

Quantification of Human IgE Immunoreactive Soybean Proteins in Commercial Soy Ingredients and Products

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ABSTRACT: The objective of this study was the detection and quantification of human IgE immunoreactive soybean proteins in commercially available soy ingredients and products. Optimum dilutions of primary antibody and antigens as well as detection sensitivity were determined for the implementation of a sandwich ELISA method using plasma from soy sensitive subjects (IgE ranging from 0.35 to 98.7 IU/mL). Human IgE immunoreactivity of commercial soybean ingredients showed that the plasma of subjects with strong allergic reaction to soybean presented proportionally higher immunoreactive response. Soy protein isolate and soy protein concentrate contained less immunoreactive proteins than soy flour and grits. As expected, a hypoallergenic soybean product presented the lowest IgE immunoreactivity. Hydrolyzed and fermented soy ingredients showed negligible human IgE immunoreactivity when proteins and peptides were < 20 kDa. The IgE immunoreactivity of soymilk samples ranged from 3.4 to 68.9 ng IgE/mg extracted protein. Tofu contained about 20-fold higher IgE immunoreactivity than soymilk products (median 171 ng IgE/mg extracted protein). Furthermore, soy cheese products presented twice the IgE immunoreactivity than tofu products (median 359 ng IgE/mg protein). Meat analogues presented considerably high extracted protein concentration (median 67.9 mg/g product). The findings of the current investigation demonstrate sandwich ELISA as a reliable immunochemical method with good repeatability, sensitivity, and low detection limit to quantify IgE immunoreactive proteins in soy ingredients and products. Quantitative measurement of specific IgE is likely to become an increasingly valuable tool for soybean industry to comply with food labeling for manufacturers, thus protecting soy-sensitive consumers.

Keywords: allergen quantification, immunoreactivity, sandwich ELISA, soy ingredients, soybean products

Introduction

Soybean (*Glycine max* L.) is the world's foremost source of protein and oil for human food and animal feed purposes (Clarke and Wiseman 2000). In 2006, soybean production represented 39% in the United States, 23% in Brazil, 18% in Argentina, and 7% in China, which accounted for 88.5% of the global soybean production (FAO 2006). Yet, the United States is one of the largest soybean producers in the world (L'Hocine and Boye 2007). Soybean acceptance has increased due to the low cost, nutritional quality, technological properties, and health benefits of soybean protein products (Friedman and Brandon 2001; L'Hocine and Boye 2007; Singh and others 2008). However, one of the difficult problems limiting the expanded use of soy protein products is the presence of allergenic proteins. The Food Allergen Labeling and Consumer Protection (FALCP) Act of 2004 includes soy in its definition of the "big 8" (FAO 2004), which comprises those foods that cause the 90% of all food allergies (Taylor and Hefle 2000; Kabourek and Taylor 2003; Cordle 2004).

Food allergy is an immunological, usually immunoglobulin E (IgE)-related reaction resulting from the exposure (ingestion, skin contact, or inhalation) to exogenous food macromolecules (generally proteins) known as allergens or antigens (Mills and others 2003; Beyer and Teuber 2004). Although most food allergies cause

relatively mild and minor symptoms, some food allergies can cause severe reactions and may even be life-threatening (US FDA 2007). The estimated prevalence of soy allergies is about 0.5% of the total U.S. population (Stephan and others 2004; Sicherer and Sampson 2006). Clinical manifestations of soy allergies can provoke digestive disorders, respiratory (rhinitis, asthma), skin reactions (urticaria, atopic eczema), and IgE-mediated systemic multisystem reactions (Batista and others 2007). A recent clinical multicenter study on soy allergy in Europe determined the threshold levels for clinical reactivity to soy, predicting that 1% of subjects with soy allergy would react subjectively and objectively with 0.21 and 37.2 mg of soy protein, respectively (Ballmer-Weber and others 2007).

Soy allergy has become a public health problem that continues to challenge both the public community and the food industry, because soybeans are widely used in processed foods and, therefore, represent a source of hidden allergens. Prophylactic treatments are still under investigation, but currently there is not a cure for food allergies caused by soy-based food products (Besler and others 2001). Currently, the only possible solution to prevent allergenic reactions is the strict avoidance of the offending allergen containing food (U.S. FDA 2007). In the United States, on March 2004, the Food Allergen Labeling and Consumer Protection Act (FALCPA) amended the Federal Food, Drug, and Cosmetic Act (FFDCA) and required that the label of a food product that is or contains an ingredient that bears or contains a "major food allergen" declare the presence of the allergens specified by FALCPA (Ben Rejeb and others 2004; US FDA 2007). This labeling law is effective since January 1, 2006. All ingredients derived or containing the 8 major food allergens require

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declaration of specific allergens in food labels (US FDA 2007). It is important to facilitate compliance with food labeling for manufacturers and also to protect soy-sensitive consumers of serious or fatal outcome by soy allergy. Therefore, this study has been focused on the detection and quantification of the major soy proteins in com-

mercially available soybean ingredients and products with the use of an implemented sandwich ELISA immunochemical method using plasma from soy-sensitive subjects.

Material and Methods

Table 1—Description of nonfermented and fermented soybean protein ingredients.

Soybean ingredients	Producer/provider
Nonfermented soybean ingredients	
Defatted soy flour—Baker's soy	Archer Daniels Midland
Defatted soy flour—toasted soy	Archer Daniels Midland
Defatted soy grits 20 to 80	Archer Daniels Midland
Defatted soybean meal 48% (feed grade)	Archer Daniels Midland
Soy protein concentrate	Archer Daniels Midland
Isolated soy protein PRO-FAM 646	Archer Daniels Midland
Isolated soy protein PRO-FAM 930	Archer Daniels Midland
Soycomil-P 65% (hypoallergenic feed grade)	Archer Daniels Midland
Ardex AF 82% (hypoallergenic feed grade)	Archer Daniels Midland
Soy beginnings—low fat soy flour	Thumb Oilseeds
Soy beginnings grits 8 to 18+ low fat soy flour	Thumb Oilseeds
Soy beginnings grits 18+ low fat soy flour	Thumb Oilseeds
Defatted soybean meal 48% (feed grade)	Thumb Oilseeds
Defatted soybean meal 48% (feed grade)	Univ. of Illinois Feed Mill Plant
20% Pulmotyl formula	Univ. of Illinois Feed Mill Plant
Fermented soybean ingredients	
Fermented PepSoyGen (feed grade)	SAI Intl.
Fermented soy protein concentrate (feed grade)	SAI Intl.
Hydrolysed soybean ingredients	
Hydrolyzed defatted soy flour	SAI Intl.
Hydrolyzed defatted soy concentrate	SAI Intl.
Hydrolyzed defatted soy protein isolate	SAI Intl.
Hydrolyzed defatted soy concentrate peptides	SAI Intl.
Predibaseats, soy peptides, and other ingredients	SAI Intl.

Table 2—Commercially available soybean products.

Soy product	Brand	Ingredients
Milk		
Soymilk	Yeo's	Whole soybean
Soymilk	Organic Valley	Organic whole soybean
Soy Dream	Imagine Foods	Organic whole soybean, rice syrup
SilkSoy	Silk	Whole organic soybean
Organic soymilk	Eden	Organic soybean, naturally malted organic wheat and barley extract, kombu seaweed
Soy powder—Original	American Natural & Specialty Brands	Tofu
Tofu		
Organic baked tofu	Soyhealth-Soy Deli	Nigari tofu (whole organic soybean), tamari (soybean, wheat)
Tofu cutlet Atsu—age	House Foods American Corp.	Whole organic soybean
Firm organic tofu—Nigari	Soyhealth-Soy Deli	Whole organic soybean
Cheese		
Vegan	Galaxy Nutritional Foods	GMO-free isolated soy protein, soymilk solids, nonhydrogenated soybean oil
Cream	Tofutti	Expeller processed natural oil blend (soybean, palm fruit, olive), soy protein, tofu
Tofutti	Tofutti	Partially hydrogenated soybean oil, tofu, soy protein
Soysation shredded	Lisanatti	Crushed organic soybean, casein, rice flour
Meat analogues		
The Good Ground Veggie	Yves	Textured soy protein, textured wheat protein
Veggie sausage		Textured soy protein

Commercial soy ingredients and products

Soybean ingredients were provided by Archer Daniels Midland Co. (ADM, Decatur, Ill., U.S.A.), Thumb Oilseeds producers (Uby, Mich., U.S.A.), SAI Intl. Co. (Geneva, Ill., U.S.A.), and Univ. of Illinois Feed Mill plant (Savoy, Ill., U.S.A.) (Table 1). Commercially available soybean products ($n = 15$) were purchased from a local market (Urbana, Ill., U.S.A.) (Table 2).

Human plasma

Plasmas were obtained from several donors selected on the basis of clinical soy allergy (Table 3). Specific IgE levels were measured by the ImmunoCAP IgE method (PlasmaLab Intl., Everett, Wash., U.S.A.) ranging from 0.35 to 98.7 IU/mL. The standard 75/502 pooled plasma IgE from 9 donors suffering from allergic disorders was obtained from the World Health Organization (WHO). This pool of lyophilized plasma was collected by Professor J. Pepys, at the Brompton Hospital, London, U.K.

Protein extraction from soybean commercial products

Protein extraction was performed by the procedure described by de Mejia and others (2004) consisting of placing 50 mg of the sample and 1 mL of 0.05 M Tris-HCl buffer, pH 8.2 (Sigma-Aldrich, Saint Louis, Mo., U.S.A.) in an Eppendorf tube. After the extract was mixed, the samples were placed in an ultrasonic bath (Bransonic model 2510, Branson Ultrasonic Corp., 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 by using a recirculating bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, N.H., U.S.A.). Following extraction, the samples were centrifuged at 20000 × g for 30 min at 4 °C in an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, N.Y., U.S.A.) and the obtained supernatant was transferred to a new Eppendorf tube. This material was then diluted (1:2) with extracting buffer (0.05 M Tris-HCl buffer, pH 8.2) for ELISA analysis.

Total soluble protein quantification

Soluble protein was quantified by the DCTM protein assay kit (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) for microplates. The reaction fundamentals are based on the Lowry assay and a microassay procedure was developed by adding 25 μ L of reagent A, followed by 5 μ L of either protein extract or bovine serum albumin (Sigma-Aldrich) for standard curve performed in the range from 10 to 1600 μ g/mL ($y = 0.186x + 0.006$, $R^2 = 0.99$). Once the protein extract was added, immediate mixing was required. Then, 200 μ L of reagent B were added, followed by 15 min incubation at room temperature. Microplates were read at 690 nm. Total soluble protein concentrations of the soybean extracts were quantified based on the standard curve. Liquid samples such as soymilk and yogurt were only diluted as indicated by Franck and others (2002) and analyzed for protein concentration.

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

SDS-PAGE electrophoresis was carried out in a Phastsystem Kit (Amersham-Pharmacia Inc., N.J., U.S.A.) with an 8% to 25% polyacrylamide gradient gel. Extracted samples were centrifuged (20000 $\times g$) to eliminate any precipitate that might have formed during freezing or thawing. The supernatant (20 μ L) was added to 20 μ L of Laemmli sample buffer (Bio-Rad Laboratories) with 5% β -mercaptoethanol (Sigma-Aldrich). Samples were boiled for 5 min. The samples and molecular weight standard were cooled to room temperature and 1.5 μ g of soybean protein per well were loaded into PhastSystem ready gels (Amersham-Pharmacia Inc.). A broad-range protein standard (Bio-Rad Kaledoscope prestained) containing pure recombinant proteins of the following molecular weights, 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa, were used. The gels were run at 55 mA constant for 20 min (60 to 125 V), fixed for 20 min in methanol/acetic acid/water (5:5:2), and stained with Coomassie Brilliant G-250 for 1 h. Then, gels were destained with 2 washes of 5 min with acetic acid/water (40%) and finally washed once again with deionized water. They were read in a Kodak Image Station 440 CF (Kodak, New Haven, Ct., U.S.A.), where their respective molecular weights and band intensities were recorded.

Western blotting

Unstained gels were soaked in 20 mL of blotting buffer pH 8.3, which consisted of 10% (v/v) methanol, 25 mM Tris base, 192 mM glycine, for 3 min. A western sandwich was assembled by placing a sponge, a filter and the gel, the Immun-Blot PVDF membrane (Bio-radTM), another filter, and sponge, avoiding the formation of bubbles. After the transfer was completed, the membrane was then sat-

urated by incubation in 5% nonfat dry milk (NFDm) in 0.01% Tween 20 (Sigma-Aldrich) in Tris-buffered saline (TTBS) buffer (Sigma-Aldrich) for 1 h, followed by overnight incubation at 4 °C in a 1:5 dilution of the soybean positive plasma from soy-sensitized subjects (PlasmaLab Intl.) in 3% BSA, 0.01% Tween 20-TBS (TTBS) buffer. The membrane was washed 3 times in TTBS buffer and was then incubated for 1 h at room temperature at 1:1000 dilution with goat antihuman IgE alkaline phosphatase (Bethyl Lab Inc., Tex., U.S.A.) prepared in 3% NFDm in TTBS buffer. The membrane was washed 3 times for 5 min with 0.01% TTBS buffer and prepared for detection using an Immun-Star Chemiluminescence Alkaline Phosphatase substrate solution (Biorad) following manufacturer's recommendations. After adding the substrate to the blot, the intensity of the bands was read in a Kodak Image Station 440 CF with a total exposure time of 2 min and 8 captures.

Optimization of the immunological method by sandwich ELISA

The sandwich ELISA measures the amount of antigen between 2 layers of antibodies, and the rate of color formation is proportional to the amount of antigen. To determine the optimum dilution for the primary antibody (98.7 IU/mL) composed of soy-allergic subject's plasma (PlasmaLabs Intl.), a titration curve was created by serially diluting the primary antibody. From this curve, the titer selected was the point located in the half maximum optical density (OD_{405nm}) value corresponding to the linear portion of the curve for soybean-positive plasma sample (98.7 IU/mL) against a representative soy protein isolate sample (ADM) used as antigen to determine the right antibody dilution with low variability (CV), a good working range of OD_{405nm}, and a good repeatability. A similar procedure was followed for different human plasma samples to determine the right dilutions for all antibodies used. The negative plasma (< 0.35 IU/mL) was used as a control.

Furthermore, following the determination of the antibody titer, the optimum dilutions of the antigens were evaluated. From a pre-screening of several soybean ingredients, a soybean meal ingredient (ADM-Bakers defatted soy flour) was selected as a representative protein extract to establish antigen dilution. A titration plot was prepared by making serial dilutions of the protein extract while 2 different titers for the primary antibody were used (1:5 and 1:10). Finally, the previously determined antibody/antigen titer was confirmed.

A 5% bovine serum albumin (BSA) was used for the blocking step in order to avoid interferences and nonspecific binding without altering the assay accuracy. Also, 2 controls, empty well and sample buffer (0.05M Tris-HCl buffer, pH 8.2), were included. The use of an automatic washer was kept at the slowest rate to avoid loss of soybean protein, and a 3% BSA was used for antibody dilutions. Microtiter plates NUNC-ImmunoTM MaxisorpTM (Nalge Nunc Intl., Rochester, N.Y., U.S.A.) were used to increase adsorbing affinity during the binding of the protein into the plate's surface.

Sandwich-type ELISA

A sandwich-type, enzyme-linked immunosorbent assay (ELISA) was developed for the detection of selected soy proteins in soy protein ingredients and products by adapting existing methodologies described by Hefle and others (1994) and Taylor (2006). Plasma from soybean sensitive subjects (Plasmalab Intl.) was used as the capture antibody. Plasma from a not soy-sensitized individual was used as a negative control. Microtiter plates were coated with 100 μ L of the human anti-soy IgE (1:5) diluted in PBST antibody buffer [1% Tween-20; 3% BSA; 1 tablet TBS in 15 mL total

Table 3—Characteristics of human plasma used in immunological experiments.^a

Plasma	Age	Gender	Soybean specific IgE by ImmunoCAP analysis (IU/mL) ^b
A	36	m	98.7
B	36	m	71.6
C	42	f	34.2
D	48	f	11.3
E	59	m	< 0.35
F			5000

^aDonors were selected on the basis of clinical allergy to soybean and *in vitro* IgE evaluation. A to D = subjects reporting soybean allergies; E = non atopic subject; F = WHO Standard 75/502 pooled plasma IgE obtained from the World Health Organization. Professor J. Pepys, Brompton Hospital, London collected lyophilized pooled sera from 9 donors, from subjects suffering with allergic disorders.

^bThe presence of soybean-specific IgE was measured by immunoCAP analysis (PlasmaLab Intl.).

volume corresponding to 0.05 M Tris HCl buffer, pH 7.6 ± 0.2 (25 °C) with 0.15 M NaCl] onto the Maxisorp™ immunoassay plate for 14 h at 4 °C overnight. The unoccupied space on the 96-well immunoassay microplate was blocked for 1 h at room temperature with a solution containing 5% BSA, 1% Tween-20, 1 tablet of TBS in 15 mL of deionized water corresponding to 0.05 M Tris HCl buffer, pH 7.6 ± 0.2 (25 °C) with 0.15 M NaCl (300 μ L/well). Between each step in the procedure, the plates were washed 4 times in PBST buffer at pH 7.4 (same procedure for each washing step) using a Biotek® Instruments Elx50 autostrip washer (Winooksi, Va., U.S.A.). One-hundred milliliters of the soy protein samples (extract, standard, or blank) were added onto the plate at various dilutions in triplicate and incubated for 1 h at 37 °C, followed by 1 h incubation with 100 μ L of the human anti-soy IgE (1:5) diluted in PBST buffer [1% Tween-20; 3% BSA; 1 tablet TBS in 15 mL total volume corresponding to 0.05 M Tris HCl buffer, pH 7.6 ± 0.2 (25 °C) with 0.15 M NaCl]. Hundred microliters per well of goat antihuman IgE alkaline phosphatase (Bethyl Labs Inc.) were added, and allowed to incubate in the plate for 1 h at room temperature. Goat antihuman

IgE alkaline phosphatase was detected by addition of 100 μ L of color reagent p-nitrophenyl phosphate (Sigma-Aldrich) at room temperature that was added to each well containing the immuno-complexes for 45 min at room temperature. The plate was read with an automatic reader Elx80810 ultra microplate reader (Biotek Instruments) at 405 nm using K.C. Junior™ computer program, exported and analyzed in Microsoft Excel™. The concentration of the allergenic proteins in each sample was calculated using the average optical density at 405 nm, based on triplicate samples and multiple dilutions.

To refer the readings obtained by ELISA as equivalent nanograms of IgE per milliliter, a titration curve was constructed by performing serial dilutions of the antibody based on the plasma potency provided by ImmunoCAP (PlasmaLabs Intl.) of 98.7 IU/mL. The IU units were transformed into nanograms of IgE per milliliter by considering the conversion factor 2.42 ng/mL of pure IgE per international units. Then, a standard curve was built considering the OD_{405nm} readings with the nanograms of IgE per milliliter obtained from human plasma samples.

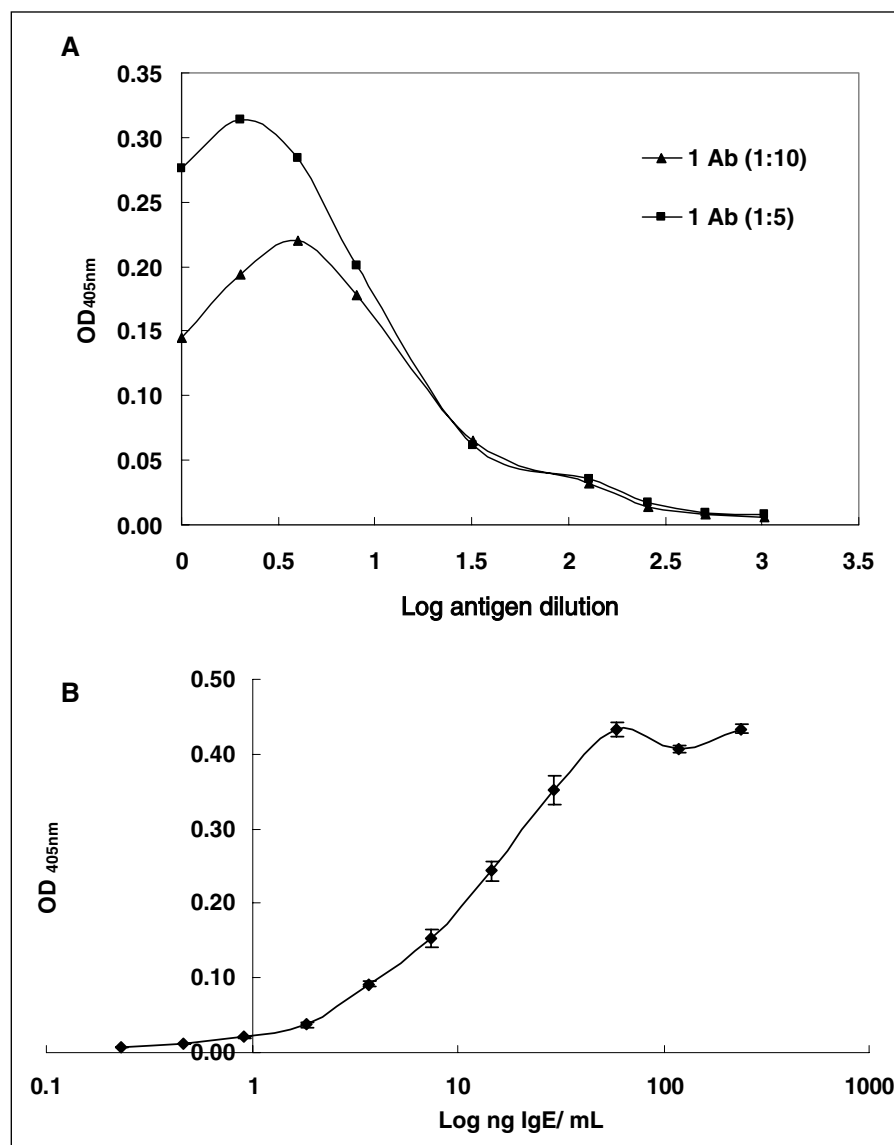


Figure 1 – (A) Titration curve of soybean protein extract with 2 dilutions of IgE (1:5 and 1:10) from plasma A (98.7 IU/mL) of soybean sensitive subject. (B) Standard curve for conversion into ng IgE/mL. Goat antihuman IgE alkaline phosphatase was used as secondary antibody and final detection with p-nitrophenyl phosphate was done. Error bars indicate standard deviation.

Statistical analysis

All experiments were done at least in duplicate. Data are reported as mean $\pm$ standard error. The data were analyzed for statistical significance ($P < 0.05$) using 1-way ANOVA procedures and means were generated and adjusted with LSD's post test provided by SAS statistical program (SAS for Windows XP, SAS Inst. Inc., version 9.1, Cary, N.C., U.S.A.).

Results and Discussion

Immunoassay performance

Figure 1 shows the results of ELISA in the presence of soy protein extract (antigen) at 2 different plasma dilutions (A) and the standard curve for conversion of OD_{405nm} values into nanogram of IgE per milliliter (B). Figure 1A shows that the primary antibody dilution factor of 5 presents higher OD_{405nm} by sandwich ELISA. The protein extract dilution (1:2) shows a higher OD_{405nm} than direct samples of the protein extract, probably due to a better antibody/antigen interaction. Therefore, the optimum titer determined for the human plasma from individuals sensitive to soybean (primary antibody) and the antigen were 5 and 2, respectively. Figure 1B shows the standard curve for conversion into nanograms of IgE per milliliter. The linear range from 3 to 90 ng IgE/mL, expressed in logarithmic units, was used to build the standard curve ($y = 0.011x + 0.039$; $R^2 = 0.95$) to relate OD_{405nm} values with immunoreactivity expressed as ng IgE/mL. The detection limit (10.79 μ g of IgE binding soy protein) was obtained as the concentration of soy protein corresponding to the mean OD_{405nm} of the negative control plus the standard deviation. These optimized conditions were subsequently used to evaluate the efficacy of the sandwich ELISA methodology in detecting allergenic proteins in the products tested.

Detection and quantification of IgE immunoreactive proteins in nonfermented, fermented, and hydrolyzed soybean ingredients

SDS-PAGE electrophoresis showed that, except for the glycinin basic 1 subunit (20 kDa), the rest of the immunodominant proteins were present with similar intensities in all samples, excluding Ardex and Soycomil-P (Figure 2). Taking into account the processing flow of soybean, soy protein isolate (SPI) is obtained from protein extraction and precipitation and soy protein concentrate (SPC) after carbohydrate removal (Singh and others 2008). Because not all the initial materials can be 100% recovered in each of the processing steps, some proteins get lost, damaged, or modified into new proteins and peptides throughout these processing steps. This may be the reason for the less intense glycinin basic subunit (20 kDa) in both SPI and SPC ingredients. Soy flour samples and soy grits presented a similar protein profile with α - and β -subunits of β -conglycinin (72 and 52 kDa, respectively), intensely identified soybean vacuolar protein P34 (34 kDa), and acidic and basic subunits of glycinin (33 and 20 kDa, respectively) (Figure 2). One of the differences between these 2 ingredients is the particle size. Soy flours are prepared by grinding soybean flakes to 100 mesh or finer, whereas soy grits are coarser than 100 mesh (Singh and others 2008). The soybean meal samples presented similar protein profile as soybean flours. The α and β subunits of β -conglycinin, P34, and acidic and basic glycinin were also identified. For the 20% pulmotyl formula, the immunodominant soybean proteins could be identified in a less intense manner due to the blending with other protein sources such as fish protein, and corn. Interestingly, the 2 hypoallergenic ingredients, 82% Ardex isolate and 65% Soycomil-P, presented distinct protein profiles. P34 was less intensified in Soycomil-P ingredient showing protein bands less than 40 kDa in size. Furthermore, the 82% Ardex isolate ingredient was still showing the α and β subunits of β -conglycinin in a less intensified manner than the soybean meal samples.

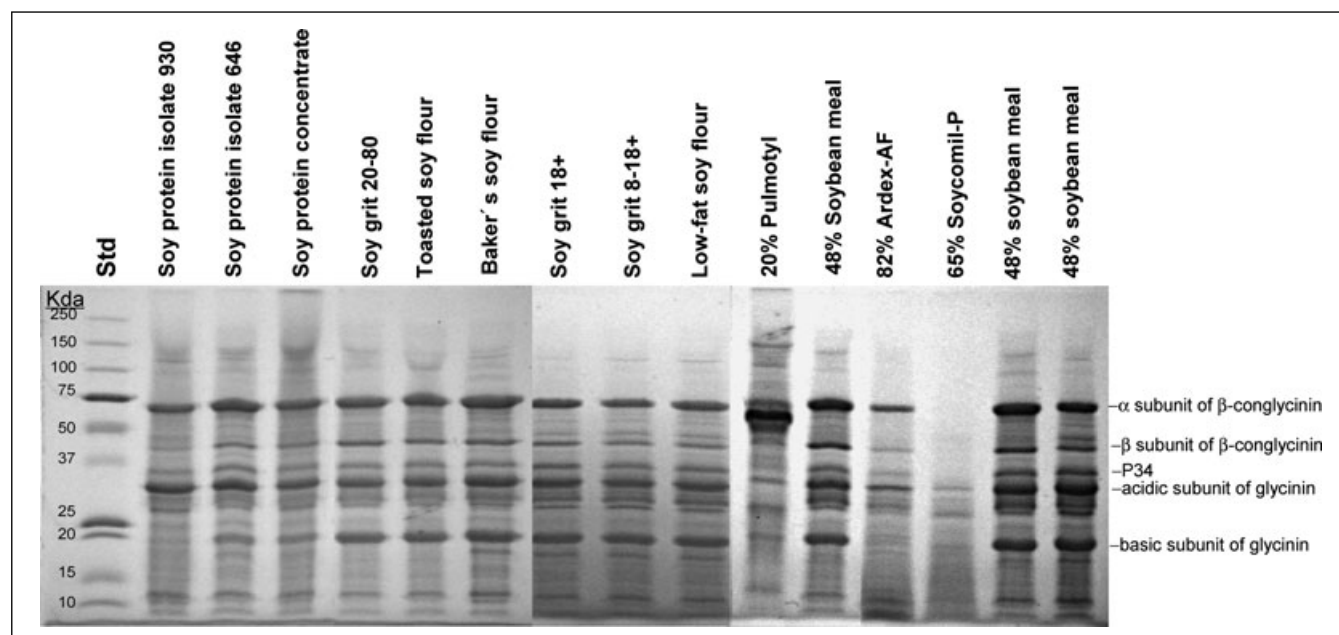


Figure 2—SDS-PAGE with 8% to 25% linear acrylamide gradient electrophoretic profile of proteins presented in different commercial soy ingredients. The following proteins have been identified: α -subunit of β -conglycinin (72 kDa); β -subunit of β -conglycinin (53 kDa); soybean vacuolar protein P34 (34 kDa); acidic subunit of glycinin (33 kDa); and basic subunit of glycinin (20 kDa).

Table 4 shows the soluble protein concentration expressed as milligrams of extracted protein per gram of dry product. Extracted proteins ranged from 360.4 ± 34.8 (82% Ardex isolate) to 14.9 ± 2.9 (Soycomyl-P hypoallergenic product) mg/g product. The degree of processing and particle size had an effect during the protein extraction. These values were used to express the results of nanogram IgE immunoreactive proteins per milligram of extracted protein.

Figure 3 shows the IgE immunoreactivity measured by sandwich ELISA with diverse potency of human plasma. *In vitro* IgE immunoreactivity of soy ingredients was tested by performing a sandwich ELISA with human plasma from 4 officially diagnosed allergic subjects to soybean. Each individual had a different sensitivity to soybean, with plasma IgE levels ranging from 98.7 IU to < 0.35 IU/mL. As expected, the plasma sample belonging to subjects who had a stronger allergic reaction to soybean presented proportionally higher absorbances at 405 nm (OD_{405nm}). The higher the potency of the human plasma, the higher the response observed ($y = 0.0019x - 0.013$, $R^2 = 0.61$). For instance, the 98.7 IU/mL plasma showed higher readings, whereas plasma 0.35 IU/mL presented lower IgE immunoreactivity or even zero. A significant difference was observed among the immunoreactivity within different intensities of human plasma samples ($P < 0.0001$). Plasma A (98.7 IU/mL) was statistically different from plasma C (34.2 IU/mL), D (11.6 IU/mL), and F (< 0.35 IU/mL), whereas plasma D (11.6 IU/mL) and plasma F (0.35 IU/mL) were not.

Figure 3A shows that soy protein isolate and soy protein concentrate contain less immunoreactive proteins than soy flour and soy grits products. These results agree with the electrophoretic profiles for these products presented in Figure 2. The high OD_{405nm} values present in soybean meal by the sandwich ELISA technique (Figure 3B) might be affected by the fact that this ingredient is an initial by-product of the oil extraction and it is used mainly as an important source of high protein feed ingredient. Pulmotyl is a formula containing 20% of soybean meal, mixed with other nutritious ingredients to fulfill the animal's nutritional requirements. Some diluting effect is produced by other sources of protein contained in this product, and therefore, the immunoreactive soy proteins were present with less intensity (Figure 3B). As expected, the hypoallergenic soybean ingredient Ardex AF presented the lowest absorbance (Figure 3B) even though it had the high-

est extracted protein (360.4 mg/g). This may be related to the way Ardex AF was processed to improve removal of immunoreactive soy proteins.

Figure 4 presents the immunoreactivity obtained by ELISA in soybean fermented and hydrolyzed ingredients. These diverse soybean ingredients, subjected to different processing conditions, were reactive to soybean positive human plasmas A, pooled, and B. Interestingly, partially fermented PepSoyGen presented the highest IgE immunoreactivity against all plasma samples probably due to the high molecular weight proteins still present. PepSoyGen is a soy-based peptide product currently available as a swine feed ingredient, produced by a solid-state fermentation with a mixture of bacterial and fungal strains. This fermented product provides the advantage of a considerably inexpensive high protein vegetable source that it is believed to elicit less antigenicity than nonfermented soybean meal and reduced antinutritional factors, such as trypsin inhibitors (Min and others 2004). Prediabeats product, a commercially available soybean meal replacer for individuals challenged by diabetes, showed readings of < 0.25 OD_{405nm} units against plasma A, B, and pooled. In this product, soybean is the major component but it also contains other protein sources that may cause a diluting effect by lowering the sensitivity of IgE immunoreactivity toward soybean proteins. Finally, fermented soybean concentrate showed the lowest OD_{405nm} values against plasmas A, pooled, and B. Hydrolyzed soy flour, hydrolyzed soy concentrate, hydrolyzed soy isolate, and ultrafiltrated peptides (SAI Co., Geneva, Ill., U.S.A.) showed OD_{405nm} values below the detection limit ($10.79 \mu\text{g}$ IgE) of allergic protein.

Figure 5A shows the SDS-PAGE electrophoresis of soybean protein extracts of various fermented and hydrolysed commercially available soybean ingredients. Nonfermented soybean meal ingredient (lane 1) showed the main bands of soy proteins: intense α and β subunits of β -conglycinin (72 and 52 kDa, respectively), soybean vacuolar protein P34 (34 kDa) and acidic and basic subunits of glycinin (33 and 20 kDa, respectively). The partially fermented PepSoyGen ingredient (lane 2) showed an intense P34 band (34 kDa), and acidic and basic subunits of glycinin fractions, eliminating peptides above 75 kDa. The fermented soybean concentrate (lane 3) presented only peptides below 25 kDa in size. The electrophoretic profile of the hydrolyzed soy flour (lane 4), hydrolyzed soy concentrate (lane 6), hydrolyzed soy protein isolate (lane 7) and soy concentrated peptides (lane 8), showed that hydrolysis was able to produce peptides with a molecular weight below 25 kDa.

Figure 5B, 5C, and 5D show IgE immunoreactive proteins detected in fermented and hydrolysed soy ingredients by western blotting using human plasma with 98.7, 71.6 IU/mL, and F pooled plasmas from different soy-sensitized subjects, respectively. By means of this qualitative test, it can be observed in this figure that several soy proteins in products 1 and 2 interacted strongly with human plasma, and others present in products 3, 4, 6, 7, and 8 had null immunoreactivity. Fermented PepSoyGen ingredient (lane 2) showed IgE immunoreactive proteins with similar patterns to soybean meal (lane 1), but its response was more diluted, showing a less allergenic response. The fermented soy concentrate (lane 3) shows almost no immune response above 10 kDa for the pooled plasma (Figure 5D). It can be observed that there is a diluting effect in the pooled plasma (Figure 5D) that reflected weaker immunoreactivity than the 71.6 IU/mL human plasma individually (Figure 5C). In contrast, plasmas 11.3 IU/mL and < 0.35 IU/mL were unable to detect reactivity in this method (data not shown). The IgE results of the pooled plasma (composed by combining plasmas A to D) used in this study perfectly agreed with the WHO pooled plasma F properly diluted (data not shown).

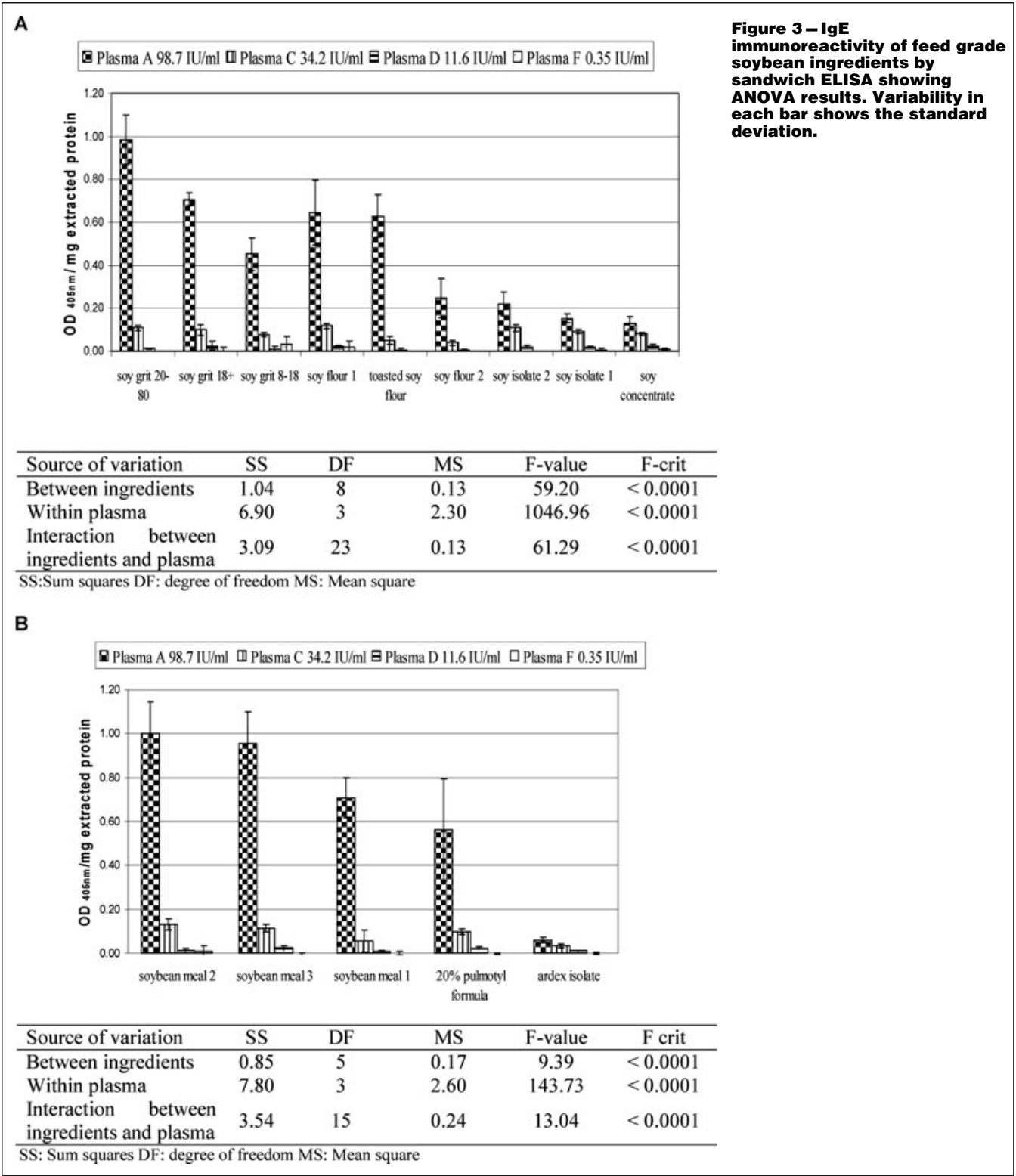
Table 4—Total soluble protein quantification in soybean ingredients.

Ingredient	mg extracted protein/g product ^{a,b}
ADM—82% Ardex AF hypoallergenic	$360.4^a \pm 34.8$
ADM—soy protein concentrate	$284.9^b \pm 18.9$
ADM—defatted soy flour-Baker's soy flour 1	$273.4^b \pm 17.3$
ADM—soy isolate 1	$271.9^b \pm 30.8$
ADM—48% soybean meal 1	$210.3^c \pm 25.7$
Thumboil—soy flour 2	$183.7^d \pm 14.3$
Thumboil—soy grits 18+	$163.9^e \pm 16.7$
ADM—soy grits 20 to 80	$154.2^{ef} \pm 19.1$
ADM—toasted soy flour	$156.6^{ef} \pm 0.0$
Thumboil—soy grits 8 to 18	$144.1^{fg} \pm 9.7$
ADM—soy isolate 2	$136.1^{gh} \pm 16.8$
Thumboil—48% soybean meal 2	$127.7^{h} \pm 7.1$
U of I—48% soybean meal 3	$122.4^{h} \pm 8.2$
U of I—20% Pulmotyl piglet formula	$104.6^{i} \pm 34.5$
ADM—65% Soycomyl-P (hypoallergenic)	$14.9^{j} \pm 2.9$

^aThe data are expressed as the mean $\pm$ standard error. Values are from 5 independent protein extractions. Different superscripts within the column indicate significant difference ($P \leq 0.05$). ^bProteins were extracted with 0.05 M Tris-HCl buffer, pH 8.2.

In summary, the estimated detection limit for plasma IgE levels was up to 11.3 IU/mL, showing detectable immunoreactivity by western blot technique. Hydrolysed soybean ingredients (lanes 4, 6, 7, and 8) exhibited a negligible IgE immunoreactivity to soy proteins and peptides for all plasma samples tested. This observation was in accordance with the ELISA results, reflecting absolutely no immunoreactivity generated by any of the hydrolyzed soy ingredients. Among the hydrolyzed ingredients, ultrafiltrated

concentrated peptides (lane 8) were able to remove immunodominant peptides below 10 kDa in size. In this case, the residual immunoreactivity of the product depends on the degree of hydrolysis applied. The addition of the costly but effective ultrafiltration step was able to eliminate most immunodominant residual proteins present in soy. Therefore, the results by ELISA and western blots confirmed the hypoimmunoreactive properties of the hydrolyzed soybean ingredients. Maldonado and others (1998)



have found that acid soluble proteins are extensively hydrolyzed (> 80%) and unlikely to elicit allergenic reactions. The same researchers suggest that heat treatments affect the conformational epitopes, while enzymatic hydrolysis destroys the sequential epitopes. More recently, Lee and others (2007) reported that pep-

tic and chymotryptic hydrolyzed fragments of 11S globulin of less than 20 kDa were no immunoreactive to IgE of human plasma.

Detection and quantification of IgE immunoreactive proteins in commercially available soybean products

Table 5 also presents the extracted protein concentration and the respective IgE immunoreactivity by sandwich ELISA of commercially available samples. Soymilk products had a soluble protein concentration that ranged from 12.1 to 54.7 mg extracted protein per gram product. Among the soymilk products, the highest soluble protein concentration was given by a powder based product that had approximately 2 times higher protein concentration, comparable to that of dried tofu samples ranging from 51.2 to 58.3 mg extracted protein per gram product. The IgE immunoreactivity of soymilk samples ranged from 3.4 to 68.9 ng IgE/mg extracted protein. Tofu contained about 20-fold higher IgE immunoreactivity than soymilk products (median 171.1 ng IgE/mg extracted protein). Furthermore, soy cheese products presented twice the IgE immunoreactivity of tofu products (median 359 ng IgE/mg protein). Meat analogues presented considerably high extracted protein concentration (median 67.9 mg/g product). Despite this high protein concentration, the IgE immunoreactivity corresponding to soybean protein was comparable to that of tofu products (median 127.8 ng IgE/mg extracted protein). The median ng IgE/g product was higher in tofu > meat analogues > soy cheese > soymilk products. Therefore, soybean subjected to different manufacture processes can give rise to products with variable IgE immunoreactivity. During food processing, chemical changes of food proteins may occur, altering their antigenic activity (Lee and others 2008). Soy proteins could interact with compounds from the food matrix being

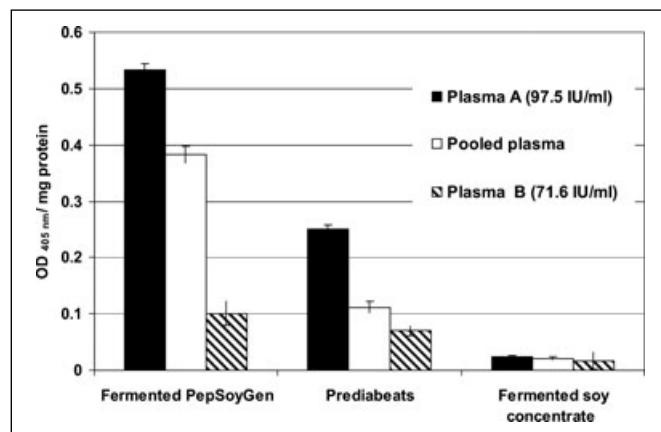


Figure 4—IgE immunoreactivity of commercially fermented and hydrolyzed ingredients by sandwich ELISA. ELISA plates were coated with IgE antibody from different plasmas (A, pooled plasma, and B) of soybean sensitive subjects and incubated with Prediabeats, fermented PepSoyGen, and fermented soy concentrate protein extracts, binding again with IgE antibody from different human plasmas and finally, goat antihuman IgE alkaline phosphatase monoclonal antibody. Data are expressed as the mean of OD_{405nm} values of 3 independent determinations. Error bars indicate standard deviation.

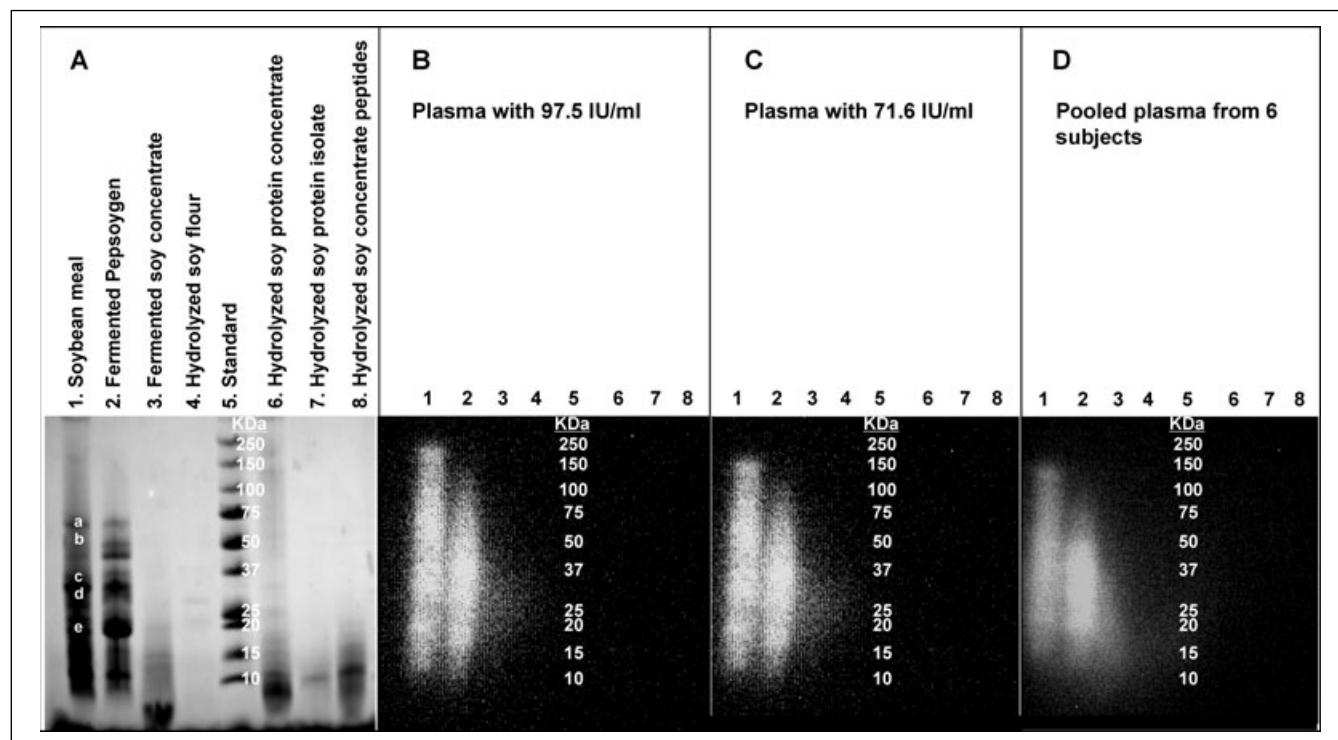


Figure 5—(A) SDS-PAGE electrophoresis of soybean protein extracts of various fermented or hydrolyzed commercially available soybean ingredients. Numbers refer to soy ingredients: 1 = soybean meal; 2 = fermented PepSoyGen; 3 = fermented soy protein concentrate; 4 = hydrolyzed soy flour; 5 = MW precision plus protein standards (Biorad, Hercules, Calif., U.S.A.); 6 = hydrolyzed soy concentrate; 7 = hydrolyzed soy protein isolate; and 8 = hydrolyzed soy concentrate peptides. Lower case letters refer to the following identified proteins: a. α -subunit of β -conglycinin (72 KDa); b. β -subunit of β -conglycinin (53 KDa); c. soybean vacuolar protein P 34 (34 KDa); d. acidic subunit of glycinin (33 KDa); e. basic subunit of glycinin (20 KDa). (B) Western blot carried out with 98.7 IU/mL human plasma. (C) Western blot carried out with 71.6 IU/mL human plasma. (D) Western blot carried out with pooled human plasma.

Table 5—Protein, fat, moisture, extracted protein, and IgE immunoreactivity by sandwich ELISA in commercial soybean products.

Soybean products	mg protein/g product ^a	mg fat/g product ^a	Moisture (%)	Extracted protein (mg /g product) ^b	OD _{405nm} /mg extracted protein ^c	ng IgE/mg extracted protein	ng IgE/g product
Soymilk							
Soymilk — Yeo	24.0	28.0	86.0 ± 0.7	12.1 ± 0.4	3.9 ± 0.3	8.7 ± 0.8	10.0 ± 0.8
Soymilk — Organic Valley	29.2	12.5	90.0 ± 0.3	16.7 ± 1.2	4.4 ± 0.5	10.5 ± 1.0	18.9 ± 2.1
Soydream — Imagine	29.2	16.7	88.8 ± 0.3	18.1 ± 0.1	1.8 ± 0.1	3.4 ± 0.2	5.9 ± 0.2
Soymilk — Silk	29.2	16.7	92.1 ± 0.7	18.9 ± 0.6	3.1 ± 0.3	7.8 ± 0.7	14.9 ± 1.7
Organic soymilk — Eden	44.0	16.0	88.2 ± 0.2	24.7 ± 0.1	2.3 ± 0.2	5.4 ± 0.5	13.0 ± 1.2
Soy powder — Original	87.0	108.7	2.7 ± 0.2	54.7 ± 2.5	0.9 ± 0.03	68.9 ± 2.9	367.0 ± 27.4
Median	29.2	16.7	88.5	18.5	3.1	8.2	14.0
Tofu							
Organic baked tofu	200.0	70.6	24.1 ± 4.2	51.2 ± 2.3	1.8 ± 0.2	138.1 ± 16.4	533.9 ± 40.3
Tofu cutlet Atsu — age	157.1	71.4	45.0 ± 6.2	53.1 ± 2.4	2.4 ± 0.4	171.0 ± 20.0	496.1 ± 89.3
Firm organic tofu — Nigari	176.5	41.2	46.5 ± 1.4	58.3 ± 0.5	3.4 ± 0.8	249.2 ± 8.7	775.9 ± 35.4
Median	176.5	70.6	45.0	53.1	2.4	171.0	533.9
Cheese							
Vegan cheese	52.6	105.3	25.8 ± 5.4	7.4 ± 0.2	7.1 ± 0.2	495.5 ± 18.6	273.1 ± 14.1
Cream cheese	33.3	233.3	48.9 ± 1.5	12.8 ± 0.7	10.3 ± 2.6	710.7 ± 64.4	473.1 ± 79.2
Tofutti cheese	105.3	263.2	11.7 ± 0.6	14.5 ± 0.9	3.16 ± 0.3	222.5 ± 19.7	283.8 ± 17.6
Soysation shredded cheese	250.0	107.1	15.8 ± 1.4	52.0 ± 3.1	1.46 ± 0.4	98.7 ± 2.5	437.3 ± 31.2
Median	79.0	170.2	20.8	13.7	5.1	359.0	360.5
Meat analogues							
The Good Ground Veggie	181.8	9.1	62.0 ± 4.2	116.9 ± 8.2	2.0 ± 0.4	146.9 ± 27.4	399.0 ± 17.1
Veggie sausage	193.0	35.1	64.0 ± 4.7	18.9 ± 1.0	1.4 ± 0.1	108.7 ± 7.7	457.7 ± 40.1
Median	187.4	22.1	63.0	67.9	1.7	127.8	428.4

^aThese values were taken from the product's label.^bProteins were extracted with 0.05 M Tris-HCl buffer, pH 8.2.^cSandwich ELISA OD_{405nm} values were determined using plasma A (98.7 IU/mL).

modified through various manufacturing processes (L'Hocine and Boye 2007). Brandon and Friedman (2002) found that mild nonenzymatic browning or exposure to alkali reduced the antigenicity of soy proteins. Babiker and others (1998) also suggested that the antigenic sites of soy proteins can be altered by reaction with carbohydrates or by enzyme-induced cross-linking. Saitoh and others (2000) found that passage of soybean hypocotyls meal through a twin-screw extruder reduced soy globulin antigenicity to 1% of its original activity.

Conclusions

The findings from this investigation demonstrated that sandwich ELISA is a reliable immunochemical method with good repeatability, sensitivity, and low detection limit to quantify IgE immunoreactive proteins in soy ingredients and soy-based products. The detection limit achieved by the developed method was 10.79 μ g IgE of allergic protein. Human plasma exhibiting the highest potency (98.7 IU/mL) gave a more accurate detection and quantification of soybean IgE immunoreactive proteins. IgE immunoreactivity of commercial soy ingredients and products depended on factors such as food processing conditions and food chemical composition. Fermented and hydrolyzed products were less immunoreactive, and this effect was related to the molecular weight of the proteins remaining in the product. However, specific IgE quantification cannot be used as an absolute diagnostic parameter but rather as a tool to assess risk of allergic reaction. Despite the limitations, quantitative measurement of specific IgE is likely to become an increasingly important parameter in the clinical investigation of food allergy (Asero and others 2007) and a valuable tool for soybean industry to comply with food labeling, thus, also protecting soy-sensitive consumers.

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