Impact of High Pressure Processing on Immunoreactivity and Some Physico-chemical Properties of Almond Milk

THESIS

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By

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ABSTRACT

Almond milk as a beverage has recently gained attention with increase in consumer awareness about health benefits of plant based beverages. The objective of this research was to investigate the influence of high pressure processing (HPP) on residual immunoreactivity, protein solubility, and other selected quality attributes. Almond milk was prepared by disintegrating 10% almonds with water and were subjected to high pressure processing (HPP; 450 and 600 MPa at 30°C up to 600 s) and thermal processing (TP; 72, 85 and 99 °C, 0.1 MPa up to 600 s).

After HPP (for all holding times), amandin, a key thermally resistant almond allergen, can no longer be detected by the anti-conformational epitopes MAb in ELISA while signal generated from the anti-linear epitopes MAb was reduced by half (P < 0.05). On the other hand, most TP samples did not show significant reductions in immunoreactivity (P > 0.05) unless processed at 85 and 99 °C for 300 s. Western blot (applicable for soluble proteins) and dot blot (applicable for soluble and insoluble proteins) also confirmed the loss of immunoreactivity by both antibodies for HPP almond milk. The reduced band intensity of the 61 and 63 kDa polypeptides and concomitant appearance of high molecular weight polypeptides in Western blot indicated that the observed decrease in immunoreactivity was partly due to the aggregation of amandin. Within the tested
conditions of the study, HPP and TP treatments respectively caused a maximum of ~70% and ~75% reduction in protein solubility in almond milk. Within the experimental ranges of the study, particle size analysis of pressure treated almond milk revealed the increase in volumetric diameter ($D_{4,3}$), surface area diameter ($D_{3,2}$) and volume median diameter ($D_{0.5}$) with increase in pressure level and treatment time possibly due to protein aggregation. The apparent aggregation rate constant for 450 MPa and 600 MPa processed samples were $k_{450\text{MPa}, 30 \degree C} = 0.0045 \text{ s}^{-1}$ and $k_{600\text{MPa}, 30 \degree C} = 0.0095 \text{ s}^{-1}$ respectively. Similarly, TP also caused alteration in particle size distribution with net effect of increase in various particle diameters when treated at least at 85 °C for 300s. On the other hand, heat-treated samples did not fit with any of the kinetic models tested in our study.

Confocal laser scanning microscopy (CLSM) examination and UV spectral data of almond milk proteins also confirmed the aggregation of proteins and fats present in almond milk. Pressure treatment also enhanced the whiteness and greenness of almond milk but these values were not significantly different in heat treated (72 and 85 °C) samples for equivalent treatment time.

The study provided preliminary evidence that pressure treatment has the potential to develop hypoallergenic almond based beverages with modified protein structures. However, it is worth note that aggregation and loss of protein solubility, rather than the epitope destruction, may be responsible for the observed decrease in immunoreactivity of the tested amandin epitopes. Future studies on immunoreactivity of variety of soluble and insoluble protein fractions are necessary to further understand mechanism of inactivation of almond allergenic proteins by high pressure processing.
Dedicated to my dad, mom, brother, wife and daughter
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CHAPTER 1

INTRODUCTION

The demand for non-dairy milk beverages has been gaining popularity in recent years with increased consumer awareness of plant-based milk health benefits. In addition, consumers’ health concerns including lactose intolerance, milk allergy, and use of growth hormones in cow’s milk were also attributed as some of the factors influencing growth of non-dairy beverage market (Zacharias, 2012).

1.1. Plant based milk beverages

Plants based milk beverages can be made from cereals such as rice, oats, and barley, nuts such as almonds, hazelnuts and walnuts and legumes (Anonymous, 2013). Soy, rice and almond milk are popular examples of non-dairy milks in the USA while oat milk is common in the Europe. These beverages vary in taste and texture and are available in different formulations in the market. Amongst the different dairy alternatives beverages, almond milk has been gaining popularity which can be manifested from its highest sales value in 2011. As reported by Latif (2013), almond milk, the most popular non dairy milk after soymilk, occupied 21% of the retail market for dairy alternative market. About 9%
of US adults consume almond milk, contributing substantial sales volume and that is why the dairy alternative beverages market reached $1.33 billion in 2011 (Latif, 2013).

1.2. High pressure processing

Conventional thermal processing can destroy health promoting nutrients and bioactive compounds. Food engineers and scientists have been striving to design, develop novel food processing methods that are environment friendly, cost effective and sufficient to meet consumers’ health expectation. Advanced thermal and non-thermal technologies are being investigated. Among the nonthermal processing methods, high pressure processing (HPP) is a novel non thermal processing technology which has gained wide spread industry acceptance for processing variety of value added products. Essentially, HPP is a pasteurization process, wherein pressure up to 87000 psi or ~600 MPa at ambient or chilled temperatures to inactivate vegetative spoilage and pathogenic microorganisms and extend the shelf life of the product. The process causes subtle effect on lower molecular weight food constituents. Such pressure treated product usually results with improved or better appearance, taste and texture than thermally treated products. Some commercially available high pressure processed products include salsa, guacamole, ham, fruit juices, guacamole, oysters and poultry products. Annual market share of high pressure processed products has been estimated to be ~ $ 2 billion (Balasubramaniam et al. 2008). Very limited knowledge is available on the impact of pressure treatment in preserving fresh-like quality attributes of almond milk.
Besides, high pressure processing can also alter molecular structures of proteins that may produce significant impact on immunoreactivity, biological activity and functionality of protein rich foods and beverages.

1.3. Objectives

The objective of this research was to investigate the influence of high pressure processing (HPP) on residual immunoreactivity and characterize its influence on protein profiles and solubility, in addition to other selected quality attributes.

I. Immunoreactivity

Almond which is the major ingredient in almond milk belongs to the tree nuts, and thus belongs to one of the eight allergenic foods. Research has shown that thermal processing is insufficient to eliminate or reduce the allergenicity of almonds. So, the study of allergenic potential of pressure treated almond milk was the primary interest of this study.

II. Physico-chemical properties

The second part of the study is related with the impact of pressure treated almond milk in modifying its physicochemical properties, more specifically particle size distribution and characterization. Particle size distribution of almond milk is also the most important parameter to be considered while optimizing the processing conditions and modifying the functional properties of food constituents.
References


CHAPTER 2

PROCESSING EFFECTS ON IMMUNOREACTIVITY & PHYSICOCHEMICAL PROPERTIES OF PLANT BASED MILK BEVERAGES

2.1. Introduction

The proliferation of almonds (*Prunus dulcis*) has been believed to be evolved in its wild form around the Mediterranean regions and then across the globe when distributed by the explorers. Since 1977, USA, has become the largest producer of almond in the world and the recent annual production has exceeded 730000 MT, accounting 80% of global production (FAO 2011). Its economic importance has been increased because of its versatility in use including foods ranging from ready to eat roasted nuts to the important ingredients in many processed foods, pharmaceuticals and cosmetics.

Almonds nuts have gained more attention since last decades due to high nutrient density and health benefits associated with their consumption. Almonds nuts are valued for their protein, essential fatty acids, dietary fibers, and several important micronutrients like potassium, magnesium, calcium, tocopherols and arginine. Moreover, almonds belonging
to the group of tree nuts also contain other wide range of phytonutrients such as carotenoids, phenolics acids, phytosterols, polyphenols, spingolipids and lignin. Due to these array of essential and non essential nutrients in almonds nuts, consumption of almonds have also been linked with reduction of heart disease, certain cancer risks, gallstones formation and many beneficial metabolic effects (Chen, and Blumberg 2008; Ros 2010; Bolling et al. 2011). Moreover, U.S. Food and Drug Administration (FDA) has also recognized tree nuts and thus almonds as “heart-protective” foods (FDA 2003).

Almonds milk refers to aqueous extracts of almond nuts. Almond milk is not milk in real sense, what is why these products are tagged with multiple generic names such as non-dairy beverage, plant based beverages, vegetable milk and nut milk /drinks depending upon their convenience. In past the primary role of almond milk was to provide nutrients in addition to quench the thirst. Generally almond milk beverages were alternatives to dairy milk for cholesterol conscious, lactose intolerance consumers who are sensitive to dairy proteins. In addition, due to consumers’ concern regarding the use of the antibiotics or growth hormones often found in dairy milk, plants based beverages are gaining popularity and has also become a choice for other groups of people like vegans and vegetarians. With the advent of functional foods, non dairy beverages and hence almond milk has also graduated from just refreshment and rehydration to functional beverages, thus rendering potential health benefits to the consumers in delicious way if not in mundane.

According to the report from Packaged Facts, plant based milk beverages increased its market in recent years amounting to $1.33 billion in 2011 (Latif 2012). Almond milk had the highest sales among alternative dairy beverages in 2011 and currently dairy
alternative beverages accounted for 21% of retail market. It has been estimated that ~ 9% of the U.S adults consume almond milk (Latif, 2012).

The purpose of this review article is two fold: (a) to review concepts associated with almond proteins and allergens, and discuss basic principle of thermal and non thermal processing (high pressure processing) approaches for preserving almond milk and their impact on physiochemical properties and allergen.

2.2. Food Allergy

Food allergies are considered to be the fourth most important public health problems by World Health Organization. It has also been estimated that ~ 2 % adults and 5-8% children are affected by some kind of food allergies (Kirsh et al., 2009). According to Food Allergen Labeling and Consumer Protection Act (FALCPA) 2004, the food manufacturers are required to disclose the presence of the allergens on the label. The top 8 major allergenic food groups described in the act are milk, eggs, fish, shellfish, peanuts, tree nuts, soybeans and wheat. These foods groups cover about 90% of the allergen foods from more than 160 foods (FDA, 2004).

Food allergy is defined as an immune response to food proteins. Food allergies may be classified in a) IgE-mediated (b) cell-mediated such as T cells and mostly involve the gastrointestinal tract; c) mixed IgE and non IgE mediated (Sicherer, et al., 2009).

Immunoglobulin E (IgE) are the most common food allergy reactions and is the leading cause of anaphylaxis in Western Europe and the United States . IgE-mediated classic food allergic reactions are usually immediate, reproducible, and readily diagnosed by detection of food-specific IgE. In IgE mediated food allergenic reactions, the allergens at
first sensitizes the memory cells and later on during subsequent exposure the production of IgE is triggered followed through release of histamines and cytokins, the chemicals responsible for allergenic symptoms, by mast cells (Sicherer et al., 2009).

It is further worth to point out that food allergens are almost always proteins but not all food proteins are allergens (Bannon, 2004). Food qualities can be affected during preparation, processing, and storage and food protein functionality modifications due to processing may also plays an important role in determining qualities. For instance, proteins can affect different functional properties like color, odor, flavor, mouthfeel, solubility, viscosity and other rheological properties (Kinsella et al., 1994).

**2.2.1. Almond allergens**

It has been recognized that consumption of almonds, which belongs to tree nuts, may lead to allergenic reactions. It was estimated that approximately 0.7% adults and 0.2% adults suffer from tree nut allergenic reactions (Sampson, 2004).

Allergenic proteins in almonds vary depending upon the molecular weight and protein family. In his review article, Boye et al. (2012) has pointed out different types of almonds allergens and their characterisitics as shown in Table 2.1. More comprehensive lists of almond allergens have also been cited by Costa et al. (2012) totaling up to eight groups of allergens namely, PR-10 (Pru du 1), TLP (Pru du 2), prolamins (Pru du 2S albumin, Pru du 3), profilins (Pru du 4), 60sRP (Pru du 5), and cupin (Pru du 6, Pru du γ-conglutin).

Amandin, the most abundant protein in almonds (protein accounts for approximately 65–75% of total almond protein), is a protein group belonging to 11S seed storage proteins belonging to globulin family. This allergen group is also called as almond major
protein (AMP) which is also known to be thermally stable (Roux et al., 2001) but so far the IgE epitopes (the region of allergenic proteins recognized by antibody) for amandin is not known (Jin and others 2009). Similarly, pressure sensitivity of amandin is not known. The globular protein is further divided into 7S vicilin type and the 11S legumin type. Amandin belongs to the later type and exists in hexameric form possessing total molecular weight of 360 kDa (Breitender, & Radauer, 2004). The different proportion of secondary structures in native amandin and thermally treated amandin has been studied by Albillos et al. (2009). The native amandin has been found to be primarily composed of \( \alpha \)-helix, \( \beta \)-sheet and unordered structure (Table 2.2). Pru-1 and Pru-2 are two seed storage proteins obtained from cDNA and belongs to the major component of functional almond amandin when these are assembled by disulfide bonds, conferring an elevated thermal stability to the entire protein. Partial unfolding of amandin occurs at minimum 94 °C and then can form aggregate of different structures within foods (Gekko, & Timasheff, 1981).

2.2.2. Epitopes and monoclonal antibodies for detecting almond allergens

Epitopes can be defined as that portion of allergenic molecule that can recognize and bind to the IgE molecule. B cell epitopes can be of two types; sequential and conformational. Sequential epitopes are also called as linear epitopes corresponding to stretch of amino acids while conformational epitopes corresponding to specific orientation of proteins (Kshirsagar et al., 2011). Kshirsagar et al. (2011) also described the use of monoclonal antibody 4F10, the antibody that targets the linear epitope during characterization of almonds and cashew proteins. Willison and et al. (2013) also produced a monoclonal antibody 4C10 from amandin-specific mouse
monoclonal antibodies 4C10 that can recognize conformational epitopes of amandin through epitope mapping study.

2.3. **Methods of preserving liquid beverages**

Almond milk is a low acid plant based beverage having pH > 4.6 (Valencia-flores et al., 2013). Consumption of unsafe low-acid chilled foods may lead to human diseases due to variety of pathogenic spores (such as *C. botulinum, Bacillus cereus*) and vegetative pathogens (such as *L. monocytogenes, E. coli* serotype O157:H7, *Salmonella enteritidis*) (Silva et al., 2010). Salmonella can be the major problems in unprocessed almonds, so for added safety, almonds processors in California are required to blanch the almonds during processing but still the products can be labeled as raw almonds (Salin, 2012). Food preservation involve steps to destruction of pathogenic microorganisms that cause food borne illness as well as any spoilage organisms that may cause economic spoilage. Similarly, food preservation also inactivates enzymes that may spoil the foods. Thermal processing is the commercially wide practiced technology. Depending upon the end preservation goals and intensity of treatment, thermaly processing may involve pasteurization, commercial sterilization, and evaporation. Thermal pasteurization, is a mild thermal treatment designed to inactivate harmful pathogenic vegetative microorganisms of public safety concern as well as any spoilage organisms that may cause economic spoilage. Pasteurization often requires a secondary barrier for preservation including refrigeration, fermentation or other means such that the environment is not suited for growth of spoilage and pathogenic microorganisms (Lund, 1988).
With the recent advancement of novel nonthermal processing methods such as high pressure processing, pulsed electric field processing, and irradiation, pasteurization is now can be defined as any thermal or non thermal process, treatment, or combination thereof that is applied to food to reduce the most resistant microorganism of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage (NACMCF, 2006).

Commercial sterilization involve severe heat treatment of foods such that it renders the product free of microorganisms that are capable of reproducing in the food under normal non-refrigerated conditions of storage and distribution (Awuah et al., 2007).

In conventional thermal processing, heat is generated with direct or indirect contact on the outside of the product to be heated by combustion of fuels or electrical heating devices and the heat is transferred into the product through conduction and convection (Pereira, and Vincete, 2010). The main goals of conventional food processing is to ensure microbiological safety and extend the shelf life of food through destruction of enzymes and toxins sometimes at the cost of sensorial and nutritional deterioration. While trying to reach the desirable microbial lethality at cold point, food constituents in other areas may be over processed leading to destruction of nutrients and sensory attributes (Pereira, and Vincete, 2010).

In recent years, novel thermal technologies such as ohmic heating and microwave heating for inactivating microorganisms have also been developed. These novel thermal technologies enables foods to heat volumetrically, quickly and uniformly because thermal energy is generated directly inside the food (de Alwis, and Fryer, 1990; Sastry, and Barach, 2000).
Emerging non thermal food-processing technologies such as high pressure processing, irradiation or pulsed electric filed processing can also be employed for preservation of foods. They use alternative lethal agents (such as pressure, electric field, or ionizing radiation) to kill the bacterial instead of heat without compromising product quality. (Balasubramaniam, et al., 2008).

Thermal food processing and non-thermal food processing can alter the immunoreactivity and physicochemical properties of foods in different ways (Shriver, and Yang 2011). Likewise, several studies have demonstrated that thermal processing (Kwok et al., 2002 and Kwok, and Niranjan, 1995) and high pressure processing (HPP) (Barba et al., 2012; Wang et al., 2008) modifies the physicochemical properties of liquid foods.

2.3.1. Thermal Processing

Thermal processing is defined as the combination of temperature and time required to eliminate a desired number of microorganisms from a food product and is intended primarily for preserving and extending the useful shelf-life of foods. Thermal processing is effective at destroying vegetative, foodborne pathogenic bacteria. There are different ways of pasteurizing foods based on time temperature relationship. In Low-Temperature-Long-Time Treatment (LTLT) method the foods are heated at lower temperature for a longer time to kill pathogenic bacteria. For example, milk is pasteurized at 145° F (63° C) for 30 minutes. Alternatively, High-Temperature-Short-Time Treatment (HTST) employs higher heat for less time to kill pathogenic bacteria. For example, milk is pasteurized at 161° F (72° C) for 15 seconds (US PHS/FDA, 1999).
The composition of added ingredients such as additives should also be taken into consideration when calculating the process time. In a study with soy milk enriched with bovine immunoglobulin, the heat treatment of 78.2 °C for 120 s caused 85% reduction of bovine Ig G for simultaneous 5 log reductions of natural flora in soy milk (Li et al., 2006).

Heat treatment and pH also play an important role in reducing the final soluble solids content in some non dairy beverages due to structural changes in proteins. Heat may cause denaturation, improved digestibility, increase in surface sulfhydryl content, surface hydrophobicity, protein aggregation and reduction in protein solubility of nuts and legumes (Lee, and Beuchat, 1992; Venkatachalam et al., 2006; Shimoyamada et al., 2012).

Food engineers use thermal process calculations to establish the desired time-temperature needed for safe processing of foods. This include knowledge of D-value, F-value and z value. D-value is the time required to reduce target microbial population by 90% at a given temperature. D-value can be obtained from semi log plot of (number of survivors) against time. F- value is defined as time required for stated number of log reduction of microorganisms at specified temperature to achieve the microbial safety. Thus F- value can be used to calculate total lethality of microorganism under the given condition. z-value represent the temperature sensitivity of microorganisms. Z-value can be estimated from the plot of D values against temperature. The z value thus obtained can be utilized to integrate the effect of heat treatment over a range of temperatures, and for calculation of equivalent pasteurization or sterilization values at other temperatures (Lund et al., 2002; Ahn et al., 2007).
Since thermal processing is primarily carried out with food safety in mind, its impact on food allergens are often overlooked. In addition, to inactivating microorganisms, heat treatment can also adversely influence product quality attributes. Subjective quality loss like taste can be impaired and quantifiable factors such as nutrient degradation may also occur even at minimum safety level (Subramaniam et al., 2006)

2.3.1.1. Effect of thermal processing on proteins and allergenicity

To understand the antigenic potential of heat treated allergenic proteins, it is crucial to know how thermal processing affects the structure of the proteins. In its native state, a globular protein exists in a highly compact stable form determined by the primary, secondary, tertiary and quaternary structure. Formation of peptides bond (covalent) between amino acids leads to the formation of primary structure, involvement of non covalent bonds, primarily hydrogen bonds between the atoms makes the secondary structure such as α-helix and β-sheets, and additional involvement of other non covalent bonds stabilize to form 3D folded structure called as tertiary structure. Quaternary structure are composed of sub units of proteins molecules to form conformational fitting with each other and sometimes with the help of ligands. All these structures are responsible for optimum protein functions. During denaturation, higher order proteins (other than primary structure) structure may be temporarily or permanently altered and this phenomena transforms the protein from native to less –ordered state (Bischof, and He, 2006).

Melting temperature (T_m) or transition temperature is defined as the temperature during which half of the protein denatures. During the process of denaturation, there may be
intramolecular or intermolecular proteins interactions which may result in aggregation, coagulation, and gelation. A type of protein – protein interactions that result with the formation of complexes of higher molecular weight is termed as aggregation.

Coagulation can be defined as the thermally irreversible randomly aggregation of protein molecules after denaturation. Finally, gelation is described as formation of three dimensional network due to orderly aggregation of proteins (Bischof, and He, 2006).

From thermodynamics point of view, denaturation can be explained as the conversion of native to non-native structure due to supply of suitable energy. Fig 2.1 shows the energy required and state of protein during denaturation. Activation energy barrier (kinetic energy barrier) and the enthalpic (total heat absorption or release) are two different regime where the former determines the time-temperature dependency for the denaturation and the later gives the energy required for phase transition. When the protein gains sufficient energy to exceed the free-energy barrier of activation, the denaturation process is thermodynamically favorable. The denatured protein may be at a higher or lower total energy state in comparison to the original one. Exothermic process such as coagulation, aggregation, and/or gelation result in lower energy than the initial value (Bischof, and He, 2006).

2.3.1.1. Thermal denaturation and aggregation

There is only marginal energy difference between native protein conformation and unfolded conformations in terms of thermal stability, usually about 5–20 kcal/mole. When proteins are exposed to increasing temperature, losses of solubility or enzymatic activity occurs over a fairly narrow range. Depending upon the protein studied and the
severity of the heating, these changes may or may not be reversible. As the temperature is increased, a number of bonds in the protein molecule are weakened and as heating continues, some of the cooperative hydrogen bonds that stabilize helical structure will begin to ruptures and hydrophobic groups are exposed to the solvent. These may destabilize the net stability of protein that arise from hydrophobic interactions, hydrogen bonding, van der Waals’ forces, electrostatic forces, and intrinsic propensities (local peptide interactions). On the other side there will also be the increase in entropy upon unfolding of the proteins. The net effect can result in aggregation of proteins (Chi et al., 2003). The mechanisms of protein folding and aggregation has been described in detail by Gsponer and Vendruscolo (2006). It is based on the concept of minimizing free energy. During folding small globular proteins appear to be characterized by a funnel-like landscape that rapidly and reliable reaches the bottom of the funnel where there is minimum free energy. The unfolded protein possess both high entropy (large number of possible conformational states) and free energy at the beginning, which means more unstable and more alternatives of conformational states. Formation of aggregates are possible due to intermolecular interactions on their pathways and are trapped at different locations (Figure 2.2).

2.3.1.1.2. Effect of temperature on protein solubility

Structure and solubility of proteins are quite interrelated. Temperature can influence the structure and solubility of protein depending upon the nature and composition of protein in solution. The solubility of almonds proteins decrease with increase in thermal treatments (Sathe, and Sze, 1997; Venkatachalal et al., 2002). It has been well
established that thermal treatment may cause protein unfolding due to distortion in secondary, tertiary and quaternary structure that may result in aggregation and/or precipitation which ultimately shows decrease in protein solubility (Balny et al., 1989; Gross, and Jaenicke, 1994).

2.3.1.1.3. Measurement of protein solubility and denaturation

The measurement of protein solubility helps to give information on protein denaturation although it does not explain structural changes within the structure (Cherry et al., 2006). Different methods of colorimetric methods for estimation of proteins are available such as Bradford method (Bradford, 1976), UV absorption (Whitaker, & Granum, 1980), Biuret method (Ruth et al., 1964) and Lowry method (Lowry et al., 1956).

The Bradford assay is based upon the shift of absorbance maximum of a dye, Coomassie Brilliant Blue G-250, from 465 nm to 595 nm for an acidic solution when it binds the protein. The anionic form of the dye is stabilized by hydrophobic and ionic interactions with conspicuous change in color. The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues.

Most proteins absorb maximum radiation in near UV region due to Trp and Tyr (Phe and disulfide bonds also absorb to a very small extent) (Aitken, and Learmonth, 1997). So, the absorption spectra of most proteins show a band with peak at 280 nm and minimum at 250 nm. However, some nucleoproteins also show a maximum absorption in the region of 260 nm due to purine and pyrimidine molecule (Goldfarb et al., 1951). The processing stability, high pressure processing and thermal processing, can be investigated by using UV/Vis spectroscopy (Herberhold et al., 2003).
2.3.1.1.4. Thermal effects on allergens

Processing conditions can alter the structure of epitopes which in turn can influence the protein allergenic properties. Accordingly, the allergenicity may be reduced when existing epitopes on proteins get destroyed or it may generate neo allergens with the net effect of altered protein conformation. Neo allergens have also been described in the literature in few foods such as pecans (Malanin et al., 1995) and wheat flour (Leduc et al., 2003). Heating have been associated with decreased allergenicity such as pollen-related fresh fruit and vegetable or sometimes with no significant effect (shrimp upon heating). Conformational epitopes are typically expected to be more susceptible to processing-induced destruction than the linear epitopes on the same allergen. Thermal processing exists in different forms such as dry heat (e.g., oven roasting, oil roasting, infra-red heating, and ohmic heating) or wet heating conditions moist heat (e.g., in cooking in aqueous media, microwave cooking, pressure cooking (autoclaving), extrusion, blanching, boiling, and steaming). So, alteration in protein structure by thermal processing can lead to epitope destruction, modification, masking, or unmasking thereby decreasing, increasing or having no effect on allergenicity. This can be attributed to protein denaturation, aggregation, and structure disruption by thermal processing and therefore have a potential to modify the allergenic properties of almond proteins. (Sathe et al., 2005).

Studies have shown that conformational epitopes of cherry allergen Pru av 1 can be irreversibly destroyed due to dry heating as a result of browning reaction products (Gruber et al., 2004). In another study, hazelnut allergens, Cora 1~18kDa, Cora 8~9kDa,
Cora 9 ~35-40kDa and Cora 11 ~47-48 kDa have been reported to be decreased as a result of autoclaving (Lopez et al., 2012). On the other hand, thermal processing has also been shown to increase the IgE binding activity of two major peanut allergens Ara h 1 and Ara h 2 (Maleki et al., 2000). This also suggests that the thermal processing may enhance peanut allergenicity.

2.3.1.2. Effect of temperature on particle size distribution

The heating effect of particle size in colloidal food systems are largely dependent upon the structure of colloidal particles. For some foods like soymilk, there is a net effect of decrease in particle size diameter due to disruption of large protein subunits (Ono et al., 1991) whereas thermal denaturation and aggregation of whey proteins leads to net increase in particle diameters (Raikos, 2010).

2.3.1.3. Effect of temperature on color

Thermal processing can change the color of foods mainly due to Maillard reactions. In white liquid foods like soymilk, there can be a decrease in lightness as a consequence of browning induced by heat. Change in color of soymilk can thus also explains the severity of heat treatment (Kwok, and Niranjan, 1995). Trimulus Hunter L(lightness)-, a(redness)- and b(yellowness)-values of milk shows that lightness decrease following 1st order kinetics while redness and yellowness increases following zero order kinetics. It has also been reported that denaturation of proteins can play a role in lightness of milk color (Pagliarini et al., 2006). Change in greenness has also been correlated with the amount of protein in bovine milk (Quinones et al., 1998).
2.3.2. High pressure processing

High-pressure processing (HPP) is a alternative processing technology in which the solid or liquid foods are pressurized up to 400 – 600 MPa at room temperature or refrigerated conditions to achieve pasteurization due to pressure rather than heat. Thus, pressure pasteurization can result in destruction of vegetative cells including spoilage and pathogenic microorganisms, inactivation of many enzymes, allowing most foods to be preserved with minimal negative impact on nutritional and sensory quality attributes. Only non-covalent and semi-covalent bonds can be effected by high pressure treatment and has less influence on low molecular weight food components such as flavor compounds, vitamins and pigments. This means HPP retains most of the food quality and can be used as an alternative for thermal processing and thus reduce the risk of deterioration in nutritional and sensory quality attributes due to over processing by thermal treatment (Balasubramaniam et al., 2008).

Application of high pressure pasteurization (up to 600 MPa at 5 - 120 °C) for extending the shelf life of many refrigerated and shelf-stable products (Evrendilek, & Balasubramaniam, 2011; Smith, Mendonca, & Jung, 2009; Li et al., 2006) have been investigated. Researchers have evaluated efficacy of high pressure processing in variety of fluid foods including human milk (Contadar et al. 2013; Molto-puigmarti et al., 2011), dairy milk (Voigt et al., 2012; Al-Nabulsi et al., 2012), tomato juice (Daryaei, & Balsubramaniam, 2012; Gupta et al., 2010), orange juice (Guignon et al., 2012; Plaza et al., 2011), soymilk (Guerrero-Beltran et al., 2009; Jung, Murphy, & Sala, 2008) and beverages (Barba et al., 2012; Tadapaneni et al., 2012). Very limited studies evaluated
the impact of pressure treatment on almond milk allergens and its physico chemical properties.

2.3.2.1. Basic principles of high pressure processing

Briefly, HPP is governed by 3 principles and/or rules. The first principle is called Le Chatelier’s Principle which explains that HPP is favorable a phenomena which results with decrease in volume and vice versa. Accordingly, the equilibrium shifts in a direction which results with decrease in volume. The second rule/principle, called as isostatic rule describes the characterisites of high pressure processing at constant temperature. At this condition, the distribution of pressure is uniform throughout the pressure vessel and the process is independent of the volume of sample. The third principle describes the effect of HPP on equilibrium shifts of food systems due to ionization effect on water molecules that may also results with negative shift in pH value of foods in solution.

It has been found that HPP delivers very subtle energy in comparison to thermal process and thus the covalents bonds (other than disulfide bonds) usually remain unaltered. It has been found that pressurization results with concomitant rise in temperature that depends upon the compressibility of food components. Each food component has specific heat of compression values (example, water ~3°C/100 MPa, fats and oils ~6 °C-8 °C/-100 MPa). Heat of compression of water and high moisture foods increases with increasing initial temperature. Heat of compression of fats and oils do not change with initial product temperature (Rasanayagam et al., 2003).

2.3.2.2. Effect of HPP on proteins
The effect of pressure on proteins structure can be explained thermodynamically as a change of state from native (N) to denatured state (D) with the consequence of change in molar volume (ΔV) at constant temperature. The standard free energy for an equilibrium reaction can be written as (Mozhaev et al., 1996):

\[ \Delta G = -RT \ln K = \Delta E + P \Delta V - T \Delta S \] ................................. (1)

Differentiating equation (1) with respect to pressure, at constant temperature (T), yields,

\[ \frac{\delta \Delta G}{\delta P} = -RT \frac{\delta \ln K}{\delta P} = \Delta V \] ........................................................... (2)

where \( \Delta V \) is called reaction volume or the variation of the molar volume of the system. If the variation of reaction volume is negligible, then \( \Delta V \) can be obtained from above equation from the following equation (Balny, and Masson, 1993):

\[ \ln K = -\Delta V x P/RT + \text{constant} \] ........................................................... (3)

Native proteins are stabilized in narrow zone by delicate balance between different non covalent (mostly hydrophobic and electrostatic interactions) interactions within the polypeptide chains and with the solvent. The volume of the native proteins are due to sum of the contribution from constituent atoms, internal cavities due to imperfect packing and with the solvent (solvation of peptides and aminoacid residues). Thus the pressure functions by altering the delicate balance of intra molecular and solvent protein interactions (Heremans, 1982). Proteins show the positive compressibility by compensating the opposite effect caused from the hydration of the charged residues due
to dominant negative volume change that is occurred from packing of internal cavities (Heremans, 1982).

The effect of pressure and temperature on equilibria or kinetics can described as two different parameters that may cause different impact on proteins structure. Pressure, from the principle of microscopic ordering, leads to reduction in entropy while increase in temperature leads to increase in entropy due to vibration of molecules and thus it involves volume change as well as change in thermal energy (Mozhaev et al., 1996).

The phase diagram represents the pressure–temperature curve with characteristic ellipsoidal shape for protein native and denatured state (Figure 2.3). Protein stability depends on the balance between the enthalpic and entropic changes. This finding also illustrate the different mechanisms of denaturation for different combination of temperature and pressure (Heremans, and Smeller, 1998).

Pressure induced denaturation at 25-37 °C occurs generally near 400 MPa but moderate pressure like 200 MPa can cause dissociation of native oligomers (Royer, 2002). Like temperature proteins can also form aggregates by high pressure processing (Randolph et al., 2002).

2.3.2.2.1. Effect of pressure on protein solubility

High pressure processing depending upon the severity of treatment may cause protein unfolding, denaturation and formation of aggregates that ultimately reduces protein solubility (Messens et al., 1997). For a small magnitude of pressure (<300MPa), the unfolding can be reversible mainly due to disruption of internal hydrophobic bonds and salt bridges but beyond this point irreversible denaturation occurs in many proteins. The
irreversible denaturation is supposed to be due to formation of disulfide linked aggregates via covalent interactions such as oxidation of sulfhydryl groups present in polypeptides chain. Moreover, some structural changes such as conformational transition of proteins are also governed by pressure induced volume changes (Balny et al., 1989). In pressure treated (up to 600 MPa and 20 min) foods proteins like walnut, soy protein and meat, the solubility of proteins have been found to be reduced (Puppo et al., 2004; Marcos et al., 2010; Quin et al., 2012).

2.3.2.3. Effect of HPP on allergenic proteins

HPP can cause alteration in allergenic protein structure and functions such partial unfolding, complete unfolding and denaturation (Somkuti et al., 2012). Due to the diversity of allergenic proteins, the effect of high-pressure treatment on immunoreactivity varies from study to study. In one study, HPP at 300-600 MPa for 5 min has been reported to decrease the allergenicity of several birch pollen allergy related foods including hazel nut, apple, celery, and peach (Meyer-Pittroff, Behrendt, & Ring, 2007). In addition, apple allergen Mal d 3 (Johnson, Van der Plancken, Balasa, Husband, Grauwet, Hendrickx, Knorr, Mills, & Mackie, 2010; Husband, Aldick, Van der Plancken, Grauwet, Hendrickx, Skypala, & Mackie, 2011), peanut allergen Ara h 2 (Hu, Chen, Gao, Luo, Ma, & Tong, 2011), bovine gamma globulin (Yamamoto, Mikami, Matsuno, Hara, Odani, Suzuki, & Nishiumi, 2010), soybean sprout (Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011), and soybean protein isolate (Li, Zhu, Zhou, & Peng, 2012) were also shown to be altered by pressure treatments. On the other hand, when subjected to HPP, the cod allergen Gad m1 (Somkuti, Bublin, Breiteneder, & Smeller, 2012),
largemouth bass allergens (Liu, Tao, Liu, Chen, & Xue, 2012), and almond allergens (Li, Yang, Chung, Chen, Ye, Teixeira, Gregory, Welt, & Shriver, 2013) did not show a significant decrease in immunoreactivity.

2.3.2.4. Effect of pressure on particle size distribution

Like thermal treatment, studies have shown that high pressure have ability to alter the structure of cells that may bring changes in solubility, permeability and micellarization (Gupta et al., 2011). So, high pressure processing (at 400 – 600 MPa) (Anon and others 2012; Zhang et al., 2005) may increase or decrease the average particle size diameters depending upon the type of constituents, pH, amount of pressure, temperature and treatment time.

It has been reported that at mild pressure, 200 MPa and 10 °C, the casein micelle size in skim milk decreases primarily due to dissociation of casein proteins but as the pressure is increased upto 300 MPa, both disintegration and aggregation of caseins can be observed (Anema et al., 2005). Very limited literature is available on pressure effects on particle size distribution of almond milk.

2.3.2.5. Effect of pressure on color

A decrease of lightness value, and an increase of greenness and yellowness has been reported in ewe’s milk when treated in the pressure range (100 to 500 MPa) and temperature (4, 25, and 50 °C) for 10 min. This changes have been attributed due to structural changes in caseins and lipid content (Gervilla et al., 2001). Changes in lightness and greenness in the pressure range (50 -600 MPa, at 10 °C for 20 - 300s) has
also been correlated with structural modification on bovine muscle proteins (Jung et al., 2003).
References


Figure 2.1. Energy states of protein denaturation. (A) Hydrogen bonds of a simple alpha helix are disrupted and formation of random coil of denatured protein. (B) Protein is denatured after supplying activation energy followed by irreversible aggregation/coagulation. B is just a cartoon representation of the relationship between energy and state (i.e., not to scale). Source: Bischof, and He (2006).
Figure 2.2. A schematic of how minimizing the free energy of a molecule could lead to protein folding native state (left) and aggregation (right). Source: Gisponer, & Vendruscolo (2006).
Figure 2.3. Effect of temperature and pressure on denaturation of protein. The protein is stable in its native state inside the ellipse. Letters (h, p and c) respectively represents heat, pressure and cold for denaturation. Source: Messens et al., 1997.
Table 2.1. Type and characteristics of almond allergens. Source: Boye et al., (2012).

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Molecular Weight (kDa)</th>
<th>Protein Family</th>
<th>Allergen Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ber e 2</td>
<td>22 and 35</td>
<td>Legumin (11S)</td>
<td>Minor</td>
</tr>
<tr>
<td>Not recognized</td>
<td>45</td>
<td>Vicilin (7S)</td>
<td>Major</td>
</tr>
<tr>
<td>Not recognized</td>
<td>20-22 and 38-42</td>
<td>Legumin (11S)</td>
<td>Major</td>
</tr>
<tr>
<td>Not recognized</td>
<td>12</td>
<td>Albumin (2S)</td>
<td>Major</td>
</tr>
</tbody>
</table>
Table 2.2. Percentages of Secondary Structures Predicted with CD Pro Software Package among Native Amandin and Its Acidic and Basic Polypeptides at 20 °C. Source: Albillos et al., (2009).

<table>
<thead>
<tr>
<th>Secondary Structure</th>
<th>Native amandin</th>
<th>42kDa (acidic)</th>
<th>20 kDa (basic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>34.3</td>
<td>5.5</td>
<td>20.0</td>
</tr>
<tr>
<td>β-sheet</td>
<td>25.0</td>
<td>37.4</td>
<td>26.7</td>
</tr>
<tr>
<td>β-turn</td>
<td>12.8</td>
<td>24.1</td>
<td>21.3</td>
</tr>
<tr>
<td>Unordered</td>
<td>27.2</td>
<td>32.8</td>
<td>32.0</td>
</tr>
</tbody>
</table>
CHAPTER 3

EFFECT OF HIGH PRESSURE PROCESSING ON THE IMMUNOREACTIVITY OF ALMOND MILK

ABSTRACT

Influence of high pressure processing (HPP) at 450 and 600 MPa, 30 °C for various holding times (0, 30, 60, 180, 300 and 600 s) on almond milk amandin (the most thermally stable allergenic protein) was investigated. The immunoreactivity of pressure treated almond milk was compared with raw and thermally processed (TP) almond milk (72, 85 and 99 °C for 0 to 300 s) using a sandwich enzyme-linked immunosorbent assay (ELISA), Western blot and dot blot. Monoclonal antibodies (MAbs) targeting linear (4F10) and conformational (4C10) epitopes on amandin were used to assess amandin immunoreactivity. To determine the aggregation of almond proteins, almond milk protein solubility was quantified after 300 s of HPP (up to 600 MPa, 30°C) and TP (at 72, 85 and 99 °C, 0.1 MPa). After HPP (for all holding times), amandin can no longer be detected by the anti-conformational MAb in ELISA while signal generated from the anti-
linear epitopes MAb was reduced by half (P < 0.05). On the other hand, most TP samples did not show significant reductions in immunoreactivity (P > 0.05) unless processed at 85 and 99°C for 300 s. Western blot and dot blot also confirmed the loss of immunoreactivity by both antibodies for HPP almond milk. The reduced band intensity of the 61 and 63 kDa polypeptides and concomitant appearance of high molecular weight polypeptides in Western blot (suitable for soluble protein sample) indicated that the observed decrease in immunoreactivity was partly due to the aggregation of amandin. Within the tested conditions of the study, HPP and TP treatments respectively caused a maximum of ~70% and ~75% reduction in protein solubility of almond milk. The study demonstrated that the aggregation and loss of protein solubility, rather than the epitope destruction, may be responsible for the observed decrease in immunoreactivity of the tested amandin epitopes. The study provided preliminary evidence that pressure treatment has the potential to develop hypoallergenic almond based beverages with modified protein structures.

3.1. Introduction

Since its origination from the Mediterranean region, almond and the beverages made from it have been consumed for centuries. Almond milk is a colloidal dispersion obtained by disintegrating almonds with water. In recent years, almond milk has emerged in the market targeting consumers suffering from lactose intolerance, hypersensitive to cow milk and soymilk, and those who seek for plant-based beverages as an alternative to dairy milks (Anonymous, 2013, Lacono, Lospalluti, Licastro, Scalici, & Pediatrica, 2008;
Almond milk is also rich in essential and non-essential nutrients like α-tocopherol, essential fatty acids, dietary fiber, and wide range of other phytochemicals. The consumption of almond milk can also be linked to reduction of the risk of coronary heart disease by decreasing the plasma and LDL cholesterol level (Chen, Milbury, Lapsley, & Blumberg, 2005). In spite of health benefits, of tree nut consumption, they are one of the eight major allergenic foods affecting 0.5% of the adults in U.S. (Sampson, 2004). The total population of allergenic individuals has been increasing in recent years (Jin, Albillos, Guo, Howard, Fu, Kothary, & Zhand, 2009). The Food Allergen Labeling and Consumer Protection Act (FALCPA) requires that the presence of any items causing allergy in any form should be mandatorily labeled (Tiwari, Venkatachalam, Sharma, Su, Roux, & Sathe, 2010). Almond seeds contain 188 different proteins as detected by two-dimensional electrophoresis (Li and He, 2004). Amongst these proteins, almond major protein (AMP) or amandin, accounts for approximately 65% of total soluble proteins (Sathe, 2004). Amandin belongs to the 11S globulin family and is reported to be stable after thermal treatments such as blanching, roasting, and autoclaving (Roux, Teuber, Robotham, & Sathe, 2001). It is therefore not surprising that the ultra-high temperature processed almond milk available in the market (Berger, Bravary, & Berger, 1997) retains considerable allergenicity.

High pressure processing (HPP) is an alternative food preservation technique that has the potential to preserve organoleptic, textural, and nutritional qualities of food ingredients. HPP inactivates pathogenic microorganisms through elevated pressures (Balasubramaniam, Farkas, & Turek, 2008). It has been reported that HPP can modify the
characteristics of some proteins depending upon the magnitude of pressure, temperature, and pressure holding time (Heremans, 1982; Balny & Masson, 1993; Gross & Jaenicke, 1994). Since HPP affects mostly the non-covalent bonds, it may cause irreversible changes in quaternary, tertiary and secondary structures of proteins. Such alteration could potentially destroy the existing epitopes or, possibly, generate neo-allergen in foods (Sathe, Teuber, & Roux, 2005).

Due to the diversity of allergenic proteins, the effect of high-pressure treatment on immunoreactivity varies from study to study. In one study, HPP at 300-600 MPa for 5 min has been reported to decrease the allergenicity of several birch pollen allergy related foods including hazel nut, apple, celery, and peach (Meyer-Pittroff, Behrendt, & Ring, 2007). In addition, apple allergen Mal d 3 (Johnson, Van der Plancken, Balasa, Husband, Grauwet, Hendrickx, Knorr, Mills, & Mackie, 2010; Husband, Aldick, Van der Plancken, Grauwet, Hendrickx, Skypala, & Mackie, 2011), peanut allergen Ara h 2 (Hu, Chen, Gao, Luo, Ma, & Tong, 2011), bovine gamma globulin (Yamamoto, Mikami, Matsuno, Hara, Odani, Suzuki, & Nishiumi, 2010), soybean sprout (Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011), and soybean protein isolate (Li, Zhu, Zhou, & Peng, 2012) were also shown to be altered by pressure treatments. On the other hand, when subjected to HPP, the cod allergen Gad m1 (Somkuti, Bublin, Breiteneder, & Smeller, 2012), largemouth bass allergens (Liu, Tao, Liu, Chen, & Xue, 2012), and almond allergens (Li, Yang, Chung, Chen, Ye, Teixeira, Gregory, Welt, & Shriver, 2013) did not show a significant decrease in immunoreactivity. In one study, it was reported that the
immunoreactivity of bovine β-lactoglobulin increased after HPP (Zhong, Liu, Liu, Cai, Tu, & Wan, 2011).

The inconsistent findings in the literature are probably due to a) the distinct characteristics of the various allergenic proteins in the investigated foods, b) type of epitope(s) investigated, c) methods used for investigating the immunoreactivity of the targeted protein(s), d) food matrix effects on the targeted immunoreactive protein(s), e) effects of food processing on the protein solubility, f) or a combination thereof. Since there are very limited studies documenting the influence of pressure treatment on almond allergens, we chose to evaluate the impact of HPP on the immunoreactivity of almond milk using antibodies targeting both conformational and linear epitopes of the major almond allergen- amandin.

3.2. Material and Methods

3.2.1. Reagents and chemicals

Microtiter plates (12×8-well) were from Costar I (Cambridge, MA). Nitrocellulose membrane and blotting papers were from Schleicher and Schuell, Inc. (Keene, NH). Sathe (2001), described sources of chemicals for electrophoresis. All other chemicals and supplies were of reagent or better grade and were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich Corporation (St. Louis, MO).

3.2.2. Almond processing and milk preparation
Raw almond seeds were purchased from a local supermarket and stored at room temperature for few weeks. Almonds (200 g) were soaked overnight (15-18 h) in 600 mL tap water at 4°C. The soaked almonds were drained and rinsed with cold water and re-weighed to determine the amount of water absorbed by the seeds. The skins of soaked almonds were manually removed (unless otherwise specified). One part, by weight, of deskinned almonds was mixed with nine parts, by weight, of water and disintegrated in a Waring Laboratory blender (Torrington, CT) for 3 minutes low speed (~ 15000 rpm). The resulting mixture was then put through two layers of muslin cloth and the filtered milky almond suspension was passed through 180-micron (µ) sieve. This final filtered aqueous suspension of almond milk was then loaded into 500 mL bulk plastic pouches, heat sealed and stored at 4°C for not more than 24 h before processing. All analyses were performed at least in triplicate for independent samples.

3.2.3. High pressure processing

Almond milk was processed in a high-pressure kinetic tester (PT-1, Avure Technologies Inc., Kent, WA). The procedure described by Gupta, Mikhayleno, Balasubramanian and Tang (2011) was used. Briefly, aliquots of 2.5 mL almond milk were placed in a high barrier pouch made from sterile filter bag (# 01-002-57, Fisher Scientific), air bubbles were removed, and the pouch heat-sealed. Two pouches were placed inside a 10 mL polypropylene syringe (model 309604, Becton Dickinson and Co., Franklin Lakes, NJ), within a sample carrier, and the remaining space inside the syringe filled with cold water (2-5°C). The syringes loaded with samples were kept in ice water to precool before
loading into the pressure chamber. The pressurization was started when the samples reached the predetermined temperature, taking into account the heat of compression of the test samples (Patazca, Koutchma, & Balasubramaniam, 2007). The almond milk samples were processed at 30°C at 450 MPa and 600 MPa, each with pressure holding time of 0, 30, 60, 180, 300 and 600 s. The equipment had a pressurization rate of about 20 MPa/s and rapid depressurization rate (~1 s).

3.2.4. Thermal processing

Aliquots of 2.5 mL almond milk at room temperature were placed into sterile plastic pouches as described in section 2.3. The pouches were then loosely wrapped with the muslin cloth and dipped in a steam kettle set at different temperatures (72, 85 and 99°C) for four different time intervals, 0, 30, 180 and 300 s at each temperature. The thermally treated samples were immediately removed and cooled in ice water (~0°C). The HPP and TP samples were immediately stored at -30°C until further use. The samples were then shipped under frozen condition (-30°C) to Florida State University (Tallahassee, FL) for immunoreactivity analyses.

3.2.5. Determination of protein content

Protein concentration was determined by the method of Lowry, Rosebrough, Farr, & Randall (1951), using bovine serum albumin (BSA, 0-200 μg/mL) in borate saline buffer (BSB, 0.1 M boric acid, 0.025 M sodium borate, 0.075 M sodium chloride, pH 8.45) as
the standard protein to normalize the samples in SDS-PAGE, Western blots, dot blots, and ELISA.

3.2.5.1. Determination of protein solubility

To study the influence of HPP on protein solubility, the Bradford (1976) method was selected. Briefly, 1 mL almond milk sample was dissolved in 10 mL borate buffer and stirred for 30 minutes in magnetic stirrer. It was then centrifuged at 7900 g at 4 °C for 15 minutes and the supernatant was collected. Fifty microliter of the supernatant was then mixed with 3 mL of Bradford reagent and 100 μL of borate buffer. Then, the absorbance was measured at 595nm using bovine serum albumin (BSA) as a standard and borate buffer as a blank. Protein solubility of thermally treated and high pressure treated almond milk was determined as the amount of soluble protein over initial soluble protein content of raw almond milk, expressed as percent.

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

SDS-PAGE was done by the method of Fling and Gregerson as described by Sathe (1993). Briefly, almond milk samples were boiled in SDS-PAGE sample buffer (50 mM Tris-HCl, 1% SDS, 0.01% bromophenol blue, and 30% glycerol, pH 6.8) for 10 min. Aliquots of samples were loaded on a 4% stacking gel and an 8–25% separating gel along with molecular weight markers and electrophoresed at a constant current of 8–12 mA/gel. Gels were subjected to 0.25% w/v Coomassie Brilliant Blue R (CBBR) staining and Western blot.
3.2.7. Production of antibodies

Rabbit anti-whole almond polyclonal antibodies (pAb) [Acosta, Roux, Teuber, & Sathe (1999)] and mouse anti-amandin monoclonal antibodies (mAb) 4C10 and 4F10, targeting conformational and linear epitopes, respectively, were produced, purified, and characterized as described previously by Sathe, Teuber, Gradziel, & Roux (2001).

3.2.8. Sandwich mAb-based Enzyme Linked Immunosorbent Assay (ELISA) for amandin detection and immunoreactivity

Rabbit anti-whole almond pAb (604 ng/well) in coating buffer (48.5 mM citric acid, 103 mM sodium phosphate, pH 5.0) were coated onto 96-well polyvinyl microtiter ELISA plates and incubated at 37°C for 2 h. The plates were washed three times with Tris buffered saline-Tween 20 (TBST, 10 mMTris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6) and blocked with 5% (w/v) non-fat dry milk (NFDM) in TBS-T at 37°C for 1 h. The plates were again washed three times with TBST and almond milk samples (10 ng protein/well) were added and incubated at 37°C for 1 h. Following washing, appropriately diluted mAbs in TBS-T with 1% NFDM were added and incubated at 37°C for 1 h. Subsequently, the plates were washed three times with TBS-T and incubated with alkaline phosphatase (AP) labeled goat anti-mouse IgG (28 ng/well) at 37°C for 1 h. Following the incubation and another washing step, 50 μL/well of phosphatase substrate (5 mg p-nitrophenyl phosphate in 5 mL substrate buffer containing 0.0049% w/v MgCl₂, 0.096% v/v diethanolamine, pH 9.8) were added for color development (37°C, 30 min). The reaction was stopped by adding 50 μL/well of 3 M NaOH and the absorbance in each
well was measured using a microplate scanning spectrophotometer at 405 nm (Venkatachalam, Teuber, Roux, & Sathe, 2002).

### 3.2.9. Western blot and dot blot analyses

Following SDS-PAGE, the proteins were transferred onto a 0.22 μm nitrocellulose membrane as described by Towbin et al. (1979) at 4°C for 4 h. The transferred polypeptides were visualized by Ponceau S staining. For dot blot, almond milk samples containing 1 µg protein were dotted on the nitrocellulose membrane and allowed to air dry. The unbound sites on the nitrocellulose membranes were blocked with TBS-T containing 5% (w/v) NFDM for 1 h at room temperature. Subsequently, the membranes were washed with three changes of TBS-T for 5 min each. The membranes were then incubated with suitably diluted mAbs overnight at 4°C on a rocker. After washing three times with TBS-T for 15 min each, the membranes were incubated with horseradish peroxidase labeled goat anti-rabbit secondary antibody for 1 h at room temperature on a rocker. The membranes were washed again as described above. Polypeptide bands or sample dots reactive to mAbs were visualized by mixing luminal with p-coumaric acid. The mixture was spread evenly to cover the entire membrane and left for 5 min. The membranes were dried, covered by a transparent plastic wrap, and exposed to X-ray film for auto-radiographic visualization (Venkatachalam et al., 2002).

### 3.2.10. Data analysis and statistical procedures
All data, except for protein solubility, were analyzed for significance (One-way ANOVA) using SPSS 9.0 (SPSS Inc., Chicago, IL). Fisher’s protected LSD ($p = 0.05$) values were calculated for appropriate data. All experiments were performed at least in duplicate and data expressed as mean + SEM. Comparisons between samples for protein solubility measurements were done using Tukey Honest Significant Difference (HSD) with family wise error rate of 5% using Minitab 15 (Minitab Inc., State College, PA). Protein solubility assays were performed at least in triplicate and the data expressed as mean with positive standard error.

3.3. Results and Discussion

3.3.1. Effect of high pressure and temperature on protein solubility of almond milk

The protein concentration of raw almond milk, using the borate as extraction buffer at room temperature (25°C), using magnetic stirrer for 30 minutes, was found to be 1.44g/100g almond milk. The soluble protein content of almond milk treated at 150, 300, 450 and 600 MPa for 300 s at 30°C is shown in Figure 3.1. It was found that protein solubility decreased with increase in pressure. There was no change in protein solubility at 150 MPa but the reduction in solubility was ~30, ~68 and ~70 % for almond milk treated at 300, 450 and 600 MPa. Also, thermal processing at 72°C for equivalent time did not cause significant reduction in protein solubility but at 85°C and 99°C there was significant reduction (~50 and ~75 % respectively) in protein solubility (Figure 3.1). The change in protein solubility in pressure and thermal treated almond milk in comparison to control was further confirmed by a decrease in total soluble solids (data not shown).
To date, nothing has been published on the effect of HPP on almond milk protein solubility. However, our result showed effects similar to those for other high pressure treated food proteins such as walnut (Qin et al., 2012), soy protein isolates Puppo et al., 2004), and meat, (Marcos, Kerry, & Mullen, 2004). Our results are also in agreement with previous studies (Venkatachalam, Teuber, Roux, & Sathe, 2002; Sathe & Sze, 1997) that showed the almond protein solubility decreased with extensive thermal treatments (boiling and autoclaving). It has been well established that HPP and thermal treatment may cause protein unfolding due to distortion in secondary, tertiary, and quaternary structure and may result in aggregation and/or precipitation leading to decrease in protein solubility (Balny et al., 1989, and Gross et al., 1994). When proteins in their lowest free energy state are transferred to another state by suitable pressure, protein unfolding/denaturation can occur at transition state as a result of overcoming the free energy barrier. At low pressure (<300 MPa), the protein unfolding can be reversible mainly due to disruption of internal hydrophobic interactions and salt bridges while at higher pressures (>300 MPa), irreversible denaturation occurs in proteins (Balny, Masson, & Travers, 1989; Gross & Jaenicke, 1994). The irreversible denaturation can be due to formation of disulfide linked aggregates via covalent interactions as a result of oxidation of sulfhydryl groups in polypeptide chains. Moreover, some structural changes, such as conformational transition of proteins, are also governed by pressure induced volume changes, an important thermodynamic quantity (Balny et al., 1989).

The result in our current study indicates that HPP and TP have different effects on almond milk proteins. The subtle effect of pressure (150 MPa) might have maintained the
protein solubility due to increased solvation through favorable ionic interactions that resulted from deprotonation of polar amino acids. Almond proteins are known to be rich in Glx (34.6-36.1%), Asx (11.9-13.2%) and Arg (9.3-9.7%) accounting for 55.8-59.0% of total amino acids, among the tested almond seeds- Carmel, Mission, and Nonpareil (Ahrens, Venkatachalam, Mistry, Lapsley, and Sathe, 2005). As the pressure increased from 300 MPa to 600 MPa, hydrophobic interactions may have been dominant due to possible increased exposure of buried non-polar residues in proteins.

It is known that pH of the food and buffer solutions transiently decrease with increase in pressure and revert back close its initial value upon depressurization (Pardes-sabja, Gonzalez, Sarker, & Torres, 2007). Since experimental techniques for measuring in situ pH under pressure are not readily available, we measured pH of the almond milk samples before and after pressure treatment. The pH of raw and pressure treated (600 MPa, 10 min) were 6.49 and 6.57 respectively. It has been reported that the majority of almond proteins have isoelectric point (pI) values in the range of 4.5-5.5 (Li and He, 2004). Thus, during pressure treatment, the pH of almond milk shifted towards pI (pH 4.5-5.5), destabilizing the net charges in protein and making the almond proteins potentially vulnerable to conformational changes. More research is needed to understand the impact of pressure treatment on isoelectric point of proteins and its impact on conformational structure.

On the other hand, it can be observed that the solubility of protein in TP almond milk was not changed at 72°C (Figure 3.1) most probably because possible partial unfolding of proteins may have facilitated protein solvation. The unfolding and subsequent surface
hydrophobicity might have increased when almond milk was treated at 85°C and 99°C resulting in ~50% and ~75% reduction of protein solubility for equivalent treatment time of 300 s.

Thermodynamic studies in proteins have shown that denaturation temperature and pressure are governed by different mechanisms. It has been explained from the phase diagram (p-T) study that temperature denaturation is entropy driven while the driving force for denaturation pressure is the change in volume (δV), i.e., activation volume, that results in a more compact structure (Smeller, 2002). Thus the same protein can have different hydrophobic and electrostatic states under two different thermodynamic parameters; temperature and pressure, which may result in different structural modifications in proteins that may or may not affect the epitopic regions.

3.3.2. Effect of high pressure and temperature on protein profiles of almond milk from SDS-PAGE

The SDS-PAGE profiles of high pressure treated (HPP) and thermal processed (TP) almond milk were compared as shown in Figure 3.2A and 3.2B, respectively. In the absence of reducing agent, amandin has two major polypeptides with estimated MWs of 61 and 63 kDa. Each polypeptide is composed of an acidic subunit (42-46 kDa) and a basic subunit (20-22 kDa) linked by disulfide bonds (Sathe, Wolf, Roux, Teuber, Venkatachalam, & Sze-Tao, 2002). The 61 and 63 kDa amandin bands can be clearly seen in the lanes of control (raw almond milk, coded 1) and almond protein extract (positive control, coded 0). After pressure treatment at 450 and 600 MPa, these bands
were diminished with concomitant appearance of the 20 kDa and 40 kDa bands as well as a smear of high molecular weight polypeptides. On the other hand, most thermally treated samples (72, 85 and 99°C) showed similar protein profiles as the control and almond protein extract unless treated at 85°C for 300 s or at 99°C for 30, 180 and 300 s. Protein aggregation after HPP treatment is likely due to intermolecular disulfide bond formation. It has also been observed that HPP promotes disulfide cross-linking in myosin (Chan, Omana, & Betti, 2011; Jantakoson, Kijroongrojana, & Benjakul, 2012), sarcoplasmic proteins (Marcos, Kerry, & Mullen, 2010), β-lactoglobulin (Chicón, Belloque, Alonso, & López-Fandiño, 2009; Zhong, Liu, & Liu, 2011), and soybean β-conglycinin and glycinin (Speroni, Beaumal, Lamballerie, Anton, Añón, & Puppo, 2009). HPP treatment probably unfolded the proteins and exposed the buried sulfhydryl groups. Degradation of the protein, on the other hand, likely involves the disruption of the native disulfide bonds. The cleavage and rearrangement of disulfide bonds have been observed in gluten following HPP treatment (Kieffer, Schurer, & Wieser, 2007). It is also interesting to note that micro fluidization (up to 180 MPa) of peanut protein extracts (Hu et al., 2011) also showed similar degradation.

3.3.3. Effect of high pressure (HPP) and thermal processing (TP) on immunoreactivity of almond milk using Western blot

Following SDS-PAGE, Western blotting was performed to examine the immunoreactivity of amandin subjected to pressure and thermal processing as shown in Figure 3. 2 A and 3. 2B. In addition to whole almond extract (coded 0), raw almond milk
without skin (coded 1) and raw almond milk with skin (coded 14) for TP samples were used as references. The mAb 4C10 was chosen to detect the immunoreactivity as this antibody is highly reactive to native, but not processed amandin i.e., it recognizes a conformational epitope (Kshirsagar, Fajer, Sharma, Roux, & Sathe, 2011). It has also been shown that the 4C10 epitope overlaps with the epitope that is targeted by amandin-reactive patients’ sera (Willison, Zhang, Su, Teuber, Sathe, & Roux, 2013). Another mAb, 4F10, which recognizes a stable linear epitope, was selected for comparative purposes. 4C10 recognized 61 and 63 kDa polypeptides whereas 4F10 recognized 38, 50, 61, and 63 kDa polypeptides. In Western blot, the band intensity of all targeting polypeptides detected by the two antibodies was reduced dramatically with increase in pressure (450MPa and 600 MPa) and pressure holding time. After HPP treatment, 4F10 weakly recognized a 48 kDa band and a 40 kDa band, which is consistent with the pattern of almond protein extract in Western blot under reducing conditions demonstrated in our previous study (data not published). On the other hand, almond milk samples were stable to most thermal treatments. The reductions in immunoreactivity were observed only for almond milk samples heated at 85°C for a minimum of 300 s and at 99°C for 30s. The reduction of immunoreactivity could potentially be due to the destruction of the native disulfide bond. A disulfide bond located within the large subunit of amandin (residues 32 and 65) plays a critical role in sustaining the 4C10 conformational epitope (Willison et al., 2003). As demonstrated in the SDS-PAGE, the disulfide bond linking the large and small subunits (residues 108 and 374) was broken in some of the amandin molecules after HPP treatment. Although this disulfide bond has only a minor effect on
4C10 reactivity (Willison et al., 2003), the results suggest the possibility that the crucial disulfide bond was also disrupted by the high pressure. Similar reduction in immunoreactivity due to disruption of disulfide bond by high-pressure microfluidization was observed in the peanut allergen Ara h 2 (Hu et al. 2011). Another factor that can contribute to the decrease in immunoreactivity is protein aggregation. After HPP treatment, amandin was cross-linked via covalent bond as illustrated by the occurrence of high molecular weight protein smears recognized by 4C10 and 4F10 in Western blot. As some large molecular weight protein aggregates fail to enter the separating gel, they cannot be observed in SDS-PAGE nor detected by the antibodies in Western blot. In addition, excess aggregation may interfere with epitope recognition by the antibodies due to steric hindrance. Similar reduction in immunoreactivity due to allergen aggregation by thermal processing was shown in peanut extract (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010).

3.3.4. Effect of high pressure (HPP) and thermal processing (TP) on immunoreactivity of almond milk using dot blot

Dot blots incorporating the same two mAbs were used to evaluate the roles of both soluble and insoluble fractions of treated and untreated almond milk. Dot blots (Figure 3.2A) with 4C10 as the detection antibody still showed reduction in immunoreactivity with pressure treated almond milk at 450 MPa for 600 s and 600 MPa for 180 s and above. These results support the notion that protein solubility is not the sole factor for reduction in immunoreactivity of HPP treated almond milk. The cleavage of the critical
disulfide bond on the conformational epitope and aggregation induced blocking of the epitopes may also contribute to the decrease in immunoreactivity. Dot blots (Figure 3.2A) with 4F10 as the detection antibody did not reveal any immunoreactivity reduction in HPP treated almond milk except at 450 MPa for 60 s and 600 s (sample number 4 and 11, respectively). Others have found that protein aggregates can be formed at intermediate protein folding stage during the depressurization steps and some also reported that some proteins are prone to form non-native disulfide bonds leading to the formation of gels and precipitates during both pressurization and depressurization steps (Randolph, Seefeldt & Carpenter, 2002; Rastogi, Raghavarao, Balasubramaniam & Niranjan, Knorr, 2007). In addition, the protein aggregates may not necessarily block the binding sites for primary antibodies, but result in steric blockage of the secondary antibodies or the associated enzyme. For the thermally processed samples, dot blots (Figure 3.2B) with 4C10 did not show any reduction in immunoreactivity except for those heated at 99°C for 180 s and 300 s. As expected, reduction of immunoreactivity in TP almond milk was detected only at 99°C for 300 s using 4F10 as detection antibody.

**3.3.5. Effect of HPP on immunoreactivity of amandin using ELISA**

For further confirmation, the immunoreactivities of high pressure treated and raw almond milk were quantitatively determined by means of indirect ELISA. Figure 3.3A and 3B demonstrated the effect of pressure treatment (450 and 600 MPa, 30°C) with holding times up to 600 s on the immunoreactivity of almond milk using 4C10 and 4F10 as detection antibodies, respectively. In all assays, almond protein extract in BSB was
used as the positive control and BSB itself was used as the negative control. The residual immunoreactivity of 4C10 reactive epitope in high pressure treated almond milk was less than 5% in comparison to raw almond milk at all pressures and pressure holding times whereas 4F10 reactive epitope retained ~50% of its immunoreactivity. These data are consistent with the results of Western blot and dot blot and indicating that the decrease in immunoreactivity after HPP was most likely due conformational alteration, possibly including disulfide bond disruption, in the case of the 4C10 epitope and excessive protein-protein interaction-induced loss in protein solubility limiting the access of the 4F10 Mab to its otherwise stable epitope. In contrast to our results, Li et al. (2013) have reported that HPP did not reduce the immunoreactivity of almond allergens with patients’ IgE. Because Li et al. assayed a polyclonal source, multiple allergenic proteins and constituent epitopes may have been targeted. In contrast our study focused on a Mab to a single linear and single conformational epitope and may not be representative. However, we did target the major allergenic almond protein and the conformational epitope significantly inhibited the binding of patient IgE to amandin suggesting that the molecule and epitopes targeted by the MAbs is representative. Thus, because HPP partially to fully interfere with the binding of both types of MAbs to their respective epitopes, we find it somewhat surprising that Li et al. did not observe at least some epitope denaturation in their assays.

Even if HPP is not able to eliminate the immunoreactivity of all almond proteins, by limiting the reactivity to the major allergen in almond, HPP may reduce the risk for some hypersensitive individuals (Willison et al., 2013). Perhaps additional adjustments in pH,
chemical treatment, and processing conditions could have similar positive effects on allergenicity in model and real food system.

3.3.6. Effect of TP on immunoreactivity of amandin using ELISA

ELISA assays on thermally processed (TP) almond milk samples (72, 85 and 99°C for up to 300 s) were also conducted to evaluate the residual immunoreactivity with mAbs 4C10 and 4F10. The results showed that the immunoreactivity detected by 4C10 was reduced only in those samples heated at minimum 85°C for 300 s or 99°C for 30 s (Figure 3. 4A). Overall, the extent in reduction of relative immunoreactivity of TP (10 to 85% reductions) was not as effective as HPP treatment. When mAb 4F10 was used as the detection antibody, the residual reactivity was higher than with 4C10 (Figure 3. 4B). This reduction in immunoreactivity of TP treated almond milk at high temperature was not unexpected in light of a previous reported by Bargman, Rupnow and Taylor (1992) that blanching and roasting eliminated the reactivity of a 70 kDa band of almond protein extracts. Roux, Teuber, Robotham and Sathe (2001) also observed slight reduction in allergenicity due to blanching and roasting of whole almond protein extracts. This reduction of immunoreactivity in heat-treated almond milk at extreme temperature (~99 °C) may due, in part, to interactions of allergenic proteins with other food constituents. For example, a reduction of buckwheat and hazel nut immunoreactivity due to thermally derived Maillard reactions has been reported (Yang, C. Li, Y.Y. Li, & Wang, 2013; Iwan et al.2011).
Combined pressure-thermal treatments (up to 700 MPa, 118°C for 10 min) has been shown to significantly reduced the immunoreactivity of the apple allergen Mal d 1, which is resistant to chemical modification and thermal processing alone (Husband et al. 2011). A similar approach might further reduce the immunoreactivity of almond milk.

3.4. Conclusions

High pressure and temperature, two different thermodynamic variables, appear to influence almond milk amandin allergens in different ways. After pressure treatment, amandin in almond milk could not be detected by the anti-conformational MAb in ELISA while signal generated from the anti-linear epitopes MAb was reduced by half. With the exception of samples processed at 85 or 90°C for 300 s, TP samples did not show significant reductions in immunoreactivity (P > 0.05) unless processed at 85 and 99°C for 300 s. Both the treatments reduced almond milk protein solubility, caused aggregation and degraded disulfide bonds. This impact of processing causes alteration in almond milk proteins structures due to which the amandin epitopes present in almond milk becomes less available during in vitro immunoreactions. Systematic studies are needed to comprehensively understand the mechanism and efficacy of combining pressure, heat and other process variables in reducing various food allergens.


Figure 3.1. Effect of high pressure processing (HPP) and thermal processing (TP) on soluble protein of almond milk treated for 5 minutes. Raw milk (0.1MPa) was used as a control. Data were reported as mean ± standard error of mean. Means between samples with no common letters differ significantly with Tukey Honest Significance Difference (HSD) family error rate (5%), individual error rate (0.26%) and pooled standard deviation (0.1626).
Figure 3.2. SDS-PAGE, Western blots and Dot blots using two different monoclonal antibodies 4C10 (targeted for conformational epitopes) and 4F10 (targeted for linear epitopes) against almond major proteins (AMP) of almond milk without skin. The coded samples on horizontal axis (top) represent process condition. (A) High pressure processed (HPP) at 30 °C. The coded samples are whole almond extract (0) used as positive control, raw milk without skin (1), 450 MPa 0 sec (2), 450 MPa 30 sec (3), 450 MPa 60 sec (4), 450 MPa 180 sec (5), 450 MPa 300 sec (6), 450 MPa 600 sec (7), 600 MPa 0 sec (8), 600 MPa 30 sec (9), 600 MPa 60 sec (10), 600 MPa 180 sec (11), 600 MPa 300 sec (12) and 600 MPa 600 sec (13). (B) Thermally processed (TP) almond milk at 0.1 MPa. The coded samples are whole almond extract/positive control (0), raw milk without skin (1), raw milk with skin (14), TP 72 ± 1 °C 0 sec (15), TP 72 ± 1 °C 30 sec (16), TP 72 ± 1 °C 180 sec (17), TP 72 ± 1 °C 300 sec (18), TP 85 ± 1 °C 0 sec (19), TP 85 ± 1 °C 30 sec (20), TP 85 ± 1 °C 180 sec (21), TP 85 ± 1 °C 300 sec (22), TP 99 ± 1 °C 0 sec (23), TP 99 ± 1 °C 30 sec (24), TP 99 ± 1 °C 180 sec (25) and TP 99 ± 1 °C 300 sec (26).
Figure 3.3. Relative immunoreactivity showed by almond major proteins (AMP) of almond milk without skin after high pressure processing (HPP) at 450 and 600 MPa at 30 °C by ELISA method. (A) Monoclonal antibody (mAb) 4C10 specifically targeted for conformational epitopes. (B) mAb 4F10 specifically targeted for linear epitopes. Data were reported as mean ± standard error of mean (n = 12). Means between samples with no common letters differ significantly (P < 0.05). The coded samples are wa: whole Nonpareil almond borate saline buffer (1:10 w/v) extract/positive buffer; and control: almond milk prepared without almond skin.
Figure 3.4. Relative immunoreactivity showed by almond major proteins (AMP) of almond milk without skin after thermal processing (TP) at 72, 85 and 99 °C (0.1MPa) by ELISA method. (A) Monoclonal antibody (mAb) 4C10 specifically targeted for conformational epitopes. (B) mAb 4F10 specifically targeted for linear epitopes. Data were reported as mean ± standard error of mean (n = 12). Means between samples with no common letters differ significantly (P < 0.05). The coded samples are wa: whole Nonpareil almond borate saline buffer (1:10 w/v) extract/positive buffer; skin: almond milk prepared with almond skin and no skin: almond milk prepared without almond skin.
CHAPTER 4

INFLUENCE OF HIGH PRESSURE PROCESSING ON SOME PHYSICOCHEMICAL PROPERTIES OF ALMOND MILK

ABSTRACT

Almond milk is gaining attention with increased consumer awareness about health benefits of plant-based beverages. The objective this research is to investigate the influence of high pressure processing (HPP) selected almond milk quality attributes and compare against raw and thermally processed product. Almond milk was prepared by disintegrating 10% almonds with water. Almond milk samples were subjected to high pressure processing (HPP; 450 and 600 MPa at 30°C up to 600 s) and thermal processing (TP; 72, 85 and 99 °C at 0.1 MPa up to 600 s). Results demonstrated that the physicochemical characteristics of almond milk, especially particle size, can be modified by high pressure processing and thermal processing. Extent of the modifications varies depending upon the magnitude of pressure, temperature and treatment time. Pressure treated almond milk exhibited net increase in average particle diameters D_{4,3}(volumetric
diameter), $D_{3.2}$ (surface area diameter) and $D_{v0.5}$ (volume median diameter) with increase in treatment time and pressure intensity when studied by using the dynamic light scattering technique. Likewise, TP also caused alteration in particle size distribution with net effect of increase in these parameters when treated at least at 85 °C for 300s. The apparent aggregation rate constant for 450 MPa and 600 MPa processed samples were $k_{450\text{MPa, } 30 ^\circ C} = 0.0045 \text{ s}^{-1}$ and $k_{600\text{MPa, } 30 ^\circ C} = 0.0095 \text{ s}^{-1}$ respectively. On the other hand, heat-treated samples did not fit with any of the kinetic models tested in our study. Further examination of treated samples using confocal laser scanning microscopy (CLSM) confirmed the aggregation of proteins and fats present in almond milk. Protein unfolding, denaturation and loss of solubility that led to aggregation were also supported by UV spectral data of almond milk proteins. Pressure treatment enhanced the whiteness and greenness of almond milk but these values were not significantly different in heat treated (72 and 85 °C) samples for equivalent treatment time. The instrumental color difference in both pressure and heat treated almond milk were below 1.5 units.

The study demonstrated that physicochemical characteristics of raw almond milk, especially particle size distribution, can be modified by HPP and TP.

4.1. Introduction

Almond milk is a colloidal dispersion made from aqueous extraction of almond nuts. It is a non dairy milk beverages that contains array of essential and non-essential nutrients which is suitable for different groups of people including those who are lactose intolerance and hypersensitive with milk proteins. It also resembles cow’s milk in many
aspects such as composition, color and acceptable flavor. Besides, like in dairy milk, the
growth inhibitory substances like trypsin inhibitor and haemagglutinins were also not
detected in sweet almonds (Ahrens, Venkatachalam, Mistry, Lapsley, & Sathe, 2005).
Almond milk has potential to reduce the plasma and LDL cholesterol levels, reduce the
risk of coronary heart disease and also provide added health benefits due to higher α
tocopherol levels, essential fatty acids and fibers present in its ingredients, almonds
(Chen & Blumberg, 2008; Bolling, Chen, McKay, & Blumberg, 2011).
Almond milk had the highest sales among alternative dairy beverages in 2011 and
currently dairy alternative beverages accounted for 21% of retail market where 9% of the
U.S adults consume almond milk (Latif, 2012). The commercial method of almond milk
production uses ultra-high temperature (UHT) processing (Berger, Bravay, & Berger,
1997). But the thermal processing of nuts based beverages also causes some adverse
effects on sensory and nutritional quality attributes (Kwok & Niranjan, 1995).
High pressure processing (HPP) is a novel technology that enables processing of food at
ambient or even at chilled condition and has potential to preserve organoleptic, textural
and nutritional qualities (Balasubramaniam, Farkas, & Turek 2008). HPP effectively
alters non covalent bonds and inactivates or inhibits the microorganisms and quality
deteriorating enzymes through physical effects of static pressure but have minimum
effects on covalent structures of low molecular weight compounds such as color, and
flavor (Koca, Balasubramaniam, & Harper, 2011; Nguyen, Tay, Balasubramaniam,
Legan, Turek, & Gupta, 2010; Gupta, Kopec, Schwartz, & Balasubramaniam, 2011).
Application of high pressure pasteurization (up to 600 MPa at 20 - 75 °C) for extending the shelf life of many refrigerated and non-refrigerated products (Evrendilek & Balasubramaniam, 2011; Smith, Mendonca, & Jung, 2009 and Li, Zhang, Balasubramaniam, Lee, Bomser, Schwartz, & Dunne, 2006) have been investigated. Researchers have evaluated efficacy of high pressure processing in variety of fluid foods including human milk (Contadar, Delgado-Adamez, Delgado, Cava, & Ramirez, 2013; Molto-Puigmarti, Permanyer, Castellote, & Lopez-Sabater, 2011), dairy milk (Voigt, Chevalier, Donaghy, Patterson, Qian, & Kelly, 2012; Al-Nabulsi, Shaker, Osaili, Clark, Harte, & Barbosa-Canovas, 2012), tomato juices (Daryaei & Balsubramaniam, 2012; Gupta, Balasubramaniam, Schwartz, & Francis, 2010), orange juices (Guignon, Aparicio, Sanz, & Otero, 2012; Plaza, Sanchez-Moreno, Ancos, Elez-Martinez, Martin-Belloso, & Cano, 2011), soymilk (Guerrero-Beltran, Estrada-Giron, Swanson, & Barbosa-Canovas, 2009; Jung, Murphy, & Sala, 2008) and mix beverages (Barba, Cortez, Esteve, & Frigola, 2012; Tadapaneni, Banaszewski, Patazca, Edirinsinghe, Cappozzo, Jackson, & Burton-Freeman, 2012).

Limited research also evaluated pressure effects on almonds including inactivation kinetics of β-glucosidase (Terefe, Sheenan, Fernando, & Versteeg, 2013), destruction of *salmonella* Enteridis (Goodridge, Willford, & Kalchyanand, 2006) and antigenicity (Li, Yang, Chung, Chen, Ye, Teixeira, Gregory, Welt, & Shriver, 2013). Very limited studies are available on the impact of pressure treatment on physicochemical properties of almond milk.
4.1.1. Objective

The objective of this study was to evaluate the impact of high pressure processing (450 and 600MPa) on almond milk at 30 °C by characterizing particle size distribution and evaluating microstructure, color and pH changes.

4.2. Materials and Methods

4.2.1. Reagents and Chemicals

All chemicals were of analytical grade and were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA) and Fisher Scientific (Mississauga, ON, Canada) unless specified otherwise.

4.2.2. Almond Processing and Milk Preparation

Raw, shelled almonds were purchased from local supermarket. These were stored at room temperature for few weeks. Almonds (200g) were soaked overnight (15-18hrs) in 600 ml water at 4°C. The soaked almonds were drained and rinsed with cold water and re-weighed to determine the amount of water absorbed by the seeds. The skin of soaked almonds were removed (unless specified). One part by weight of almonds were mixed with nine parts by weight of water and disintegrated in a Waring Laboratory blender (Torrington, CT, USA) for 3 minutes. The resulting mixture was then put into two layers of muslin cloth and the filtered milky almond suspension was then passed through 180µ sieve. This final aqueous suspension was then packaged into 500 mL bulk plastic
pouches, heat sealed and then stored at 4°C for not more than 24 hrs before processing. All analysis was performed at least in triplicate for independent samples.

4.2.3. High pressure processing

Almond milk was processed in a laboratory scale high-pressure unit (model Avure PT-1; Avure Technology Inc, Kent, WA, USA) with DASYLab ® 7.0 software (National Instruments Corp., Austin, Tex., and U.S.A) as described by Ramaswamy, Balasubramaniam and Sastry (2007). Briefly, 2.5 ml of chilled (2-5°C) almond milk was packaged in a high barrier pouch (# 01-002-57, Fisher Scientific) excluding air bubbles and then heat-sealed. Two pouches were placed inside a 10-mL polypropylene syringe (Model 309604, Becton Dickinson and Co., Franklin Lakes, N.J., U.S.A.), and the remaining space inside the syringe was filled with cold water (2-5°C). The syringe loaded with sample was then kept in ice water to equilibrate syringe content to reach initial temperature before loading into the pressure chamber. The pressurization was started when the sample temperature reached the predetermined point taking into account the heat of compression of the test samples (Ramaswamy, Balasubramaniam, & Sastry, 2007; Patazca, Koutchma, & Balasubramaniam, 2007). The almond milk samples were processed at ~30°C for 450MPa and 600MPa, for different pressure holding times (0, 30, 60, 180, 300 and 600s). The pressurization rate (17 MPa/s) and decompression time (~1s) were not included in process time.
4.2.4. Thermal Processing

2.5 mL of the almond milk at room temperature were filled into sterile plastic pouches as described in section 3.2.3. Five pouches at a time were then loosely wrapped with the muslin cloth and constantly stirred once these were dipped in water bath set at different temperatures (72, 85 and 99 °C) for 300 and 600s. The estimated come up time (~30s) was not included in the process time. The thermally treated samples were immediately removed and cooled into ice water ~ 0 °C.

The HPP and TP samples were immediately stored at 4°C. Particle size, microstructure and color of almond milk samples were determination within 24 hours after processing. Samples for composition analysis were stored at ~ -30 °C for not more than 1 week before analysis.

4.2.5. Chemical Composition

The pH and °Bx of almond milk was measured using digital pH meter (Accumet XL 15, Fisher Scientific, Mississauga, ON, Canada) and digital brix/RI refractometer (Reichert Analytical Instrument, Japan) respectively. These parameters were measured before and after processing.

The lipid, moisture and ash content of the raw almond milk were also estimated. A Bligh, and Dyer (1959) method was carried out to determine the total lipid content. The moisture was determined by oven method (AOAC 1995; official method 925.40) at ~105 °C (28.5 inch of Hg) until the constant weight was obtained. For ash content the sample (0.5g) was first dried in a hot air oven at 105 °C before being transferred to a muffle
furnace at 550 °C, for 5 h until a white or light gray ash resulted (AOAC 1995; official method 923.03).

Bradford (1976) method was followed for total soluble protein determination. Briefly, one mL almond milk sample was dissolved in 10mL borate buffer (0.05M, pH 8.5) and stirred for 30 minutes in magnetic stirrer. It was then centrifuged at 7900 g at 4 °C for 15 minutes and the supernatant was collected. 100 μL of borate buffer (0.05M, pH 8.5) was put into 100 μL of supernatant in a 15mL test tube. It was then mixed with 5 mL of Bradford reagent and then, the final volume of 6mL was made with distill water. The absorbance was measured at 595nm after 10 minutes in a 1.5 mL disposable cuvette. Bovine serum albumin (BSA) was used as a standard and borate buffer (0.05M, pH 8.5) was used as a blank.

The readings are the mean of at least nine readings performed in triplicates in three independent experiments.

4.2.6. Particle Size Distribution

The particle size distribution of almond milk was measured with a laser light scattering instrument (Master sizer micro plus, Malvern Instruments Ltd., Malvern, U.K.) using the software version 2.19 in polydisperse analysis model. The default size range was 0.05 – 555.71µm and instrument presentation set in 50HD. The refractive index (RI) of dispersed phase (almond milk particles) was taken as 1.529, absorption RI 0.1 and the corresponding values for dispersant (deionized water) were 1.33 . ~ 2.0 mL sample was diluted in the 500 mL dispersant and the speed of the motor was set at 2000 rpm. All
samples were measured in the obscuration range of 15 - 23% and the residual obtained was less than 0.85.

The particle size were characterized by $D_{4,3}$, $D_{3,2}$ and $D_{v0.5}$. The specific surface area, SSA ($m^2/g$) of the samples was also determined.

The volume mean diameter ($D_{4,3}$) was calculated as:

$$D_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$$

(1)

where, $D_{4,3}$ (µm) is the diameter of the sphere of equivalent volume to measured particles (volume mean diameter), $n$ is the number of particles and $d$ is the linear diameter (µm) of the particle.

The surface area diameter ($D_{3,2}$) was calculated as:

$$D_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$$

(2)

where, $D_{3,2}$ (µm) is the particle diameter that has the same specific surface as that of the full distribution.

$D_{v0.5}$ (µm) is the volume median diameter below/above which 50% of the volume of the particles is found.

Span (width of the distribution) was also calculated for raw almond milk as follows.

$$\text{Span} = \frac{(D_{v0.9} - D_{v0.1})}{D_{v0.5}}$$

(3)

where, $D_{v0.9}$ (µm) and $D_{v0.1}$ are respectively the diameter below which 90% and 10% of the volume of the particles is found.
All measurements were average of at least nine readings performed in triplicates from three independent experiments.

4.2.7. Confocal Laser Scanning Microscopy (CLSM)

A confocal laser scanning microscope, Olympos, Fluoview FV1000, (Olympus Optical Co., Tokyo, Japan), 2.1.2.5 system version with Plapon 60X O SC NA 1.4. Magnification of 60X oil immersion objective lens was used for all samples. CLSM was set up to image Nile red in channel 1 with dye analog Alexa Fluor 488 (Excitation 488nm, Emission 520) and to image Fast Green FCF in channel 2 with dye analog Alexa Fluor 633 (Excitation 635 nm, Emission 647). The samples were diluted 10 fold with 10mL borate buffer (0.05M, pH 8.5) Nile Red was prepared in the concentration of 0.02g/L in acetone to stain the proteins and Fast Green FCF was used in the concentration of 0.2g/liter in distill water to stain the fats. Protein is stained in red, lipids core in green and the dark regions represent unstained species filled with water molecules.

The two dyes were then mixed in equal proportion. Then 100 µL of the dye mixture was mixed in 400µL of the diluted sample and kept for 10 minutes. About 25 µL of these dyed samples were put on the plain glass slide and after putting the cover slip the edges were sealed. The images were captured in 512 x 512 pixel size.

4.2.8. Modeling of processing effect on protein aggregation kinetics

The study of kinetics of aggregation in colloidal system is complex. Factors such as treatment intensity, particle size, shape, polydispersity, physicochemical interactions
between different constituents and aggregate hydrodynamic behavior (Kyriakidis, Yiantsios, & Karabelas, 1997) can influence aggregation kinetics.

There are two different limiting regimes for kinetic aggregation of aqueous colloids. First is called as diffusion limited cluster aggregation (DLCA) which occurs when there is negligible repulsive force between the colloidal particles. Another, the reaction limited cluster aggregation (RLCA) kinetics, which involves some sorts of repulsive force between the particles, and the aggregation, can occur when the particles overcome this residual interaction energy barrier (Lin, Lindsay, Weitz, Ball, Klein, & Meakin, 1989). HPP and TP may help overcome such energy barrier and promote aggregation of the particles. Studies in model colloidal system have also shown that each of these limiting regimes is universal, irrespective of the chemical nature of the colloids (Lin et al., 1989). Depending upon the intensity of the pressure or thermal treatment, almond milk proteins can aggregate due to reaction limited colloidal aggregation (RLCA).

We have modeled rate constant for aggregation of almond milk colloidal system at different high-pressure processed conditions by adapting the method described by Euston, Finnigan and Hirst (2000), for heated whey protein–stabilized emulsions. We assumed that almonds proteins and fats will take part in aggregation and thus, the contribution of other constituents in the aggregation process is negligible. Proteins may also exist in adsorbed form or in non-adsorbed forms in dispersion. We also assumed that amount of almond milk proteins used in our study was at very dilute concentration and this was insufficient to form gel by HPP or TP. This analysis only considered the impact
of process on protein aggregation and did not consider the subsequent influence of storage time.

Rate constant for aggregation of almond milk particles was determined by identifying the order of the reaction that fits best with the rate law equation. To check the first order reaction the following equation proposed by Das and Chattoraj (1982) was be used:

\[ N_t = N_0 \exp (-kt) \]  

where \( N_0 \) and \( N_t \) are the number of free particles at time zero treatment time and after specific process time \( t \) respectively, and \( k \) is the reaction rate constant. For \( n^{th} \) orders (1.5 and 2), the following rate equation (Euston, Finnigan, & Hirst, 2000) was used:

\[ \left[ \frac{N_t}{N_0} \right]^{1-n} = 1 + (n - 1)kt \]  

where, \( n \) is the order of the reaction.

Since number of free particles in the dispersion could not be experimentally determined using dynamic light scattering instrument, we replaced the term \( [N_t/N_0] \) by another equivalent ratio \( [D_{3,0t=0}/D_{3,0t=t}]^3 \) as suggested by Euston et al. (2000).

i.e., \( [N_t/N_0] = [D_{3,0t=0}/D_{3,0t=t}]^3 \)  

\( D_{3,0t} \) is the volume average diameter calculated by software from Malvern Master sizer as given below

\[ \ln D_{3,0} = 6 \ln D_{3,2} - 3 \ln D_{4,3} \]  

Substituting the value of \( [N_t/N_0] \) from equation (6) to (4), we have

\[ [D_{3,0t=0}/D_{3,0t=t}]^3 = \exp (-kt) \]  

The slope from the plot of \( \ln [D_{3,0t=0}/D_{3,0t=t}]^3 \) vs. \( t \) estimated the value of apparent aggregation rate constant for first order reaction.

Similarly, the equation for higher order will be,

\[ [D_{3,0t=0}/D_{3,0t=t}]^{3(1-n)} = 1 + (n - 1)kt \]
The slope of the plot \([D_{3,0t=0}/ D_{3,0t=t}]^{1.5}\) vs. \(t\) and \([D_{3,0t=0}/ D_{3,0t=t}]^{-3}\) vs. \(t\) (obtained from equation 9) will give apparent aggregation rate constant value for 1.5 and 2 order kinetics respectively.

Linear regression was carried out using a Microsoft Excel 2010 program. Rate constant will be chosen from the linear regressions that possess the highest correlation coefficient value \((r^2)\).

4.2.9. UV scanning of protein extract of almond milk

The procedure described by Zhang, Jiang, Miao, Mu and Li (2012) was followed. The supernatant obtained from raw and processed almond milk were diluted with equal volume of distill water and scanned using a UV –visible light Spectrophotometer 2450 (Shimadzu, Columbia, Md., U.S.A.). The absorbance was measured with light source wavelength of 282 nm in wavelength range of 250 nm to 350 nm with 1-cm path length UV disposal cuvettes. The borate buffer (0.05M, pH 8.5) was used as a control. UV spectra measurements were taken at ambient temperatures.

4.2.10. Color Measurements

A Hunter Color Quest XE (Hunter Associates Lab., Reston, Va.,U.S.A.) was used to measure the color of almond milk. Almond milk samples were gently stirred in a test tube and then the \(\sim 2.5\) mL samples were put in the plastic cell (path length 2mm) and put on specimen holder. The instrument was calibrated by using instrument standard white tile \((X = 80.20, Y = 85.11, Z = 90.14)\) and light trap.
Raw milk was used as a control for high pressure and thermally processed samples. $L^*$ value was used to measure the lightness ranging from 0 to 100. $a^*$ represents red (+) to green (-) while $b^*$ represents yellow (+) to blue (-). D65 was the source of light and the angle of observation was $10^\circ$. The mode of measurement was reflectance excluded and the area of view was 1 inch diameter. The total color difference between raw milk and processed milk was calculated using the following formula:

$$\Delta E^* = \left[\Delta L^* + \Delta a^* + \Delta b^*\right]^{1/2}$$

(10)

where $\Delta E^*$ is the net difference in color, $\Delta L^*$ is the mean difference in lightness/darkness between difference control and processed samples, $\Delta a^*$ is the mean difference in red/green color between control and processed samples and $\Delta b^*$ is the mean difference in yellow/blue color between control and processed samples.

The readings were taken at three different positions on either side of each sample. The readings are the mean of three independent samples.

### 4.2.11. Statistical analysis

All the statistical analysis was done by using the Minitab (Minitab Inc, State College, PA, USA). Comparisons between means were done using Tukey Honest Significance Different (HSD), where applicable. A significance level of $\alpha=0.05$ was used for all analysis.

### 4.3. Results and Discussions

#### 4.3.1. Composition and properties
Table 4.1 shows major compositions and some characteristic properties of raw almond milk. The extraction rate of almond milk (data not shown here) was also fairly constant ranging from 85 to 88 % (w/w) with total solids ranging from 7 to 9 % (w / w). These total solids value including proteins and lipids are lower than that reported by Gallier, Gordan and Singh (2012) but these are higher than those reported by Valencia-Flores, Hernandez-Herrero, Guamis and Ferragut (2013). Variation in composition of almond milk are not unexpected due to ingredients variability and processing conditions such as the ratio of almonds and water, soaking time and temperature, disintegration method and filter size.

The values obtained for pH and °Brix of raw almond milk 6.49 and 4.8 respectively (Table 4.1). Almond milk after high pressure processing (450 and 600MPa,30 °C) as well as thermal processing(72,85 and 99 °C, 0.1MPa) treated for 600s showed slight increment in pH value up to 6.62. On the other hand, almond milk after high pressure processing (450 and 600MPa,30 °C) as well as thermal processing (72,85 and 99 °C, 0.1MPa) treated for 600s showed marked reduction in °Brix (~1.5). These changes can be mainly due to decrease in soluble protein content. In an earlier study, we found, HPP (600 MPa,30 °C) and TP (99 °C,0.1MPa) treated for 300s decreased almond milk protein solubility by ~70 and 75% respectively (Dhakal et al., 2013). Similar reduction in protein solubility due to high pressure processing (Anon, Lamballerie, & Speroni, 2012 ; Lakshmanan, De Lamballerie, & Jung, 2006) and thermal processing (Kasera, Singh, Kumar, Lavasa, Prasad, & Arora, 2012 ; Albillos, Al-Taher, & Maks, 2011) has also been reported for nuts and legumes.
4.3.2. Particle size characterization

4.3.2.1 Particle size distribution of raw and processed almond milk

The study of particle size can aid in the understanding and controlling of the functionality and quality of almond milk during processing. Like thermal treatment, studies have shown that high pressure have ability to alter the structure of cells that may bring changes in solubility, permeability and micellarization (Gupta et al., 2011). So, high pressure processing (at 400 – 600 MPa) (Anon, Lamballerie, & Speroni, 2012 ; Zhang, Li, Tatsumi, & Isobe, 2005) and thermal processing (at 50 -125 °C) (Raikos, Kapolos, Farmakis, Koliadima, & Karaiskakis, 2009 ; Ren, Tang, Zhang, & Guo, 2009) may increase or decrease the size of particles depending upon the type of constituents, pH, amount of pressure, temperature and treatment time.

The particle size distribution of raw (control), high pressure processed (450 and 600MPa, 30 °C) and thermally processed (72 and 85°C, 0.1 MPa) almond milk treated for 300s represented the mono modal distribution as shown in Fig 4.1. In comparison to raw almond milk, both of the processing methods alter the particle size distribution.

Raw almond milk had the particle distribution span of 1.560 ± 0.057 that is fairly consistent with the previous studies (Gallier, Gordon, & Singh, 2012). D_{4,3} , D_{3,2} , Dv_{0.5} and SSA values of raw almond milk were found to be 2.98 ± 0.48 ,1.82 ± 0.19, 2.354 ± 0.107 and 3.36 ± 0.38 respectively (Table 4.2). The values were in agreement with previous study conducted by Valencia-Flores et al. (2013) ; Beisson et al. (2001).

Pressure treatment at 450 MPa and 600 MPa for 300s at 30 °C caused the change in the distribution of particles making it broader with increase in span size 1.95± 0.02 and
1.921± 0.025 respectively (Figure 4.1). There was also a shift in peak towards the larger units (negatively skewed distribution) with increase in pressure.

On the other hand thermal processing at 72 °C did not affect the particle size distribution and the curve almost coincided with the curve of the raw almond milk. However, thermal processing at 85°C for equivalent treatment time (300s) at 0.1MPa caused the change in the distribution of particles with increased in span size 2.3 ± 0.076 that was also manifested with broader distribution and reduction in peak height. The shift was slightly on the left side of pressure treated samples (Figure 4.1).

The processing also impacted average particle diameters (Table 4.2). Pressure treated almond milk at 450 and 600 MPa increased particle diameters in comparison to raw almond milk. In case of thermal processing, it was found that that average particle diameters of almond milk treated at 72 °C (for 300 and 600s) did not alter significantly when compared with raw almond milk (Table 4.2). When the temperature was increased to 85 °C, average particle diameters were markedly increased and the particles were even precipitated when treated at 99 °C (data not shown here). This effect of heat treatment on particle diameter was also reported in dairy milk (Raikos et al., 2009).

4.3.2.2. Aggregation of almond milk by high pressure processing

Fig. 4.2A-C show the effect of high pressure on particle diameter $D_{4,3}$, $D_{3,2}$ and $D_{v 0.5}$ respectively with various holding time 0 – 600 s using raw almond milk as a control. When treated at two different pressures, 450 MPa and 600 MPa, the diameter of the particle at first linearly increased with increase in pressure holding time and then the
diameter remained constant after the curve reached the peak point. $D_{4,3}$ which is more sensitive to particles with large size was found in the range $8.6 \pm 1.3$ (0 s holding time) to $15.92 \pm 2$ (600s holding time). $D_{3,2}$ and $D_{v0.5}$ values under these conditions were $2.98 \pm 0.25$ to $4.54 \pm 0.32$ and $7.2 \pm 1.03$ to $14.16 \pm 1.15$ respectively. Fig.4.2D shows the effect of high pressure on SSA and its value decreased with increase in pressure holding time that ranged from $2.064 \pm 0.15$ (highest value, 0s holding time) to $1.296 \pm 0.109$ (lowest value, 600 s holding time). There was no significant difference for SSA between the two pressure treated samples (450 MPA and 600 MPa) treated for equal holding time.

Thus, from Fig. 4.2A-C, although some dissociation of molecules may take place during pressure treatment, it implies that association/aggregation of the particles have dominated the volume distribution of the particles in high-pressure treated almond milk. Amongst the different constituents in foods, researchers have shown that proteins aggregate can be formed with high-pressure treatment (Qin et al., 2012; Tang, & Ma, 2009). The major factor that resulted with increase in particle size diameters $D_{4,3}$, $D_{3,2}$ and $D_{v0.5}$ of almond milk at different pressure and treatment time can also be related with the aggregation of proteins. Thus the data suggested that high pressure processing might have caused the unfolding and denaturation of protein, which favored hydrophobic and other weak interactions between exposed groups of proteins to associate and form higher molecular weight protein products. Moreover, this increase in hydrophobicity of proteins might have also favored interaction with lipids, and as the treatment time increases, the rate of aggregation is hindered by lipid phase. With further increase in time, very fewer proteins are left in the liquid phase for aggregation.
4.3.2.3. Microstructure

To investigate the mechanisms of structural modifications of almond milk particle due to high-pressure treatment, micrographs were obtained through confocal laser scanning microscopy. Fig. 4.3 A-C corresponds to micrographs of raw and processed almond milk samples stained with Fast Green FCF and Nile red to visualize lipids and proteins respectively.

Fig 4.3A shows the distribution of proteins and fats in raw almond milk. In raw almond milk, red colored proteins and the fat globules in green color can be seen in sparse distribution. Also some globules were found to be covered by thin layer of protein as shown by white pointed arrow. This type of distribution is in agreement with the distribution reported by Gallier, Gordon and Singh (2012), regardless of the staining specificity of the dyes.

The pressure treatment of almond milk at 600 MPa for 300s (Fig. 4.3C-D) resulted in aggregation of fat and proteins with less available free proteins in aqueous phase, showing dark background in micrograph (micrograph also available in appendix A). Also due to masking of red and green color (overlay image), some protein coated oil droplets appeared in yellow color. Such masking of red and green color due to colocalization of pixels has also been reported in micro fluidized (150 MPa) low fat stirred yoghurt (Ciron, Gee, Kelly, & Auty, 2010). Heat treatment of almond milk at 85 °C for equivalent treatment time (300s) also showed the similar effects on aggregation of protein and lipids compared with HPP at 600 MPa (Fig. 4.3C). The fat droplets were found integrated in the aggregated protein matrix, possibly, due to hydrophobic interactions. Although, the
particle size analyzer showed drastically increased value (5 to 7 times greater than raw almond milk) of particle size in processed samples, it is obvious from the micrograph observation with CLSM that such very large increment in individual fat droplets was not noticeable. It should be noted that although some fat droplets increased in size after processing there were still large number of small droplets, which were conspicuous in aggregate form along with larger fat globules in protein matrix. Thus, we can reasonably assume that the aggregated structures of proteins and fats might have been calculated by instrument as equivalent spherical diameter, the measurement characteristics of the instrument (Bowen, 2002). These results, on the whole, supported that hydrophobicity of proteins might have increased during protein unfolding/denaturation with either high pressure or heat treatment that ultimately caused these molecules to aggregate and form cluster with fat droplets which are hydrophobic in nature.

4.3.2.4 Modeling particle aggregation kinetics in pressure treated almond milk

Table 4.3 presents the kinetic constants of pressure treated almond milk. Within the conditions of the study, second order kinetics better described pressure treated almond milk samples as evident from correlation coefficient for samples processed at 450 and 600 MPa at 30 °C (Table 4.3). The apparent aggregation rates (k) for almond milk treated at 450 and 600 MPa were 0.0058 s\(^{-1}\) and 0.0095 s\(^{-1}\) respectively.

4.3.3. UV absorption spectra of protein solutions
The structural information on almond milk proteins can be studied conveniently from the UV absorption spectrum (both shape of the peak and wavelength of maximum absorbance) obtained from protein samples. Fig. 4.4 presents the UV spectra of raw and processed almond milk proteins. The wavelength in the range 275 to 290 nm represents mostly the absorption spectra of phenyl group in Tryptophan (Trp) and Tyrosine (Tyr) residue and to a very small extent on the phenylalanine (Phe) and disulfide bonded cystine residues (Gill, & von Hippel, 1989). Generally, absorption spectrum of protein is the sum of absorption spectra of these amino acid residues. As the spectrum of raw almond milk (Fig. 4, label a) moved from higher wavelength to lower side, the steepness of the curve increased showing maximum inflection at 276 nm with absorbance of 1.1. The absorption spectra of the curve at mild pressure treatment (150 MPa) showed similar shape with peak at 276.5 nm and having absorbance 1.2 (Figure 4.4 label b). When the pressure was increased to 300 MPa (Fig 4.4, label c), the maximum absorbance was drastically reduced to 0.6, indicating a cooperative process during structural changes in proteins. The unfolding of residues converted protein from ordered to disordered state resulting in loss of protein solubility. However, the sample was still able to maintain the peak in reduced form at 276.5 nm, probably due to slight changed in absorption by phenylalanine residue (Permyakov, 2012). The shape of the curve at 450 and 600 MPa (Figure 4.4, label d and e) is rather interesting. The absorption spectra of the curves showed drastic change in shape with blue shift showing flattened peak 268 and 265 nm for samples treated at 450 and 600 MPa respectively. The absorbance values for both of these samples were slightly reduced
(0.5) in comparison to samples processed at 300 MPa. Figure 4.4 also indicates that at pressure above 450 MPa the shape of the curve remains unchanged and these curves represent the pressure denatured protein due to instability in protein structure, the consequence of protein unfolding and hydrophobic interactions. Zhang et al., (2012) has also reported such structural information related with alteration of protein structure at high pressure in chickpea protein isolate.

The structural changes in protein was also studied in heat treated almond milk in the same wavelength range, 250 – 350 nm (Figure 4.4, label c, e and h). No changes were observed in the shape of spectra of samples treated at 72 °C(Figure 4.4, label f) when compared with the raw almond milk (Figure 4.4, labeled g). As the temperature was increased to 85 °C (Figure 4.4, labeled h), there was a drastic reduction in absorbance (0.6) due to decrease in protein solubility, possibly through hydrophobic effects and protein unfolding but still was able to maintain the peak at 275.5 nm . With further increase in temperature to 99 °C (Figure 4.4, label h), denaturation was almost complete showing slight decrease in absorbance (0.4) but the shape of the curve was dramatically changed and the curve showed a blue shift having peak at 265nm.

The above result shows that almond milk proteins are sensitive to pressure as well as temperature denaturation. Both treatments caused the reduction in absorbance, reflecting loss of soluble proteins due to their conformational disorder. However, there can be difference in type of structural deformation of protein molecules by these two different thermodynamic parameters, pressure and temperature, as indicated by the shape and position of the spectrum in denatured state. The difference between temperature
denaturation and pressure denaturation of chignolin, an artificial protein (β-hairpin) composed of 10 residues, has also been reported by Okumura, (2012). Further research is necessary to get detail structural deformation, regarding the location and proportion of conformational changes in higher order structures on almond milk protein denaturation.

4.3.4. Color analysis

Table 4.4 shows the effect of TP and HPP on appearance of almond milk for equal holding time of 600 s. Thermal processing did not show significant difference between each other or with the raw almond milk with respect to any of the color parameters. On the other hand, in comparison with the control, there was a slight increase in lightness (L*) and greenness (a*) without difference in yellowness (b*) for almond milk processed at 450 and 600 MPa. Such mild effect on L* (Lakshmanan. De Lamballerie, & Jung, 2006 ) and a* (Koca, Balasubramaniam, & Harper, 2011) value by HPP has also been reported in soymilk and white- brined cheese respectively. This slight increase in L* implies the corresponding increment in reflectance value. This can be attributed mainly due to pressure denatured compact structure of almond milk proteins that permitted less light to pass through them. Increase in lightness due to protein denaturation has also been reported in pressure treated sarcoplasmic proteins (Bak, Lindahl, Karlsson, & Orlien, 2012). Likewise, increase in greenness can be due to reduction of soluble protein in almond milk assuming negligible effect due to enzymatic browning in pressurized and unpressurized samples within a short storage time where almond milk was stored at 4 °C.
for not more than 24 h before analysis. Change in greenness has also been correlated with the amount of protein in bovine milk (Quinones, Barbano, & Philips, 1997).

When we compared the color difference (Δ E) between the samples, there was no significance difference between two pressure treated samples for L*, a* and b* values ΔE on the whole turned out to be significantly different in these samples. In order to further substantiate the above findings, sensory and quality evaluation from consumer’s perspective is necessary.

4.4. Conclusion

The study demonstrated that physicochemical characteristics of raw almond milk, especially particle size distribution, can be modified by HPP and TP. The particles size increase due to aggregation of proteins and lipids by HPP and TP to different extent depending upon the magnitude of pressure, temperature and treatment time. Aggregation of HPP processed almond milk particles follows second order kinetics. Pressure treatment at 450 and 600 MPa also enhance the whiteness and greenness of almond milk. HPP and TP have less negative impact on color (ΔE) (<1.5 units) immediately after processing. Further study on sensory and quality evaluation from consumer’s perspective is necessary to substantiate the above findings regarding the objective evaluation of almond milk color.


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Figure 4.1. High pressure processing (HPP) and thermal processing (TP) effects on particle size distribution of almond milk treated for 300s. Raw almond milk was used as the base product (control, 30°C, 0.1 MPa).
Figure 4.2. High pressure processing (HPP) effects on particle size of almond milk. (A) Volume mean diameter, \( D_{4,3} \). (B) Surface area mean diameter \( D_{3,2} \). (C) Median particle diameter \( D_{v, 0.5} \). (D) Specific surface area (SSA). Raw almond milk was used as the base product (control, 0.1 MPa). The error bars represent ± Standard Deviation (SD) of at least 9 readings from three independent experiments. Pairwise comparisons between the means were done by Tukey Honest Significant Difference (HSD) method with significance level of \( \alpha=0.05 \). Means with at least one common letter within/between the same pressure treated samples and/or control are not significantly different.
Figure 4.2. continued

(C) Median particle diameter (D_{0.5}) in µm

(D) Specific surface area (SSA) in m²/g

Legend:
- Control (0.1 Mpa)
- 450 MPa
- 600 MPa
Figure 4.3. Confocal laser scanning microscopy (CLSM) images (zoom 8X) of samples with 60X objective lens and its NA 1.4 (A) Raw milk (Control, 30 °C, 0.1 MPa). (B) Pressure treated (600 MPa, 300s at 30 °C) (C) Heat treated (85 °C, 300s at 0.1 MPa). The Nile red stained fat appears red (Excitation 488nm, Emission 520) and Fast Green FCF stained protein appears green (Excitation 635 nm, Emission 647) in these 2D images with (512 x 512 (pixel)). The scale bars accounts for 20 µm in all samples.
Figure 4.3. Continued
Figure 4.4. Effects of processing on the UV absorption spectra of supernatant obtained from almond milk proteins when scanned over the range of 250 - 350 wavelength (nm) with light source 282 nm and slit width 1.0 nm at slow speed. (a) Control (raw almond milk) (b) 150 MPa (c) 300 MPa (d) 450 MPa (e) 600 MPa (f) 72 °C (g) 85 °C and (h) 99 °C. All the samples were processed for 300s. The borate buffer (0.05M, pH 8.5) was used as a blank.
Table 4.1. Some major composition and physico-chemical characteristics of raw almond milk.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH at 20 °C</td>
<td>6.5 ± 0.06</td>
</tr>
<tr>
<td>Total solids (g/100 g wet basis)</td>
<td>8.2 ± 0.8</td>
</tr>
<tr>
<td>Total soluble solids (°Bx)</td>
<td>4.8 ± 0.42</td>
</tr>
<tr>
<td>Refractive index at 20 °C</td>
<td>1.345 ± 0.085</td>
</tr>
<tr>
<td>Total soluble protein(^b) (g/100 g wet basis)</td>
<td>1.44 ± 0.2</td>
</tr>
<tr>
<td>Lipids (g/100 g wet basis)</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>Ash (g/100 g wet basis)</td>
<td>0.65 ± 0.09</td>
</tr>
</tbody>
</table>

\(^a\)Values are mean ± SD of three independent experiments.

\(^b\)Soluble proteins are extracted with borate buffer (0.05M, pH 8.5)
Table 4.2. Average particle size parameters and specific surface area (SSA) of raw, pressure treated and heat treated almond milks.

<table>
<thead>
<tr>
<th>Sample/time (s)</th>
<th>$D_{4,3}$ (μm)</th>
<th>$D_{3,2}$ (μm)</th>
<th>$D_{0.5}$ (μm)</th>
<th>SSA (m$^2$/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control / -</td>
<td>2.977 ± 0.48$^a$</td>
<td>1.82 ± 0.19$^a$</td>
<td>2.354 ± 0.11$^a$</td>
<td>3.363 ± 0.38$^a$</td>
</tr>
<tr>
<td>450 MPa/300s</td>
<td>12.58 ± 1.39$^b$</td>
<td>3.9 ± 0.39$^b$</td>
<td>11.48 ± 1.36$^{bc}$</td>
<td>1.526 ± 0.20$^b$</td>
</tr>
<tr>
<td>450 MPa/600s</td>
<td>13.326 ± 1.68$^{bcd}$</td>
<td>4.07 ± 0.21$^{bd}$</td>
<td>12.38 ± 2.05$^{bd}$</td>
<td>1.43 ± 0.14$^{bc}$</td>
</tr>
<tr>
<td>600 MPa/300s</td>
<td>14.95 ± 1.06$^c$</td>
<td>4.485 ± 0.32$^{cd}$</td>
<td>13.83 ± 1.17$^d$</td>
<td>1.30 ± 0.09$^{bc}$</td>
</tr>
<tr>
<td>600 MPa/600s</td>
<td>15.01 ± 1.16$^c$</td>
<td>4.54 ± 0.32$^{cd}$</td>
<td>14.16 ± 1.13$^d$</td>
<td>1.296 ± 0.11$^{bc}$</td>
</tr>
<tr>
<td>72°C/300s</td>
<td>3.212 ± 0.10$^a$</td>
<td>1.77 ± 0.27$^a$</td>
<td>2.512 ± 0.09$^a$</td>
<td>3.435 ± 0.46$^a$</td>
</tr>
<tr>
<td>72°C/600s</td>
<td>3.965 ± 0.88$^a$</td>
<td>1.88 ± 0.32$^a$</td>
<td>2.66 ± 0.15$^a$</td>
<td>3.25 ± 0.54$^a$</td>
</tr>
<tr>
<td>85°C/300s</td>
<td>11.235 ± 0.89$^{bd}$</td>
<td>3.432 ± 0.61$^b$</td>
<td>9.22 ± 0.80$^c$</td>
<td>1.75 ± 0.11$^c$</td>
</tr>
<tr>
<td>85°C/600s</td>
<td>19.935 ± 3.93$^c$</td>
<td>5.195 ± 0.2$^c$</td>
<td>17.675 ± 2.8$^e$</td>
<td>1.196 ± 0.15$^c$</td>
</tr>
</tbody>
</table>

$^a$Values (mean ± SD) are of at least 6 samples from three experiments and these values within a column followed by the same letter are not significantly different $p < 0.05$.
$^b$Treatment time
$^c$Raw almond milk (untreated, 30°C, 0.1 MPa)
$^d$Volume mean diameter.
$^e$Surface mean diameter.
$^f$The diameter below which 50% of the volume of particles is found.
Table 4.3. Kinetic parameters for determination of the order of particle size aggregation of high pressure processed almond milk under isothermal conditions for various time 

<table>
<thead>
<tr>
<th>Treatment Condition (30 °C)</th>
<th>Order of reaction</th>
<th>Plot</th>
<th>( (r^2)^b )</th>
<th>((k_a)^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>450 MPa</td>
<td>1</td>
<td>( \ln \left[ \frac{D_{3,0t=0}}{D_{3,0t=t}} \right]^3 ) vs t</td>
<td>0.88</td>
<td>0.0016</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>( \left[ \frac{D_{3,0t=0}}{D_{3,0t=t}} \right]^{-1.5} ) vs t</td>
<td>0.92</td>
<td>0.0030</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>( \left[ \frac{D_{3,0t=0}}{D_{3,0t=t}} \right]^{-3} ) vs t</td>
<td>0.95(^d)</td>
<td>0.0058</td>
</tr>
<tr>
<td>600 MPa</td>
<td>1.5</td>
<td>( \ln \left[ \frac{D_{3,0t=0}}{D_{3,0t=t}} \right]^3 ) vs t</td>
<td>0.83</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>( \left[ \frac{D_{3,0t=0}}{D_{3,0t=t}} \right]^{-3} ) vs t</td>
<td>0.86</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

\(^a\) Treatment time (0, 30, 60, 180, 300 and 600s).
\(^b\) \( r^2 \): coefficient of determination
\(^c\) \( k_a \): apparent aggregation rate constant
\(^d\) Best fit among the three assumed order of reaction (1, 1.5 and 2)
Table 4.4. Effects of high pressure processing (HPP) and thermal processing (TP) on color of almond milks treated for 600s.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>( L^aC )</th>
<th>( a^D )</th>
<th>( b^E )</th>
<th>( \Delta E^F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^B)</td>
<td>85.38 ± 0.495(^a)</td>
<td>-1.02 ± 0.17(^a)</td>
<td>6.82 ± 0.523(^a)</td>
<td></td>
</tr>
<tr>
<td>450 MPa</td>
<td>86.60 ± 0.098(^b)</td>
<td>-1.35 ± 0.035(^b)</td>
<td>6.76 ± 0.095(^a)</td>
<td>1.274 ± 0.098(^a)</td>
</tr>
<tr>
<td>600 MPa</td>
<td>86.21 ± 0.323(^b)</td>
<td>-1.38 ± 0.137(^b)</td>
<td>6.83 ± 0.225(^a)</td>
<td>0.980 ± 0.189(^b)</td>
</tr>
<tr>
<td>72 °C</td>
<td>84.98 ± 0.189(^a)</td>
<td>-0.91 ± 0.048(^a)</td>
<td>6.43 ± 0.105(^a)</td>
<td>0.570 ± 0.156(^c)</td>
</tr>
<tr>
<td>85 °C</td>
<td>85.58 ± 0.179(^a)</td>
<td>-0.96 ± 0.065(^a)</td>
<td>6.73 ± 0.210(^a)</td>
<td>0.232 ± 0.114(^c)</td>
</tr>
</tbody>
</table>

\(^A\) Values (mean ± SD) within a column followed by the same letter are not significantly different \( p < 0.05 \) using Tukey Honest Significant Difference (HSD) method.

\(^B\) Raw almond milk (untreated, 30 °C, 0.1 MPa)

\(^C\) \( L^* \) represents the lightness with values from 0 (black) to 100 (white).

\(^D\) \( a^* \) positive values are red and negative values are green.

\(^E\) \( b^* \) positive values are yellow and negative ones are blue

\(^F\) \( \Delta E \) is color difference between control and treated sample.
CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Effect of high pressure processing on the immunoreactivity of almond milk

- High pressure processing (HPP; 450 and 600 MPa, 30 °C) and thermal processing (TP; 72, 85, 99 °C, 0.1MPa) can influence almond milk allergens through different mechanisms.

- High pressure and thermal treatments can cause aggregation of proteins and reduce the almond milk protein solubility to different extent depending upon pressure, temperature and treatment time.

- High pressure and thermal treatments can cause alteration in conformational structures that may reduce the availability of epitopes in almond milk during in vitro immunoreactions.

- Our research provided preliminary evidence that high pressure processing has the potential to produce hypoallergenic almond based beverages. Further studies are necessary to evaluate immunoreactivity of variety of soluble and insoluble fractions.
• of almond milk proteins. In addition, it may be worthwhile to study HPP induced aggregated proteins with respect to bioactivity, digestibility, enzyme activity and potential immunoreactivity. Study on the impact of immunoreactivity by high pressure processing at elevated temperature and/or in combination with enzymatic hydrolysis.

• Study of effect of other food components such as proteins, lipids and sugars on the stability of allergenic proteins in pressure treated almond milk.

Influence of high pressure processing on some physicochemical properties of almond milk

• High pressure processing (HPP) at 450 and 600 MPa (30 °C) and thermal processing (TP) at minimum 85 °C may help modify the particle size distribution with net effect of increase in particle diameters.

• Aggregation of proteins and lipids are the major cause of increase in particle diameters due to processing depending upon the magnitude of pressure, temperature and treatment time.

• Aggregation of HPP processed almond milk particles follows second order kinetics.

• HPP and TP treatments under the conditions of the study have minimum impact on browning reactions (ΔE <1.5 units).

• Alterations in protein structures produce whitening and greening effect on almond milk immediately after HPP processing. Additional studies are necessary to document consumer acceptance of pressure treated almond milk.
- Efforts can also be made to develop novel almond milk based products (through cold gelation) by treating the product at chilled conditions under pressure.

- Study on the aggregation mechanism and the effect of HPP on the morphology of aggregated structure to improve food functionality.

- Pressure treatment effects on almond milk proteins secondary structure and aggregation can be investigated by chemical structure and identification methods such as circular dichroism (CD) spectroscopy, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and size exclusion chromatography. Additionally, a study to improve understanding on intra molecular characterization of HPP processed almond milk proteins such as enthalpies, crystallization events and glass transition are desired.
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Appendix A

Confocal laser scanning microscopy (CLSM) images of samples with 60X objective lens with its NA 1.4

Figure A. 1. Control (raw almond milk, at 30 °C, 0.1MPa)
Figure A.2. Almond milk processed at 600 MPa, 30 °C for 300s.
Figure A.3. Almond milk processed at 85 °C, 0.1MPa for 300s.