



Effect of high pressure processing on the immunoreactivity of almond milk



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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 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 indicated that the observed decrease in immunoreactivity was partly due to the aggregation of amandin. The tested HPP and TP treatments respectively caused a maximum of ~70% and ~75% reduction in protein solubility. The study demonstrated that the loss of protein solubility, rather than the epitope destruction, may be responsible for the observed decrease in amandin immunoreactivity.

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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 as an alternative non-dairy beverage in the U.S., Europe and Australian market. This plant-based beverage is targeted to consumers suffering from lactose intolerance, and hypersensitive to cow milk, and those who seek for plant-based beverages as an alternative to dairy milk (Anonamous, 2013; Lacono, Lospalluti, Licastro, Scalici, & Pediatra, 2008; Salpietro, 2005). Almond milk is also rich in essential and non-essential nutrients like α -tocopherol, essential fatty acids, dietary fiber, and a 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 LDL cholesterol level (Chen, Milbury, Lapsley, & Blumberg, 2005). In spite of health benefits, they are one of the eight major allergenic foods affecting 0.5% of the adults in the U.S. (Sampson, 2004). The total population of allergenic individuals has been increasing in recent years (Jin et al., 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 et al., 2010). Almond seeds contain 188 different proteins as detected by two-dimensional electrophoresis (Li & He, 2004). Amongst these proteins, almond major protein (AMP) or amandin accounts for approximately 65% of total soluble proteins (Wolf, & Sathe, 1998). 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, Bravay, & Berger, 1997) retains considerable allergenicity.

High pressure processing (HPP) is an alternative food preservation technique that has the potential to preserve the organoleptic, textural,

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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 (Balny & Masson, 1993; Gross & Jaenicke, 1994; Heremans, 1982). Since HPP affects mostly the non-covalent bonds, it may cause irreversible changes in the 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 hazelnut, apple, celery, and peach (Meyer-Pittroff, Behrendt, & Ring, 2007). In addition, apple allergen Mal d 3 (Husband et al., 2011; Johnson et al., 2010), peanut allergen Ara h 2 (Hu et al., 2011), bovine gamma globulin (Yamamoto et al., 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 major protein allergens (Li et al., 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, 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.

2. Material and methods

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, Teuber, Gradziel, and Roux (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).

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 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 deskinced almonds was mixed with nine parts, by weight, of water and disintegrated in a Waring Laboratory blender (Torrington, CT) for 3 min in low speed (~15,000 rpm). The resulting mixture was then put through two layers of muslin cloth and the filtered milky almond suspension was passed through a 180-micron (μ) sieve. This final filtered aqueous suspension of almond milk was then loaded into 500 mL 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.

2.3. High pressure processing

The schematic of experimental procedures and analysis conducted during the study is presented in Fig. 1. Almond milk was processed in a high-pressure kinetic tester (PT-1, Avure Technologies Inc., Kent, WA). The procedure described by Thai Nguyen, Rastogi, and Balasubramaniam (2007) was used. Briefly, aliquots of 2.5 mL almond milk were placed in a high barrier pouch made from a sterile filter bag (# 01-002-57, Fisher Scientific), air bubbles were removed, and the pouch was 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 was 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). Representative pressure–time–temperature profile obtained during high pressure processing of almond milk is presented in Fig. 2. The almond milk samples were processed at 30 °C (± 2 °C) at 450 MPa and 600 MPa, each with pressure holding time of 0, 30, 60, 180, 300 and 600 s. Then the immunoreactivity of the samples was determined (see Sandwich mAb-based Enzyme Linked Immunosorbent Assay (ELISA) for amandin detection and immunoreactivity and Western blot and dot blot analyses sections). Additional experiments were also carried out at 30 °C (± 2 °C) over 150–600 MPa for 300 s to estimate pressure effects on protein solubility (Fig. 1). The equipment had a pressurization rate of about 20 MPa/s and rapid depressurization time (~1 s).

2.4. Thermal processing

Aliquots of two 2.5 mL almond milk at room temperature were placed into sterile plastic pouches as described in the High pressure processing section. The pouch were then loosely wrapped with the muslin cloth and dipped in a steam kettle at different temperatures (72, 85, and 99 °C) for four different time intervals, 0, 30, 180 and 300 s at each temperature. During a preliminary experimentation, sample temperature was monitored by using a k-type thermocouple temperature data logger (DCC Corporation, Pennsauken, NJ, model#3211) and come up time (105 to 115 s) was determined. The reported thermal process holding times do not include come-up time. The thermally treated samples at specified process temperature and holding times were then 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.

2.5. Determination of pH, °Bx and protein content

The pH and °Bx of almond milk were measured using a pH meter (Accumet XL 15, Fisher Scientific, Mississauga, ON, Canada) and brix/RI refractometer (Reichert Analytical Instruments, model #13940000, Japan) respectively. pH and brix values were measured before and after processing.

Protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (BSA, 0–200 μ g/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.

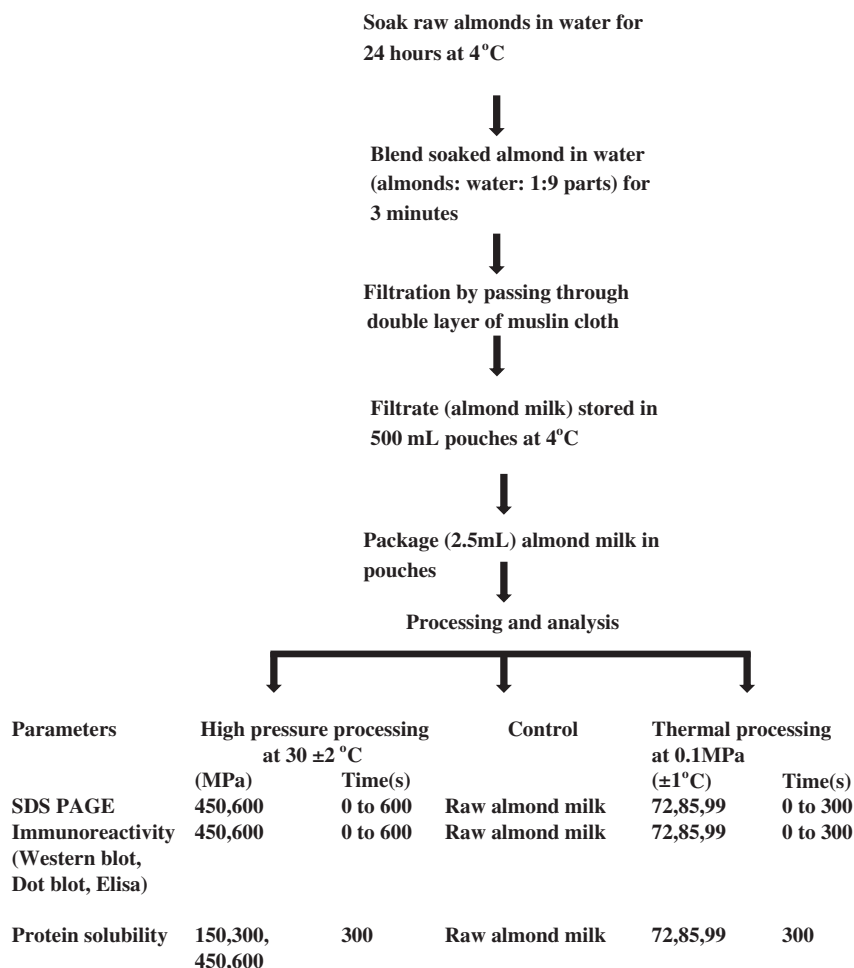


Fig. 1. Schematic of almond milk preparation, processing and analysis.

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 min in a magnetic stirrer. It was then centrifuged at 7900 g at 4 °C for 15 min and the supernatant was collected. Fifty microliters of the supernatant was then mixed with 3 mL of Bradford reagent and 100 µL of borate buffer. Then, the absorbance was measured at 595 nm 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.

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

SDS-PAGE was done by the method of Fling and Gregerson (1986) as described by Sathe (1991). 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.

2.7. Production of antibodies

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

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

Immunoreactivity of amandin in unprocessed and processed almond milk was determined by a sensitive, specific, and robust ELISA (Su et al., 2013).

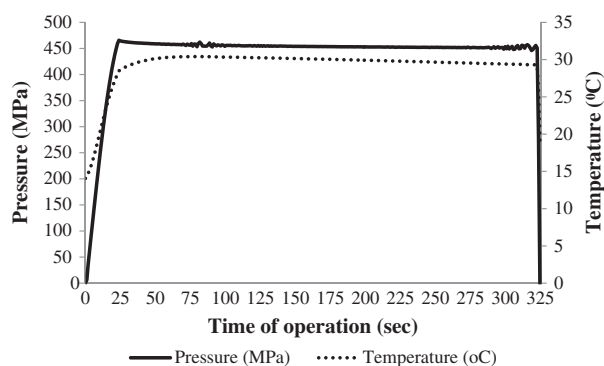


Fig. 2. Pressure–temperature–time profile during high pressure processing of almond milk.

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 mM Tris, 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 μ L/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) was added for color development (37 °C, 30 min). The reaction was stopped by adding 50 μ L/well of 3 M NaOH and the absorbance in each well was measured using a microplate scanning spectrophotometer at 405 nm.

2.9. Western blot and dot blot analyses

Following SDS-PAGE, the proteins were transferred onto a 0.22 μ m nitrocellulose membrane as described by Towbin, Staehlin, and Gordon (1979) at 4 °C for 4 h. The transferred polypeptides were visualized by Ponceau S staining. For dot blot, almond milk samples containing 1 μ g 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 then was 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, Teuber, Roux, & Sathe, 2002).

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 were expressed as mean $\pm$ SEM. Comparisons between samples for protein solubility measurements were done using Tukey Honest Significant Difference (HSD) with a 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 were expressed as mean $\pm$ standard error.

3. Results and discussion

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

The protein concentration of raw almond milk, at 25 °C was found to be 1.44 g/100 g 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 Fig. 3. It was found that protein solubility decreased with increase in pressure. However, there was a slight increase in protein solubility at 150 MPa, but not statistically significant ($P > 0.05$) and then at 300,

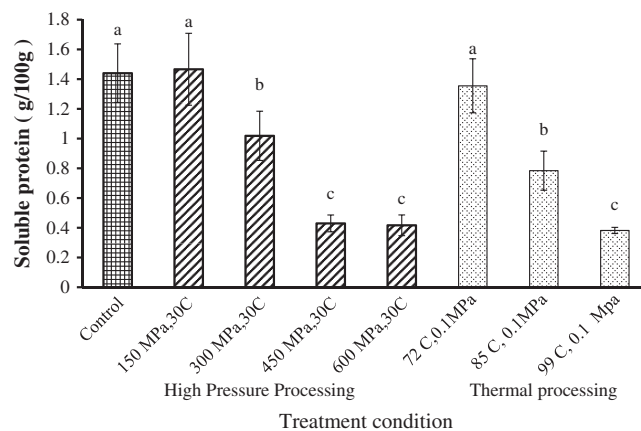


Fig. 3. Effect of high pressure processing (HPP) and thermal processing (TP) on soluble protein of almond milk treated for 5 min. Raw milk (0.1 MPa) was used as a control. Data were reported as mean $\pm$ standard error of mean. Means between samples with no common letters differ significantly with Tukey Honest Significant Difference (HSD) family error rate (5%), individual error rate (0.26%) and pooled standard deviation (0.16).

450 and 600 MPa at 30 °C, the reduction in solubility was ~30, ~68 and ~70% respectively. Thermal processing at 72 °C did not cause significant reduction in protein solubility but at 85 °C and 99 °C there was a significant reduction (~50 and ~75% respectively) in protein solubility (Fig. 3). The change in protein solubility in pressure and thermal treated almond milk in comparison to control (raw almond milk) 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, 2010). Our results are also in agreement with previous studies (Sathe & Sze, 1997, and Venkatachalam et al., 2002) reported the almond protein solubility decreased with thermal treatments (boiling and autoclaving). It is known that HPP and thermal treatment may cause protein unfolding due to distortion in their secondary, tertiary, and quaternary structure(s) and may result in protein aggregation and/or precipitation leading to decrease in protein solubility (Balny, Masson, & Travers, 1989, and Gross & Jaenicke, 1994). When proteins in their lowest free energy state are transferred to another state by pressure, protein unfolding/denaturation can occur as a result of overcoming the free energy barrier. For pressures >300 MPa, irreversible denaturation occurs in proteins (Balny et al., 1989, and Gross & Jaenicke, 1994). The irreversible denaturation has been attributed to the 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).

HPP and TP may also have different effects on almond milk protein structures and this difference is also supported from the SDS-PAGE protein profile presented in Fig. 4.

Fig. 3 shows that at relatively low pressure (150 MPa), HPP slightly, increased protein solubility in almond milk. This increase in solubility may be due to improved protein solvation as a result of deprotonation of polar amino acids under hydrostatic pressure. Carmel, Mission, and Nonpareil almond seed 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 (Ahrens, Venkatachalam, Mistry, Lapsley, & Sathe, 2005). The pH of raw and pressure treated (600 MPa, 10 min) almond milk samples was 6.49 and 6.57 respectively. The differences were not statistically significant (data not shown). There can be a transient decrease in pH of the food and buffer solutions during

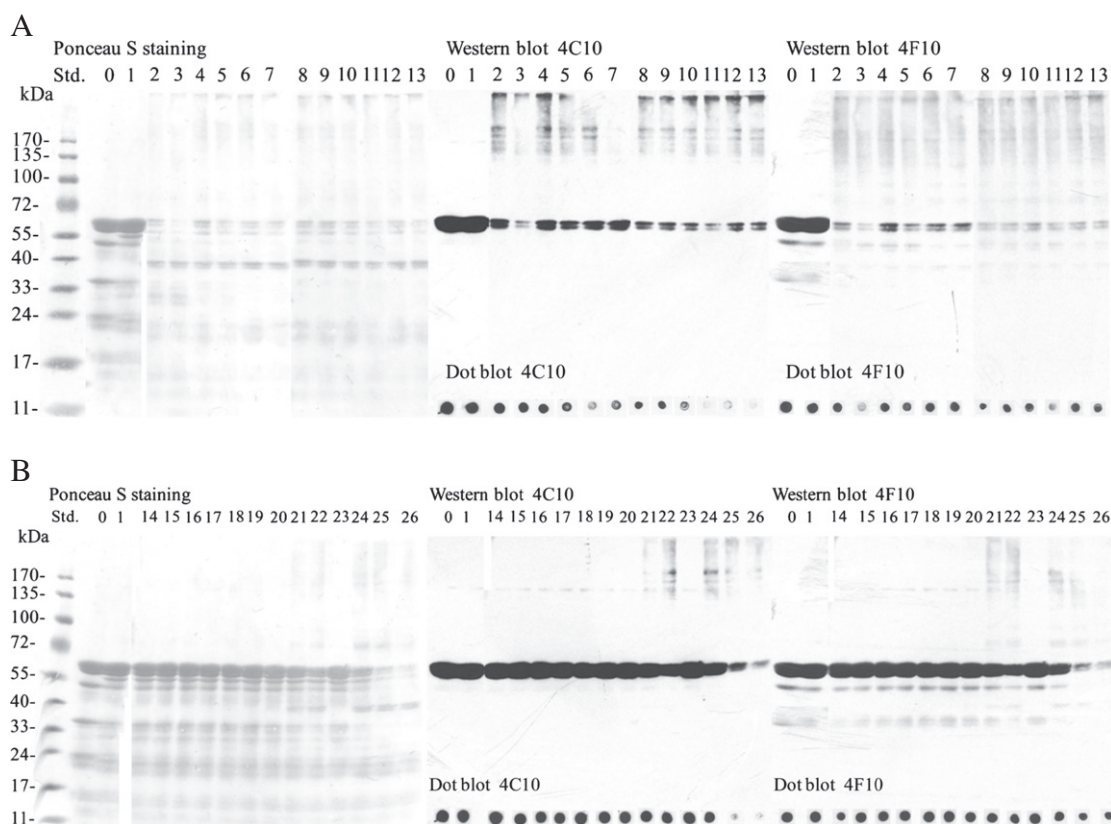


Fig. 4. SDS-PAGE, Western blots and Dot blots using two different monoclonal antibodies 4C10 (targeted for conformational epitopes) and 4F10 (targeted for linear epitopes) against almond major proteins (AMP) of almond milk without skin. The coded samples on horizontal axis (top) represent process condition. **(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 s (2), 450 MPa 30 s (3), 450 MPa 60 s (4), 450 MPa 180 s (5), 450 MPa 300 s (6), 450 MPa 600 s (7), 600 MPa 0 s (8), 600 MPa 30 s (9), 600 MPa 60 s (10), 600 MPa 180 s (11), 600 MPa 300 s (12) and 600 MPa 600 s (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 s (15), TP 72 ± 1 °C 30 s (16), TP 72 ± 1 °C 180 s (17), TP 72 ± 1 °C 300 s (18), TP 85 ± 1 °C 0 s (19), TP 85 ± 1 °C 30 s (20), TP 85 ± 1 °C 180 s (21), TP 85 ± 1 °C 300 s (22), TP 99 ± 1 °C 0 s (23), TP 99 ± 1 °C 30 s (24), TP 99 ± 1 °C 180 s (25) and TP 99 ± 1 °C 300 s (26).

pressurization and then the pH returns close to its initial value upon depressurization as reported by [Paredes-Sabja, Gonzalez, Sarker, and Torres \(2007\)](#). The effect of high pressure on pH of foods due to change in dissociation constants of attendant acids and bases has also been reported in the literature ([Stippl, Delgado, & Becker, 2004](#)). Almond proteins have a isoelectric pH (pI) range of 4.5–5.5 ([Sathe 1991; Li & He, 2004](#)). Protein deprotonation may transiently decrease the sample pH thereby shifting it towards the pI and decrease protein solubility. Since experimental techniques for measuring in situ pH under pressure were not readily available, future efforts in this line of research are beneficial.

The thermally processed almond milk protein solubility did not decrease at 72 °C ([Fig. 3](#)) probably because possible partial unfolding of proteins may have facilitated protein solvation. At 85 °C and 99 °C and 300 s treatment time protein unfolding and subsequent increase in surface hydrophobicity may increase protein–protein interactions leading to the observed ~50% and ~75% reduction of protein solubility.

Thermodynamic studies of 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 major driving force for pressure-induced denaturation 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.

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

The SDS-PAGE profiles of high pressure treated (HPP) and thermal processed (TP) almond milk were compared as shown in [Fig. 4A](#) and [Fig. 4B](#), respectively. In the absence of reducing agent, amandins 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 et al., 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. HPP has been reported to promote disulfide cross-linking in myosin ([Chan, Omana, & Betti, 2011; Jantakoson, Kijroongrojana, & Benjakul, 2012](#)), sarcoplasmic proteins ([Marcos et al., 2010](#)), β -lactoglobulin ([Chicón, Belloque, Alonso, & López-Fandiño, 2009; Zhong et al., 2011](#)), and soybean β -conglycinin and glycinin ([Speroni et al., 2009](#)). HPP treatment probably unfolded the proteins and exposed the buried sulfhydryl groups. The cleavage and rearrangement of disulfide bonds have also been observed in gluten following HPP treatment ([Kieffer, Schurer, Köhler, & Wieser,](#)

2007). Similar behavior in micro-fluidized (up to 180 MPa) peanut protein extracts (Hu et al., 2011) has been noted.

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 Fig. 4A and Fig. 4B. 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 amandin as it recognizes a conformational epitope (Kshirsagar, Fajer, Sharma, Roux, & Sathe, 2011). The 4C10 recognizes amandin epitope that overlaps with the epitope also recognized by amandin-reactive patients' sera IgE (Willison et al., 2013). Another mAb, 4F10, that recognizes a linear epitope on amandin, 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 (450 MPa 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 reported earlier (Su et al., 2013). Almond milk proteins 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 30 s.

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., 2013). As seen in 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. 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).

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).

3.4. Effect of high pressure processing (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 (Fig. 4A) 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 (Fig. 4A) 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 numbers 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 (Fig. 4B) 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.

4. 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. Fig. 5A and 5B 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,

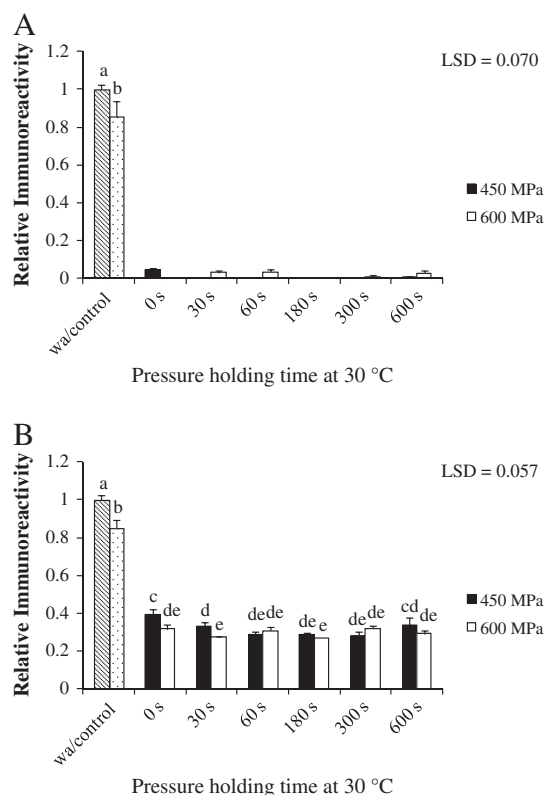


Fig. 5. Relative immunoreactivity showed by almond major proteins (AMPs) of almond milk without skin after high pressure processing (HPP) at 450 and 600 MPa at 30 °C by ELISA method. (A) Monoclonal antibody (mAb) 4C10 specifically targeted for conformational epitopes. (B) mAb 4F10 specifically targeted for linear epitopes. Data were reported as mean $\pm$ 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.

in the case of the 4C10 epitope and to 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 interferes 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 reduce the immunoreactivity of all almond proteins, by limiting the reactivity to the major allergen (amandin) 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.

4.1. 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 (Fig. 6A). 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 (Fig. 6B). This reduction in immunoreactivity of TP treated almond milk at high temperature was not unexpected in light of a previous study reported by Bargman, Rupnow, and Taylor (1992) that blanching and roasting eliminated the reactivity of a 70 kDa band of almond protein extracts. Roux et al. (2001) also observed a slight reduction in allergenicity due to blanching and roasting of whole almond protein extracts. Besides the aggregation of almond proteins, this reduction of immunoreactivity in heat-treated almond milk at extreme temperature (~99 °C) may also be due, in part, to interactions of allergenic proteins with other food constituents. For example, a reduction of buckwheat and hazelnut immunoreactivity due to thermally derived Maillard reactions has been reported (Yang, Li, & Wang, 2013; Iwan et al. 2011).

Combined pressure-thermal treatments (up to 700 MPa, 118 °C for 10 min) have 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.

5. 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 epitope 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 ($P > 0.05$). Both treatments reduced almond milk protein solubility, caused aggregation and degraded disulfide bonds. This impact of processing causes alteration in almond milk protein structures due to which the amandin epitopes present in almond milk become 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.

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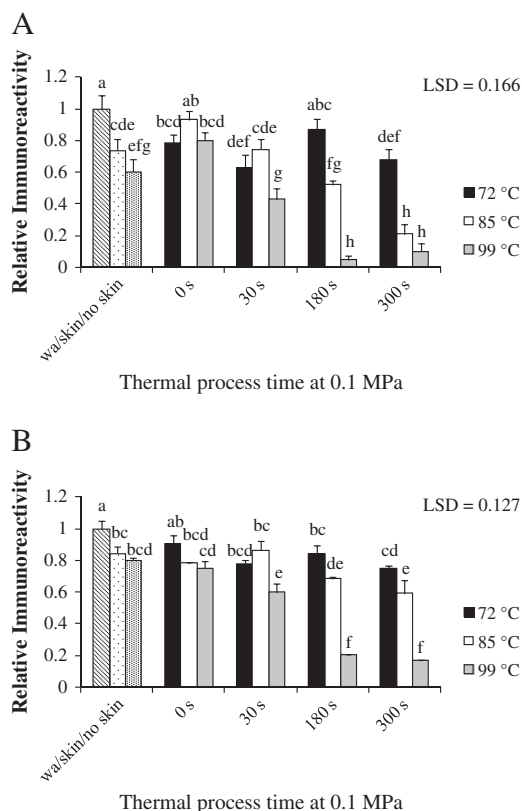


Fig. 6. Relative immunoreactivity showed by almond major proteins (AMPs) of almond milk without skin after thermal processing (HPP) at 72, 85 and 99 °C (0.1 MPa) by ELISA method. (A) Monoclonal antibody (mAb) 4C10 specifically targeted for conformational epitopes. (B) mAb 4F10 specifically targeted for linear epitopes. Data were reported as mean $\pm$ 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.

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