent complexes. Yada (2004) documented that β-conglycinin was more

unstable to heat and easy to unfold structurally compared to glycinin because of the large number of SH and SS groups.

The SDS-PAGE and western blot results of the thermal treatments were not compatible with the results obtained from the PU treatment. The thermal treatments showed a constant pattern and intensity of glycinin subunits, which are known for their heat stability. Also, P34 did not change during the treatments. In contrast, PU was more effective to reduce all allergens in soymilk including P34. It is important to mention that the principle of PU is different from thermal treatment, because heat is generated by PU as an indirect effect of acoustic energy absorption. In addition, there are possibly more factors, such as chemical and mechanical alterations, that contributed the reduction of allergens by PU.

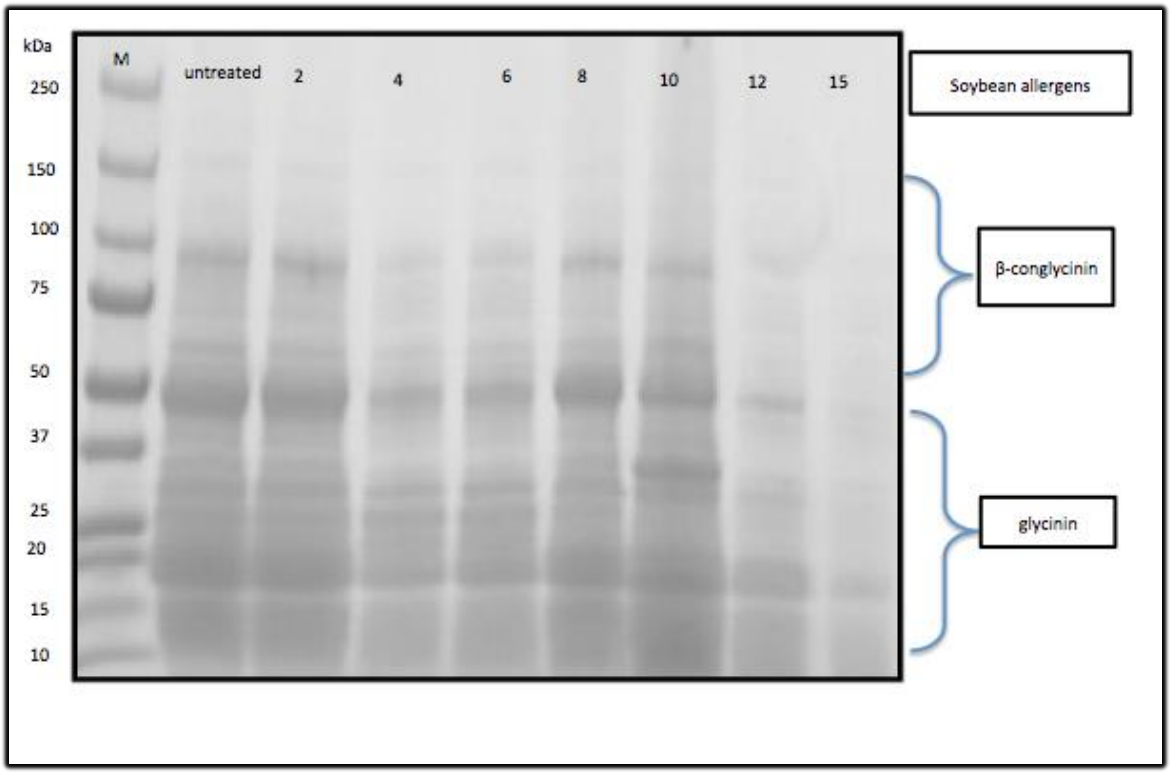

Figure 5-1. SDS-PAGE profile of soymilk treated with power ultrasound for 2, 4, 6, 8, 10, 12, and 15 min, compared to untreated soymilk. Molecular weights are expressed in kDa**.**

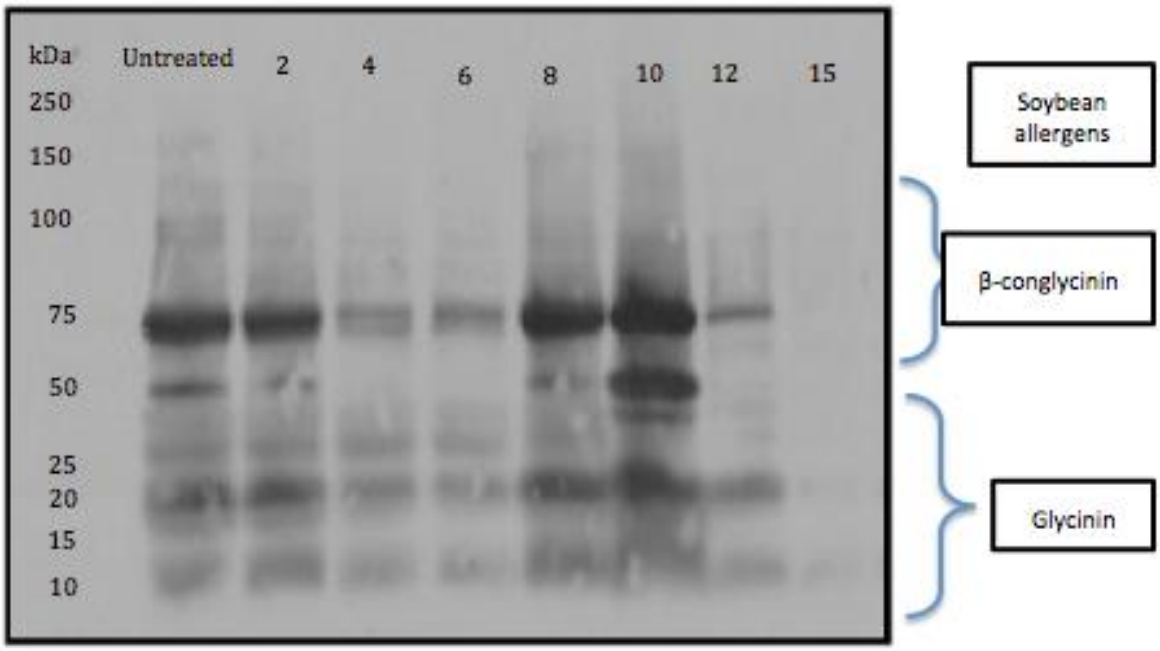

Figure 5. 2. Western blot profile of power ultrasound treated soymilk for 2, 4, 6, 8, 10, 12 and 15 min, compared to untreated sample. Molecular weights expressed are in kDa**.**

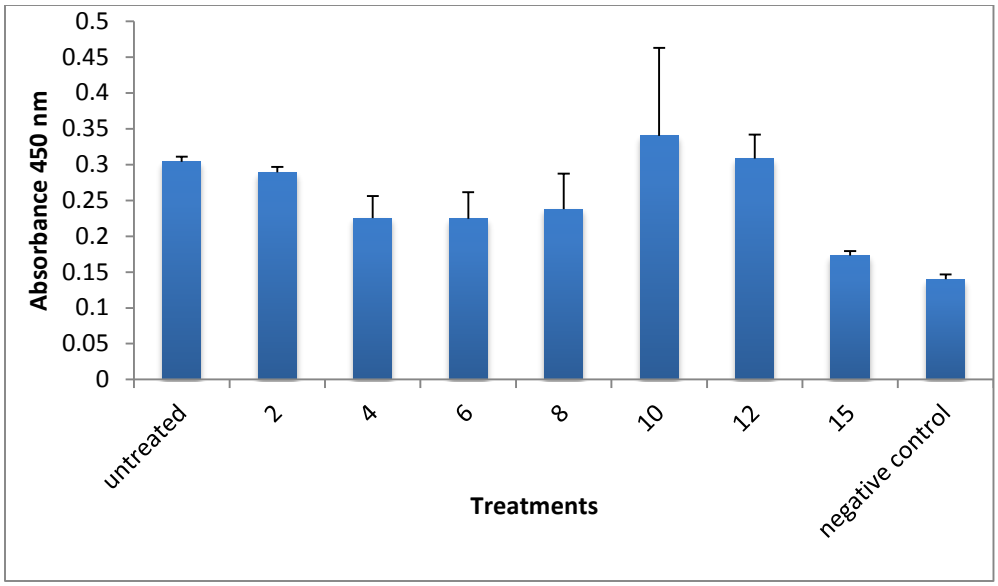

Figure 5-3. Results from indirect ELISA conducted on soymilk treated with power ultrasound for 2, 4, 6, 8, 10, 12, and 15 min, compared to untreated soymilk. ELISA was performed with human plasma from patients allergic to soybean, and normal plasma (from humans without soybean allergy) was used as a negative control. The values show the mean absorbance of three replicates with standard deviation.

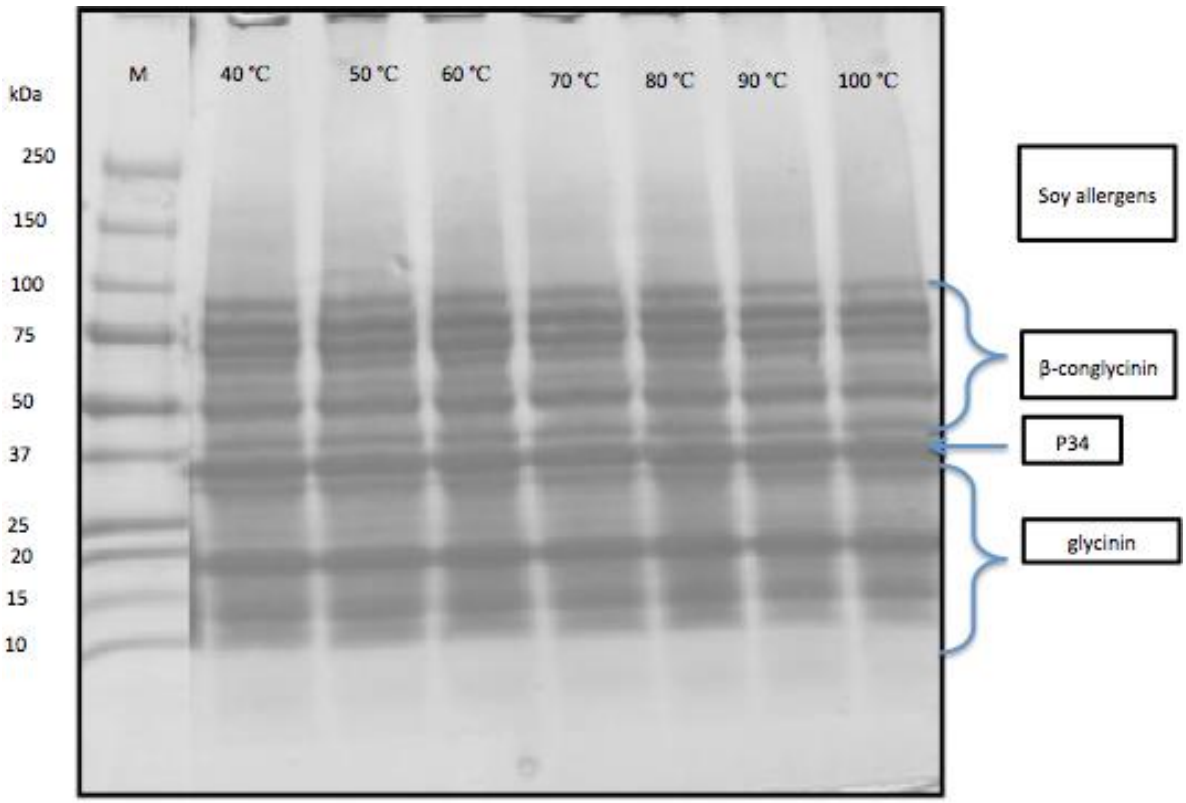

Figure 5-4. SDS-PAGE of soymilk after thermal treatments at 40, 50, 60, 70, 80, 90, and 100 °C for 10 min. Molecular weights are expressed in kDa.

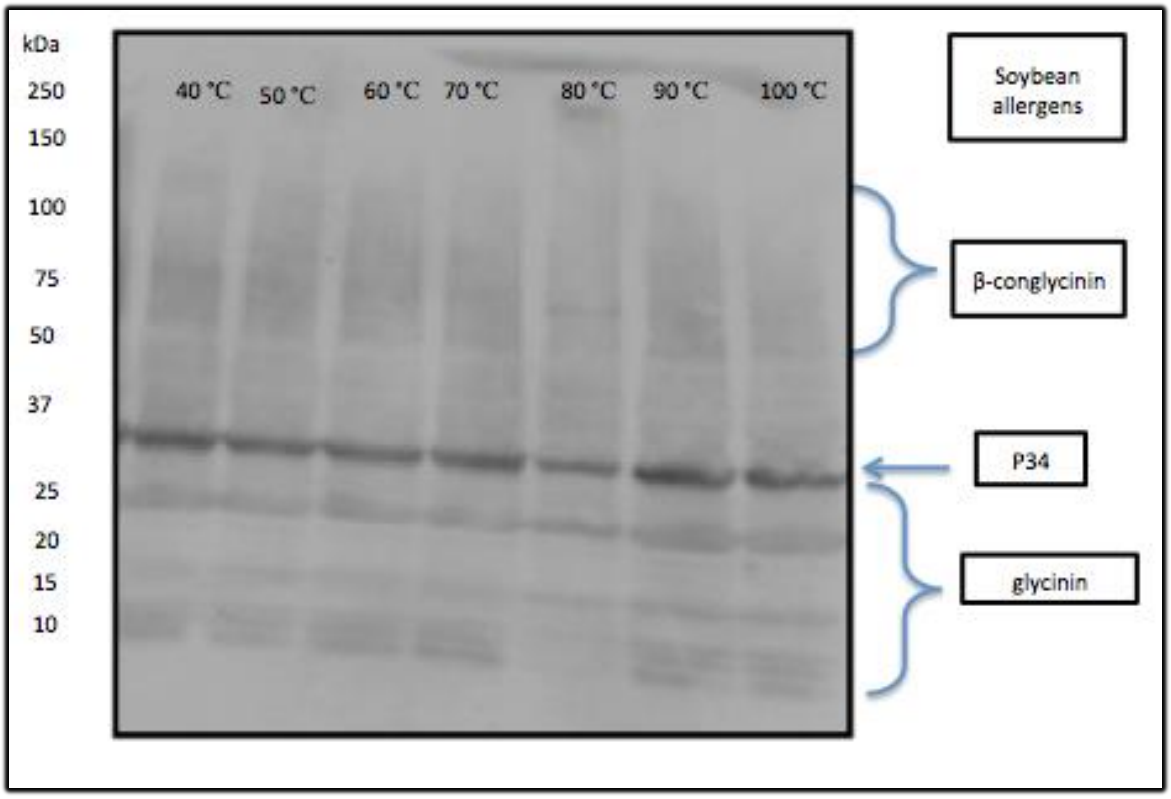

Figure 5-5. Western blot profile of thermal treatments treated soymilk at 40, 50, 60,70,80,90, and 100 °C for 10 min, compared to untreated sample. Molecular weights expressed are kDa.

CHAPTER 6
.THE EFFECT OF POWER ULTRASOUND ON PHYSICOCHEMICAL PROPERTIES OF SOYBEAN EXTRACT AND SOYMILK

Background

The physicochemical properties of a product are defined as attributes related to its physical and chemical characteristics. In the food industry, some of the important physicochemical functions of soy protein are color control, solubility, emulsification and gelatinization. These functional properties are crucial to determine the efficiency of food processing and the final quality of a food product (Yada 2004). Power ultrasound (PU) is a growing technology in the fields of research and food processing. It has been documented that PU has a broad range of benefits in food processing methods, such as extraction, separation, filtration, enzyme and microbial inactivation, and viscosity alteration (Patist and Bates 2008). The use of PU improved the commercial oil production and reduced the time required to extract oils from soybean (Li et al. 2004). Karki et al. (2010) reported that the use of PU improved the release of protein from defatted soy flakes.

The objective of this study was to investigate the effect of PU on the physicochemical properties of soybean extract and soymilk, which include the following: color, viscosity, pH, and temperature changes.

Materials and Methods

Soybean Extract Preparation

Raw soybeans were ground and defatted in hexane (1:4 w/v). The defatted soybeans (1 g) were mixed with 10 mL of 0.01 M Phosphate Buffer Saline (PBS) pH 7.4 and stirred for 30 min at room temperature. Untreated and treated samples with PU were centrifuged (Mini centrifuge, Fisher Scientific, USA) at 2200 g for 10 min.

Preparation of Soymilk

Soymilk was prepared according to Shun-Tang et al. (1997) with slight modification. Raw soybeans were soaked in deionized water for overnight at 4 °C. The swollen beans were ground by adding deionized water (w/v 7:1) and homogenized using kitchen aid blender (St. Joseph, Michigan, USA). The homogenate was filtered through a defatted cotton sheet. The resulting raw soymilk was heated in a water bath at 80 °C for 10 min and then quickly cooled to approximately 24 °C. The temperature was measured before and after the treatment using a thermometer.

Power Ultrasound Treatment of Soybean Extract and Soymilk

The samples were treated with PU at 20 kHz and 1,500 W (Sonics and Materials Inc., CT, USA). The ultrasonic system consisted of an air-cooled converter (CV 294), fabricated with piezoelectric lead zirconate titanate (PZT) crystals and a standard autoclavable titanium alloy probe (Tl-6Ai-4V, 2.5 cm diameter and 25 cm length. Samples were placed in 400 mL aluminum beakers and treated for 2, 4, 6, 8, 10, 12, and 15 min.

Measurement of Temperature Changes, pH, Viscosity

The temperature (°C) of the samples was measured in the untreated and treated samples with an infrared thermometer (Omega OS423-LS, Omega Engineering, Inc., Stanford, CT, USA). The surface temperature was recorded immediately after PU, and the values were reported as an average of triplicate measurements (one measurement per replicate sample) of the final temperature.

The pH of the untreated and treated samples was determined at room temperature with a pH meter (Accumet Basic AB 15/157, Fisher Scientific, USA). The values were reported as an average of triplicate samples.

The viscosity of untreated and treated soybean extracts and soymilk was measured with a Brookfield digital viscometer (model DV-II, Brookfield Engineering Laboratory, USA). Approximately 400 mL of untreated and treated samples were measured using a spindle no. 1 at a speed of 100 rpm. The viscosity values were reported as the average of the readings taken from triplicate samples over a period of 20 s.

Color Analysis

The effect of PU treatment on the color of soybean extracts and soymilk was measured with a color machine vision system (CMVS). The values L* (lightness), a* (redness), and b* (yellowness) were determined. The instrument was standardized to a standard white tile (L*=96.59, a*=-0.02, b*=1.95). A total of three readings were taken (one reading from each of three replicate samples), and the average was reported

Statistical Analysis

Statistical analysis was conducted using one way analysis of variance (ANOVA) with SAS version 9.0 (Cary, NC, USA) and Tukey's comparison of means. Differences are considered at $\alpha=0.05$. All experiments conduct in triplicate.

Results and Discussion

Since the physicochemical properties, such as color, viscosity, pH, and temperature, are important parameters in food production, it was necessary to examine their behavior after PU treatment. Table 6-1 shows the viscosity of untreated and PU-treated soybean extract samples. Results demonstrated that the PU treatment caused a

significant (p<0.05) increase in the viscosity of the samples, which was noticed at all treatment times. Table 6-2 shows the viscosity of untreated and PU-treated soymilk samples. Similar to soybean extracts, soymilk samples exhibited changes in viscosity according to the duration of the treatment. The PU treatment caused a small but statistically significant increase in the viscosity with increasing treatment time. It should be noted, however, that these differences were small and that the variation between replicate samples was low as indicated by small standard errors (Table 6-1 and 6-2). It is known that PU, depending on the ultrasound intensity, causes a temporary or permanent increase or decrease in the viscosity of food samples. For instance, during the ultrasound treatment, cavitation phenomena can cause shear specifically of thixotropic fluids (less viscous liquids) resulting in a temporary decrease in the viscosity. In contrast, when enough energy is applied, decrease in the molecular weight may raise the permanent viscosity diminution (Seshadri et al. 2003). Our results agree with those of Fahmi et al. (2011), who reported that PU slightly increased soymilk viscosity when samples were treated in a 35 kHz ultrasonic cleaning bath. Also, they noticed that the increase in viscosity was associated with increased protein concentration. The PU can increase the penetration of moisture into the fiber network and thereby increase the viscosity in certain vegetable purees (Bates et al. 2006). An increase in viscosity and consistency coefficient after PU was reported to be accompanied by changes in the binding capacity of water, because the hydrophilic groups of amino acids were oriented toward water surroundings and facilitated binding to water molecules (Kresic et al. 2008). However, Huang et al. (2007) showed that the opposite could occur during the treatment of cornstarch granules. The authors found a decrease in viscosity as a

consequence of degradation and decrease of molecular weight of starch molecules caused by partial cleavage of the glycosidic linkages.

The pH values of soybean extracts changed significantly ($p<0.05$) after the PU treatment compared to the untreated samples (Table 6-3). A decrease in pH values was observed with increasing treatment times. Table 6-4 shows the significant ($p<0.05$) decrease in pH values of PU-treated soymilk samples, which was also more pronounced with longer treatment times than with short treatment times. It should be noted that the standard errors between replicate samples were low and that the differences in pH values were small (Tables 6-3 and 6-4). The pH values ranged from pH 6.57 to pH 6.31 for soybean extracts and from pH 7.34 to pH 7.15 for soymilk samples. These small changes did not change the general slight acidity and slight alkalinity, respectively, of the tested soybean extracts and soymilk samples. Chandrapala et al. (2012) studied the effect of ultrasound on casein micelles and reported that small and temporary changes of pH values were observed and that the changes increased with the duration of the treatment. Decrease in pH values also was observed after treatment of cauliflower, Brussels sprouts, and button mushrooms with PU (Jambrak et al. 2007). In contrast, treatment of whey protein with PU (20 kHz, 600 W) did not significantly change the pH values of the samples. Our system was more powerful (20 kHz, 1,500 W) than that used by Jambrak et al. (2007), which may explain the enhanced chemical effects of PU in our experiments. The decrease of pH may be due to the cavitation phenomena that occurred during PU. It was reported that PU caused formation of hydroxyl radicals ($OH^{\bullet}$) and other compounds resulting from cavitation. The combination of the high pressure and high temperature during

ultrasonication can break water molecules and generate highly reactive free radicals that may react with and modify other molecules (Knorr et al. 2004; Soria and Villamiel 2010). Kentish and Ashokkumar (2011) reported that sonication of air-saturated solutions often leads to dropping of the pH values as a result of nitric acid formation. During the sonication, several reactions can occur involving oxygen and nitrogen, which form NO_2 and subsequently nitric acid.

The surface temperature of soybean extract and soymilk exposed to PU increased significantly compared to the untreated samples (Table 6-5 and Table 6-6, respectively). An increase in the temperature was also obvious after short treatments. For instance, after 2 min of treatment, the temperature of soybean extract almost doubled compared to the untreated samples (Table 6-5). Based on the literature review of PU treatment, important issues should be considered in the evaluation of the presented results: Firstly, the temperature was not recorded during the treatment (measurements were taken after the treatment). Secondly, only surface temperature was measured, and it is possible that the temperature was higher inside the liquid media. Li et al. (2011) emphasized that an accurate recording of temperature during the treatment could be important for the correct interpretation of results obtained from non-thermal treatments. An increase in the temperature was observed when whey protein suspensions were treated with a 20 kHz probe (Jambrak et al. 2008). Both temperature and pressure can increase rapidly after a short ultrasound treatment (Knoor et al. 2004). This might happen because the application of ultrasound in liquid can create many thousands of cavitational bubbles whose collapse during prolonged treatment can

generate extreme temperatures of about 5,000 K and pressures of about 1,000 atm (Cullen et al. 2012).

The impact of PU treatment on the color parameters of soybean extract and soymilk is summarized in Table 6-7 and Table 6-8, respectively. There was no change in the lightness (L*), redness (a*), and yellowness (b*) after PU treatment for short times (2 and 4 min) compared to the untreated samples. However, significant (p<0.05) decreases in lightness and increases in redness and yellowness were observed after longer treatment times. Decrease of L* values may occur because of the production of brown pigments as a result of the Maillard reaction induced by high temperature generated during the PU treatment. Kwok and Niranjan (1999) reported that L* values of soymilk samples decreased after heat treatments (ranging from 80-140 °C for 0.5-180 min) because of the formation of brown pigments.

Table 6-1. Effect of PU on the viscosity of soybean extract

Treatment	Viscosity (cp)
Untreated sample	11.67 ± 0.12^{f}
2 min	13.30 ± 0.15^{e}
4 min	14.10 ± 0.10^{cd}
6 min	13.87 ± 0.03^{de}
8 min	14.67 ± 0.13^{c}
10 min	14.27 ± 0.06^{cd}
12 min	15.60 ± 0.20^{b}
15 min	16.73 ± 0.29^{a}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p<0.05$.

Table 6-2. Effect of PU on the viscosity of soymilk

Treatment	Viscosity (cp)
Untreated sample	17.13 ± 0.13^{d}
2 min	17.33 ± 0.07^{d}
4 min	16.56 ± 0.03^{e}
6 min	19.13 ± 0.13^{b}
8 min	18.47 ± 0.07^{c}
10 min	19.63 ± 0.13^{a}
12 min	18.57 ± 0.07^{c}
15 min	18.23 ± 0.07^{c}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p<0.05$.

Table 6-3. Effect of PU on pH values of soybean extract

Treatment	pH
Untreated sample	6.84 ± 0.02^{a}
2 min	6.79 ± 0.01^{ab}
4 min	6.76 ± 0.01^{bc}
6 min	6.76 ± 0.01^{bc}
8 min	6.71 ± 0.01^{cd}
10 min	6.70 ± 0.01^{cd}
12 min	6.66 ± 0.01^{d}
15 min	6.56 ± 0.02^{d}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p< 0.05$.

Table 6-4. Effect of PU on pH values of soymilk

Treatment	pH
Untreated sample	7.50 ± 0.04^{a}
2 min	7.46 ± 0.03^{b}
4 min	7.41 ± 0.02^{abc}
6 min	7.35 ± 0.02^{bc}
8 min	7.35 ± 0.03^{bc}
10 min	7.31 ± 0.02^{cd}
12 min	7.22 ± 0.02^{de}
15 min	7.15 ± 0.01^{e}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p< 0.05$.

Table 6-5. Effect of PU Treatment on the Temperature Profile of Soybean Extract

Treatment	Temperature (°C)
Untreated sample	19.4 ± 0.26^{g}
2 min	33.03 ± 0.20^{f}
4 min	61.30 ± 0.26^{e}
6 min	70.90 ± 0.06^{d}
8 min	85.87 ± 0.26^{c}
10 min	86.80 ± 0.86^{c}
12 min	91.27 ± 0.49^{b}
15 min	97.03 ± 1.13^{a}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p< 0.05$.

Table 6-6. Effect of PU Treatment on Temperature Profile of Soymilk

Treatment	Temperature (°C)
Untreated sample	18.50 ± 0.30^{g}
2 min	39.03 ± 0.67^{f}
4 min	58.37 ± 2.94^{e}
6 min	69.87 ± 1.18^{d}
8 min	80.40 ± 0.53^{bc}
10 min	76.13 ± 2.38^{cd}
12 min	83.70 ± 0.35^{ab}
15 min	89.03 ± 0.31^{a}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p< 0.05$.

Table 6-7. Effect of PU on color characteristics of soybean extract

Treatment	L*	a*	b*
Untreated Sample	89.14±0.34^{a}	-0.41±0.10^{d}	13.63±0.16^{b}
2min	88.45±0.69^{a}	-0.46±0.14^{d}	13.45±0.16^{b}
4min	88.75±0.63^{a}	-0.44±0.08^{d}	13.47±0.13^{b}
6min	86.83±0.37^{ab}	2.88±0.23^{c}	11.91±0.01^{c}
8min	86.19±1.20^{ab}	2.39±0.35^{c}	17.34±0.13^{a}
10min	83.00±0.76^{bc}	4.08±0.20^{b}	17.73±0.17^{a}
12min	80.82±0.48^{bc}	5.69±0.28^{a}	18.04±0.49^{a}
15min	83.22±1.38^{c}	4.48±0.06^{b}	13.39±0.18^{b}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p<0.05$.

Table 6-8. Effect of PU on color characteristics of soymilk

Treatment	L*	a*	b*
Untreated sample	90.59±0.19^{ab}	-0.01±0.02^{cd}	10.57±0.27^{cd}
2min	89.67±0.21^{bc}	-0.15±0.03^{d}	10.71±0.26^{cd}
4min	90.17±0.20^{b}	0.19±0.17^{bcd}	10.77±0.29^{bc}
6min	91.70±0.13^{a}	0.57±0.15^{ab}	9.63±0.15^{d}
8min	88.94±0.27^{c}	0.43±0.02^{b}	10.70±0.16^{cd}
10min	86.93±0.41^{d}	0.38±0.08^{bc}	12.31±0.34^{a}
12min	85.95±0.24^{de}	0.22±0.01^{bcd}	11.85±0.04^{ab}
15min	85.59±0.16^{e}	1.09±0.18^{a}	11.96±0.04^{a}

Values are expressed as a mean±SD error from three independent experiments. Means with different letters within the same column are significantly different at $p<0.05$.

CHAPTER 7
EVALUATION OF THE ALLERGENICITY OF TRANSGENIC AND NON - TRANSGENIC SOYBEANS

Background

In the United States, approximately 90% of currently produced soybeans are genetically modified. The genetically modified soybeans in the market are transgenic soybeans targeted toward insect protection or herbicide resistance to improve the quality of the product (Betz et al. 2000).

However, there is a public fear related to consuming transgenic foods due to an early study that raised concerns about transgenic technology. It was reported that enhancing the quality of soybean proteins led to introducing a new allergen, 2S, from Brazilian nuts to soybean. For safety reasons, the development of this crop was stopped because of the major risk of increasing the allergenicity (Nordlee et al. 1996).

There are several ways by which a novel protein or neoallergen (new allergen) may increase the allergenicity. These include: cross-reacting with another allergen, being a neoallergen never exposed to the immune system, or acting as an allergen per se (Panda 2012). So far, most studies have focused on investigating the allergenicity of 5-Enolpyruvylshikimate-3-Phosphate Synthesis CP4-EPSPS, which is the most common gene in transgenic crops that is transferred to increase herbicides resistance (Wu et al. 2012). However, there is a lack of data to explain whether the allergenicity of transgenic soybean increases under the conditions of food processing.

Hence, this experiment was designed to assess the allergenicity of transgenic soybeans during thermal treatments, which are common processes in the food industry.

Materials and Methods

Sample Preparation

We obtained the non-transgenic soybean (IA2101) samples from Iowa State University. Commercial transgenic soybean samples were obtained from Bunge Company. Soybeans were ground and defatted in hexane (w/v 1:4). The defatted soybeans (1 g) were stirred with 10 mL 0.01 M phosphate buffered saline (PBS) pH 7.4 for 30 min at room temperature. The untreated and treated samples were centrifuged at 3,200 rpm for 15 min and divided into 10 mL tubes for further use.

Thermal Treatments

The experiment was designed to treat the soybean extracts by three types of methods: heating, steaming, and roasting. All processing steps were carried out in triplicate.

Heat Treatment: Soybean extract samples were placed in 10 mL plastic tubes. Heat treatment was conducted for 10 min at 50, 70, and 100 °C in a water bath (DW-Ac 920, D.W. Industries, Seoul, Korea). After the treatment, samples were removed immediately from the water bath and cooled to stop any further denaturation

Steam: Soybean extract samples were subjected to steam pressure processing. The samples were placed in 10 mL plastic tubes, and steaming was performed in a Deni 9700 Electric 5 quart pressure vessel (Keystone Manufacturing Company, Inc, NY, USA) at a steam pressure of 103 kPa and a temperature of 121 °C for 10 min.

Roasting: The roasting process was carried out in a conventional oven (General Electric oven, model JRP 36 GIV 3 SB). The samples (10 g) were roasted at 100 °C for 10 min.

Protein Concentration

Untreated and treated samples were subjected to protein determination using the Bradford assay. Ten mL of each sample (treated and untreated) were added to 300 mL Bio-Rad Bradford reagent. The absorbance of the mixture was read at 595 nm in a Spectramax 340^{384} (Molecular Devices, Sunnyvale, CA, USA) and quantified using bovine serum albumin as standard.

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The samples were analyzed by SDS-PAGE according to the protocol described by Chung et al. (2008). After PU treatment, samples with equal amounts of protein in Laemmli sample buffer containing 0.05% 2-mercaptoethanol were heated in a water bath (100 °C) for 10 min. The samples were loaded into (4-10%) Tris-glycine precast gels (Bio-Rad Laboratories, Hercules CA, USA) and subjected to electrophoresis for 1.5 h at 150 V. The gels were stained with GelCode Blue solution, de-stained with water, and scanned using a Canon scanner (Canon Inc., Sunnyvale, CA, USA).

Western Blot Analysis

After separation of the protein by SDS-PAGE, unstained gels were washed with distilled water and then transferred onto an Immun-Blot PVDF membrane at 15 V for 30 min. After the transfer was completed, the membrane was washed with TBST, and the unbound membrane was blocked using 5% nonfat dry milk in 0.01% Tween 20 in TBS buffer for 1 h. The membrane was washed with TBST 3 times and incubated with a pooled patient plasma (cap no 50 KU/L), which was diluted in 5% nonfat dry milk in TBS buffer (1:6), for 4 h at room temperature with gentle shaking. After 3 washes with TBST buffer, the membrane was incubated at room temperature for 1 h with a goat anti-human IgE HRP (diluted in TBST at 1:1,000). The membrane was washed again 3

times with TBST for 5 min. Super Signal West Pico Chemiluminescent substrate was used for detection.

Results and Discussion

To identify and compare the protein composition of non-transgenic soybeans and transgenic soybeans, SDS-PAGE was preformed. The untreated non-transgenic (Figure 7-1) and transgenic soybeans (Figure 7-3) showed similar electrophoretic patterns. The β-conglycinin and glycinin subunit bands were observed in both sets of cultivated soybeans. It is known that soybeans are subjected to thermal treatments in food processing. To investigate the effects of thermal treatments on soybean allergenicity, we studied three methods (each separately): heating, steaming, and roasting. Heat treatment of both non-transgenic and transgenic soybeans for 10 min at 50 and 70 °C showed more bands of β-conglycinin and glycinin subunits as a result of thermal treatments increasing the extractability of allergens. Albillos et al. (2011) showed that thermal treatments at 60 °C improved the extractability of proteins for allergen detection as a result of increased solubility of protein at this temperature. A slight decrease in β-conglycinin subunits was observed at 100 °C (Figures 7-1 and 7-3). The steam treatment was more effective in reducing the bands in transgenic soybeans compared to non-transgenic soybeans. The largest decrease was achieved in both cultivars during the roasting process, possibly due to protein denaturation and aggregation.

The next step was to analyze the immunoreactivity of non-transgenic and transgenic soybean after thermal processing. Western blot results of non-transgenic soybean extracts (Figure 7-2) and of transgenic soybean extracts (Figure 7-4) were similar and showed β-conglycinin and glycinin subunit bands. However, the intensities of bands, mainly of glycinin subunits, were higher in transgenic soybean extracts

compared to the non-transgenic soybean extracts. Mild thermal treatments (50 and 70 °C) led to a slight decrease in P34 in both cultivars. For both cultivars, samples that were exposed to a higher temperature (100 °C) showed that most of the allergens were present. However, a new band could be detected only in the transgenic soybean extract (Figure 7-4, lane 4) and likely corresponded to 2S, a major allergen in soybean. Steam treatment showed a more intense effect than heat treatment on β-conglycinin and glycinin subunits in both cultivars. The profiles of western blots of roasted non-transgenic (Figure 7-2, lane 6) and transgenic (Figure 7-4, lane 6) soybeans revealed the absence of any binding between the plasma and allergens.

The findings of the SDS-PAGE and western blot analyses showed that the non-transgenic and transgenic soybeans contained the same allergens, particularly β-conglycinin and glycinin subunits. We did not observe any neoallergens (new allergens) in transgenic soybean under the conditions of thermal treatments. Our results are in agreement with those of Sten et al. (2004), who showed that there was no significant difference in allergenicity of 18 different varieties of soybean (10 genetically modified, 8 wild types). Similarly, Panda (2012) reported that the IgE binding to proteins of three transgenic soybean lines was not extensively different from that to proteins of tested non-transgenic soybean lines. In addition, Panda (2012) pointed out that a variation of allergen content was natural due to the genetic variability of the seeds.

There is no rigid evidence that transgenic foods may affect human health. In addition, to introduce transgenic crops in the market, the crops must undergo a difficult safety assessments described in the Codex Alimentarius Guidelines (Codex, 2003). With regard to our study, it is important to consider that pooled plasma was used in the

immunology methods instead of individual plasma samples from donors allergic to soybeans. This approach may have masked potential presence of (neo) allergens in transgenic soybean, because some individuals may express higher sensitivity to certain proteins than others. Also, the experiment did not include near-isoline soybeans or other varieties that are genetically similar; the results therefore cannot conclusively confirm that transgenic soybeans will not increase the allergenicity of soybeans.

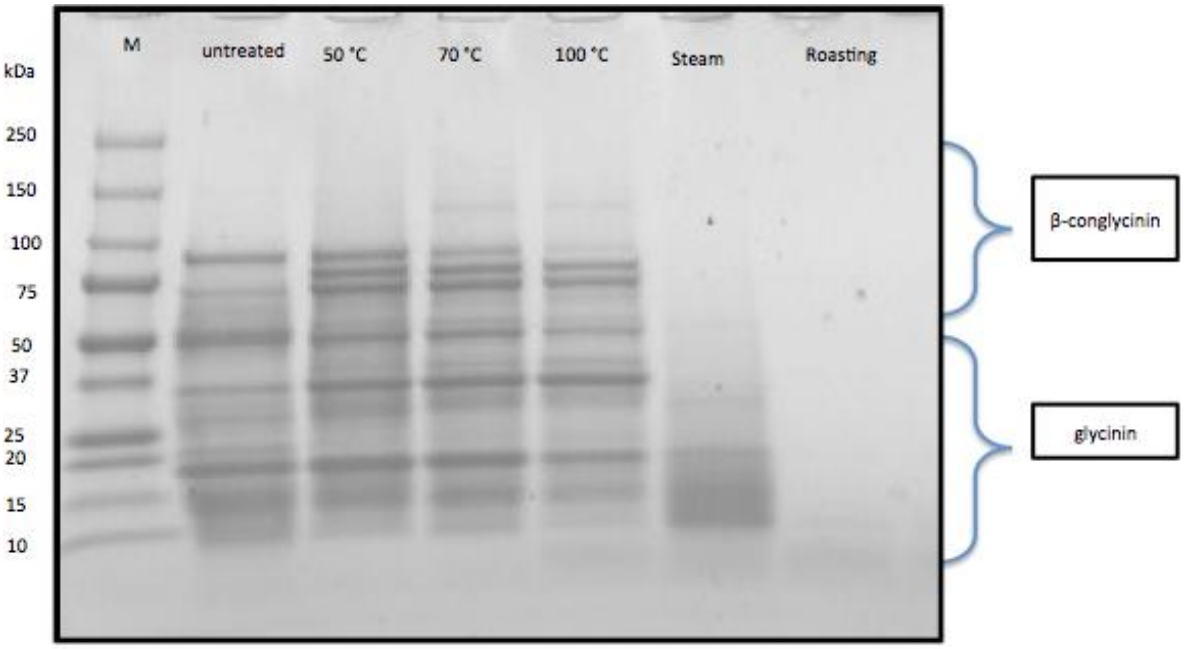

Figure 7-1. SDS-PAGE profile of non-transgenic soybean extracts exposed to different thermal treatments and compared to untreated extract. Molecular weights are expressed in kDa**.**

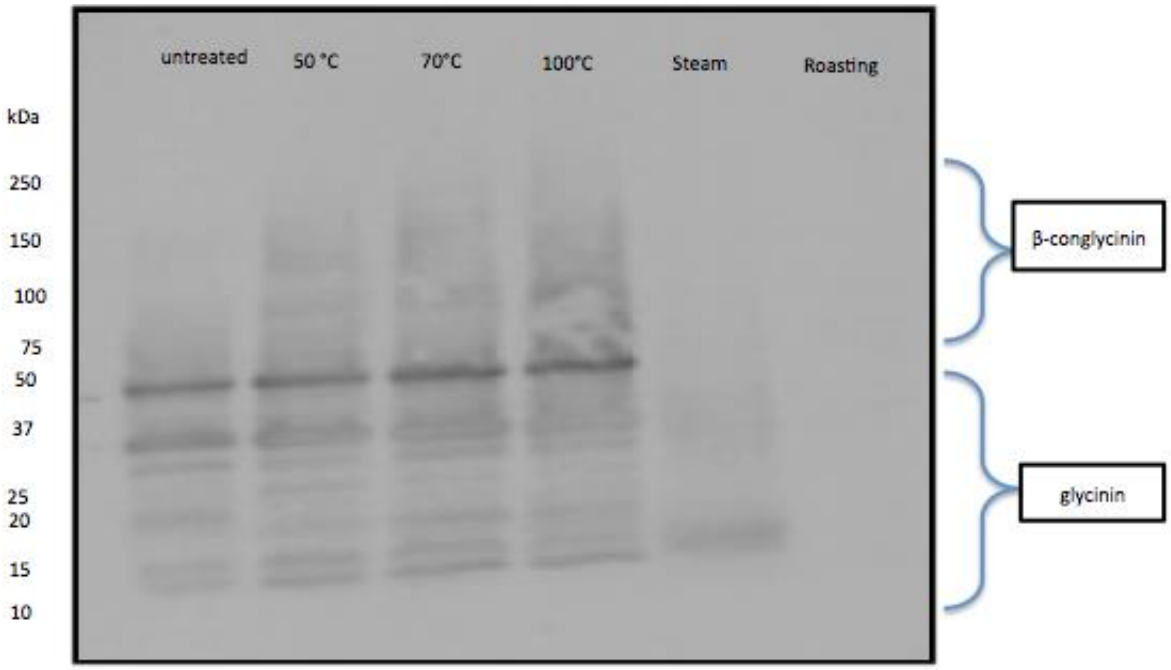

Figure 7-2. Western blot profile of non-transgenic soybean extracts exposed to different thermal treatments and compared to untreated extract. Molecular weights are expressed in kDa.

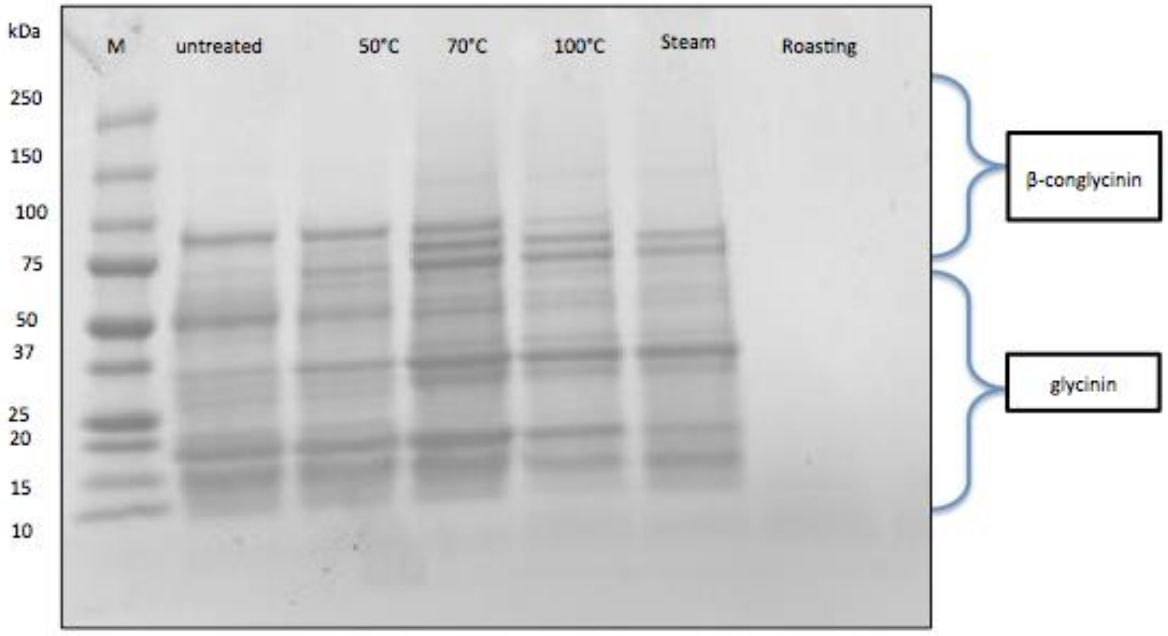

Figure 7-3. SDS-PAGE profile of transgenic soybean extracts exposed to different thermal treatments and compared to untreated extract. Molecular weights are expressed in kDa**.**

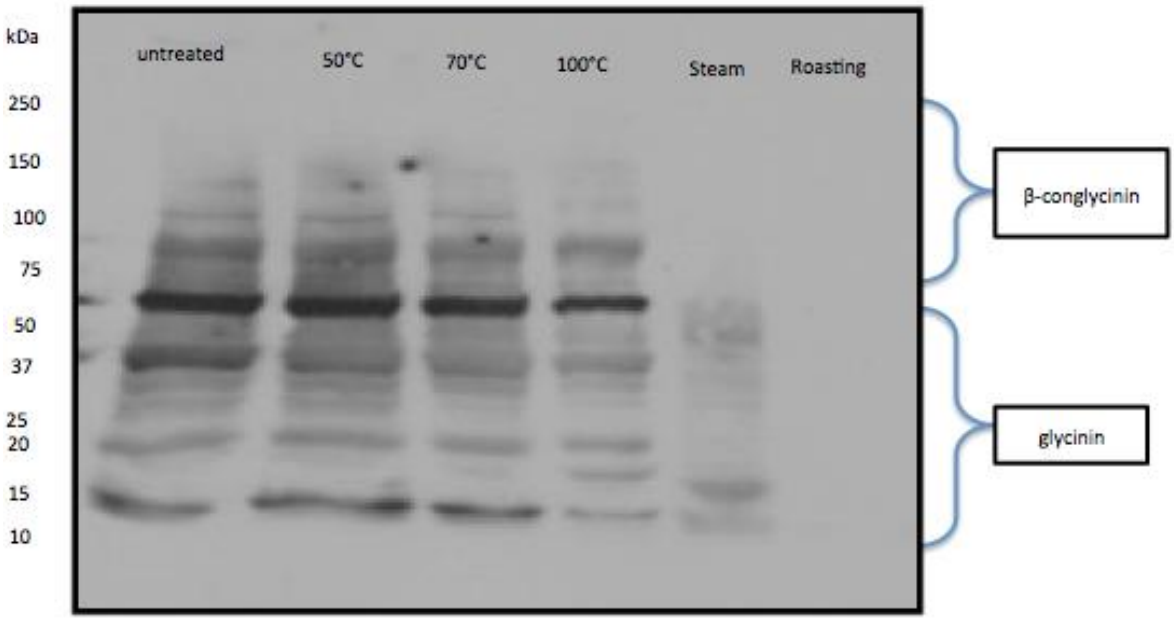

Figure 7-4. Western blot profile of transgenic soybean extracts exposed to different thermal treatments and compared to untreated extract. Molecular weights are expressed in kDa.

CHAPTER 8
CONCLUSIONS AND RECOMMENDATIONS

The results of non-thermal treatments indicate a promising technology to reduce the allergenicity of soybeans. Pulsed light reduced the allergenicity of soymilk over the time of treatment with significant increase of temperature. Based on the results, we assume that pulsed light can reduce allergenicity of soymilk. However, the penetration of pulsed light was an issue during the treatment.

The findings from this study also demonstrated that PU reduced the major and minor allergens in soybean extracts and soymilk as illustrated by SDS-PAGE and western blot. There was a remarkable reduction in allergens after 10 min and longer treatment showed no IgE binding. Thermal treatments did not show any reduction in soymilk allergens. Furthermore, there was a reduction of lightness (L) during the treatment in soybean extracts and soymilk. The treatment demonstrated a significant ($p<0.05$) increase in temperature and viscosity. However, the values of pH decrease over the treatment. The suggestion of this study that the application of power ultrasound can be applied to reduce the allergens in soybean extract and soymilk.

Further investigation of the effect of thermal treatments on the allergenicity of non-transgenic and transgenic soybeans was also necessary. There was no difference in allergenicity between non-transgenic and transgenic soybeans. Therefore, the transgenic variety should be considered as safe as the non-transgenic soybean. However, it is essential to conduct these experiments with different non-transgenic and transgenic soybean lines already are found in the market. Also, in vitro methods should consider to use a single plasma instead of pooled plasma to answer the question of whether transgenic soybeans will increase the allergenicity of soybeans.

Further work is necessary to explain the mechanism of PU and PL using circular dichroism and fluorescence studies, where the major and minor allergens would be isolated and changes in protein structure could be clarified. Also, in vivo studies are necessary to verify the validity of our results. Finally, clinical trial will be useful to determine whether the treated soybeans and soymilk with PU and PL still remain and lead to develop the allergy.

LIST OF REFERENCES

Albillos, S. M., Al-Taher, F., & Maks, N. (2011). Increasing extractability of protein for allergen detection after food processing. *Food Chemistry*, *127*, 1831-1834.

Amigo-Benavent, M., Silván, J. M., Moreno, F. J., Villamiel, M., & Del Castillo, M. D. (2008). Protein quality, antigenicity, and antioxidant activity of soy-based foodstuffs. *Journal of agricultural and food chemistry*, *56*(15), 6498-6505.

Amnuaycheewa, P., & de Mejia, E. G. (2010). Purification, characterisation, and quantification of the soy allergen profilin (Gly m 3) in soy products. *Food Chemistry*, *119*(4), 1671-1680.

Anugu, A.; Yang, W. & Krishnamurthy, K. (2009). Efficacy of pulsed ultraviolet light for reduction of allergenicity in isolated milk proteins. 2009 FIT Annual Meeting Abstract, June 6-9, Anaheim, CA.

Anugu, A.; Yang, W.; Shriver, S.K.; Chung, S.-Y. & Percival, S.S. (2010). Efficacy of pulsed ultraviolet light on reducing the allergenicity of isolated egg proteins. 2010 IFT Annual Meeting Abstract, July 17–20, Chicago, IL.

Barbosa- Canovas, G. V., Schaffner, D. W., Pierson, M. D., & Zhang, Q. H. (2000). Pulsed light technology. *Journal of food science*, *65*(s8), 82-85.

Batista, R., & Oliveira, M. M. (2009). Facts and fiction of genetically engineered food. *Trends in Biotechnology*, *27*(5), 277-286.

Baur, X., Pau, M., Czuppon, A., and Fruhmann, G. (1996). Characterization of soybean allergens causing sensitization of occupationally exposed bakers. Allergy 51, 326-330.

Betz, F. S., Hammond, B. G., & Fuchs, R. L. (2000). Safety and Advantages of Bacillus thuringiensis Protected Plants to Control Insect Pests. *Regulatory Toxicology and Pharmacology*, *32*(2), 156-173.

Breiteneder, H., & Ebner, C. (2000). Molecular and biochemical classification of plant-derived food allergens. *Journal of Allergy and Clinical Immunology*, *106*(1), 27-36.

Burks Jr, A., Brooks, J. R., & Sampson, H. A. (1988). Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *Journal of Allergy and Clinical Immunology*, *81*(6), 1135-1142.*l immunology*, *90*(6), 889-897.

Burks, A., Williams, L. W., Thresher, W., Connaughton, C., Cockrell, G., & Helm, R. M. (1992). Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. *Journal of allergy and clinical immunology*, *90*(6), 889-897.

Chung, S. Y., Yang, W., & Krishnamurthy, K. (2008). Effects of Pulsed UV- Light on Peanut Allergens in Extracts and Liquid Peanut Butter. *Journal of food science*, *73*, 400-404.

Cullen, P. P. J., Tiwari, B. K., & Valdramidis, V. P. (2011). *Novel thermal and non-thermal technologies for fluid foods* (pp.135-165). Academic Press.

Davis, P. J., & Williams, S. C. (1998). Protein modification by thermal processing. *Allergy*, *53*(s46), 102-105.

Davis, P. J., Smales, C. M., & James, D. C. (2001). How can thermal processing modify the antigenicity of proteins? *Allergy*, *56*, 56-60.

Dill, G. M. (2005). Glyphosate- resistant crops: history, status and future. *Pest Management Science*, *61*(3), 219-224.

Duke, S. O. (1990). Overview of herbicide mechanisms of action. *Environmental health perspectives*, *87*, 263.

Elmnasser, N., Dalgalarrondo, M., Orange, N., Bakhrouf, A., Haertlé, T., Federighi, M., & Chobert, J. M. (2008). Effect of pulsed-light treatment on milk proteins and lipids. *Journal of agricultural and food chemistry*, *56*(6), 1984-1991.

Fahmi, R., Khodaiyan, F., Pourahmad, R., & Emam-Djomeh, Z. (2011). Effect of Ultrasound Assisted Extraction upon the Protein Content and Rheological Properties of the Resultant Soymilk. *Advance Journal of Food Science and Technology*, *3,*245-249*.*

FARRP (Food Allergy Research and Resource Program) (2008). Searchable protein database: Soybean. <URL: http://www.allergenonline.com/db_sort.asp> [Accessed 12.12.11]

Feng, H., Yang, W., & Hielscher, T. (2008). Power ultrasound. *Food Science and Technology International*, *14,* 433-436*.*

Franck, P., DA, M. V., Dousset, B., Kanny, G., Nabet, P., Guenard-Bilbaut, L., & Parisot, L. (2002). The allergenicity of soybean-based products is modified by food technologies. *International archives of allergy and immunology*, *128*(3), 212-219.

Friedman, M., & Brandon, D. L. (2001). Nutritional and health benefits of soy proteins. *Journal of Agricultural and Food Chemistry*, *49*(3), 1069-1086.

Garino, C., Zitelli, F., Travaglia, F., Coϊsson, J. D., Cravotto, G., & Arlorio, M. (2012). Evaluation of the impact of sequential microwave/ultrasound processing on the IgE binding properties of Pru p 3 in treated peach juice. *Journal of agricultural and food chemistry*, *60*(35), 8755-8762.

Giri, S. K., & Mangaraj, S. (2012). Processing Influences on Composition and Quality Attributes of Soymilk and its Powder. *Food Engineering Reviews*, *4*(3), 149-164.

Giri, S. K., & Mangaraj, S. (2012). Processing Influences on Composition and Quality Attributes of Soymilk and its Powder. *Food Engineering Reviews*, *4*(3), 149-164.

Gomez-Lopez, V. M., Ragaert, P., Debevere, J., & Devlieghere, F. (2007). Pulsed light for food decontamination: a review. *Trends in Food Science & Technology*, *18*(9), 464-473.

Helm, R. M., Cockrell, G., Connaughton, C., West, C. M., Herman, E., Sampson, H & Burks, A. (2000). Mutational analysis of the IgE-binding epitopes of P34/Gly m Bd 30K. *Journal of allergy and clinical immunology*, *105*(2), 378-384.

Herian, A. M., Taylor, S. L., & Bush, R. K. (1993). Allergenic reactivity of various soybean products as determined by RAST inhibition. *Journal of food science*, *58*(2), 385-388.

Herman, E. M., Helm, R. M., Jung, R., & Kinney, A. J. (2003). Genetic modification removes an immunodominant allergen from soybean. *Plant Physiology*, *132*(1), 36-43.

Herrero, A. M., Jiménez- Colmenero, F., & Carmona, P. (2009). Elucidation of structural changes in soy protein isolate upon heating by Raman spectroscopy. *International Journal of Food Science & Technology*, *44*, 711-717.

Huang, Q., Li, L., & Fu, X. (2007). Ultrasound effects on the structure and chemical reactivity of cornstarch granules. *Starch - Stärke*, *59*, 371-378.

James, C. (2008). *Global status of commercialized biotech/GM crops: 2008* (Vol. 39). Ithaca, NY: ISAAA.

Johnson, P. E., Van der Plancken, I., Balasa, A., Husband, F. A., Grauwet, T., Hendrickx, M. & Mackie, A. R. (2010). High pressure, thermal and pulsed electric-field- induced structural changes in selected food allergens. *Molecular nutrition & food research*, *54*(12), 1701-1710.

Karki, B., Lamsal, B. P., Jung, S., van Leeuwen, J. H., Pometto III, A. L., Grewell, D., & Khanal, S. K. (2010). Enhancing protein and sugar release from defatted soy flakes using ultrasound technology. *Journal of Food Engineering*, *96*, 270-278.

Kentish, S., & Ashokkumar, M. (2011). The physical and chemical effects of ultrasound. In *Ultrasound Technologies for Food and Bioprocessing* (pp. 1-12). Springer New York.

Kenward, K. D., Brandle, J., McPherson, J., & Davies, P. L. (1999). Type II fish antifreeze protein accumulation in transgenic tobacco does not confer frost resistance. *Transgenic research*, *8*(2), 105-117.

Knorr, D., Zenker, M., Heinz, V., & Lee, D. U. (2004). Applications and potential of ultrasonics in food processing. *Trends in Food Science & Technology*, *15*, 261-266.

Koppelman, S. J., & Hefle, S. L. (2006). *Detecting allergens in food* (pp. 1-20). Woodhead Publishing Ltd.

Krishnamurthy, K., Irudayaraj, J., Demirci, A., & Yang, W. (2008). 11 UV Pasteurization of Food Materials. *Food processing operations modeling: design and analysis*, 281.

Krishnan, H. B., Kim, W. S., Jang, S., & Kerley, M. S. (2009). All three subunits of soybean β-conglycinin are potential food allergens. *Journal of agricultural and food chemistry*, *57*(3), 938-943.

Kwok, K. C., MacDougall, D. B., & Niranjan, K. (1999). Reaction kinetics of heat-induced colour changes in soymilk. *Journal of food engineering*, *40*(1), 15-20.

L'Hocine, L., & Boye, J. I. (2007). Allergenicity of soybean: new developments in identification of allergenic proteins, cross-reactivates and hypoallergenization technologies. *Critical reviews in food science and nutrition*, *47*(2), 127-143.

Lakshmanan, R., De Lamballerie, M., & Jung, S. (2006). Effect of Soybean- to-Water Ratio and pH on Pressurized Soymilk Properties. *Journal of food science*, *71*(9), E384-E391.

Lemaux, P. G. (2008). Genetically engineered plants and foods: a scientist's analysis of the issues (Part I). *Annu. Rev. Plant Biol.*, *59*, 771-812.

Li, H., Pordesimo, L., & Weiss, J. (2004). High intensity ultrasound-assisted extraction of oil from soybeans. *Food research international*, *37*, 731-738.

Li, X., Li, Z., LiN, H., & Samee, H. (2011). Effect of Power Ultrasound on the Immunoactivity and Texture Changes of Shrimp (Penaeus vannamei). *Czech Journal of Food Sciences*, *29*, 508-514.

Li, Y. Q., Tian, W. L., Mo, H. Z., Zhang, Y. L., & Zhao, X. Z. (2012). Effects of Pulsed Electric Field Processing on Quality Characteristics and Microbial Inactivation of Soymilk. *Food and Bioprocess Technology*, 1-10.

Li, Y., Yang, W., Chung, S. Y., Chen, H., Ye, M., Teixeira, A. & Shriver, S. (2011). Effect of pulsed ultraviolet light and high hydrostatic pressure on the antigenicity of almond protein extracts. *Food and Bioprocess Technology*, *6*(2), 431-440.

Li, Z. X., Lin, H., Cao, L. M., & Jameel, K. (2006). Effect of high intensity ultrasound on the allergenicity of shrimp. *Journal of Zhejiang University Science*, *7*, 251-256.

Liggins, J., Bluck, L. J. C., Runswick, S., Atkinson, C., Coward, W. A., & Bingham, S. A. (2000). Daidzein and genistein contents of vegetables. *British Journal of Nutrition*, *84*(5), 717-725.

Malaki Nik, A., Tosh, S., Poysa, V., Woodrow, L., & Corredig, M. (2008). Physicochemical characterization of soymilk after step-wise centrifugation. *Food Research International*, *41*(3), 286-294.

Manzocco, L., Panozzo, A., & Nicoli, M. C. (2013). Effect of pulsed light on selected properties of egg white. *Innovative Food Science & Emerging Technologies*.

Nooji,J. (2011). Reduction of wheat allergen potency by pulsed ultraviolet light, high hydrostatactic pressure and non-thermal plasma. M.S. thesis, Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL,USA 32611.

Nordlee, J. A., Taylor, S. L., Townsend, J. A., Thomas, L. A., & Bush, R. K. (1996). Identification of a Brazil-nut allergen in transgenic soybeans. *New England Journal of Medicine*, *334*(11), 688-692.

Ogawa, T., Samoto, M., & Takahashi, K. (2000). Soybean allergens and hypoallergenic soybean products. *Journal of nutritional science and vitaminology*, *46*(6), 271-279.

Omoni, A. O., & Aluko, R. E. (2005). Soybean foods and their benefits: potential mechanisms of action. *Nutrition reviews*, *63*(8), 272-283.

Panda, R. (2012). Soybean allergy: Effect of genetic modification (GM), heat and enzymatic treatment on overall allergenicity.

Patist, A., & Bates, D. (2008). Ultrasonic innovations in the food industry: from the laboratory to commercial production. *Innovative food science & emerging technologies*, *9*(2), 147-154.

Peñas, E., Gomez, R., Frias, J., Baeza, M. L., & Vidal-Valverde, C. (2011). High hydrostatic pressure effects on immunoreactivity and nutritional quality of soybean products. *Food Chemistry*, *125*, 423-429.

Rock, C., Yang, W., Goodrich-Schneider, R., & Feng, H. (2012). Conventional and alternative methods for tomato peeling. *Food Engineering Reviews*, *4*, 1-15.

Sathe, S. K., Teuber, S. S., & Roux, K. H. (2005). Effects of food processing on the stability of food allergens. *Biotechnology advances*, *23*, 423-429.

Shewry, P. R., Tatham, A. S., & Halford, N. G. (2001). Genetic modification and plant food allergens: risks and benefits. *Journal of Chromatography B: Biomedical Sciences and Applications*, *756*(1), 327-335.

Shriver, S., Yang, W., Chung, S. Y., & Percival, S. (2011). Pulsed ultraviolet light reduces immunoglobulin E binding to Atlantic white shrimp (Litopenaeus setiferus) extract. *International journal of environmental research and public health*, *8*(7), 2569-2583.

Shun-Tang, G., Ono, T., & Mikami, M. (1997). Interaction between protein and lipid in soybean milk at elevated temperature. *Journal of agricultural and food chemistry*, *45*(12), 4601-4605.

Smith, C. J., Watson, C. F., Morris, P. C., Bird, C. R., Seymour, G. B., Gray, J. E., & Grierson, D. (1990). Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. *Plant molecular biology*, *14*(3), 369-379.

Song, Y. S., Frías, J., Martinez-Villaluenga, C., Vidal-Valdeverde, C., & de Mejia, E. G. (2008). Immunoreactivity reduction of soybean meal by fermentation, effect on amino acid composition and antigenicity of commercial soy products. *Food Chemistry*, *108*, 571-581.

Song, Y. S., Martinez- Villaluenga, C., & De Mejia, E. G. (2008). Quantification of human IgE immunoreactive soybean proteins in commercial soy ingredients and products. *Journal of food science*, *73*(6), T90-T99.

Soria, A. C., & Villamiel, M. (2010). Effect of ultrasound on the technological properties and bioactivity of food: a review. *Trends in Food Science & Technology*, *21*, 323-331.

Stacey G. Genetics and Genomics of Soybean. New York: Springer,Science and Business Media, 2008. 3–17

Stanic- Vucinic, D., Stojadinovic, M., Atanaskovic- Markovic, M., Ognjenovic, J., Grönlund, H., van Hage, M., ... & Velickovic, T. C. (2012). Structural changes and allergenic properties of β- lactoglobulin upon exposure to high- intensity ultrasound. *Molecular nutrition & food research*, *56*, 1894-1905

Tanabe, S. (2007). Epitope peptides and immunotherapy. *Current Protein and Peptide Science*, *8*(1), 109-118.

Taylor, S. L., & Hefle, S. L. (2001). Food allergies and other food sensitivities. *food technology-champaign then chicago-*, *55*(9), 68-84.

Tezuka, M., Taira, H., Igarashi, Y., Yagasaki, K., & Ono, T. (2000). Properties of tofus and soymilks prepared from soybeans having different subunits of glycinin. *Journal of agricultural and food chemistry*, *48*(4), 1111-1117.

Tryphonas, H., Arvanitakis, G., Vavasour, E., & Bondy, G. (2003). Animal models to detect allergenicity to foods and genetically modified products: workshop summary. *Environmental health perspectives*, *111*, 221-222.

Vorlova, L. (2011). Important Minor Soybeans Proteins: Soybean Allergens and Enzymes Inhibitors, Soybean and Health, Prof. Hany El-Shemy (Ed.), ISBN: 978-953-307-535-8, InTech, DOI: 10.5772/17937. Available from: http://www.intechopen.com/books/soybean-and-health/important-minor-soybens-proteins-soybean-allergens-and-enzymes-inhibitors

Wal, J- M. "Thermal processing and allergenicity of foods." *Allergy* 58.8 (2003): 727-729.

Wambura, P., & Verghese, M. (2011). Effect of pulsed ultraviolet light on quality of sliced ham. *LWT-Food Science and Technology*, *44*(10), 2173-2179.

Wekhof, A., Trompeter, F. J., & Franken, O. (2001, June). Pulsed UV disintegration (PUVD): a new sterilisation mechanism for packaging and broad medical-hospital applications. In *The first international conference on ultraviolet technologies* (pp. 14-16).

Wilson, S., Blaschek, K., & Mejia, E. G. (2005). Allergenic proteins in soybean: processing and reduction of P34 allergenicity. *Nutrition reviews*, *63*, 47-58.

Wu, H., Zhang, Y., Zhu, C., Xiao, X., Zhou, X., Xu, S.;& Huang, M. (2012). Presence of CP4-EPSPS Component in Roundup Ready Soybean-Derived Food Products. *International journal of molecular sciences*, *13*(2), 1919-1932.

Yada, R.Y. (2004). Soy proteins. Proteins in Food Processing (pp. 123-145).Woodhead Publishing.

Yamanishi, R., Huang, T., Tsuji, H., Bando, N., & Ogawa, T. (1995). Reduction of the soybean allergenicity by the fermentation with Bacillus natto. *Food Science and Technology International, Tokyo*, *1*(1), 14-17.

Yang, W. W., Chung, S. Y., Ajayi, O., Krishnamurthy, K., Konan, K., & Goodrich-Schneider, R. (2010). Use of pulsed ultraviolet light to reduce the allergenic potency of soybean extracts. *International journal of food engineering*, *6*,1-12.

Yang, W. W., de Mejia, E. G., Zheng, H., & Lee, Y. (2011). Soybean allergens: presence, detection and methods for mitigation. *Soybean and Health. Rijeka: InTech*, 433-464.

BIOGRAPHICAL SKETCH

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