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Reliable Enzyme-Linked Immunosorbent Assay for the Determination of Soybean Proteins in Processed Foods

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Among allergenic foods, soybean is known as a food causing adverse reactions in allergenic patients. To clarify the validity of labeling, the specific and sensitive detection method for the analysis of the soybean protein would be necessary. The p34 protein, originally characterized to be p34 as an oil-body associated protein in soybean, has been identified as one of the major allergenic proteins and named Gly m Bd 30K. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of the soybean protein in processed foods was developed using polyclonal antibodies raised against p34 as a soybean marker protein and the specific extraction buffer for extract. The developed sandwich ELISA method was highly specific for the soybean protein. The limit of detection (LOD) and the limit of quantification (LOQ) of the developed ELISA were 0.47 ng/mL (equivalent to 0.19 μg/g in foods) and 0.94 ng/mL (equivalent to 0.38 μg/g in foods), respectively. The recovery ranged from 87.7 to 98.7%, whereas the intra- and interassay coefficients of variation were less than 4.2 and 7.5%, respectively. This study showed that the developed ELISA method is a specific, precise, and reliable tool for the quantitative analysis of the soybean protein in processed foods.

KEYWORDS: Soybean; enzyme-linked immunosorbent assay (ELISA); p34; Gly m Bd 30K; allergen; food allergy

INTRODUCTION

Food allergies are a disease affecting approximately 8% of children and 2% of adults in industrialized countries (*1–3*). The clinical manifestation of food allergies varies from mild symptoms, such as the oral allergy syndrome or mild urticaria, to severe anaphylactic reactions with fatal consequences. To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. Allergens contained in food products pose a major risk for sensitized persons. Therefore, sufficient information about potentially allergenic ingredients in a food product is necessary (*4, 5*). In 1999, the Joint FAO/WHO Codex Alimentary Commission agreed to label eight kinds of foods that contain ingredients known to be allergens including soybeans (*6, 7*). Soybeans are one of the "big eight" foods that are believed to be responsible for 90% of all food allergies (*8*). In Japan, the Ministry of Health, Labor and Welfare has enforced a labeling system for allergenic food materials since April 2002 (*3*). In this system, labeling for 5 food products including egg, milk, wheat, buckwheat, and peanuts is mandatory and is recommended for 20 other food materials such as soybeans and shrimp. For the recommended labeling method, soybean labeling would be the most important issue because of the almost unlimited uses of soybeans and the number of patients with an allergy to soybeans has been increasing (*9–12*).

Concerns about soybean allergy have become exacerbated due to increased use of soybean ingredients in processed foods (for example, miso, soy sauce, tofu, and meat alternative). For better protection of the consumer, suitable methods are required for the specific and sensitive detection of allergens. To date, the enzyme-linked immunosorbent assay (ELISA) has been the

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preferred approach for allergen detection because it detects the protein/allergen responsible for allergic reactions with its high precision, simple handling, and good potential for standardization (*13*). Some studies for the determination of soybean proteins using ELISA have been reported (*14–25*). However, reliable measurements using these methods have been considered to be difficult for application involving processed foods because the previous methods have cross-reactivity to some peas and crops and low repeatability and reproducibility in the application of processed food to ensure the validity of labeling.

In the present study, we developed a reliable sandwich ELISA method with a high sensitivity and specificity for the determination of soybean proteins using the antibody to Gly m Bd 30K, which was originally characterized to be a vacuolar protein, p34, as an oil-body associated protein with a molecular mass of 34 kDa in soybean (*26, 27*). p34 was identified as the major soybean allergen in soybeans (*28, 29*). p34 is detected in various processed foods containing soybean (for example, tofu, yuba, soybean milk, and products containing soybean-isolated proteins) due to its high absorption characteristics to lipids or other proteins (*30, 31*). The allergenicity is retained during heat processing and stable to digestion by the gastrointestinal tract (*32, 33*). In addition, it has been reported that there is no soybean variety lacking p34 (*30*). Accordingly, p34 is suitable as a marker protein for the determination of the soybean protein in processed foods possibly containing soybeans. We developed the sandwich ELISA method using a polyclonal antibody against p34 and a suitable extraction buffer for the extraction of p34 from processed foods. We showed that this detection method could be applicable to processed foods and that the trace amounts of soybean contained in commercial food products can be detected using the proposed sandwich ELISA method.

MATERIALS AND METHODS

Food Materials. The soybeans (*Glycine max* var. Enrei, Haruyutaka, Nattosyoryu, and Toyomusume) were obtained from Kinki University in 2005. The food materials and commercial processed foods were purchased at local supermarkets (Ibaraki, Japan) in 2006.

Chemicals and Reagents. Acetone, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), hexane, 2-mercaptoehanol (2-ME), methanol, polyoxyethylene−sorbitan monolaurate (Tween 20), sodium chloride (NaCl), sodium dodecyl sulfate (SDS), sodium hydrogen carbonate ($NaHCO_3$), sucrose, and sulfuric acid (H_2SO_4) were purchased from Wako Pure Chemical Industries. Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and phosphate-buffered salts (PBS) were purchased from Sigma-Aldrich Co. (St. Louis, MO) and Takara Bio, Inc. (Shiga, Japan), respectively. Superdex 75 and CNBr-activated Sepharose 4B were purchased from GE Healthcare U.K., Ltd. (Buckinghamshire, U.K.).

Preparation of the Polyclonal Antibody against p34, an Allergenic Protein Gly m Bd 30K. The reliable performance of the method for the detection of soybean proteins would require the specific and appropriate antibody to soybean protein. We chose p34, which was identified as the major soybean allergen, as a marker protein of the determination of soybean proteins. To prepare the polyclonal antibody to p34, the protein was first purified from soybeans according to the method of Kalinski et al. (*26, 27*). Three hundred grams of the soybeans was soaked in 1 L of distilled water for 24 h. The soaked soybeans were homogenized in 2 L of a 0.1 M Tris-HCl, pH 8.6, 10 mM 2-ME mixture by a juicer (MX-X6, Matsushita Electric Industrial Co., Ltd., Osaka, Japan). The homogenate was filtered through several layers of cheesecloth and centrifuged at 20000*g* to collect the oil-body pad. The oil-body pad was resuspended in a 5 times volume of 0.1 M Na_2CO_3 for 1 h on ice, and the supernatant containing the protein was collected by centrifugation. p34 was purified by gel filtration chromatography (Superdex 75, 26/90 cm) equilibrated with 0.1 M Na_2CO_3. A half aliquot of the purified p34 fraction was heated at 100 °C for 30 min and mixed with the other half nonheated p34 fraction and then used as an antigen to obtain the antibody that would be applicable for the many kinds of foods in the wide range of raw materials to the foods processed with high temperature and pressure. The antigen was mixed 1:1 with Freund's complete adjuvant and injected into Japanese white female rabbits as a first injection. After the first injection, injections of antigen with Freund's incomplete adjuvant were performed five times at intervals of 2 weeks for 8 weeks. One week after the final injection, the whole blood was collected. The antiserum was separated and dialyzed with 20 mM PBS, pH 7.0. An affinity column was prepared by fixation with 50 mg of the antigen to a Sepharose 4B column (26/5 cm). The antiserum was applied to the affinity column equilibrated with 20 mM PBS, pH 7.0, and the polyclonal antibody was eluted with 0.1 M glycine, pH 2.7. The eluted antibody was neutralized immediately after elution with 1 M Tris-HCl, pH 9.0, and was dialyzed against 20 mM PBS, pH 7.0. The care and use of the experimental animals in this study followed "The Ethical Guidelines of Animal Care, Handling and Termination" prepared by the National Institute of Health Sciences.

Preparation of Calibration Standard Solutions. For the determination of soybean protein using the ELISA method, the preparation of a calibration standard solution is necessary. The calibration standard solution was prepared according to the official guideline and the previous reports in Japan (*34–36*). The soybeans including Enrei, Haruyutaka, Nattosyoryu, and Toyomusume of equal amounts were homogenized by a food processor (IFM-700G, Iwatani International Co., Osaka, Japan), and the mixed soybean powder was collected through a 0.3 mm mesh. After removal of the fats with acetone and hexane, the defatted soybean powder (DSP) was dried for 48 h in air. The protein content in DSP was determined to be 43.1% by using the Kjeldahl method. The initial extract for the calibration standard solution was prepared from the DSP as follows: A 300 mg sample of the DSP was added to a 20 mL mixture of 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5% SDS, and 2% 2-ME. The mixture was then shaken for 16 h at room temperature for extraction. The extract was centrifuged at 10000*g* for 30 min, and the supernatant was filtered through a 0.8 μm microfilter paper (DISMIC-25CS, Toyo Roshi Kaisya Ltd., Tokyo, Japan). The protein content of the initial extract was assayed using a 2-D Quant Kit (GE Healthcare U.K., Ltd.). The amount of soybean soluble protein of per gram of DSP was approximately 225 mg. The initial extract was diluted to 50 ng/mL using 0.1 M PBS, pH 7.4, 0.1% SDS, 0.1% 2-ME, 0.1% BSA, and 0.1% Tween 20 and stored as the calibration standard solution for the ELISA at 4 °C. We confirmed the stability of the calibration standard solution in the solution containing 0.1% 2-ME for 6 months at 4 °C.

Protein Extraction from Food Samples. The food samples were homogenized using a food processor. Nineteen milliliters of extraction buffer (120 mM Tris-HCl, pH 7.4, 0.1% BSA, 0.05% Tween 20, 0.5−4% SDS, 2% 2-ME) was added to 1 g of the food homogenate and then extracted for 16 h using the shaker at 90−110 rpm. The extraction buffer was prepared with the identical composition as the extraction buffer in commercial ELISA kits for allergen tests in Japan except for the SDS concentration (*35–37*). That is, the concentration of SDS in the extraction buffer was examined in the range of 0.5% (5 g/L dissolved with buffer), 1% (10 g/L dissolved with buffer), 2% (20 g/L dissolved with buffer), and 4% (40 g/L dissolved with buffer). The food extract was centrifuged at 3000*g* for 20 min, and the supernatant was filtered through a filter paper (5A Filter Paper, Toyo Roshi Kaisya, Ltd.). The filtrate was collected as the food sample extract and used immediately in the examination.

Preparation of Model Processed Foods. The best source of information on the method performance for allergen detection methods would be model processed food samples. Therefore, five kinds of model processed foods (rice gruel, sausage, sweet adzuki bean soup, sweet potato cake, and tomato sauce) were prepared using the typical procedures described by the manufacturers. To make a final protein concentration of 10 μg/g (soybean soluble protein weight/sample weight) in the model processed foods, the amount of the DSP spiked in the model processed foods was calculated, taking into account the protein content of the DSP and the change in weight of the model processed foods during their preparation. The soybean soluble protein

amount per gram of the DSP was approximately 225 mg, so we spiked 44.4 mg of the DSP to make 1 kg of each model processed food because we estimated the ratio of the soybean soluble protein weight to the DSP weight to be 22.5%. The amount of the spiked DSP was calculated from the result using the 2-D Quant kit. The ingredients were spiked with the DSP at the ingredient stage and then cooked according to the following method.

Rice gruel consists of rice, sugar, and water. Rice, sugar, water, and the DSP were mixed in a juicer (MX-X6, Matsushita Electric Industrial Co.) and homogenized. The homogenate was packed in a retort bag (SP-1000K, Mita Rika Kogyo Co., Ltd., Osaka, Japan) and cooked at 121 °C for 10 min. The cooked rice gruel was cooled in flowing water and stored at −20 °C.

Sausage consists of pork, salt, sugar, and ice water. Pork, salt, sugar, ice water, and the DSP were mixed and homogenized using a food mixer (BLIXER-5 plus, Robot Coupe USA Inc.). The homogenate was packed in the retort bag and cooked at 80 °C for 20 min. The cooked sausage was cooled in flowing water and stored at −20 °C.

Sweet adzuki bean soup consists of adzuki beans, sugar, and water. The adzuki beans were precooked in boiling water for 40 min and then filtered through a 0.3 mm mesh. The cooked adzuki beans, sugar, water, and the DSP were mixed in the juicer and homogenized. The homogenate was packed in the retort bag and cooked at 100 °C for 10 min. The cooked sweet adzuki bean soup was cooled in flowing water and stored at −20 °C.

Sweet potato cake consists of sweet potato, sugar, and water. The sweet potato was precooked for 5 min using a microwave oven (RE-S240, Sharp Co., Osaka, Japan). The cooked sweet potato, sugar, water, and the DSP were mixed and homogenized using the food mixer. The homogenate was packed in the retort bag and cooked at 100 °C for 15 min. The cooked sweet potato cake was cooled in flowing water and stored at −20 °C.

Tomato sauce consists of tomato puree, salt, sugar, and water. Tomato puree, sugar, water, and the DSP were mixed in the juicer and homogenized. The homogenate was packed in the retort bag and cooked at 100 °C for 20 min. The cooked tomato sauce was cooled in flowing water and stored at −20 °C.

ELISA. A microtiter plate (F8 Maxisorp Nunc-Immuno module, Nunc, Roskilde, Denmark) was coated with the diluted polyclonal antibody against p34 in 1 M $NaHCO_3$, pH 8.5 (100 μL/well), and incubated for 16 h at 4 °C. After two washings with a washing buffer (100 mM PBS, pH 7.4, 1% Tween 20, 250 μL/well), the plate was blocked with a blocking buffer (100 mM PBS, pH 7.4, 1% BSA, 5% sucrose, 200 μL/well) for 3 h at 25 °C. The blocking buffer was removed, and then the plate was dried for 16 h at 25 °C. The food sample extract was diluted 1:19 in a dilution buffer A (100 mM PBS, pH 7.4, 0.1% BSA, 0.1% Tween 20, 0.1% SDS, 0.1% 2-ME). The calibration standard solution was diluted to concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 ng/mL using dilution buffer B (a mixture of the dilution buffer A and the extraction buffer at 19:1). The diluted food sample extract and the calibration standard solution were added to the plate (100 μL/well) and incubated for 1 h at 25 °C. All experiments were performed in triplicate. After five washings, a diluted biotin-labeled polyclonal antibody against p34 (100 μL/well) was added to the plate and incubated for 1 h at 25 °C. After washing, a diluted horseradish peroxidase-conjugated streptavidin (Poly-HRP streptavidin N200, Pierce Co.; 100 μL/ well) was added to the plate and incubated for 30 min at 25 °C. After washing, 3,3′5,5′-tetramethylbenzidine (TMBE-1000, Moss, Inc.; 100 μL/well) was added to the plate and incubated for 20 min at 25 °C. The reaction was stopped by the addition 100 μL of 0.25 M H_2SO_4. The absorbance was measured by a plate reader (SUNRISE 2000, Wako Pure Chemical Industries, Ltd.) at the dominant and subdominant wavelengths of 450 and 620 nm, respectively. The concentration of the soybean soluble protein in the diluted food sample extract was calculated from the calibration curve prepared by a four-parameter logistic analysis. The value of the soybean soluble protein in the foods was converted using the dilution factor (×400).

Western Blot. The sample solution was mixed 1:1 with Laemmli buffer (Bio-Rad Laboratories, Inc.) containing 2.5% 2-ME. The sample was boiled at 100 °C for 5 min and cooled in flowing water. The sample and a molecular weight marker (Precision Plus Protein All Blue Standard, Bio-Rad Laboratories Inc.) were applied at 10 μL/lane to the 15% separation gel (E-T15L, Atto Co., Tokyo, Japan) in a running buffer (10× Tris/glycine/SDS buffer, pH 8.3, Bio-Rad Laboratories, Inc.). The electrophoresis was performed at a constant electric current of 20 mA/gel. The protein was blotted onto a PVDF membrane (Hypond-P, Wako Pure Chemical Industries, Ltd.) in a blotting buffer [a mixture of 10× Tris/glycine buffer, pH 8.3 (Bio-Rad Laboratories, Inc.), methanol, and distilled water at 1:2:7] by a blotting system (Trans-Blot SD Cell, Bio-Rad Laboratories, Inc.). The blotting was performed at a constant electric current of 2 mA/cm^2 for 1 h. The membrane was blocked with 1× TBS (Bio-Rad Laboratories, Inc.) containing 0.1% BSA and 0.05% Tween 20 for 30 min at 25 °C. After three washings with a washing buffer (1× TBS containing 0.05% Tween 20), the membrane was shaken in a diluted polyclonal antibody against p34 in the blocking buffer for 1 h at 25 °C. After three washings, the membrane was shaken in a diluted biotin-labeled anti-rabbit IgG antibody (VECTASTAIN ABC-AP Rabbit kit, Vector Laboratories, Inc.) in the blocking buffer for 1 h at 25 °C. After three washings, the membrane was shaken in a diluted alkaline phosphatase-conjugated avidin (VECTASTAIN ABC-AP Rabbit kit, Vector Laboratories, Inc.) for 30 min at 25 °C. After three washings, the membrane was shaken in 100 mM Tris-HCl, pH 9.5, for 15 min at 25 °C and detected with an alkaline phosphatase substrate kit IV <BCIP/BNT> (Vector Laboratories, Inc.). The reaction was stopped by the addition of distilled water.

Single Laboratory Validation. For determination of the intra-assay precision, the mean coefficients of variation (CVs) in the five kinds of model processed foods were based on eight replicates of extraction from the same model processed food. The interassay precision was determined as the mean CVs on the basis of the triplicate analyses on eight different days. To investigate the effect of the food matrix on the determination of soybean protein, we examined the dilution linearity using the model processed foods. The extracts of these processed foods were diluted 1:1, 1:3, and 1:7 with dilution buffer B and were measured using the developed ELISA. The limit of detection (LOD) for the sandwich ELISA was calculated as 3 times the standard deviation (SD) of the buffer blank mean value after eight experiments. The limit of quantification (LOQ) was calculated as 10 times the SD of the buffer blank mean values after eight experiments. The homogeneity of the sample materials in model processed foods was verified following the procedure described in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (*38*). Twelve test portions of each sample material were analyzed using the proposal method. The obtained concentrations of soybeans protein were submitted to one-way analysis of variance (ANOVA). The F ratios for all sample materials were below the critical F value (data not shown).

RESULTS

Preparation of a Polyclonal Antibody against p34. p34 was purified from the soybean powder using the method described under Materials and Methods. **Figure 1A** shows the results of the nonheated p34 and the heated p34 using the SDS-PAGE analyses. Only a single band with a molecular mass of 34 kDa was observed in the all lanes. No difference between nonheated p34 and heated p34 appeared to be observed on SDS-PAGE. A polyclonal antibody to p34 was prepared in rabbits using the mixture of nonheated p34 and heated p34. In the Western blot analysis, the prepared polyclonal antibody reacted to the single band with the 34 kDa protein in all lanes (**Figure 1B**). These results suggest that the prepared antibody can specifically react with both the nonheated p34 and heated p34 in soybeans.

Construction of a Sandwich ELISA for Determination of the Soybean Soluble Protein. A sandwich ELISA was constructed with the polyclonal antibody to the prepared p34 according to the method described under Materials and Methods. Five kinds of model processed foods were extracted with the extraction buffer (containing 0.5% SDS, 2% 2-ME) of the commercial ELISA kits for allergen tests in Japan (*35–37*). The levels of soybean soluble protein in the five kinds of

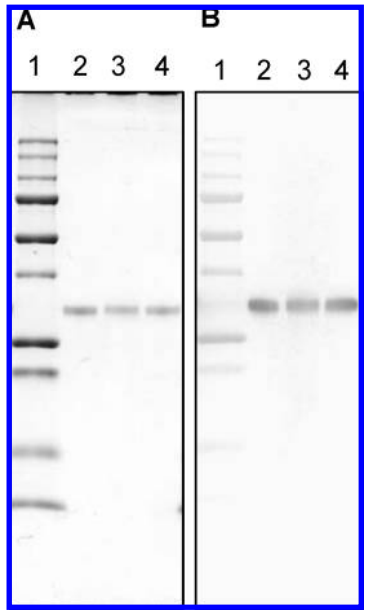

Figure 1. SDS-PAGE and Western blot of the purified p34: (**A**) SDS-PAGE (15%); (**B**) Western blot with the prepared polyclonal antibody against the mixture of heated p34 and nonheated p34. Lanes: 1, molecular weight marker (prestained); 2, nonheated p34; 3, heated p34; 4, mixture of nonheated p34 and heated p34.

Table 1. Detection of Soybean Soluble Proteins in the Model Processed Foods[a]

model processed food	mean (μg/g)	recovery (%)
rice gruel	11.24	112.4
sausage	2.87	28.7
sweet adzuki bean soup	11.16	111.6
sweet potato cake	10.11	101.1
tomato sauce	10.75	107.5

[a] Detection of soybean soluble proteins in five kinds of model processed foods (rice gruel, sausage, sweet adzuki bean soup, sweet potato cake, and tomato sauce) using the developed ELISA. The model processed foods contain soybean soluble proteins of approximately 10 μg/g. Mean = average concentration (soybean soluble protein weight/food weight) of soybean soluble proteins. Recovery = mean/10 (μg/g).

model processed foods were measured using the developed ELISA (**Table 1**). The recoveries of the soybean soluble protein were 112.4, 111.6, 101.1, and 107.5% in rice gruel, sweet adzuki bean soup, sweet potato cake, and tomato sauce, respectively. The recovery of the soybean soluble protein from sausage was 28.7%, and not satisfactory for the Japanese guideline criteria (50−150%). These results showed that the extraction buffer (containing 0.5% SDS, 2% 2-ME) does not have the ability to extract the soybean soluble protein from sausage.

To improve the extraction efficiency of the soybean soluble protein from sausage, we examined the concentration of SDS and 2-ME in the extraction buffer. The concentration of SDS in the extraction buffer was prepared at 0.5, 1, 2, and 4%. The model processed foods were used to extract the soybean soluble protein by each extraction buffer and measured by the developed ELISA (**Table 2**). The recovery of the soybean soluble protein from the sweet potato cake that was extracted by the extraction buffer (containing 0.5%−2% SDS) was 95.5−101.1%. The recovery of the soybean soluble protein from the tomato sauce by the extraction buffer (containing 0.5%−2% SDS) was 96.2−107.5%. The recovery of the soybean soluble protein from the sausage by each extraction buffer containing 0.5, 1, and 2% SDS was 28.7, 55.2, and 95.0%, respectively. These results show that an increase in the SDS concentration in the extraction buffer would induce a rise in the recovery of the soybean soluble proteins in the sausage. However, the recoveries of the soybean soluble protein from the three kinds of model processed foods using the extraction buffer including 4% SDS also were much lower than those with the 2% SDS. These results show that the highest concentration (4%) of SDS would decrease the recovery of the soybean soluble protein in the model processed foods. We also examined the concentration of 2-ME in the extraction buffer. However, the increase in the 2-ME concentration did not affect the recoveries of the soybean soluble protein from the model processed foods (data not shown). Therefore, we set the concentration of SDS in the extraction buffer to 2%, and all experiments were performed using the improved extraction buffer (containing 2% SDS, 2% 2-ME).

Sensitivity and Specificity of the Developed ELISA. Figure 2 shows the calibration curve for the determination of the soybean soluble protein using the developed ELISA. The LOD of the ELISA method using the calibration standard solution is 0.47 ng/mL, equivalent to 0.19 μg/g of food sample. The LOQ is 0.94 ng/mL, equivalent to 0.38 μg/g of food sample. One hundred kinds of foods (legumes, 9; grains, 8; nuts and seeds, 13; spices, 5; meats, 5; vegetables, 27; fruits, 6; seafoods, 25; others, 2) were examined to test the reactivity and specificity using the developed ELISA. **Table 3** shows the results for 30 kinds of foods that mainly consisted of legumes and nuts. The values of all foods except for milk, rice, and barley were less than the LOQ (0.38 μg/g of food sample) of the developed ELISA. The values of milk, rice, and barley were 0.51, 0.44, and 0.67 μg/g, respectively.

Single Laboratory Validation with the Developed ELISA and the Model Processed Foods. Table 4 shows the result of a single laboratory validation for the developed ELISA using the model processed foods. The recoveries of the soybean soluble protein were 112.4, 111.6, 101.1, and 107.5% in rice gruel, sweet adzuki bean soup, sweet potato cake, and tomato sauce, respectively. The dilution linearities using these processed foods were satisfactory ($R^2 > 0.99$). The interassay precision for the five model processed foods across eight different days was 7.5, 6.9, 7.3, 5.8, and 6.4% CV, respectively. The intra-assay precision for the five model processed foods was 2.2, 4.1, 4.2, 3.3, and 3.1% CV, respectively.

Detection of Soybean Proteins in the Commercial Processed Foods. Fifteen kinds of commercial processed foods including soybean were analyzed using the developed ELISA (**Table 5**). All 10 kinds of commercial processed foods except the soy sauce and natto showed >20 μg/g over the calibration curve range. Two kinds of natto showed 1.15 and 1.44 μg/g, respectively. Two kinds of soy sauce and soybean oil had levels less than the lowest value in the calibration standard solution.

DISCUSSION

We developed a reliable and specific ELISA method for the determination of soybean soluble proteins in processed foods. A soybean soluble protein in the processed foods could be denatured by various manufacturing processes (heating, baking, frying, acidifying, and pressurizing processes). The denatured soybean soluble protein often showed a lower solubility in water and a change in its three-dimensional structures. Therefore, we developed an antibody to that and a suitable extraction buffer for the determination of the soybean soluble proteins.

In Japan, the threshold of food allergen in processed foods for labeling has been set at the level of 10 μg/g (the corre-

Table 2. Effect of SDS Concentration on the Extraction of Proteins from the Model Processed Foods[a]

	concentration of SDS in extraction buffer							
	0.5% SDS		1% SDS		2% SDS		4% SDS	
model processed food	mean (μg/g)	recovery (%)	mean (μg/g)	recovery (%)	mean (μg/g)	recovery (%)	mean (μg/g)	recovery (%)
sausage	2.87	28.7	5.52	55.2	9.50	95.0	7.11	71.1
sweet potato cake	10.11	101.1	9.55	95.5	9.72	97.2	5.49	54.9
tomato sauce	10.75	107.5	9.92	99.2	9.62	96.2	5.22	52.2

[a] Detection of the soybean soluble proteins in three kinds of model processed foods (sausage, sweet potato cake, and tomato sauce) using the developed ELISA. The model processed foods were extracted using different SDS concentrations (0.5, 1, 2, and 4%) in the extraction buffer. The model processed foods contain soybean soluble proteins of approximately 10 μg/g. Mean = average concentration (soybean soluble protein weight/food weight) of soybean soluble proteins. Recovery = mean/10 (μg/g).

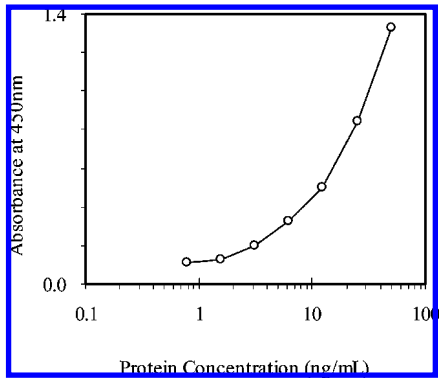

Figure 2. Calibration curve of the developed ELISA. The calibration curve was obtained from the mean value after eight experiments. Concentration of calibration standard solution (calculated soybean soluble protein weight/food weight) = 0 ng/mL (0 μg/g), 0.78 ng/mL (0.31 μg/g), 1.56 ng/mL (0.62 μg/g), 3.13 ng/mL (1.25 μg/g), 6.25 ng/mL (2.5 μg/g), 12.5 ng/mL (5 μg/g), 25 ng/mL (10 μg/g), and 50 ng/mL (20 μg/g).

Table 3. Cross-Reactivity of the Developed ELISA[a]

food	mean (μg/g)	food	mean (μg/g)
egg	<0.19	cashew nut	<0.19
milk	0.51	coconut	<0.19
wheat	<0.19	hazelnut	<0.19
buck wheat	0.35	pekan bean	0.22
peanut	<0.19	chickpea	<0.19
rice	0.44	pinto bean	<0.19
sesame	<0.19	tiger bean	<0.19
corn	<0.19	kidney bean	<0.19
barley	0.67	adzuki bean	<0.19
rye	0.24	green bean	<0.19
red pepper	0.37	field bean	<0.19
almond	<0.19	pork	0.28
macadamia nut	0.36	chicken	0.29
pistachio	<0.19	beef	<0.19
pine nut	<0.19	salmon	<0.19

[a] Cross-reactivity of the developed ELISA for 100 kinds of foods (legumes, 9; grains, 8; nuts and seeds, 13; spices, 5; meats, 5; vegetables, 27; fruits, 6; seafoods, 25; others, 2). The food extracts were prepared using the improved extraction buffer (containing 2% SDS, 2% 2-ME). The results of 30 samples are shown. Mean = average concentration (soybean soluble protein weight/food weight) of soybean soluble proteins.

sponding allergenic ingredient soluble protein weight/food weight) (*34*). Therefore, it is necessary for the detection method to be able to accurately detect the corresponding allergenic ingredient soluble protein at this level. In addition, it is also important that there is no cross-reactivity of the detection method because the cross-reactivity to food materials will cause a false-positive result.

Some immunoassays based on antibodies to soybean proteins such as the trypsin inhibitor, 7S β-conglycinin, and 11S glycinin have been reported in previous studies (*14–25*). However, these immunoassays appear to be difficult to apply to processed foods to monitor the allergen ingredients because the methods have cross-reactivity to some peas and crops and low repeatability and reproducibility in the application of processed food to ensure the validity of labeling (*14–23*). Tsuji et al. constructed a sandwich ELISA method based on a monoclonal antibody against p34 (*24, 25*). They reported that the detectable range of the immunoassay for the determination of p34 is 5−500 ng for the denatured allergen. Therefore, their immunoassay could detect the denatured soybean proteins in processed foods with high sensitivity. However, they did not show the cross-reactivity of the immunoassay to food materials. We prepared a polyclonal antibody to the prepared p34 and constructed the sandwich ELISA using the antibody.

We examined the sensitivity and the specificity of the developed ELISA method. The LOD and the LOQ of the developed ELISA were 0.47 ng/mL (equivalent to 0.19 μg/g in foods) and 0.94 ng/mL (equivalent to 0.38 μg/g in foods), respectively. This result suggests that the developed ELISA has a higher sensitivity to the soybean protein than the immunoassay developed by Tusji et al. (*24, 25*). In addition, the developed ELISA had no cross-reactivity to all of the examined food materials (legumes, grains, nuts and seeds, spices, meats, vegetables, fruits, and seafoods) except for milk, rice, and barley. The developed ELISA had slight cross-reactivity to milk, rice, and barley at level greater than the LOD. However, all of the levels were determined by the developed ELISA method to be <1.0 μg/g when the examined food materials were tested (**Table 3**). The previous ELISA methods also had high cross-reactivity to some peas and crops at the level of 0.9−3.6% to soybean protein reactivity (*17–21, 23*). The level of cross-reactivity of some peas and crops on the previous methods was much higher than that of the developed ELISA. Therefore, we consider that the developed ELISA has more specific reactivity to soybean protein than the previous method. These findings suggest that the developed ELISA has a high sensitivity and specificity for monitoring the allergen ingredients to ensure correct labeling.

An extraction buffer containing SDS and 2-ME was developed to extract the food allergen ingredient soluble proteins from the processed foods with high efficiency (*37*). The extraction buffer (containing 0.5% SDS, 2% 2-ME) has been available for monitoring of food allergens (egg, milk, wheat, buckwheat, and peanut) in the Japanese official method (*34–36*). We examined the recoveries of the soybean soluble proteins from the model processed foods by the extraction using the extraction buffer (containing 0.5% SDS, 2% 2-ME) and the developed ELISA method. The developed ELISA method showed a low recovery in sausage,although it had high recoveries from the other processed foods. A soybean protein has been widely used in processed foods as a binder due to its high adsorption characteristics (*30, 31*). In particular, p34, which is known as an oil-body associated protein, is extracted together with an oil-

Table 4. Single Laboratory Validation Using the Developed ELISA and the Model Processed Foods[a]

model processed foods	mean (μg/g)	recovery (%)	intra-assay		interassay		R^2
			SD (μg/g)	CV (%)	SD (μg/g)	CV (%)	
rice gruel	9.76	97.6	0.56	2.2	1.83	7.5	0.9967
sausage	8.77	87.7	0.86	4.1	1.50	6.9	0.9997
sweet adzuki bean soup	9.87	98.7	1.01	4.2	1.79	7.3	0.9995
sweet potato cake	9.28	92.8	0.82	3.3	1.34	5.8	0.9994
tomato sauce	8.97	89.7	0.72	3.1	1.44	6.4	0.9990

[a] A single laboratory validation for the developed ELISA and five kinds of model processed foods (rice gruel, sausage, sweet adzuki bean soup, sweet potato cake, and tomato sauce). The intra-assay precision was calculated from eight replicates of extraction from the same model processed food. The interassay precision was calculated from triplicate analyses on eight different days. The model processed foods contain soybean soluble protein of 10 μg/g. Mean = average concentration (soybean soluble protein weight/food weight) of soybean soluble proteins. Recovery = mean/10 (μg/g). SD = standard deviation. CV = coefficient of variation. R^2 = dilution linearity (correlation coefficient).

Table 5. Detection of Soybean Soluble Proteins in the Commercial Foods[a]

food	label	mean (μg/g)
tofu	soybean	>20
soybean milk	soybean	>20
fried bean curd	soybean	>20
dried bean curd	soybean	>20
boiled soybean	soybean	>20
sausage	soybean protein	>20
cookie	soybean protein	>20
ham	soybean protein	>20
miso 1	soybean	>20
miso 2	soybean	>20
natto 1	soybean	1.15
natto 2	soybean	1.44
soy sauce 1	soybean	<0.19
soy sauce 2	soybean	<0.19
soybean oil	soybean	<0.19

[a] Detection of soybean soluble proteins in 15 kinds of commercial foods, labeled as containing soybean or soybean products on package. Labels = labels of package. Mean = average concentration (soybean soluble protein weight/food weight) of soybean soluble proteins.

pad from soybeans (*26, 27*). Therefore, we considered that the concentration of SDS and 2-ME was not sufficient to extract p34 because of strong binding with the lipid or the protein in the sausage. To improve the extraction efficiency of the soybean soluble proteins from sausage, we examined the concentration of SDS and 2-ME in the extraction buffer. When the concentration of SDS in the extraction buffer was raised, the recoveries from sausage increased. The extraction buffer containing 2% SDS showed the highest recoveries from sausage. However, the extraction buffer containing 4% SDS showed a rather lower recovery than the extraction buffer containing 2% SDS for all of the model processed foods. We speculate that the high concentration of SDS would decrease the antibody stability and the binding ability and/or decrease the extraction efficiency of the soybean soluble protein in the model processed foods. On the other hand, for the examination of the concentration of 2-ME in the extraction buffer, the recoveries from the model processed foods were not changed. These results suggest that the extraction buffer (2% SDS, 2% 2-ME) is most suitable for the extraction of soybean soluble proteins from the processed foods.

To evaluate the developed ELISA method for the determination of the soybean soluble protein in processed foods, a recovery study and intra- and interassays were tested using model processed foods. The results showed that this ELISA method has good accuracy and precision. The Japanese government has established the interlaboratory validation protocol in the official guidelines published in 2006 (*34*). The outline of the interlaboratory validation protocol is as follows: number of laboratories, >8; number of incurred samples (model processed foods), >5; number of dose levels, >1 including 10 μg/g (the corresponding allergen protein weight/food weight); recovery, 50−150%; RSD_R, <25%. These criteria are based on ISO5725 (JIS Z8402), which is almost the same as that of the AOAC (*39*), and ISO5725 includes a robustness analysis in the difference between them. In the guidelines, the initial extract solution and the extraction procedure from the allergen were specified and standardized. The immunoassay has to be satisfactory for recoveries (50−150%) using the Japanese validation criteria. We examined the performance of three kinds of commercial kits for the detection of soybean soluble proteins using the model processed foods. However, most of the results using the commercial kits gave <50% recoveries, although the others gave extraordinarily high recoveries (>225%) (data not shown). This result suggests that the developed ELISA is suitable for detection in the presence of the soybean soluble protein in processed foods. Further studies of the interlaboratory validation using the processed food models are now in progress. In addition, we examined the commercial processed foods labeled as containing soybean using the developed ELISA. The developed ELISA could determine the soybean soluble protein in all 10 kinds of commercial foods except for fermented foods (soy sauce, natto) and soybean oil. The soybean soluble proteins in these fermentation foods appeared to be degraded by the protease of microorganisms during the fermentation process. The soybean soluble proteins in the soybean oil could be removed from the oil during the refining process. Accordingly, we considered that it would be difficult to determine the soybean soluble proteins in the fermented foods and soybean oil, because the soybean soluble protein is degraded or removed during the processing process.

In conclusion, the developed ELISA method is a reliable, specific, and sensitive method for determination of the soybean soluble protein in processed foods. This method has been demonstrated to be suitable for the quantitative measurement of the specific soybean soluble protein in processed foods without food matrix effects. This proposed ELISA method is able to accurately monitor the labeling system in a reliable manner and can be useful for mandatory inspections based on the Japanese regulation.

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