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Analytical Methods

Purification, characterisation, and quantification of the soy allergen profilin (Gly m 3) in soy products

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ABSTRACT

Profilins are pan-allergen proteins present in various plant foods and pollens. The objective was to develop a method for purification and characterisation of profilin from soy protein isolate. Furthermore, profilin was quantified in soy products and the effect of processing evaluated. Profilin was purified using poly-L-proline affinity chromatography, dialysis and ultrafiltration, and its quantification was implemented by indirect ELISA. Profilin in soymilks ranged from 4.37 ± 0.14 to 7.24 ± 0.30 mg/g protein, while in fermented products profilin ranged from 1.67 ± 0.02 to 5.47 ± 0.02 mg/g protein. Pasteurisation of soymilk was an ineffective method to completely eliminate profilin. Food matrix influenced thermal stability; at 100 °C, β-sheet and random coil structures were altered, while the α-helices remained intact. Induced fermentation of soybean meal by *Bifidobacterium lactic*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* resulted in 68.3% to 72.7% reduction of soy profilin. Heat treatment, fermentation and hydrolysis effectively reduced soy profilin.

1. Introduction

Food allergy is an adverse immune response to food or food additives (Taylor, 2006). Soybean is a protein-rich crop consumed worldwide. The allergens in soybean prevent some segments of the population from enjoying its nutritional and functional benefits. Soy allergies can provoke mild symptoms and can also be the cause of life threatening reactions. The threshold of protein consumption for soy allergic patients ranged from 0.0013 to 500 mg (Ballmer-Weber et al., 2007; Becker et al., 2004). Alteration of allergens before absorption impacts the potential of an allergic response (Thomas et al., 2007). Currently, 33 immunoglobulin E (IgE) binding proteins are listed on the soybean allergen online database (FARRP, 2008). In addition, another 39 kDa allergen called P39 has been recently identified as a novel allergen present in soybean (Xiang et al., 2008).

Profilin, a metabolic protein found in both plant and animal sources (Radauer & Breiteneder, 2007), is another allergen found in soybean. Profilins regulate polymerisation of actin into filaments through the formation of profilactin complexes. According to Smole, Bublin, Radauer, Ebner, and Breiteneder (2008), only plant profilins are recognised as allergens. Profilins are pan-allergens showing 30% occurrences in fruits and vegetables (Asero, Monsalvew, & Barberw, 2008; Lopez-Torrejon et al., 2005). People, who are allergic to profilin from one plant source, can also be allergic to profilins in other plant sources, which is known as cross-sensitisation. The allergenic profilin family is reported as the third most prevalent allergenic plant food behind the prolamin and Bet v 1 families (Jenkins, Griffiths-Jones, Shewry, Breiteneder, & Mills, 2005). It is believed that profilins are heat-labile proteins and become unstable after digestion (Lucas, Cochrane, Warner, & Hourihane, 2008; Scadding, 2008). Furthermore, Rihs et al. (1999) reported that fractional peptides of recombinant soy profilin (rGly m 3, 14 kDa) did not show significant binding reactivity to IgE antibodies. Food processing, such as heating, enzymatic hydrolysis, and fermentation, thus could provide the potential means to reduce antibody-binding capacity of profilin, remove allergic profilin with concomitant reduction of soy antigenicity. The goal of this study was to develop a method for purification, characterisation, and quantification of profilin in soy products and thus determine the effect of processing.

2. Materials and methods

2.1. Chemicals and materials

CNBr-activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Poly-L-proline (M_r 1,000 to 10,000), bovine

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serum albumin (BSA), β-mercaptoethanol, Trizma-hydrochloride, phosphate buffered saline with Tween (PBST) pH 7.4, Tween-20, Tris buffered saline (TBS), and *para*-nitrophenyl phosphate (pNPP) were purchased from Sigma–Aldrich (Saint Louis, MO). Native sample buffer, silver stain kit and prestained PAGE molecular weight standards were purchased from Bio-Rad (Hercules, CA); Tris–glycine running buffer, 8–25% precast polyacrylamide gradient mini-gel, buffer strips for Phastsystem mini-gels, ECL advance blocking, agent and chemiluminescent reagent were purchased from GE Healthcare (Amersham, UK). Alcalase from *Bacillus licheniformis* (E.C. 3.4.21.62), pepsin from porcine gastric mucosa (E.C. 3.4.23.1), and rabbit anti-goat IgG alkaline phosphatase conjugated were purchased from Sigma–Aldrich. Goat polyclonal antibody against a peptide mapping near the *N*-terminus of *Arabidopsis thaliana* profilin (aN-17, sc-15948), rabbit anti-goat IgG HRP, and actin-C2 mouse monoclonal IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sheep anti-mouse IgG HRP was purchased from GE Healthcare. All other reagents were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Soy ingredients and commercial soy products

Soybean was ground using 40 mesh Thomas Wiley mini mill 3383-L10 (Swedesboro, NJ). Soy protein isolate (SPI), soy protein concentrate (SPC), fermented soybean meal (FSBM), and soybean meal (SBM) were obtained from the local market in Urbana-Champaign, IL. Pasteurised soymilks with and without soaking of seeds were prepared at the Illinois Program for Soy Products (Urbana, IL). Commercial soymilks, natto, soy paste, and yogurt were purchased in local markets in Urbana-Champaign, IL. For the heating study, commercial soymilk IV and purified profilin were heated for 5 and 10 min at 100 °C. Fermented defatted soybean flours and fermented cracked soybean were prepared from *Glycine max* L. cv. Merit soybean as described by Frias, Song, Martínez-Villaluenga, de Mejia, and Vidal-Valverde (2008). FSBMs were prepared from *Glycine max* L. cv. Merill soybean as described by Song, Frias, Martinez-Villaluenga, Vidal-Valdeverde, and de Mejia (2008).

2.3. Enzymatic hydrolysis

Alcalase hydrolysis of defatted soy flour was conducted as described by Martinez-Villaluenga, Bringe, Berhow, and de Mejia (2008). Based on our previous studies, alcalase hydrolysates yield bioactive peptides; therefore, we wanted to learn about the potential reduction of profilin. Defatted soy flour would be the material used by the food industry to produce hydrolysates.

Stability of profilin to pepsin hydrolysis was studied as described by Bannon and Martino-Catt (2007). Briefly, 2 g of soy flour were dispersed in 48 ml deionised water. The mixture was heated up to 80 °C for 5 min and was adjusted to pH 2. One hundred milligrams of pepsin (EC 3.4.23.1; 662 units/mg) were added, mixed, and hydrolysis was conducted for 3 h at 37 °C. The hydrolysate was then adjusted to pH 7, ultrafiltrated, centrifuged at 14,000*g* at 4 °C for 30 min, filtered through 0.22 μm filters, and stored at −20 °C until used.

2.4. Purification of soy profilin by poly-L-proline – CNBr activated 4B affinity chromatography

Purification of profilin was performed by poly (L-proline)-Sepharose affinity chromatography at 4 °C with modifications (Tawde et al., 2006). Briefly, 5 g SPI in 100 ml washing buffer were loaded onto the column, washed out sequentially with column buffer (0.1 M KCl, 0.1 M glycine, 10 mM Tris–HCl, and 0.5 mM dithiothreitol (DTT) pH 7.8), profilactin elution buffer (column buffer with 4 M urea added), and profilin elution buffer (column buffer with 8 M urea added). The amount of profilin in each fraction was determined using indirect ELISA. Fractions containing high concentrations of profilin were pooled and dialysed against deionised water for 48 h (molecular mass cutoff, 12 kDa), concentrated by Eppendorf centrifuge model 5417R (Brinkmann Instruments, Westbury, NY) using Centricon-3 (molecular mass cutoff, 3 kDa) concentrators, freeze dried, and kept at −20 °C until used.

2.5. Total soluble protein quantification

Soluble protein was quantified by the DC Protein Assay™ (Bio-Rad) as previously described by Dia, Wang, Oh, de Lumen, and de Mejia (2009). The absorbance was read at 630 nm. Total soluble protein concentration of the fractions from affinity chromatography and soy products were quantified based on the BSA standard curve ($y = 0.0002x - 0.0098$, $r^2 = 0.99$).

2.6. Native polyacrylamide gel electrophoresis (native PAGE)

Electrophoresis was carried out in a Phastsystem (Amersham-Pharmacia Inc., Piscataway, NJ) with 8–25% polyacrylaminde gradient gel. Each protein extract was diluted (1:1 ratio) with Bio-Rad native sample buffer and vortexed. Four microlitres (equivalent to 1.5 μg of protein) of the mixture were loaded per well, run at 55 mA constant for 20 min (60–125 V), fixed for 20 min in methanol:acetic acid:water (4:1:5, v/v/v), and stained with silver stain following manufacturer's instruction (Bio-Rad). The gels were read in a Kodak Image Station 440 CF, where the respective molecular masses and band intensities were recorded for the different samples.

2.7. Immunoblotting

The PVDF membrane with the transferred proteins was blocked with ECL advance blocking agent in TBS with 1% Tween-20 (TBST) for 1 h, washed four times with TBST, and incubated with 1:1000 goat profilin polyclonal antibody for 16 h at 4 °C. The membrane was washed 4 times with TBST, incubated with 1:1000 mouse anti-goat IgG HRP for 2 h, washed four times with TBST, and detected using chemiluminescence following manufacturer's instructions (GE Healthcare). The membrane picture was taken with a Kodak Image station 440 CF. Actin (C-2) mouse monoclonal IgG towards human actin and sheep anti-mouse IgG-HRP were used in profilactin verification.

2.8. Characterisation of purified profilin

2.8.1. Reverse phase-high performance liquid chromatography (RP-HPLC)

A Beckman Coulter Gold chromatograph system (Fullerton, CA) was used for RP-HPLC analysis. A C_{12} RP-HPLC column (Jupiter 4u Proteo 90A column, 250 mm × 4.6 mm); (Phenomenex, Torrance, CA) was used. Detection of protein concentration was conducted using diode array detection at 168–280 nm with solvent A: 5% aqueous acetonitrile containing 0.008% TCA; solvent B: 95% aqueous acetonitrile containing 0.1% TCA. The separation was performed at a flow rate of 1 ml/min with a linear gradient of B% from 0% to 15% in 30 min, and from 15% to 60% for 10 min, then held at 60% for 5 min and decreased to 0% in 10 min. Profilin was calculated for its hydropathicity using the Kyte-Doolittle hydropathicity scale (kcal/mol) (Kyte & Doolittle, 1982).

2.8.2. Liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)

Purified profilin solution was dried under vacuum. Trypsin digestion was accomplished by adding 25 μl of trypsin solution

(12.5 ng of trypsin/μl in 25 mM NH_4HCO_3), carried out at 37 °C for 4–12 h. The digested peptides were extracted using 100 μl of 50% acetonitrile in 5% formic acid, and the sample mixture was sonicated for 10 min. The extraction was repeated twice, and the three extracts were pooled and dried. The dried peptides were dissolved in 10 μl of 5% acetonitrile in 0.1% formic acid for LC/MS/MS analysis. LC was carried out in digested peptides using dC18 Atlantis nanoAcuity column, 75 μM × 150 mm, 3 μm particle (Waters, Milford, MA) using aqueous 0.1% formic acid as solvent A and 50% acetonitrile with 0.1% formic acid as solvent B. A linear gradient from 1% to 90% B was run for 80 min and back to 1% B for 10 min with flow rate maintained at 0.25 μl/min. MS analysis was carried out using a Q-Tof API-US nanoAcquity UPLC (Waters) tandem mass spectrometer equipped with electron spray ion source. The Q-Tof was operated in positive ion mode. The desolvation and source temperatures were set at 120 and 80 °C, respectively.

2.8.3. Differential scanning calorimetry (DSC)

DSC Q2000 V23.12 Build 103 (TA Instruments, New Castle, DE) thermograms were recorded from 20 to 130 °C, using a heating rate of 1 °C/min with temperature precision of ±0.01 °C. The machine was equilibrated at 20 °C. Briefly, 20 μl of 0.51 mg/ml profilin was analysed. The sample was degassed prior to the experiment. Denaturation temperatures of purified profilin were analysed at pH 2, 4, and 7. The temperature with maximum peak was considered as the denaturation temperature. Enthalpy of denaturation (ΔH) was calculated by multiplication of heat capacity (Cp) in J/g °C with differences between temperature (ΔT) in °C as described by TA Instruments (Yu, Martin, & Schmidt, 2008).

2.8.4. Use of bioinformatics tools for protein homology study and prediction of epitopes of soy profilin

Sequences of recombinant soybean profilin (rGly m 3), arabidopsis profilin (Ara t 8), peanut proflin (Ara h 5), and birch pollen profilin (Bet v 2) were derived from the information provided by the National Center of Biotechnology Information (National Center for Biotechnology Information Database (NCBI), 2009) protein database (http://www.ncbi.nlm.nih.gov) with accession numbers of CAA11756, Q42499, Q5XXQ5, and P25816, respectively. Three dimensional modelling (3D) of soybean profilin secondary structure was predicted by using Swiss Model Workspace (2009): An Automated Comparative Protein Modelling Server (http://swissmodel.expasy.org). Potential epitopes of profilins were predicted by Abie Pro 3.0: Peptide Antibody Design, ChangBioscience (2009) (http://www.changbioscience.com/abie/abie.html).

2.8.5. Circular dichroism (CD)

The CD spectra in the far UV region (190–250 nm) of profilin were measured using a JASCO (Tokyo, Japan) spectropolarimeter Model J-715 fitted with a PTC temperature controller and a NesLab RTE 111 water bath. Approximately 0.13 mg/ml of profilin were analysed in a 1-cm path square quartz cuvette with a Teflon cap at temperatures of 25, 85, 90, and 100 °C (pH 7) with a speed of 50 nm/min, resolution 1 nm, bandwidth 1 nm, sensitivity 50 mdeg, response 0.5 s; 20 scans were averaged. The ellipticity values were calculated via K2D2 Website (Andrade, Chacon, Merelo, & Moran, 1993; Chacon & Andrade, 2007). Prediction of the percent of protein secondary structure from CD spectra reflected the structure of profilin upon heating.

2.9. Quantification of soy profilin in soy products and ingredients by indirect enzyme-linked immunosorbent assay (indirect ELISA)

2.9.1. Parameters in indirect ELISA

To determine the optimum conditions, a series of dilutions of the primary and secondary antibodies were conducted in indirect ELISA (primary antibody: 1:2, 1:10, 1:50, 1:100, 1:1000; secondary antibody: 1:500, 1:100, 1:2000). Goat profilin polyclonal antibody and rabbit anti-goat IgG alkaline phosphatase conjugated were used as the primary and the secondary antibodies, respectively. Briefly, 100 μl of purified profilin were plated into a 96-well microplate and kept at 4 °C for 16 h. Two controls, empty well and deionised water were also included. The plate was washed with 0.01 M PBST pH 7.4 using an automatic washer. The plate was blocked with 5% BSA in TBST, incubated for 1 h at 25 °C, washed, plated with 50 μl primary antibody, incubated for 1 h at 25 °C, washed and plated with 50 μl secondary antibody, and incubated for 1 h at 25 °C. The plate was washed, added with 100 μl of pNPP, read using an automatic reader (Elx80810 ultra microplate reader; Biotek Instruments) at 405 nm after 20 min, stopped by adding 100 μl 3 N NaOH at 25 min and read again at 35 min.

2.9.2. Quantification of soy profilin in soy products and ingredients

ELISA has been used widely in the detection of proteins in soybean (Koppelman, 2006). Purified profilin was used to prepare an indirect ELISA profilin standard curve using the optimised dilutions of the primary and the secondary antibodies. The linear points were extrapolated and the obtained standard equation was used to correlate absorbance at 405 nm with immunoreactivity. All the soy samples were quantified for protein concentration and equal amounts of protein (179 μg/100 μl) were analysed in duplicate in two independent studies. Estimation of percent reduction in profilin concentration in each soy product was calculated in comparison to the raw product as follows:

$$100 \times \frac{[\text{Profilin, mg/g}]_{\text{raw}} - [\text{Profilin, mg/g}]_{\text{processed}}}{[\text{Profilin, mg/g}]_{\text{raw}}}$$

2.10. Statistical analysis

Data of protein and profilin quantification by immune assays were analysed by ANOVA using SAS (SAS Institute Inc., Cary, NC). Means were generated and adjusted with Tukey's Studentised Range (HSD). Probability $p < 0.05$ indicated statistically significant differences.

3. Results and discussion

3.1. Purification of soy profilin

Fig. 1 illustrates the scheme of profilin purification from soy protein isolate. Unbound proteins were eluted out by a PLP column buffer. A high concentration of profilin was washed out from the column in the washing step. This can be explained by the fact that profilin can bind with actin, and the ability of profilin to form dimers, trimers, and tetramers. In fact, the binding capacity of actin to the tetramer of profilin is higher than its binding to the monomer profilin (Babich, Foti, Sykaluk, & Clark, 1996; Mittermann et al., 1998; Psaradellis, Kao, & Babich, 2000; Wopfner et al., 2002). As a result, these profilin-containing proteins could not bind with proline ligands resulting in their being eluted out from the affinity column. The actins and profilactins were then eluted by column buffer containing 4 M urea. The electrophoretic profile showed the presence of a protein with molecular mass around 50 kDa. The eluted profilactin was verified by immunoblotting with the antibodies against profilin and actin. The results showed that both profilin and actin antibodies interacted strongly with the 50 kDa protein. The presence of profilactin complex in soybean is therefore identified in this study. As reported by De Sá and

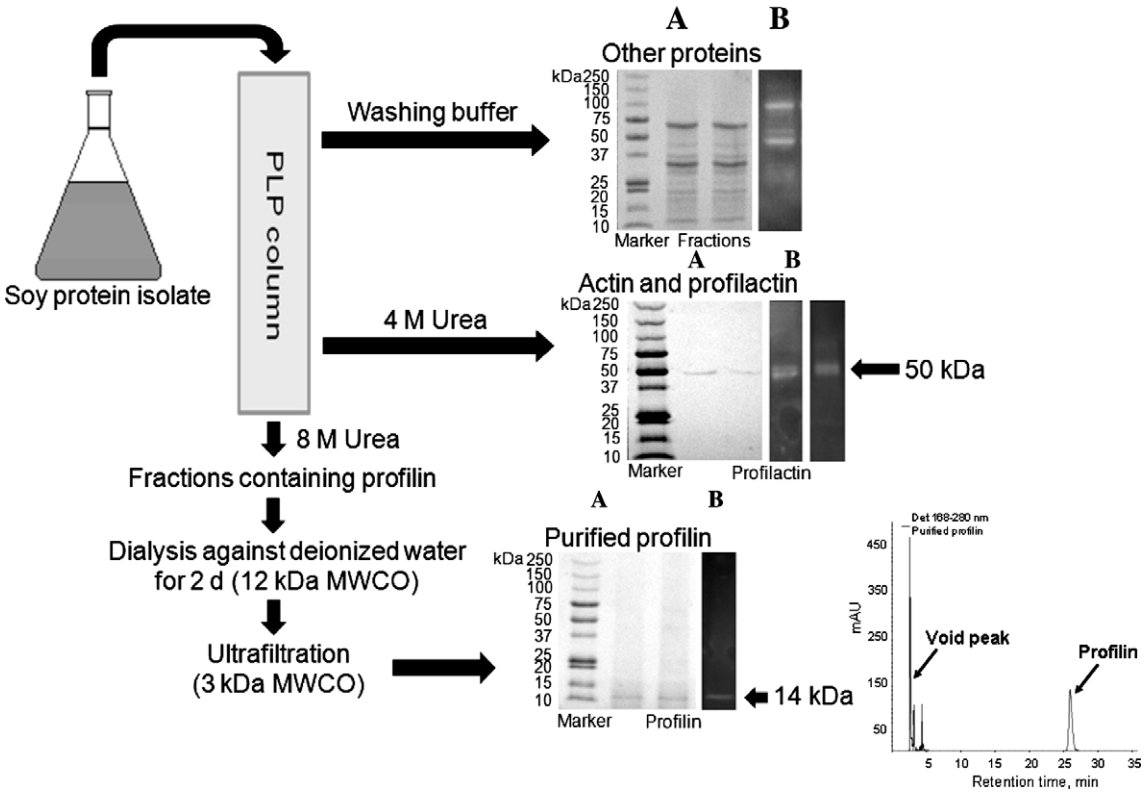

Fig. 1. Scheme of soy profilin purification showing native electrophoretic (A) and immunoblotting profiles (B) in each step of the purification. Proteins in the PVDF membrane were incubated with 1:1000 goat profilin polyclonal antibody for 16 h at 4 °C and then incubated with 1:1000 mouse anti-goat IgG HRP for 2 h and detected using chemiluminescence (see materials and methods section). RP-HPLC chromatogram of purified profilin is also shown.

Drouin (1996), soybean actin, a 336 amino acid sequence protein has a molecular mass of 36.96 kDa. The combination of soy actin and profilin yields a molecular mass of around 50 kDa for profilactin. It has been reported that profilin binds actin in various plants (Tawde et al., 2006). In the final step of the purification, profilin was eluted by the column buffer containing 8 M urea. Fold purification increased up to a value of 590 with a band intensity of 82% purity (data not shown).

3.2. Characterisation of purified profilin

3.2.1. Reverse phase-high performance liquid chromatography (RP-HPLC)

Purified profilin was eluted out from the column at 25.5 min (Fig. 1). Kyte-Doolittle hydropathicity scale (kcal/mol), indicated by the sequence of amino acids, was used to identify and verify the purified profilin. A positive value indicates hydrophobicity of protein while a negative value indicates hydrophilicity of protein (Kyte & Doolittle, 1982). In RP-HPLC, hydrophilic proteins are eluted out at lower retention times while hydrophobic proteins are eluted out at higher retention times. Hydropathicity scale of soy profilin was −0.18, indicating a hydrophilic protein. This corresponds to the fact that profilin is a water-soluble globular-type protein (Radauer & Hoffmann-Sommergruber, 2004). RP-HPLC successfully verified the purity of profilin.

3.2.2. Liquid chromatography/mass spectrometry/mass spectrometry (LC–MS/MS)

LC–MS/MS analyses of purified profilin obtained from poly-L-proline affinity chromatography were performed to further verify the identity and presence of profilin. Upon analyses, purified profilin gave three peptides that corresponded to the recombinant soy profilin (rGly m) sequence: YMVIQGEPGAVIR; KGPGGVTVK; and GPGGVTVKK.

3.2.3. Differential scanning calorimetry (DSC)

Denaturation by temperature indicated that the stability of soy profilin is important in the lowering of antibody-binding capacity, leading to a possible reduction of immunoreactivity by heat. Temperature of denaturation (T_d) was indicated by an endothermic peak in the DSC profile. Enthalpy of denaturation (J/g) is defined as the enthalpy change required to denature a protein under certain conditions. The results showed that at lower pH, the thermal stability of purified profilin increased, indicating that at lower pH more energy was required to denature profilin. At pH 2, 4 and 7, the temperatures of denaturation were 116.6 ± 1.0, 85.5 ± 1.3 and 80.3 ± 1.3 °C, respectively; the corresponding enthalpies of denaturation were 49.1 ± 1.0, 34.3 ± 1.3 and 29.7 ± 1.3, respectively. Kwok, Qin, and Tsang (1993) reported that soybean proteins appear to be more heat-labile at higher pH than at lower pH. At lower pH values, the net positive charge increased and led to increased solubility. Upon continued lowering of the pH, the protein was more protonated resulting in aggregation. Damodaran (1998) and Hermansson (1986) reported that increasing the ionic strength also elevated T_d. Moreover, it was reported that globular proteins generally are more stable to denaturation at pH values close to their pI (Prigent, 2005) and globular proteins in soybean were reported to form aggregates upon heating (Mills, Sancho, & Moreno, 2007). For soy profilin, the pI has been reported to be 4.4 (Rihs et al., 1999). Denaturation was possible at pH 7 and temperature around 80 °C. This observation showed that soy profilin is also a heat-labile protein like other profilins (Scadding, 2008). In addition, celery profilin has been reported to be unstable against heating by microwave at 100 °C for 30 min. However, the authors reported that con-

ventional cooking in water for 20 min did not affect the allergenicity of profilin, and IgE-binding activity was also detectable (Jankiewicz et al., 1996; Jankiewicz et al., 1997; Kleber-Janke, Crameri, Scheurer, Vieths, & Becker, 2004).

3.2.4. Protein homology and prediction of epitopes of soy profilin

Soy profilin shares 74.8% homology with birch profilin (Fig. 2A). Linear epitopes on birch pollen profilin were reported to be in residues 1–20, 36–47, and 108–133 which are categorised into three clusters: the *N*-terminal α-helix and the succeeding long, solvent-exposed loop and a long loop between the central β-sheet and the helix opposite to the *N*- and *C*-terminal helices, and the *C*-terminal α-helix (Fedorov, Ball, Mahoney, Valenta, & Almo, 1997). The prediction of allergenic epitopes by Abie Pro 3.0: Peptide Antibody Design indicated that soy profilin has the common antibody-binding epitopes in the regions of solvent-exposed loop and a long loop between the central β-sheet and the helix opposite to the *N*- and *C*-terminal helices, and the *C*-terminal α-helix. In addition Rihs et al. (1999) reported that recombinant soy profilin (rGly m 3) and recombinant birch pollen profilin (rBet v 2) had the common IgE-binding epitopes. The sequence homology between soy profilin and arabidopsis profilin and peanut are 77% and 84.7%, respectively, indicating that the antibody against arabidopsis profilin could be used effectively in the identification of soy and peanut profilins (Fig. 2B and C). Also, soy profilin and arabidopsis profilin have potential epitopes covering the sequence from 34–100. The predicted structure of soy profilin contains 3 α-helices, 8 β-sheets, and 12 random coils.

3.2.5. Circular dichroism (CD)

Fig. 3 shows the CD profile of purified profilin at 25, 85, 90, and 100 °C, respectively. The spectral characteristics of α-helices show a large-magnitude positive peak at ~195 nm, and two almost equivalent negative peaks around 208 and 222 nm. CD spectra of β-sheets often display a negative band in the region of 210–220 nm and a positive band in the 190–200 nm region of the spectrum (Wallace, 2000). From the results, increasing in temperature resulted in reduction of β-sheet structure and increasing in random coil structure (% β-sheet decreased from 33% to 32%, 31%, and 30% at 25, 85, 90, and 100 °C, respectively) while the α-helices remained constant. Predictions of the percent of secondary structure of profilin were calculated from the ellipticity values and indicated that α-helices were constant (data not shown).

These results indicated that α-helices of soy profilin are resistant to heating, even at temperatures up to 100 °C. This is important in predicting the stability of soy profilin epitopes since the *N*-terminal α-helix, a long loop between the central β-sheet and the middle helix, and the *C*-terminal α-helix are likely to be the epitopes of soy profilin. It is possible that most epitopes of soy profilin remain intact after heating even up to 100 °C. More research is

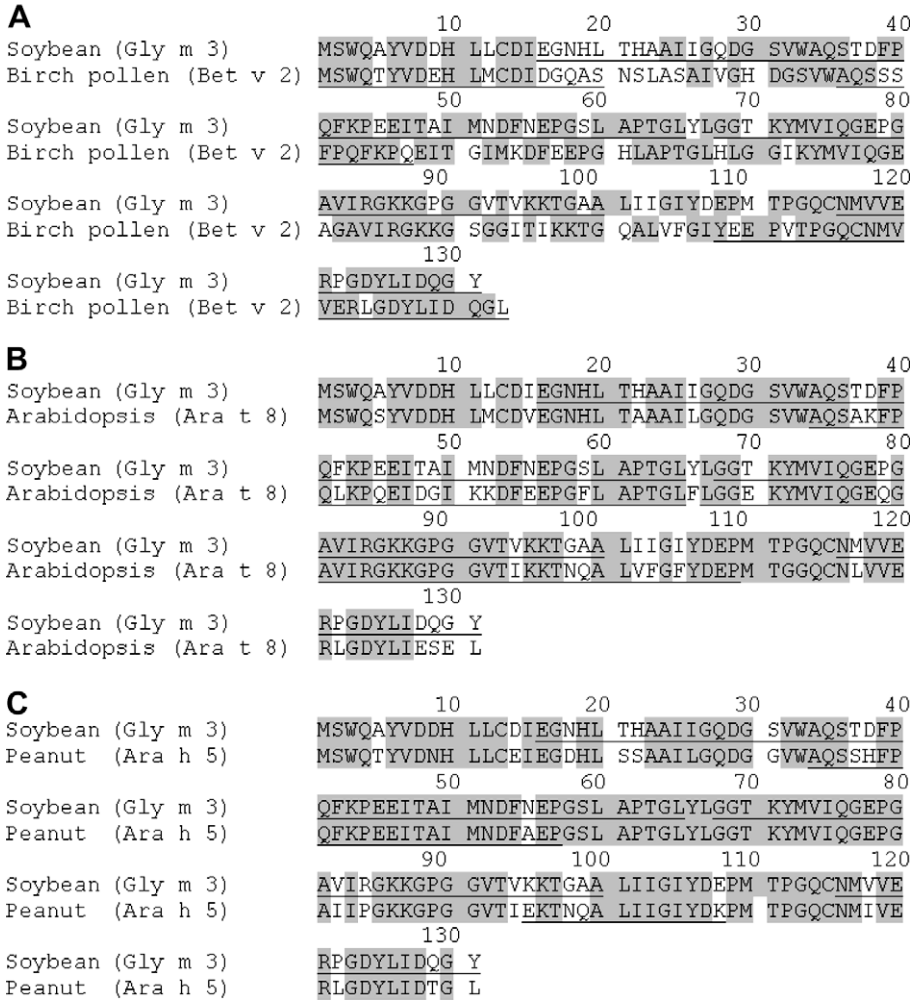

Fig. 2. Alignment of profilin sequences. (A) Sequence homology between soybean profilin (Gly m 3) and birch pollen profilin (Bet v 2) (74.8% homology), (B) Sequence homology between soybean profilin (Gly m 3) and arabidopsis profilin (Bet v 2) (77% homology). (C) Sequence homology between soybean profilin (Gly m 3) and peanut profilin (Ara h 5) (84.7% homology). Sequence homologies are shaded and linear stretches of potential epitopes are underlined.

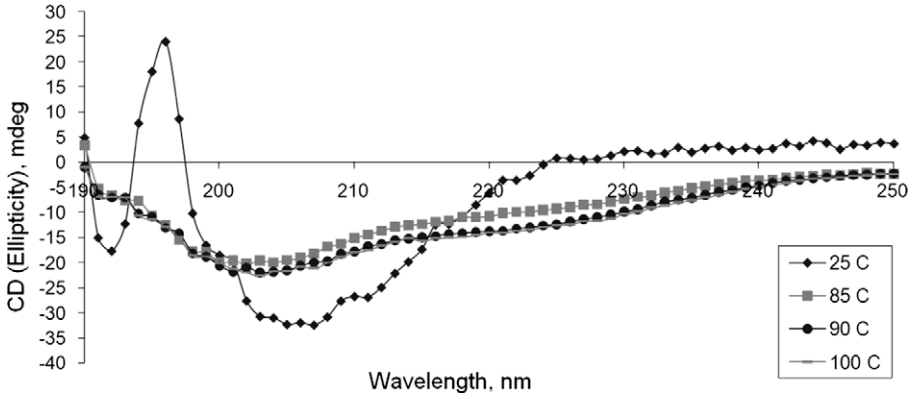

Fig. 3. Circular dichroism spectra of purified soy profilin heated at 25 °C, 85 °C, 90 °C, and 100 °C and pH 7.0.

needed to confirm these epitopes. ELISA results showed 86.1% reduction of soy profilin upon heating at 100 °C for 10 min.

3.3. Quantification of soy profilin in commercial products by indirect ELISA

3.3.1. Parameters in indirect ELISA

The optimum titres were achieved by a 1:1000 dilution of the primary antibody and 1:1000 dilution of the secondary antibody, respectively. A standard curve was prepared following the optimised indirect ELISA conditions, with a detection range of 0–45 μg/ml. The corresponding equation was as follows:

$$\text{Absorbance at } 405\text{ nm} = 0.0434\ [\text{Profilin}] - 0.0139;\ r^2 = 0.99.$$

The concentration of profilin is in μg/ml.

3.3.2. Quantification of soy profilin in soy products and ingredients by indirect ELISA

Profilin concentrations in all the soy products and ingredients studied are shown in Tables 1 and 2. SPI, the most concentrated source of soy protein, contains the highest amount of profilin. This was followed by SPC, soybean flours, and SBM, respectively. Profilin concentration varied in the soybean seed flours tested. Flours from soybean grown in the US showed higher profilin concentration than soybeans grown in Brazil, indicating that differences in growth conditions may affect profilin concentration. More studies are needed in order to establish clearly the effect of environment on profilin concentrations. Moreover, the results indicated no statistical differences in profilin concentration for the same cultivar of soybean flours when the beans were dried by natural drying method or by grain dryer. Soaking of soybean seeds before preparation of soymilk resulted in an increase in the total soluble protein. The results showed that unsoaked soybeans yielded less profilin than soaked soybeans but the difference was not statistically significant ($p < 0.05$). Profilin concentration in commercially pasteurised soymilks ranged from 4.37 ± 0.14 to 7.24 ± 0.30 mg/g protein and varied as the manufacturers used different cultivars of soybean, different ingredients, or employed different pasteurisation conditions (Mullin et al., 2001; Poysa & Woodrow, 2002; Poysa, Woodrow, & Yu, 2006). To pasteurise soymilk, it is usually heated at 65 °C for 30 min (Yazici, Álvarez, Mangino, & Hansen, 2005), and 72 °C for 15 s (Shelef, Bahnmiller, Zemel, & Monte, 1998). The percent reduction of profilin in each commercial product was estimated by comparing the raw ingredients with the processed

Table 1
Profilin concentration of soy flours, soymilks, and soy hydrolysates as determined by ELISA.

Soy products	Absorbance at 405 nm	Profilin concentration[a] (μg/ml)	Protein concentration[b] (mg/ml)	Profilin[c] (mg/g soluble protein)	
Soy flours					
Soy protein isolate (SPI)	1.15 ± 0.02	26.73 ± 0.44	5.49 ± 0.03	14.88 ± 0.24	A
Soy protein concentrate (SPC)	0.69 ± 0.01	16.15 ± 0.24	4.49 ± 0.13	8.99 ± 0.14	B
Soybean seed flour (grown in the US and dried by grain dryer)	0.61 ± 0.04	14.36 ± 0.81	4.07 ± 0.44	7.80 ± 0.45	C
Soybean seed flour (grown in Brazil and dried by natural drying)	0.48 ± 0.00	11.29 ± 0.05	3.98 ± 0.11	6.29 ± 0.03	EF
Soybean seed flour (grown in Brazil dried by grain dryer)	0.44 ± 0.00	10.38 ± 0.07	4.25 ± 0.21	5.78 ± 0.04	FG
Soybean meal (SBM)	0.51 ± 0.01	12.11 ± 0.20	4.26 ± 0.23	6.74 ± 0.11	ED
Soymilks					
Pasteurised soy milk (whole soybean with 6 h soaking)	0.48 ± 0.01	11.44 ± 0.29	4.12 ± 0.20	6.36 ± 0.16	EF
Pasteurised soymilk (whole soybean without soaking)	0.43 ± 0.00	10.26 ± 0.10	3.70 ± 0.24	5.72 ± 0.05	FGHI
Commercially pasteurised soymilk I (whole organic soybean seed)	0.55 ± 0.02	12.99 ± 0.54	4.64 ± 0.18	7.24 ± 0.30	D
Commercially pasteurised soymilk II (whole organic soybean seed)	0.45 ± 0.01	10.79 ± 0.33	5.10 ± 0.25	6.01 ± 0.18	FG
Commercially pasteurised soymilk III (whole organic soybean seed)	0.41 ± 0.01	9.84 ± 0.24	4.89 ± 0.71	5.48 ± 0.14	GHI
Commercially pasteurised soymilk IV (whole organic soybean seed)	0.38 ± 0.02	9.09 ± 0.46	4.48 ± 0.13	5.06 ± 0.25	HIJ
Commercially pasteurised soymilk V (whole organic soybean seed)	0.32 ± 0.01	7.67 ± 0.24	4.82 ± 0.33	4.37 ± 0.14	K
Boiled soymilk IV (5 min)	0.24 ± 0.01	5.76 ± 0.23	3.36 ± 0.31	3.21 ± 0.13	JK
Boiled soymilk IV (10 min)	0.08 ± 0.09	2.47 ± 0.31	2.00 ± 0.15	1.38 ± 0.17	M
Enzymatic soy hydrolysate					
Hydrolysate of SPI (Pepsin E.C. 3.4.23.1)	0.21 ± 0.00	5.17 ± 0.03	6.48 ± 0.41	2.88 ± 0.02	L
Hydrolysate of SPC (Pepsin E.C. 3.4.23.1)	0.24 ± 0.00	5.75 ± 0.03	4.26 ± 0.23	3.20 ± 0.02	L

[a] Standard curve for soybean profilin: $y = 0.0434x - 0.0139$, $r^2 = 0.99$.
[b] Standard curve for protein: $y = 0.0002\ x - 0.0098$, $r^2 = 0.99$.
[c] Columns with different superscripts indicate statistical differences ($p \leqslant 0.05$, $n = 2$).

Table 2
Profilin concentration of commercially fermented soy products, and fermented soy flours as determined by ELISA.

Fermented soy products	Absorbance at 405 nm	Profilin concentration[a] (μg/ml)	Protein concentration[b] (mg/ml)	Profilin[c] (mg/g soluble protein)
Commercially fermented soy product				
Natto (*Bacillus natto* starter culture)	0.38 ± 0.01	9.05 ± 0.18	6.48 ± 0.41	5.04 ± 0.10 IJ
Soybean paste (*Aspergillus oryzae* starter culture)	0.31 ± 0.00	7.52 ± 0.03	3.70 ± 0.24	4.19 ± 0.02 K
Soy yogurt (mixed starter culture*)	0.43 ± 0.00	10.31 ± 0.03	2.99 ± 0.15	5.74 ± 0.02 FGH
Solid-state fermentation				
Fermented cracked soybean flour (*Aspergillus oryzae*)	0.31 ± 0.01	7.51 ± 0.15	2.17 ± 0.13	4.18 ± 0.08 K
Fermented cracked soybean flour (*Rhizopus oryzae*)	0.33 ± 0.01	7.94 ± 0.07	2.13 ± 0.45	4.42 ± 0.04 JK
Liquid-state fermentation				
Fermented cracked soybean flour (Natural fermentation)	0.32 ± 0.00	7.73 ± 0.07	3.16 ± 0.51	4.30 ± 0.04 K
Fermented cracked soybean flour (*Lactobacillus plantarum*)	0.34 ± 0.00	8.17 ± 0.07	3.36 ± 0.31	4.55 ± 0.04 JK
Fermented defatted soybean flour (Natural fermentation)	0.22 ± 0.00	5.34 ± 0.08	3.19 ± 0.25	2.98 ± 0.05 L
Fermented defatted soybean flour (*Lactobacillus plantarum*)	0.21 ± 0.02	5.22 ± 0.36	3.58 ± 0.43	2.91 ± 0.20 L
Fermented soybean meal (Natural fermentation)	0.21 ± 0.00	5.10 ± 0.07	2.77 ± 0.60	2.84 ± 0.04 L
Fermented soybean meal (*Bifidobacterium lactis*)	0.15 ± 0.00	3.67 ± 0.03	2.20 ± 0.11	2.05 ± 0.02 M
Fermented soybean meal (*Lactobacillus plantarum*)	0.11 ± 0.01	3.00 ± 0.03	2.63 ± 0.15	1.67 ± 0.02 M
Fermented soybean meal (*Saccharomyces cerevisiae*)	0.13 ± 0.01	3.33 ± 0.20	1.81 ± 0.15	1.85 ± 0.11 M

[a] Standard curve for soybean profilin: $y = 0.0434x - 0.0139$, $r^2 = 0.998$.
[b] Standard curve for protein: $y = 0.0002x - 0.0098$, $r^2 = 0.996$.
[c] Columns with different superscripts indicate statistical differences ($p \leqslant 0.05$, $n = 2$).
* Soy yogurt was prepared from the mixed starter culture of *Bifidocacterium bifidum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus lactis*, *Lactobacillus rhamnosus*, *and Streptococcus thermophilus*.

products. All commercially pasteurised soymilks used in this study were prepared from whole soybeans. Based on the range of soy profilin in soybean seed flours (5.78 ± 0.04 to 7.80 ± 0.45 mg/g protein), pasteurisation process did not lead to a significant reduction of profilin.

A commercially pasteurised soymilk was selected to further heat at 100 °C for 5 and 10 min to evaluate the heating (boiling) effect, since we found that the pasteurisation process resulted in low percent reduction or not significant reduction of soy profilin. The results showed that heating of soymilk at 100 °C for 5 min resulted in a 36.6% reduction in profilin, while heating for 10 min resulted in a 72.7% reduction (Fig. 4A). For purified profilin, 40.8% and 86.1% reductions were observed after heating for 5 and 10 min, respectively (Fig. 4B). The results indicated that soy profilin is not stable when heated at 100 °C. Although the rate of reduction in profilin concentration of heated soymilk and heated pure profilin were similar, the higher percent reduction in heated pure profilin indicated that the food matrix influenced profilin stability. Boiling at 100 °C for 10 min led to effective reduction of soy profilin. However, heating can also lead to the destruction of amino acids, vitamin degradation, and other deteriorative reactions such as browning, and development of cooked flavours (Kwok, Liang, & Niranjan, 2002; Kwok et al., 1993; Amigo-Benavent, Silvan, Moreno, Villamiel, & del Castillo, 2008). The heating temperature and processing time of soybean must be optimised to reduce and destroy allergens, but also to maintain a desirable quality and maximum nutritive value.

Natto, soy paste, and soy yogurt are fermented soy products prepared from whole soybeans. The results showed that the reduction in profilin in natto fermented by *Bacillus natto* was 12.8% to 35.4% and for soy paste 12.8% to 46.3%, in comparison to soy flour. Soy yogurt showed lower profilin concentration than soybean flour with statistically significant reductions due to hydrolysis ($p < 0.05$). In regards to the fermentation process, the results already showed a high percent reduction of soy profilin. In this study, we used soy yogurt as a representative sample of enzymatic hydrolysis of soymilk. In addition, Frias et al. (2008) reported that fermentation of soybean flour with *B. subtilis* and *Lactobacillus plantarum* resulted in more than 80% reduction of total soy immunoreactivity. This can be explained by the bacterial strains utilised in fermentation of soy yogurt which are different from those used in the study by Frias et al. (2008), and also the proteolytic activities of the bacterial

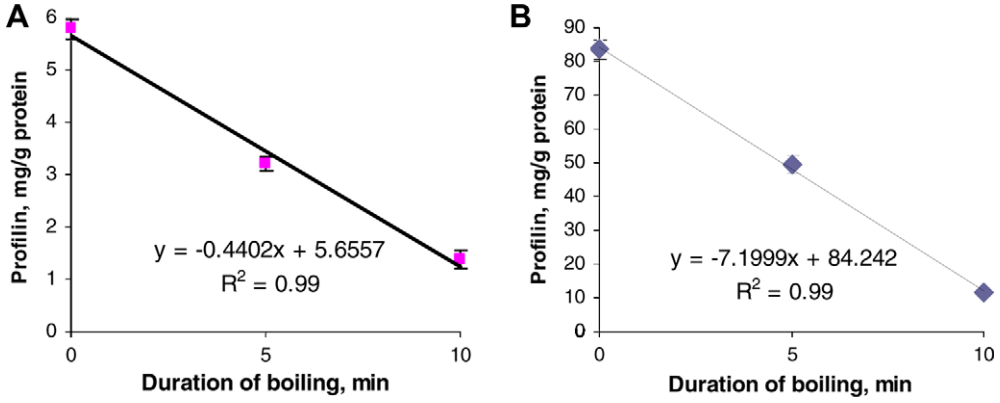

Fig. 4. Soy profilin concentration after heat treatment at 100 °C. (A) Commercially pasteurised soymilk IV, (B) Purified soy profilin.

strains used to ferment yogurt were less active. Peptic hydrolysis of soybean flours showed reductions in profilin concentration but these were not significant differences ($p > 0.05$) in peptic hydrolysates of soy protein isolate and soy protein concentrate. The hydrolysis of SPI with pepsin resulted in 80.7% reduction while in SPC the results showed a 64.4% reduction.

The results from both solid and liquid fermentations of cracked soybean flour were not statistically different in percent reduction and ranged from 21.3% to 41.7% and 27.7% to 46.4%, respectively. In these fermentations, the soybeans were cracked into pieces and fermented. After fermentation, the cracked soybeans were ground to obtain soybean flours. There was probably less soluble protein available for microorganisms to utilise in cracked soybean seeds, in comparison to ground soybean flours. The percent reductions in fermented soybean flours, therefore, were statistically greater than those in fermented cracked soybean seeds ($p < 0.05$). Liquid state fermentation of defatted soybean flour and soybean meal by natural fermentation and fermentation by *L. plantarum* resulted in non-significant differences in reduction of soy profilin and the percent reduction ranged from 55.8% to 57.9%, respectively. These fermented soy products had less reduction in profilin in comparison to the liquid state fermentation of soybean meal flours by *Bifidobacterium lactis*, *L. plantarum*, and *Saccharomyces cerevisiae* ranging from 68.3 to 71.4%. This can be explained by the fact that the *B. lactis*, *L. plantarum*, and *S. cerevisiae* strains have higher proteolytic activities than microorganisms present in natural fermentation. These results indicated that both natural and induced fermentations lead to a great reduction in soy profilin antibody-binding capacity and therefore fermentation is an effective method to reduce soy profilin. This would be important for allergic patients, as very small amounts of allergen could lead to allergic reactions; therefore, studies with human plasma and challenge human tests are needed. Song et al. (2008) also reported that fermentation of soybean flours by *L. plantarum* resulted in 96% to 99% reduction in the IgE-binding immunoreactivity of soybean allergens. In addition, Yamanishi et al. (1996) and Shreffler, Sampson, and Sicherer (2001) concluded that the allergenicity of soybean was destroyed by fermentation. Both hydrolysis and fermentation showed that soy profilin is not stable to protease hydrolysis, as confirmed by Lucas et al. (2008) who found that profilins were not stable to digestion.

3.3.3. Native polyacrylamide gel electrophoretic profiles of soy hydrolysates

Fig. 5A shows the electrophoretic profile of SPI with indication of major soybean proteins and reported soy allergens. The densitometric results indicated that the intensity of ~14 kDa band corresponding to profilin ranged from 6.04–8.31% in relation to total proteins. Fig. 5B shows the electrophoretic profiles of enzymatic hydrolysates. Lanes 1–4 represent SPI, pepsin hydrolysates of SPI,

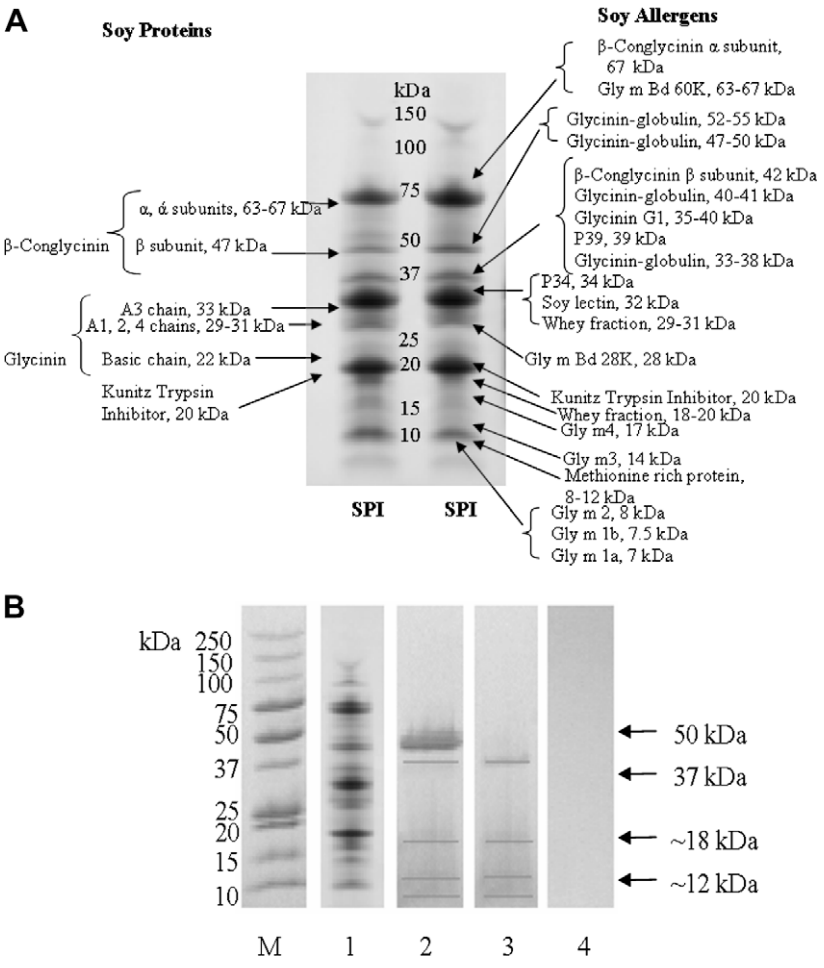

Fig. 5. (A) Electrophoresis profiles of soy protein isolate (SPI), (B) electrophoresis profiles of soy protein hydrolysates. *M* is a broad range molecular weight standard indicated in kilo Daltons (kDa) lane 1: SPI, lane 2: hydrolysate of SPI (Pepsin, E.C. 3.4.23.1), lane 3: hydrolysate of SPC (Pepsin, E.C. 3.4.23.1), lane 4: hydrolysate of defatted soy flour (alcalase from *Bacillus licheniformis*). Electrophoresis was carried out with 8–25% polyacrylamide gradient gel. For each protein, 1.5 μg were loaded per well and stained with silver.

pepsin hydrolysates of SPC, and alcalase hydrolysate of defatted soy flour, respectively. The results showed that pepsin hydrolysis altered soy proteins: ~50, ~37, ~18, ~13, and less than 10 kDa bands were present in the SPI hydrolysate, while in pepsin hydrolysate of SPC, the results showed only ~37, ~18, ~13, and less than 10 kDa bands. SPI contains a higher amount of soy protein than SPC. The results showed the absence of 50 kDa band in SPC while the band is present in SPI. In addition, hydrolysis of defatted soy flour by alcalase from *B. licheniformis* resulted in undetectable soy proteins with molecular masses higher than 10 kDa. This can be explained by the fact that only acid-soluble proteins are present in the final steps of preparation, due to the addition of TCA, while in the hydrolysis with pepsin, the final pH was adjusted to 7 and all the proteins soluble at pH 7 were present. Hydrolysis of soybean proteins with alcalase from *B. licheniformis* resulted in removal of soy profilin and other soy allergens. Pepsin hydrolysis led to a reduction in the size of soy proteins with molecular masses higher than 50 kDa. However, through pepsin hydrolysis, greater reduction of soy profilin was obtained but still the polyclonal antibody against arabidopsis profilin was able to detect profilin in the hydrolysates (data not shown). Hydrolysis of soy proteins by proteases resulted in the alteration of soy proteins (Gibbs, Zougman, Masse, & Mulligan, 2004), and reduction in profilin concentration, but antibody-binding capacity was retained.

4. Conclusions

Soy profilin is hydrophilic, and a heat-labile protein; however, as pH decreases, the thermal stability of soy profilin increases. Alteration of the secondary structure of soy profilin by heat resulted in changes of the composition of both the β-sheet and random coil structures, while the α-helices remained constant. Pasteurisation of soymilk is an ineffective method to completely eliminate soy profilin and the food matrix of soymilk affected profilin thermal stability. In commercially fermented soy products, the estimated reduction of soy profilin varies from product to product depending on the ingredients and the fermentation conditions. Alcalase hydrolysis of soy flour resulted in complete removal of soy profilin and soy proteins with molecular masses higher than 10 kDa. Soy profilin is unstable towards hydrolysis. Heating of soymilk at 100 °C for 10 min, and inducing fermentation of soybean meal by *Bifidobacterium lactic*, *L. plantarum*, and *S. cerevisiae* resulted in an estimated 68 to 72% reduction of profilin. Heat, enzymatic hydrolysis, and inducible fermentation effectively reduced antibody-binding capacity of profilin.

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