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Determination of Soy Proteins in Food Products by Enzyme Immunoassay

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Summary

Soy allergy is a common food allergy in North America and it can be fatal. Once it is diagnosed, dietary avoidance is the only sure method of management. Until recently, no analytical method was available to detect trace amounts of unintentional allergen contamination in our food supply. We have now developed polyclonal antibodies specific to soy proteins that do not cross-react with any of the 31 nuts, legumes or other common food ingredients tested. The antiserum was used to develop a sensitive immunoassay for the determination of soy proteins in foods. The concentration of soy proteins that inhibits 50% of antibody-antigen binding, IC_{50}, was 35 ng/mL and the linear range was 3 to 117 ng/mL. The detection limit was 2 ppm for the various foods tested. Recoveries ranged from 79–90%. The intra- and inter-assay coefficients of variation in this procedure were <8% for both canned fish and hamburger spiked at three levels between 13.5 to 54.0 ppm. The ELISA procedure was applied to a limited number of samples of hydrolysed vegetable protein (HVP), canned fish, hamburger and infant formula products. Soy proteins were identified in some HVP and soy-containing formulae, but not in the 29 fish, 14 hamburger and 20 infant formula products.

Keywords: soy, food allergen, immunoassay, ELISA, foods, HVP

Introduction

Soy is considered to be an important food allergen along with milk, egg, peanut and fish (*1,2*). Controlled clinical trials that estimate the prevalence of soy allergy are very limited. Bock and Atkinson (3), and Giampietro *et al.* (*4*) reported soy allergy in approximately 3% of children with food allergies, while Magnolfi (*5*) estimated it to be 6%. Sampson (*6*) reported a prevalence of 5% in a younger group of patients with atopic dermatitis.

Most of the soybeans produced are used as animal feed. However, soy-protein products are also used extensively by the food industry and therefore are increasingly consumed by humans (*7,8*). The increased exposure to allergenic soy proteins may be partly responsible for the increased development of soy allergy (*4,5*). In this regard, children are especially vulnerable, since soy-based formulas are routinely used as a substitute for feeding children who are allergic to cow's milk (*4,5,9*). Reactions to soy formulas have also been reported to occur in 17% to 47% of children with cow's milk allergy (*10,11*). Subsequently, hypoallergenic infant formulas and medical foods were developed (*12,13*).

Soybeans may be ingested as whole beans, flour or oil. In addition, soy may be used as a binder, meat filler, emulsifier, texturizer or flavouring agent, such as hydrolysed vegetable proteins (HVP). Innovations in food technology create problems such as hidden or masked food allergens. Thus, avoidance of soy in the diet is increasingly difficult (*14,15*). As with peanut oil, soy protein has also been found in small amounts in some samples of soy oil, lecithin and margarine (*16,17*). However, in a study similar to the one done in peanut-sensitive patients, soy oil was not found to cause adverse reactions to patients with soy hypersensitivity (*18*).

Soy contains multiple allergenic storage proteins. These proteins are broadly divided into the globulin (80–90%) and whey (10–15%) fractions, and both fractions contain allergenic components. The globulin fraction consists of 15, 11, 7 and 2S fractions by ultracentri-

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fugation. The whey fraction contains several bioactive substances including Kunitz trypsin inhibitor, which is also allergenic. The chemistry and allergenicity of these proteins have been extensively reviewed, and many of them have been cloned and sequenced (*19–21*).

The soybean dust aeroallergens are different from food allergy soy proteins. Inhalant soybean dust allergens have been traced to two hull-derived proteins, Gly m 1 and Gly m 2, which were responsible for an epidemic of asthma in Barcelona, Spain (*22,23*). Also, soy lecithin has been reported to be one cause of baker's asthma (*24,25*).

Many investigators have obtained polyclonal and monoclonal antibodies to soy proteins for functional studies of soy antigens (*26–28*). However, only a few were designed for quantitative analysis. Tsuji *et al.* (*29*) used monoclonal antibodies to measure Gly m Bd 30K allergen by enzyme-linked immunosorbent assay (ELISA) in a range of 5–500 ng. Unfortunately, no validation data were available. Ravestein and Driedonks (*30*) reported the use of soy specific polyclonal antibodies in the analysis of meat products with a detection limit of 0.5% (5 mg/g) soy proteins. Similar results were reported for a commercially available soya kit (Cortecs Diagnostics, U.K.) (*31–33*). Apparently, these two procedures are adequate for identifying adulteration or high levels of soy contamination in meat products.

It is imperative to ensure that our food supply is safe, and since trace amounts of offending allergens such as soy and peanuts can result in mild to life threatening reactions (*34–38*), a sensitive method of detection is urgently required. Here, we describe the development of a well characterized and validated ELISA procedure suitable for quantifying trace amount of soy proteins in various foods. This procedure is similar to the peanut immunoassay we reported recently (*39*).

Experimental

Reagents and supplies

Soybean acetone powder, bovine serum albumin (RIA grade), ovalbumin, goat anti-rabbit IgG peroxidase conjugate (second antibody), Tween 20, daidzein, genistein, polyethylene glycol (PEG, average relative molecular weight 8000), 10 mM phosphate buffer saline, and glycerol were purchased from Sigma Chemical Co., St. Louis, MO. *o*-Phenylenediamine dihydrochloride (OPD) was a product of Pierce, Rockford IL. Freund's complete and incomplete adjuvants were obtained from Gibco, Grand Island, NY. Dialysis tubing (10 mm in diameter with a 10 000 molecular mass cut-off) was purchased from Spectrum Medical Industries Inc., Los Angeles, CA. Flat-bottomed polystyrene microtiter plates were obtained from Dynatech Laboratories, Inc., Chantilly, VA. Legumes, nuts and other food ingredients were purchased in local stores. Canned fish, hamburger and other food items were sampled from various provinces in Canada.

Buffers

Phosphate-buffered saline (PBS), pH = 7.4, contained 10 mmol NaH_2PO_4, 2.7 mmol KCl and 138 mmol NaCl per litre of deionized water solution. Washing buffer (PBS-T) consisted of 0.1% Tween 20 in PBS. Coating buffer (pH = 9.6) contained 13 mM Na_2CO_3 and 35 mM $NaHCO_3$. Diluent contained 0.1% BSA, 0.1% Tween 20 and 3% PEG in PBS, while citric buffer (pH = 5.0) consisted of 51 mM Na_2HPO_4 and 24 mM citric acid per litre of deionized water. The substrate consisted of 17.5 mg OPD and 10 mL of 30% H_2O_2 in 25 mL citric buffer.

Instrumentation

Microtiter plates were washed with PBS-T using a Bio-Rad Microplate washer with five wash and soak cycles programmed for 8 s each. A 12-channel pipetter was used for dispensing liquids. Absorbances of microtiter wells were measured on a dual-beam Titertek Multiscan MCC with 492 nm sample and 620 nm reference filters. Data were transmitted to a spreadsheet program for analysis. The instrument was checked monthly by a Spectrocheck plate and software (QC Technology, New York).

Immunogen

Three soy immunogens (raw, cooked and denatured soy proteins) were prepared in the following manner:

Raw soy immunogen – Bulk soybean (100 g) was first ground. The finely ground soybean was stirred in hexane (1 L) for 30 min, then decanted. This defatting process was repeated 3 times with fresh hexane. The soy powder was vacuum filtered and air dried overnight. The soy proteins were extracted from the defatted soy powder with PBS (pH = 7.4, 4 °C, shaking over-night), centrifuged (20,000 g, 4 °C, 30 min), dialysed in PBS (MWCO 10,000, 48 h), filtered (0.45 μm membrane), diluted to 4 mg/mL, and stored frozen.

Cooked soy immunogen – A mass of 10 g of the defatted soybean powder in 100 mL PBS was simmered for 10 min prior to the extraction and purification procedures, which were the same as described for the raw soy immunogen.

Reduced and unfolded soy immunogen – A mass of 10 g of the acetone precipitated soybean powder (Sigma) was extracted in 10 mM Tris buffer, pH = 7.4, with 1% SDS (to unfold) and 10 mM mercaptoethanol (to reduce), at 4 °C for 16 h. The final extract was centrifuged, dialysed in the extraction buffer and filtered the same way as the other soy extracts.

Using SDS-PAGE, multiple bands from 10-100 kDa were observed in these 3 immunogens. All other raw legume and tree nut proteins were prepared in the same manner. Concentrations of proteins were determined by the Bradford (*40*) method (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Immunization

The immunization protocol was reported previously, except soy immunogens were used in lieu of peanut proteins (*39*). Serum titers were monitored eight days after each boosting dose by comparing the changes in absorbance at a fixed dilution of the antisera. Animals producing the highest titers of antibodies which provided the most sensitive inhibition curves were exsanguinated under anaesthesia. Optimal immunization occurred from

four to six months after the initial priming injection. Sera were kept frozen at –20 °C in 200 μL aliquots. Once thawed, an equal volume of 50% glycerol in water was added and the solution was stored at –20 °C. The glycerolated antibodies were stable for at least 24 months.

Plate coating

Microtiter plates were rinsed with deionized water and the wells filled with 200 μL solution of immunogen (1 μg/mL) plus ovalbumin (10 μg/mL) as coating protein in coating buffer. After 16 h at 4 °C, the wells were washed with PBS-T using a Bio-Rad microplate washer with five wash and soak cycles of 8 s each, followed by three wash cycles of deionized water. The sensitized plates were stored in plastic bags at –20 °C. In this study, we found these plates could be stored up to 12 months.

Sample preparation

A mass og 10 g of sample was homogenized in 100 mL PBS as an extraction buffer in a 250 mL polypropylene centrifuge bottle using a Polytron for 20 s at 50% power and then agitated at 100 rpm for 60 min at room temperature. Samples were centrifuged at 1,500 g for 30 min. The solids were removed by filtration through Whatman No.1 paper and approximately 1 to 5 mL of the filtrates were collected in polypropylene tubes for analysis.

In the recovery studies, samples were artificially contaminated with raw soy proteins at various levels (0, 13.5, 27 and 54 μg/g). These fortified samples were incubated for 30 min at room temperature prior to extraction.

Immunoassay

The competitive ELISA procedure is similar to the peanut ELISA previously reported (*39*). Briefly, 1 mL aliquot of antiserum diluted 1:50,000 with diluent was added to 25 μL of sample or standard. After mixing and incubation at 4 °C for 60 min, 200 μL of the mixture was added to the wells of the sensitized plate in triplicate. After a further 30 min incubation at 4 °C and washing, a second antibody horseradish peroxidase conjugate was added. Following further incubation at room temperature for 30 min and washing, the substrate was added. Thirty minutes later, the color reaction was stopped and the absorbance was read at 492 nm. The standard curve consisted of eight concentrations of soy proteins (0, 2.7–170 ng/mL). The raw soy protein standard was prepared according to the procedure for the immunogen except omitting the dialysis step. Soy standards were prepared in a blank sample extract of the food commodity which is going to be analyzed in order to provide a matrix- -modified standard curve. The soy protein concentrations were determined by a linear plot of the logit of the absorbance against the log of the concentration of the standards.

Results and Discussion

The antibodies generated by rabbits immunized with denatured, raw and cooked soy immunogens are able to recognize the native, and both heat or chemically denatured soy proteins. However, unlike peanut immunogens, soy proteins were much less antigenic under the same immunization conditions. Appreciable titers were observed only after the second booster doses. The chemically denatured (reduced and unfolded) soy proteins were much more effective immunogens, eliciting the highest titers and the lowest soy protein concentration that inhibits 50% of antigen-antibody binding (IC_{50}). Consequently, this antiserum was used in the remaining experiments.

To evaluate the specificity of the antibodies, 31 common legumes, tree nuts, food ingredients and phytoestrogens (Table 1) were tested for cross-reactivity by preparing standard curves in PBS and their IC_{50} values were determined in the ELISA. The polyclonal anti-soy antibodies did not recognize any constituent of the food items tested, demonstrating the high specificity of antibodies to soy proteins. Similar degree of specificity was observed in the anti-peanut antibodies that we reported previously (*39*).

Table 1. Legumes, tree nuts and common ingredients showing no cross-reactivity with the specific anti-soy antisera used in this study[a]

Legumes	Nuts	Ingredients	Phytoestrogens
peanut	hazelnut	corn	daidzein[c]
green pea	brazil nut	cocoa	genistein[c]
chic pea	pine nut	milk	
carob	pecan	chocolate	
pinto bean	almond	sugar[b]	
white bean	walnut	soy lecithin	
kidney bean	cashew	soy lectin	
navy bean	pistachio	peanut lectin	
lentil		coconut	
lupine		fish	
		egg	

[a] No inhibition was observed at concentrations of proteins ≤20 μg/mL.
[b] No inhibition at concentrations ≤100 mg/mL.
[c] No inhibition at concentrations ≤1 mg/mL.

A general purpose ELISA procedure was developed so that hidden or masked food allergens, such as soy, can be found in any food matrix. We and others have reported that the standard curves generated by different food matrices were not superimposable, although close, to those generated by PBS or water (*39,41–43*). Therefore, matrix-modified standard curves were used. We assumed the matrix extract used for the standard curve did not contain any quantifiable soy proteins when >3 negative samples obtained from different suppliers gave the same absorbance.

Clearly, tolerance limits for allergens are unknown. It could be in μg or mg range depending upon the degree of sensitivity as well as the age group being considered (*14,34,37,38*). Therefore, a low detection method is required. Our ELISA was capable of detecting 35 ng of soy per 1 mL of food extract, or 2 mg of soy per 1 g of samples, based on the lower datum point, which is at least 10% inhibition, in the standard curve. The log-logit

plot of the standard curve is linear for the whole range of 3–170 ng/mL, with $r^2 > 0.98$, consistently.

The Canadian Food and Drug Regulations (B.01.010) allow unspecific common names on food labels such as »flavour« and »seasoning«. Hydrolysed vegetable protein (HVP) is a common seasoning which is used frequently in canned fish products. This practice is of concern to soy sensitive people. The Food and Drug Regulations (B.01.010) presently require only enzymatic hydrolysed plant proteins to be labelled, unlike the United States Federal Register (1993) which now requires a source declaration on all hydrolysed proteins. The majority of the HVP are soy-based and are done by acid hydrolysis (*44*), therefore, are not required to be declared by plant source. Conclusive evidence that HVP is safe for soy sensitive people is not available. We analysed 6 commercial soy-based HVP products which are used in fish packing plants. Among them, two were detected as positive for soy by our assay. The results are tabulated in Table 2. The IC_{50} value is used for comparing the degree of inhibition exhibited by various compounds. These results indicate that the two HVP products retain their antigenicities and are possibly allergenic (*45–47*).

Table 2. Possible antigenicity in the following commercial hydrolysed soy proteins commonly used in canned fish evidenced from our specific anti-soy antisera

Brand	IC_{50}/(ng/mL)
A	113,560
B	6,310
C	1,190
D	nd[a]
E	99[b]
F	167[c]

[a] No inhibition at concentrations ≤40 μg/mL
[b] Detectable at ≥3 ppm level
[c] Detectable at ≥5 ppm level

We applied our procedure to a soy containing sample (canned tuna fish) and to a mini-survey of 29 tinned fish products which were purchased in stores or obtained from fish packing plants. No detectable soy protein residues were found (Table 3). In fortified tuna fish, recoveries ranged from 77–95% at 13.5, 27 and 54 ppm levels.

Table 3. Survey of tinned fish products

	No. of samples	ppm
Tuna	10	nd
Salmon	16	nd
Sardine	3	nd

All tuna samples obtained contained hydrolysed vegetable proteins, including a complaint sample. One sardine sample was in soy oil; nd = no detectable soy proteins.

The precision of the ELISA was determined by running the extracts of the samples 4 times per day for 4 days. The intra-assay and the inter-assay coefficient of variations were 3.5–4.2 and 2.4–5.1%, respectively (Table 4). Respective results obtained for hamburger were 3.6–8.0% and 1.5–2.6% (Table 5). In the 14 hamburger samples surveyed, no detectable soy proteins were evident. In another survey of infant formulae, initiated from an allergy related complaint, 8 soy-based products showed 24.6–66.9 ppm soy proteins. The milk-based formulae containing soy oil and the hypoallergenic soya did not have any quantifiable soy proteins. Surprisingly, one soy–based formula containing milk had no detectable soy proteins, which may have been extensively hydrolysed. The other 14 milk base formulae were soy free (Table 6). The procedure is also applicable to wiener and chicken breast which showed similar recoveries (data not shown). The recoveries and reproducibilities of the assay were comparable to that of the peanut immunoassay (*39*).

Table 4. Fraction of recovery (in %) of soy proteins from tuna fish

	Fraction of soy proteins added / ppm		
	13.5	27	54
Intra-assay	77±2.7	90±3.4	85±3.6
Inter-assay	89±3.2	95±4.8	87±2.1

Values are expressed in means ± s.d., n = 4

Table 5. Fraction of recovery (in %) of soy proteins from hamburger[a]

	Fraction of soy proteins added / ppm		
	12.5	25	50
Intra-assay	96±7.7	81±5.7	63±2.3
Inter-assay	94±1.4	79±2.1	63±1.1

Values are expressed in means ± s.d., n = 4
[a]No detectable soy proteins in any of the 14 hamburgers surveyed

Table 6. Survey on soy-based infant formulae

Brand	Type	Fraction / ppm
1	skim milk, soy oil	nd
2	skim milk, soy oil	nd
3	skim milk, soy oil	nd
4	skim milk, soy oil	nd
5	soy protein base	24.6
6	soy base	46.4
7	soy base	30.0
8	soy base	57.4
9	soy base	62.1
10	soy base	66.9
11	soy protein	48.2
12	soy protein	54.0
13	soy base, milk protein	nd
14	soya (hypoallergenic)	nd

Soy protein was not detected in another 14 milk based infant formulae.
nd = no detectable soy proteins at the detection limit of 0.25 ppm

This study demonstrates the feasibility of applying the ELISA method for the analysis of soy residues in various food products. The simplicity, sensitivity and se-

lectivity of the assay makes it suitable for routine surveillance by regulatory agencies or quality assurance programs in food industries.

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Određivanje proteina soje u prehrambenim proizvodima enzimskim imunokemijskim postupkom

Sažetak

Alergija na soju uobičajena je alergija na hranu u sjevernom dijelu Amerike, a može biti i smrtonosna. Ako je jednom dijagnosticirana, jedini siguran način je izbjegavanje prehrane sojom. Donedavno nije postojao analitički postupak za otkrivanje tragova nenamjerno prisutnih alergena u prehrani. Proizvedena su poliklonska antitijela, specifična za proteine soje, koja ne ulaze u unakrsne reakcije s bilo kojim od ispitivanih sastojaka hrane (u raznim vrstama oraha i povrću). S antiserumom je priređen osjetljiv postupak za imunokemijsko određivanje soje u hrani. Koncentracija proteina soje koja inhibira 50% vezanja antitijela s antigenom, IC_{50}, iznosila je 35 ng/mL, a linearno područje protezalo se od 3 do 117 ng/mL. Granica detekcije bila je 2 ppm pri ispitivanju različitih vrsta hrane. Iskorištenje je iznosilo od 79 do 90%. Koeficijenti varijacije unutar i između pojedinih određivanja bili su <8% za ribu u konzervi i hamburgere, a utvrđeni su na tri razine između 14-54 ppm. Postupkom ELISA provjeren je samo određeni broj uzoraka hidroliziranih vegetabilnih proteina (HVP), konzervirane ribe i hamburgera. Proteini soje pronađeni su u nekim hidroliziranim vegetabilnim proteinima, ali ne u 29 ribljih proizvoda i 14 hamburgera.