

Purification, Thermal Stability, and Antigenicity of the Immunodominant Soybean Allergen P34 in Soy Cultivars, Ingredients, and Products

S. WILSON, C. MARTINEZ-VILLALUENGA, AND E.G. DE MEJIA

ABSTRACT: Protein P34 (*Gly m Bd 30K*) is the immunodominant allergen in soybean (*Glycine max* L.). The objectives of this study were (1) to study the effect of thermal treatment on P34 antigenicity and secondary structure after isolation and purification of P34 from soybean by chromatographic techniques; (2) to identify the variability of P34 allergen within 138 accessions from a diverse USDA soybean germplasm collection by ELISA; and (3) to quantify P34 immunoreactivity in various commercial soy ingredients and products. Thermal processing decreased P34 antigenicity. Soybean accessions with the highest P34 content were ancestral (12 mg/g defatted flour) followed by modern (10 mg/g defatted flour) and exotic (8 mg/g defatted flour). The cultivar that emerged as the lowest-expressing P34 accession was PI548657 (2.3 mg/g defatted flour). Among commercial soy ingredients, soy flour yielded the highest P34 antigenicity (32 mg/g extracted protein) followed by soy protein isolate (29 mg/g extracted protein) and soy protein concentrate (24 mg/g extracted protein). Among soy consumer products, soymilk presented the highest P34 antigenicity, ranging from 7 to 23 mg/g extracted protein, followed by tempeh (8 mg/g extracted protein), soy infant formula (3.4 mg/g extracted protein), soy powder (2 mg/g extracted protein), and soy cheese products (0.50 mg/g extracted protein). Korean miso, soy sauce, soy chili mix, soy nuts, soy cream cheese, soy meat patty, texturized soy protein, and soy cereal exhibited undetectable P34 antigenicity (detection limit = 0.45 ng). Selecting soybean varieties with low levels of this allergen, or via processing, could potentially make soybean products less antigenic.

Keywords: antigenicity, *Gly m Bd 30K*/P34, soybean genotypes, soy products, thermal stability

Introduction

Soybean (*Glycine max* L.), a member of the legume family, is the world's foremost provider of protein and oil in the food industry. The use of soybean proteins has been expanded in the food industry due to their excellent nutritional and technological properties and health benefits (L'Hocine and Boye 2007; Singh and others 2008). However, in the United States, soybean ranks among the 8 most significant food allergens, to which more than 90% of all food allergies are attributed (Taylor and Hefle 2000; Kabourek and Taylor 2003; Cordle 2004). Food allergy is a relatively rare immunological, usually immunoglobulin E (IgE)-related reaction resulting from the exposure to exogenous food macro molecules (generally proteins) known as allergens or antigens (Mills and others 2004). Although most food allergies cause relatively mild and minor symptoms, some food allergies can cause severe reactions, and may even be life-threatening (USFDA 2007). According to the Natl. Inst. of Allergy and Infectious Disease, food allergies affect 6% to 8% of young children and 2% of adults (Sampson 2002).

Estimates of threshold levels for triggering adverse reaction in soy allergic individuals vary widely and have been reported to vary from 0.0013 to 500 mg (Becker and others 2004; Ballmer-Weber and others 2007). To date, 34 soybean proteins have been identified as allergens (FARRP Allergen Protein Database 2008; Xiang and others 2008). Among these, a member of the papain superfamily of cysteine proteases, P34, also referred as *Gly m Bd 30K*, have been iden-

tified as a predominant immunodominant allergen. IgE binding assays using immunoglobulins from soybean sensitive individuals indicated that 65% of the total allergenic response was accounted for by *Gly m Bd 30K* (Ogawa and others 1993; Helm and others 2000; Herman 2003). P34 is an insoluble glycoprotein that consists of 258 amino acid residues contained by disulfide linkages in the 7S globulin fraction of soybean storage protein and may play a role in protein folding (Hosoyama and others 1996; Helm and others 2000). It exhibits posttranslational production derived from a 46 to 47 kDa precursor protein by the partial removal of 122 N-terminal amino acid residues and tertiary conformation consistent with the papain family (Ji and others 1998; Yaklich and others 1999; Ogawa and others 2000). P34 is a relatively minor seed constituent comprising less than 1% of total seed protein (Helm and others 1998) and has a tendency to bind to lipid and oil, which is likely significant in its role as an allergen (Mills and others 2004).

There are 3 general approaches to manage food allergies: avoidance, medical treatment, and low or no allergen-content food. With increased labeling requirements recently enacted into U.S. law, avoidance will be more easily practiced. However, avoidance is still very difficult when the consumer has no control over the ingredients, such as in restaurants where there is no access to the supplier's labels. There are a number of strategies for medical treatment of food allergies being developed, including new drugs and immunotherapy. They are promising tests; however, when or if they will be available remains uncertain (Joseph and others 2006). The 3rd general approach is to produce or identify soybeans or soy foods that are null for allergens or have reduced content. Soybeans are processed in a number of ways, some of which may affect the allergenic potential of soy. Processing conditions that may make

MS 20080213 Submitted 3/23/2008, Accepted 5/5/2008. Authors are with Dept. of Food Science and Human Nutrition, Univ. of Illinois at Urbana-Champaign, 228 ERML, MC-051, 1201 W. Gregory Drive, Urbana, IL 61801, U.S.A. Direct inquiries to author de Mejia (E-mail: edemejia@uiuc.edu).

epitopes less available to antibody receptors would be valuable to mitigate food allergies (Wilson and others 2005). Therefore, the objectives of this research were (1) to study the effect of thermal treatment on P34 antigenicity and secondary structure after P34 isolation and purification from soybean by chromatographic techniques; (2) to identify P34 allergen variability in soybean cultivars within 138 accessions from a diverse USDA germplasm collection; and (3) to quantify P34 antigenicity in various commercial soy ingredients and consumer products subjected to different types and degrees of processing.

Materials and Methods

Materials

Non-GMO soybeans were obtained from the Illinois Center for Soy Products, Univ. of Illinois at Urbana-Champaign (UIUC). They were grown in Cerro Gordo, Ill., U.S.A. and contained 11.2% humidity. Soybean cultivars ($n = 138$) from the USDA Soybean Germplasm Collection at UIUC were kindly provided by Dr. Randall Nelson and subclassified as exotic, modern, and ancestral. Commercially available soybean ingredients were provided by Archer Daniels Midland Co. (Decatur, Ill., U.S.A.) and included several soy flours, soy protein isolates (SPI), and soy protein concentrates (SPC). Commercially available soybean consumer products were purchased from a local organic market (Urbana, Ill., U.S.A.) and included soymilk, tempeh, infant formulas, soy powders, soy cheeses, Korean miso, soy sauce, soy and rice granules, soy chili mix, soy nuts, cream cheese, tofu, texturized soy protein, and soy cereal. Primary monoclonal mouse α -P34 antibody (P34 protein specific monoclonal antibody P4B5) was kindly provided by Dr. Eliot Herman, Donald Danforth Plant Science Center, St. Louis, Mo., U.S.A. (Herman and others 1990). Secondary goat anti- α -mouse alkaline phosphatase-conjugated polyclonal antibody was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Isolation of P34 protein

Isolation of P34 was carried out according to the method of Ogawa and others (1993) with modifications. In brief, soybeans (approximately 50 g) were soaked in water overnight at 4 °C, strained, and homogenized with buffer 0.1 M Tris-HCl, pH 8.6, containing 10 mM 2-mercaptoethanol for 4 min (1:3, v/w). The homogenate was squeezed through cheesecloth and the filtrate was centrifuged at $18000 \times g$ for 30 min at 4 °C. The oil pad (float layer) was recovered using cheesecloth, suspended in buffer 0.1 M Tris-HCl, pH 8.6, containing 10 mM 2-mercaptoethanol (2.5:1, v/w), homogenized using a hand-blender, and centrifuged at $18000 \times g$ for 30 min at 4 °C. Further, it was resuspended (2.5:1, v/w) in buffer 0.1 M Tris-HCl, pH 8.6, containing 10 mM 2-mercaptoethanol, and 0.1 M NaCl, homogenized, and centrifuged at $18000 \times g$ for 30 min at 4 °C. This step was repeated twice and the subsequent oil pad was recovered and suspended in 1 M sodium carbonate (2.5:1, v/w), stirred at 4 °C overnight, and centrifuged at $18000 \times g$ for 30 min at 4 °C. Ammonium sulfate (Sigma) was added to supernatant making 70% saturation and stored overnight at 4 °C. This precipitated solution was centrifuged at $18000 \times g$ for 20 min at 4 °C and then the precipitate was dissolved in buffer 30 mM Tris-HCl, pH 8.0. This solution was transferred into a dialyzing tube (Sigma) against the same buffer for 2 to 3 d at 4 °C, changing the buffer every 12 to 24 h. After centrifugation for 15 min at 4 °C, the supernatant containing P34 was then recovered. Volumes for all supernatants were recorded and samples retained for purity analysis. The yield was calculated as amount of total protein extractable from 1 g of soybean and stored in aliquots at -20 °C until needed.

Purification of P34 protein

Size exclusion chromatography (SEC). A BioGel P-60 fine resin (Biorad, Hercules, Calif., U.S.A.) was hydrated with 11 mL/g of 0.1 M potassium phosphate buffer pH 7.4 containing 0.05 M NaCl and 0.02% sodium azide for 12 h at room temperature and loaded into an empty column (2.5 cm i.d.) with 40 mL maximum capacity. The column was run by gravity at a flow rate of 2.4 mL/h. Each fraction (approximately 1 mL) was read at 280 nm to determine protein concentration. Fractions were collected every 25 min until no absorbance at 280 nm reading was detected. Western blot and ELISA were used to confirm the presence of P34. The fractions with the highest P34 content were concentrated using Millipore stirred ultrafiltration cell (Millipore, Billerica, Mass., U.S.A.) and filtered through a 10 kDa molecular weight cutoff membrane (Millipore) under vacuum supplied by nitrogen gas at 20 to 30 psi. Further, fractions were applied to a reverse-phase chromatography column to eliminate any remaining contaminants.

High-performance liquid chromatography (HPLC). A Beckman Coulter Gold chromatograph system was used for HPLC analysis consisting of a 508 autosampler, programmable solvent, and UV detector module. Data acquisition and processing was performed with a 32 Karat software for data analysis. A Vydac reversed-phase HPLC C_4 column (4.6 $\times$ 250 mm length, 10 μ m particle size) was used to perform HPLC analysis, with a flow rate of 1 mL/min. Acetonitrile containing 0.1% trifluoroacetic acid (TFA) was used as solvent at various gradients: 50% acetonitrile + 0.1% TFA from 0 to 10 min, 60% acetonitrile + 0.1% TFA from 10 to 30 min, and 80% acetonitrile + 0.1% TFA from 30 to 40 min. Detection of protein concentration was conducted at 280 nm. Samples were collected at 1 min/tube. The fraction that indicated 1 main peak in HPLC was concentrated to one-quarter of its original volume using a 10 kDa centrifugal filter (Millipore) and filtered with a 0.22 μ m syringe filter (Fisher Scientific, Ill., U.S.A.) to remove any remaining low-molecular-weight proteins, salts, and residual aqueous solution.

Heat treatment of P34

Purest P34 fractions (1 mL) were pooled and diluted 1:100 in 30 mM Tris-HCl, pH 8.0. Aliquots of 500 μ L were transferred to Eppendorf tubes and heated in a boiling water bath, subsequently removed at various time intervals: 3, 5, and 7 min; every 2 min from 10 to 56 min, and at 60 min. These samples were kept at -20 °C to further study P34 antigenicity by ELISA and secondary structure by circular dichroism techniques.

Total protein determination

In brief, 50 μ L of each sample were added to 1.5 mL Biorad Bradford reagent, mixed, and incubated for 5 min. This mixture was read at 595 nm with a Beckman DU640 spectrophotometer and quantified using a bovine serum albumin (Sigma) standard curve ($y = 0.66x + 0.11$, $R^2 = 0.99$). Total protein concentration was used to further quantify P34 per gram of extracted protein.

Total protein extraction of soybean genotypes and commercial soy products

The dried mature seeds from 138 genotypes were kept at 4 °C in a dry environment. Seeds were ground into flour with a laboratory mill fitted with mesh size of 40 to obtain a uniform particle size material and stored at -20 °C until used. This ground meal material was then defatted overnight in a Soxhlet extractor using hexane as solvent. The defatted meal was air-dried in a hood for 8 h and kept at 4 °C until further use. The extraction procedure consisted of placing 0.05 g of the defatted flour and 1 mL of the extracting buffer (0.05 M Tris-HCl buffer, pH 8.2) in an Eppendorf tube. After being

mixed, the samples were placed in an ultrasonic bath (Bransonic Model 2510, Danbury, Conn., U.S.A.) for 70 min, mixing again every 10 min to avoid settlement. The temperature of the water bath was controlled at 40 °C. At least 2 independent extractions were performed for each sample. Following extraction, the samples were centrifuged at $20000 \times g$ for 30 min at 4 °C in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, N.Y., U.S.A.), and the resulting supernatant was then transferred to an Eppendorf tube.

The procedure outlined by Franck and others (2002) was used for protein extraction of all commercial products. Commercial dried samples were ground in a mill with a nr 40 mesh and added to the extraction buffer (0.05 M Tris-HCl buffer, pH 8.2). Liquid samples were concentrated using Millipore Ultrafree sterile centrifugal filter units (Millipore), 10000 nominal molecular weight limit. A 200 to 400 μ L aliquot of each sample was loaded into the filter and centrifuged for 60 min at $2822 \times g$ and 4 °C. Larger volumes (≥ 5 mL) were concentrated in a Millipore stirred ultrafiltration cell and filtered through a 10 kDa molecular weight cutoff membrane (Millipore) under vacuum supplied by nitrogen gas at 20 to 30 psi. The filtrate was discarded and the concentrated sample inside the cell was recovered using a Pasteur pipette and then stored at -20 °C until needed.

Protein gel electrophoresis

Protein extracts were centrifuged at $20000 \times g$ for 5 min. The supernatant (50 μ L) was added to 150 μ L of tricine sample buffer (Biorad Laboratories) and boiled for 5 min. After the samples had cooled down to room temperature, the same amount of protein for each sample was loaded into 16.5% Tris-Tricine polypeptide ready gels (Biorad Laboratories). Broad-range protein standard (Biorad Precision Plus Standards) containing pure recombinant proteins of the following molecular weights (MW), 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa, and another one with a 32 kDa protein that served as a comparative standard were also loaded into the gel. The gels were run in Mini Protean-3 Cells (Biorad Laboratories) using Tris-Tricine-SDS running buffer (Biorad Laboratories) at 55 mA constant for 100 min (60 to 125 V). Further, gels were fixed for 30 min in methanol/acetic acid/water (1:4:5, v/v/v), stained with Coomassie Brilliant G-250, destained for 1 h with acetic acid/water (40%, v/v), and finally, washed once again with distilled water. They were read in a Kodak Image Station 440 CF (Kodak, Rochester, N.Y., U.S.A.), where their respective molecular weights and band intensities were recorded. Each protein band was normalized by dividing its intensity by the intensity of the 32 kDa standard on the same gel and then applied to a P34 standard curve ($y = 870.77x - 670.59$, $R^2 = 0.99$).

Western blot analysis

Unstained gels were soaked for 3 min in 20 mL of blotting buffer, which consisted of 10% (v/v) methanol, 25 mM Tris-HCl, 192 mM glycine. A western sandwich was assembled by placing a sponge, a filter, and the gel, the Immun-Blot polyvinylidene fluoride (PVDF) membrane (Biorad), another filter and sponge, avoiding the formation of bubbles. The transfer of proteins was completed overnight at 40 V constant in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) at 4 °C. After the transfer was completed, the membrane was washed in Tris-Buffered Saline Tween-20 (TBST) solution (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% Tween-20) for 15 min. The membrane was blocked with 5% nonfat dried milk in TBST solution for 1 h and subsequently incubated with purified mouse anti-P34 antibody diluted (1:4000) in TBST containing 1% nonfat dried milk at room temperature for 1 h. After extensive washes (3 $\times$ 10 min) with TBST, the membrane was in-

cubated for 1 h at room temperature with alkaline phosphatase conjugated to goat antimouse IgG antibody diluted 1:2000 in TBST containing 1% nonfat dried milk. The membrane was washed as mentioned previously with TBST and prepared for detection using an Immun-Star Chemiluminescence Alkaline Phosphatase substrate solution (Biorad) and imaged using a Kodak Image Station 440 CF.

Enzyme-linked immunosorbent assay (ELISA)

In the indirect ELISA method used in this study, the antigen (pure P34 or soy samples) is coated to the well plate, monoclonal P34 antibody binds to the antigen forming a complex, then enzyme-linked secondary antibody binds to specific P34 monoclonal antibody. Substrate was then added and converted by enzyme into colored products. As a result, the rate of color formation is proportional to the amount of specific antibody that interacted with the antigen. A 50- μ L aliquot of each sample (extract, standard, or blank) was coated onto a Maxisorp immunoassay plate at various dilutions in triplicate and incubated for 14 h at 4 °C. The plate was automatically washed using a Biotek Instruments Elx50 autostrip washer with PBS-Tween buffer (Sigma) at pH 7.4. The plate was blocked (300 μ L in each well) for 1 h at room temperature with a blocking solution containing 5% bovine serum albumin (BSA), 1% Tween-20, 1 tablet of Tris buffer saline (TBS) in bidistilled water. The wells were washed, incubated for 1 h at room temperature with mouse P34 monoclonal antibody (50 μ L) diluted 1:2800 in antibody buffer (1% Tween-20, 3% BSA, 1 tablet TBS), and washed again. A goat anti- α -mouse alkaline phosphatase secondary antibody at 1:2000 (diluted with antibody buffer) was added at 50 μ L per well, incubated for 1 h at room temperature, and washed. Subsequently, 100 μ L of the color reagent *p*-nitrophenyl phosphate (PNPP) were added to each well and the plate incubated for 30 min at room temperature. The plate was read with an automatic reader (Biotek Instruments, Winooksi, Va., U.S.A.) at 405 nm using the KC Junior. The concentration of P34 (mg/mL) in each sample was computed using the average optical density at 405 nm, based on triplicate samples and multiple dilutions, and applied to a standard curve prepared with pure P34 ($y = 0.0136x + 0.0835$, $R^2 = 0.95$).

Secondary structure analysis—circular dichroism (CD)

The purest fractions from size exclusion (SEC) and HPLC were pooled, concentrated, and applied to CD, performed with Jasco J720 spectropolarimeter (Easton, Md., U.S.A.) and analyzed with CDSSTR software. A 200- μ L sample of approximately 0.16 mg/mL P34 in solution was added to a 1-mm pathlength temperature-jacketed cuvette, and the absorbance of right and left circularly polarized light was read at 180 to 260 nm at 200 nm/min with 0.5 nm intervals, 5.0 nm bandwidth, 0.5 nm resolution, 50 mdeg sensitivity, 0.25 s response, and 1 accumulation. The secondary structural analyses used the DICHROWEB interactive webserver (Lobley and others 2002; Whitmore and Wallace 2004) with the CDSSTR algorithm (Han and others 1984) and database 3. The normalized root mean square deviation (NRMSD) parameter was calculated as a means of assessing the goodness of fit. Delta epsilon ($\Delta\epsilon$ mdeg/M/cm) values were calculated for CDSSTR from the far-UV CD readings (θ , mdeg) using the following formula: $\Delta\epsilon = \theta \times [(0.1 \times \text{mean residue weight}) / (\text{pathlength} \times \text{concentration} \times 3298)]$, where mean residue weight = 34 kDa MW/257 amino acid residues = 132.3; pathlength = 0.1 cm; 3298 = a correction factor. The percent α -helix was determined and used as reflect of secondary structure.

Statistical analysis

One-way analysis of variance (ANOVA), with Tukey's comparison of means for statistical significance, was used for statistical analysis. A probability of $P < 0.05$ indicated significant differences.

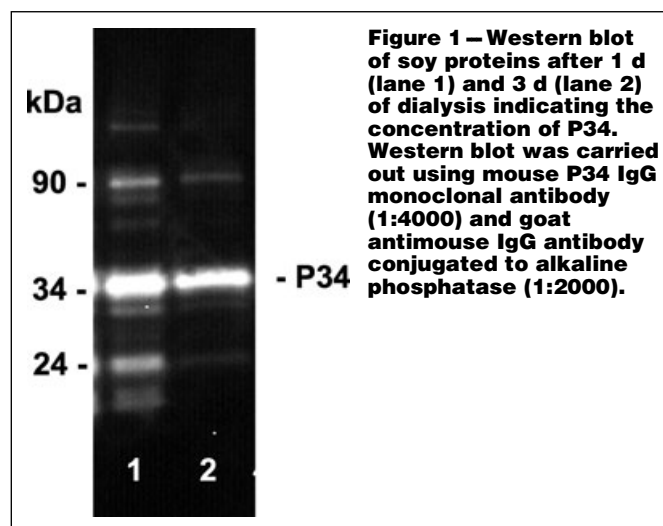
Results and Discussion

Isolation and purification of P34

Table 1 shows total protein, P34 concentration, and yield throughout the isolation procedure. The most efficacious purification step was dialysis (80-fold increase) followed by $(\text{NH}_4)_2\text{SO}_4$ and Na_2CO_3 (13-fold increase). Na_2CO_3 functioned to remove P34 from its other oil body protein constituents while dialysis removed salts added to precipitate proteins and lower molecular weight proteins. Figure 1 shows Western blot of protein solutions after 1 d (lane 1) and 3 d (lane 2) of dialysis. Protein solution exhibited bands at 90, 34, and 24 kDa after 1 d of dialysis. The highest intensity band at 34 kDa was identified as P34. The band at approximately 90 kDa may represent the α' subunit of conglycinin. P34 is associated with the α -subunits of β -conglycinin within the 7S globulin through disulfide bonds (Hirano and others 1987). Samoto and others (1996) reported that the addition of a reducing agent aided in removing conglycinin into the supernatant and P34 into the precipitate. This was attributed to the cleavage of the disulfide bond between P34 and conglycinin subunits. Therefore, it is possible that 2-mercaptoethanol used in the present study did not completely cleave this bond. The band approximately 24 kDa could be assigned to oleosin, another oil body protein (Kalinski and others 1990). P34 associates with this protein before cell lysis. After 3 d of dialysis a 60-fold yield increase in P34 was achieved (Table 1). At this step, P34 concentration represented 22% of the total protein content indi-

Table 1 – Total protein, P34 content, and yield within different steps of isolation procedure.

Process	Volume (mL)	Total protein (g)	P34 (ng/g protein)	Fold Increase	Fold yield Increase
Crude extract	100	7.5	0.2	1.0	1.0
Buffer 2, NaCl, Step I	80	5.4	0.5	1.1	1.6
Buffer 2, NaCl, Step II	55	3.1	1.4	1.3	2.4
Na_2CO_3	29	0.2	236.5	12.5	22.3
$(\text{NH}_4)_2\text{SO}_4$	8.5	0.05	1706.3	13.4	45.5
Dialysis	3	0.003	36400	79.8	60.7



cated by the net intensity of the 34 kDa band compared with the total net intensity of all protein bands showed by gel electrophoresis (data not shown). Therefore, further purification by size exclusion chromatography (SEC) and reverse-phase high-performance liquid chromatography (RP-HPLC) was needed. Figure 2A shows the protein elution profile obtained by absorbance readings at 280 nm of different fractions collected from SEC column. P34 identification in each protein fraction was performed by western blot using mouse P34 IgG monoclonal antibody. Figure 2B presents the western blot of 23 to 33 protein fractions collected from SEC column. The results revealed that fractions 31 to 33 contained the purest P34, which eluted at approximately 13 to 14 h. These results were confirmed by

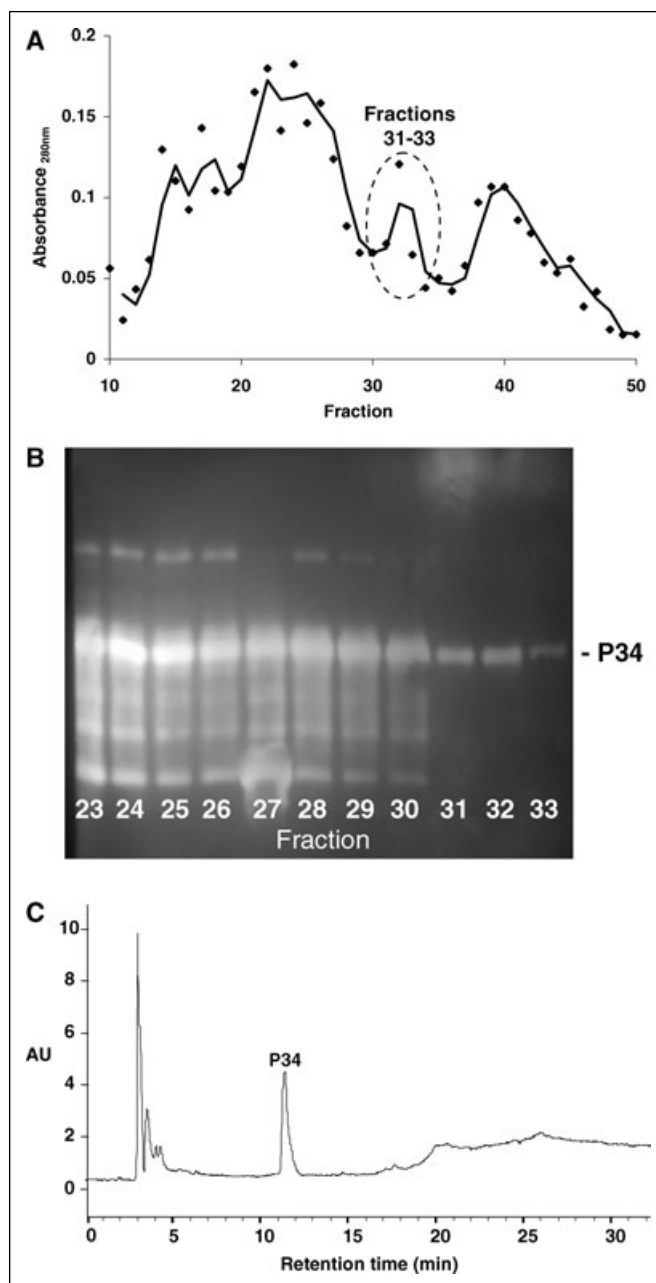


Figure 2 – A. Protein elution profile obtained by absorbance readings at 280 nm of different fractions collected from size exclusion chromatography. B. Western blot of SEC 23 to 33 fractions. C. High-performance liquid chromatography (HPLC) profile of pooled 31 to 33 fractions showing pure P34 (see Materials and Methods section for details).

gel electrophoresis and silver staining detection (data not shown), which indicated 1 single band. The detection limit of silver stain was < 1 ng. It was inferred that if any other contaminating proteins were present in the sample, they would have been detected. Purest P34 fractions (31 to 33) were pooled and analyzed by HPLC (Figure 2C). HPLC chromatographic profile showed that peaks prior to 5 min corresponded to buffer absorbance, and the main peak with a retention time of approximately 12 min was identified as P34 protein. The purity of the P34 in the pooled 31 to 33 fractions was approximately 98%. This sample was used to further study the effect of heat treatment on P34 antigenicity and secondary structure.

Effect of heat treatment on P34 antigenicity

Commercial use of soybean proteins in food products often involves thermal processing; it was hence important to investigate the effect of heat treatment on P34 antigenicity and secondary structure determined as percentage of α -helix (Figure 3). The results showed that P34 antigenicity increased at 5 min of boiling and gradually decreased as the duration of boiling approached 60 min. The constant rate of denaturation was 1191 ng/mL/min (calculated as decrease of protein concentration per time of heating). The sample boiled for ≥ 30 min exhibited no detectable antigenicity. Secondary structure of P34 initially consisted of 40% of α -helix and suffered changes during time of boiling, exhibiting 60% of α -helix after 5 min, while longer times (≥ 10 min) returned α -helix to the native state. These results correlated with P34 antigenicity, suggesting that the modifications in secondary structure, after 5 min boiling, may have exposed linear epitopes that could be responsible for the increased in P34 antigenicity observed. To date, it is unknown whether P34 contains conformational epitopes in addition to its linear epitopes (L'Hocine and Boye 2007). Burks and others (1991) reported that cooking soy protein at 80 to 120 °C for 60 min de-

creased IgE binding. Beyer and others (2001) reported that the main allergen in peanuts (Ara h 1), which shares sequence homology with P34, was reduced in fried and boiled preparations of peanut products and consequently resulted in a significant reduction of IgE-binding intensity. However, for some proteins, IgE reactivity may increase (that is, appearance of new epitopes or unmasking of existing epitopes) subsequent to processing, which suggests a potential increase in allergenicity (Thomas and others 2007).

In general, food allergens are resistant to thermal processing and digestion (Mills and others 2004). If a processing method can denature the allergenic protein's secondary structure by heat treatment or otherwise, it is possible that the conformational epitopes would not be recognized by IgE antibodies *in vivo*, thus reducing the immunoreactivity of the protein. Thomas and others (2007) concluded that food processing may impact the allergenicity potential of proteins; however, there are no general rules regarding how different allergenic foods respond to physical (that is, thermal, mechanical), chemical, or biochemical processing methods. For some, the epitopes are destroyed, but for others they are unaltered. Furthermore, it has been reported that loss of secondary structure at 55 to 70 °C, cleavage of disulfide bonds at 70 to 80 °C, formation of new intra- or inter molecular interactions, rearrangements of disulfide bonds at 80 to 90 °C, and the formation of aggregates at 90 to 100 °C follow destruction of tertiary structure. These modifications eventually result in loss of organized structure and denatured protein (Wal 2003). However, P34 has been reported to be highly heat stable. In fact, autoclaving the protein has been shown to increase antigenicity with human sera (Yamanashi and others 1995). It is also not known how a change in structure affects linear epitopes of the allergen. For example, epitopes from casein, another immunodominant allergen in cows' milk, that are previously hidden can be unraveled by processing (Lehrer and others 2002; Fiocchi and

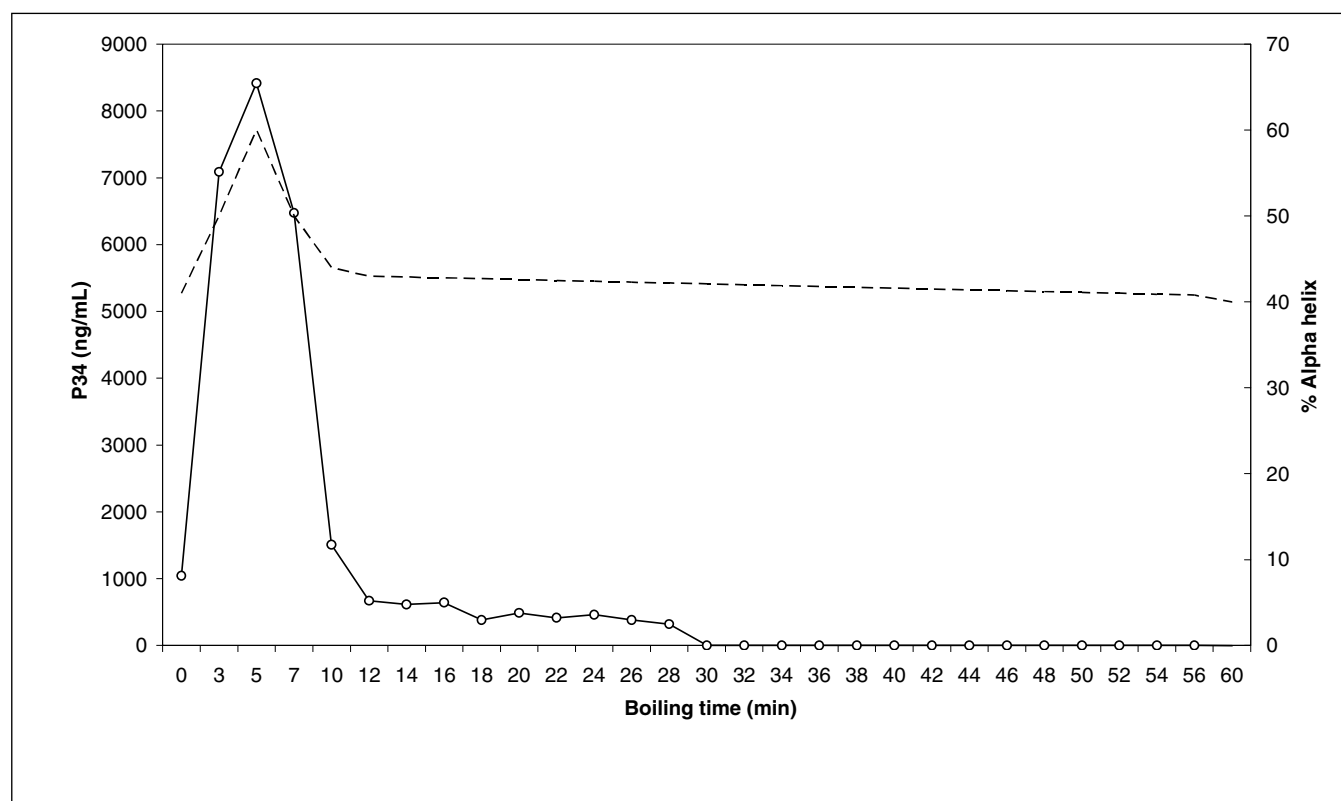


Figure 3 – Effect of heat treatment (100 °C) during different time periods on P34 antigenicity and secondary structure (α -helix).

others 2004). It is important to note that P34 has 12 identified linear epitopes (Hosoyama and others 1996); for this reason, even after denaturation due to thermal treatment, antibodies may recognize and bind to remaining antigenic sites. It seems that changes in secondary structure acted to shield (as in the samples boiled for > 30 min) or expose (as in the sample boiled for 5 min) the linear epitopes on the P34 antigen, changing the way the antigen is recognized by the antibody.

Quantification of P34 in soybean cultivars

Table 2 presents P34 content determined by ELISA in 138 soybean cultivars from a diverse germplasm collection from 10 different countries. The results indicated that allergen P34 was present in all soybean accessions studied and its concentration was highly variable. Soybean genotypes with the highest concentration of P34 were ancestral (12 mg/g defatted flour) followed by modern group (10 mg/g defatted flour) and exotic accessions (8 mg/g defatted flour). However, statistical analysis among different groups showed nonstatistical differences ($P \geq 0.05$). The cultivar of particular interest due to its lowest P34 concentration was the modern accession PI548657 grown in Jackson (US) (2.32 mg/g defatted flour). SDS-PAGE electrophoretic profile of 4 low-expressing P34 soybean cultivars is presented in Figure 4. The allergen P34 was readily detectable at 34 kDa molecular weight and no differences in the total protein profile among P34 low-expressing soybean cultivars were observed.

Our results show that diversity for P34 concentration does exist within commercial soybean cultivars, which agrees with previous literature findings. However, quantitative analysis of allergen P34 in soybean genotypes was not available in previous studies. Us-

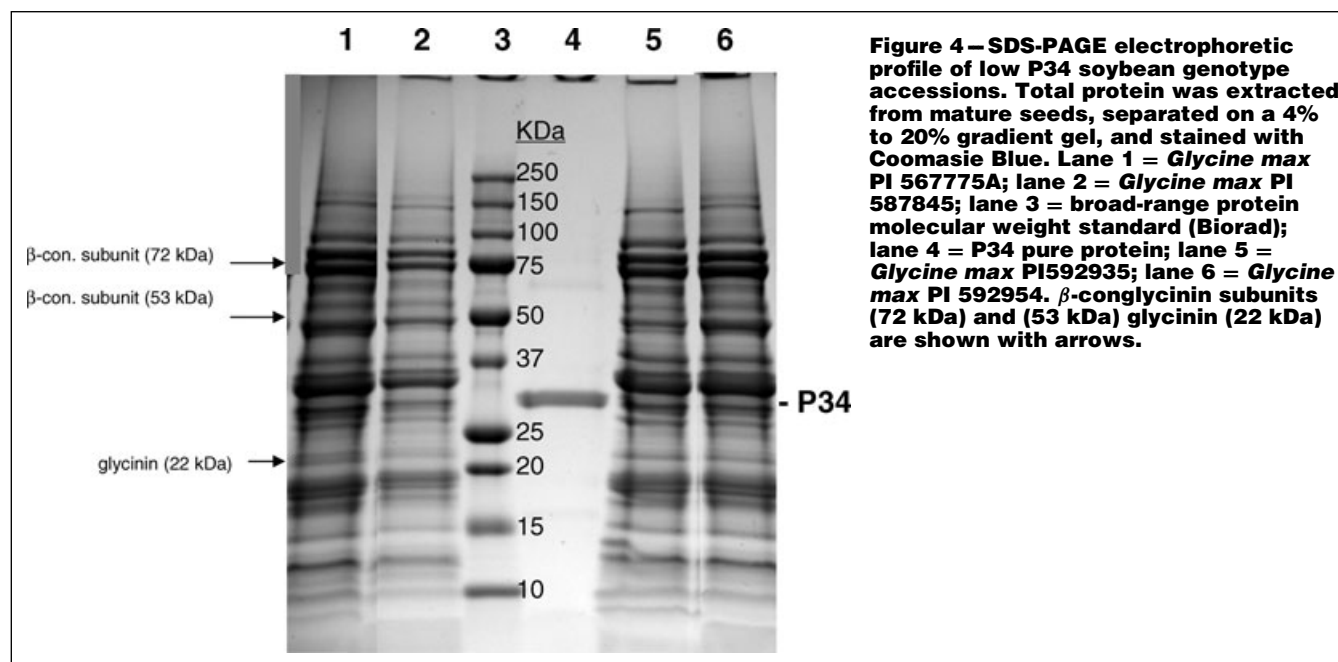
ing a monoclonal antibody Yaklich and others (1999) found that P34 was almost uniformly distributed in the genetic diversity of soybean and naturally occurring varieties lacking P34 allergen protein were not found when U.S. cultivars released between 1947 and 1988 were screened. A similar study of Japanese lines did not reveal any P34 null lines (Ogawa and others 2000). Xu and others (2007) also showed that P34 was present in 16 soybean genotypes examined by 2D SDS-PAGE electrophoresis and MS-spectrometry analysis. However, P34 spots showed weak intensity in the wild genotype compared to that in the landrace genotype. A bigger study screening 16266 accessions of USDA soybean germplasm identified only 2 P34 null lines (Joseph and others 2006) containing only a very small amount of P34 protein shown in the 2D IEF/SDS-PAGE immunoblots. Joseph and others (2006) stated that reduction of P34 could be due as a mutation likely that only affects the production of the P34 protein rather than a mutation in a transcription factor that caused massive expression differences in the seed protein profile. That mutation affected to 4 amino acid residues in the P34 protein sequence, specifically the change from serine to cysteine. Since this amino acid is important in disulfide bond formation, which in turn ensures correct overall 3-dimensional structure, it was postulated that with the addition of a cysteine residue the protein misfolds and hence would be inactive in the soybean seed.

Herman (2005) and Herman and others (2003) previously showed that biotechnology is technically suitable to greatly reduce the endogenous allergenicity. However, regulatory requirements and other factors make it difficult to introduce genetically modified allergen-reduced foods into the market, especially for uses in infant formula or food where both producer and consumers have resisted using any biotechnology-derived components.

Table 2 – P34 concentration in various soybean (*Glycine max* L.) genotypes from the USDA germplasm collection.

Genotype	n	(mg P34/g extracted protein) ^a		(mg P34/g defatted flour, dry basis) ^a	
		Range	Average $\pm$ SD	Range	Average $\pm$ SD
Ancestral	26	5.16 to 24.39	11.64 $\pm$ 6.87	4.71 to 22.28	11.95 $\pm$ 6.27
Modern	15	5.23 to 30.38	9.82 $\pm$ 5.94	4.78 to 27.75	10.37 $\pm$ 5.43
Exotic	97	2.54 to 22.84	7.65 $\pm$ 4.26	2.32 to 20.86	7.92 $\pm$ 3.89

^aData are expressed as mean $\pm$ SD. No statistical difference among genotypes, $P > 0.05$.



Quantification of P34 antigenicity in commercial soy ingredients and products

Table 3 presents the P34 antigenicity found in 51 commercial soy ingredients and consumer products determined by ELISA. Soy flour yielded the highest P34 antigenicity (32 mg/g extracted protein) followed by soy protein isolate (SPI) (29 mg/g extracted protein), although they were not statistically different ($P = 0.26$). Soy protein isolates are produced with a dilute alkali clarification and extraction of protein curd at a pH of approximately 4.5 (Singh and others 2008). This mild processing would not significantly modify P34 antigenicity. However, soy protein concentrates (SPC) exhibited P34 antigenicity of 24 mg/g extracted protein, which was found statistically lower than that for soy flour ($P = 0.00076$). SPC is produced by an extensive processing consisting of aqueous alcohol washing or acid leaching with moist heat (66 to 93 °C) to remove sugars, ash, and other minor components (Singh and others 2008) that may lead to destruction of conformational epitopes of P34 in a small extent.

Commercial soy consumer products exhibited significantly lower P34 antigenicity than soybean flour, probably due to extensive processing. In this group, soymilk presented the highest P34 antigenicity, ranging from 7 to 23 mg/g extracted protein, although P34 content was significantly lower than that found in soy flour. Selective removal of P34 from soymilk and defatted soymilk has been reported by centrifugation into the oil pad layer formed in the presence of reducing agents (Samoto and others 1994; Obata and others 1998). Commercial soymilks ranged in protein concentration from 18% to 50% protein from different providers, containing different ingredients in some cases, which may account for some of the P34 content variability. Tempeh presented P34 antigenicity (8 mg/g extracted protein), statistically lower than that of soy flour ($P = 0.001$). These results agree with those found by other researchers using radioallergosorbent test (RAST) inhibition studies with human sera (Herian and others 1993). Tempeh is produced as a cake of cooked

and fermented soybeans bound together by a mycelium of *Rhizopus oligosporus*. Soy infant formula showed 3.4 mg P34/g extracted protein. The infant formulas currently on the market are all free of cow's milk protein and lactose, and recommended for those infants, and others, who are allergic to milk protein and those who have lactose intolerance or lactase deficiency. P34 antigenicity in soy powder accounted for 2 mg/g extracted protein. This product is a granulated SPC that has been subjected to partial carbohydrate (soluble sugars) and oil removal, and results in a product with minimally 10% moisture and 68% protein. The protein extraction for this product yielded 105 mg protein/g material on average for soy powders, while the protein extracted from SPC on average was 250 mg/g material, almost 3 times as much protein as the soy powders. Consequently, the average P34 antigenicity for SPC is 30 times higher than those for soy powders when expressed as mg P34/g product, which agrees with the results found in our previous study (Gonzalez de Mejia and others 2004). Another explanation may be that soy powders are more extensively processed than traditional concentrates and P34 antigenicity has been reduced to a greater extent. The average value calculated for P34 antigenicity in soy cheese products was as low as 0.50 mg/g extracted protein. Nondairy cheese alternatives can be produced in a number of different ways using SPI, tofu, or soy beverage as the soybean source, and may include milk proteins, various gums, cornstarch, and tapioca to create a cheese-like texture (Singh and others 2008). Mozzarella soy cheese was subjected to lactic acid fermentation, which could contribute to lower P34 antigenicity (0.005 mg/g extracted protein) compared to the other cheese products. It has been reported that fermentation of cows' milk using mesophilic and thermophilic lactic acid bacteria results in a 99% reduction in antigenicity of the protein allergens (Jedrychowski and Wroblewska 1999). Korean miso, soy sauce, soy chili mix, soy nuts, soy cream cheese, soy meat patty, texturized soy protein, and soy cereal exhibited undetectable P34 antigenicity levels (ELISA detection

Table 3 – P34 concentration in selected commercial soy ingredients and products by ELISA.

Ingredients ^A		n ^B	mg P34/g product (d.m.) ^C	mg P34/g extracted protein
Soy ingredients				
Soy flour	Defatted soybean	4	11.00 ± 1.00	32.00 ± 4.00 ^a
Soy protein isolate (SPI)	90% soy protein	2	8.00 ± 0.20	29.00 ± 9.00 ^{a,b}
Soy protein concentrate (SPC)	Acid-leached soy flakes	5	6.00 ± 0.80	24.00 ± 5.00 ^b
Soy products				
Soymilk		4	8.00 ± 3.00	15.00 ± 3.00 ^c
1. Energy drink	Casein, spirulein, SPI	1	20.00 ± 0.01	23.00 ± 4.00
2. Vanilla, liquid	Corn, barley, seaweed, SPI	1	9.00 ± 0.40	19.00 ± 5.00
3. Dry powder	Tofu, corn maltodextrin	1	0.45 ± 0.01	10.00 ± 1.00
4. Plain, liquid	Soymilk	1	3.00 ± 0.01	7.00 ± 0.3
Tempeh	Cooked & fermented soybeans	1	0.30 ± 0.01	8.00 ± 0.20 ^d
Soy infant formula	SPI	4	0.20 ± 0.09	3.40 ± 1.60 ^e
Soy powder	SPC	2	0.20 ± 0.03	2.00 ± 0.50 ^e
Soy cheese		3	0.02 ± 0.01	0.50 ± 0.30 ^f
1. Parmesan	Texturized soy protein	1	0.06 ± 0.01	1.00 ± 0.2
2. Shredded Mozzarella	Lactic acid cultured soymilk	1	0.003 ± 0.010	0.005 ± 0.004
3. Gouda	Casein, SPI, cornstarch	1	0.005 ± 0.003	0.200 ± 0.05
Korean miso	Fermented soybean	1	0.004 ± 0.001	0.002 ± 0.000 ^g
Soy sauce	Fermented soybeans	2	ND ^D	ND ^h
Soy and rice granules	Treated soy flour	1	ND	ND ^h
Soy chili mix	SPC	1	ND	ND ^h
Soy nuts	Roasted soybeans, HSP ^E	1	ND	ND ^h
Soy cream cheese	SPI	1	ND	ND ^h
Tofu	SPC, HSP	1	ND	ND ^h
Texturized soy protein	Extruded soy protein	10	ND	ND ^h
Soy cereal	18% soy protein	1	ND	ND ^h

Data are expressed as mean ± SD of at least a triplicate analysis. Different superscript letters in the column indicated statistical significance ($P < 0.05$).

^A Ingredient information was taken from the product's label; ^B n = different commercial brands of the same type of product; ^C d.m. = dry matter; ^D ND = not detected. Limit of detection was 0.45 ng allergen. ^E HSP = hydrolyzed soy protein.

limit = 0.45 ng antigen). Like tempeh, miso and soy sauce are produced by fermentation, a process that has been investigated with regard to its potential on production of hypoallergenic products (Yamanashi and others 1995; Frias and others 2008; Song and others 2008). Hydrolysis of soy proteins into peptides by microbial enzymes may undergo destruction of both conformational and linear epitopes, consequently reducing or eliminating the antigenicity of soy proteins (Frias and others 2008; Song and others 2008). Soybean miso is traditionally produced by adding koji (from various strains of mold, yeast, or lactic acid bacteria) to soybeans to create a fermented soybean paste, generally used as soup bases throughout Asia (Golbitz 1995). Pressing the liquid out of the miso produces soy sauce. Tsuji and others (1995, 1997) reported undetectable levels in miso using sandwich ELISA. Another study determined the RAST inhibition of miso and soy sauce to be far lower than their nonfermented counterparts (Herian and others 1993). Fermentation conditions such as particule size, microorganism used, and extent of fermentation may have different impacts on reduction of soybean immunoreactivity (Frias and others 2008; Song and others 2008). Also, the possible inclusion of cooked, nonfermented soybeans could explain the higher P34 immunoreactivity of tempeh compared to miso and soy sauce. Texturized soy protein (TVP) is produced by extruders that manipulate soy ingredients using pressure and temperature to make them flow, expand, and then collapse to produce a meat-like extender (Lusas and Riaz 1996; Singh and others 2008). The ELISA values suggest that combinations of thermal and mechanical pressure processes are responsible for destruction of conformational and linear P34 epitopes, which underwent a negligible P34 antigenicity.

Processing conditions, variability of the allergen composition in the food, structural and inherent stability of the allergen, food matrix, or the multiplicity of conformational/linear epitopes on a given allergen can all influence immune responses to individual allergens and may impact the overall allergenicity of the food (Thomas and others 2007).

Conclusions

Heating of P34 first increased antigenicity and then decreased it substantially to nondetectable levels. Thermal processing underwent changes in secondary structure of P34, increasing its antigenicity after 5 min of boiling, through exposure of linear epitopes hidden in the native secondary structure. Further heating time completely decreased P34 immunoreactivity. P34 concentration varied according to soybean genotype class. There were not statistically different concentrations of P34 among cultivars. However, the cultivar that emerged from the screens of particular interest was P34 lowest-expressing accession PI548657. This naturally occurring accession can be crossed into other germplasm to produce elite or stacked trait lines that can be incorporated into products needed by consumers without any biotechnology-derived component. The method of soybean processing employed has a dramatic effect on the remaining immunoreactivity of P34. The results of this study suggested that soybean processing, particularly fermentation and hydrolysis, can have a dramatic effect on the subsequent antigenicity of the soybean product. Elimination of P34 by selecting soybean varieties with low levels of this allergen, or via processing, could potentially enhance the safe use of soybean products, making them available to soy-sensitive individuals. Other allergenic proteins in soybean need to be studied to optimize the effect of processing and thus provide the consumer with low allergenic soy products.

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