JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

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Among food allergens of tree nuts, walnuts are a frequent cause of adverse food reactions in allergic patients. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of walnut soluble proteins in processed foods was developed. The sandwich ELISA is highly specific for walnut soluble proteins. The recovery ranged from 83.4 to 123%, whereas the intra- and interassay coefficients of variation were less than 8.8 and 7.2%, respectively. This study showed that the proposed method is a reliable tool for detection in the presence of hidden walnut proteins in processed foods.

KEYWORDS: Walnut; enzyme-linked immunosorbent assay (ELISA); 2S albumin; food allergy

INTRODUCTION

It is estimated that up to 8% of children and 2% of adults are affected by food allergies in industrialized countries (*1*−*3*). The clinical manifestation of food allergies varies from mild symptoms, such as oral allergy syndrome or mild urticaria, to severe anaphylactic reactions with fatal consequences.

Tree nuts are regarded as one of the most potent of all known food allergens and are often attributed to the cause of food anaphylaxis and anaphylaxis death. Walnut allergy is the most common tree nut allergy and can be observed in all age groups (*4*). In addition, the walnut allergy is extremely potent, inducing life-threatening allergic reactions similar to the peanut allergy (*5*−*7*). Walnuts have been used as a food ingredient for processed foods such as biscuits, breads, and ice cream. To avoid the onset of allergic reactions from walnuts, regulatory authorities want walnuts to be listed in food products. In 1999, the Joint FAO/WHO Codex Alimentary Commission agreed to label eight kinds of foods that contain ingredients known to be allergens that included tree nuts (*8, 9*). According to the recommendation, in the United States, European Union (EU), Canada, and Australia/New Zealand, walnut labeling has been mandatory. Also, in Japan, the ministry ordinance has recommended that walnuts would be appropriately labeled. Despite the labeling precautions, walnuts remain quite dangerous, as they are often present in commercial foods as a hidden allergen due to cross-contamination during food processing. In most factories, many different products are manufactured with various ingredients, as they sometimes even run on the same production line.

Even small amounts of walnuts can cause severe reactions, and thus strict management is required to minimize the potential harm. To practically and effectively manage walnuts in foods and food ingredients, especially hidden traces of walnuts, a simple and reliable detection method for hidden walnuts is necessary. To date, only a reported ELISA for the detection of walnuts (*10*) and a presentation of the validated ELISA of undeclared walnut residues in foods at the 2003 Institute of Food Technologists (IFT) annual meeting in Chicago are available, with the other option being a tedious DNA-based method (*11, 12*).

Most plant food allergens can be found among pathogenesis-related proteins, seed storage albumins and globulins, and α-amylase and protease inhibitors (*13*). The walnut allergens so far identified are *Juglans regia* Jug r 1, Jug r 2, Jug r 3, and Jug r 4, corresponding to walnut 2S albumin, vicilin-like protein (7S), PR-14(LTP), and legumin (11S), respectively (*14*). Among these walnuts allergens, some researchers have already shown that a major allergen in the English walnut is a 2S albumin seed storage protein, Jug r 1, that possesses important homologies in the amino acid sequence with other 2S albumin proteins from the Brazil nut, cottonseed, castor bean, and mustard (*15*−*17*). Jug r 1 is synthesized as a precursor protein and cleaved into a large subunit and small subunit, these being joined by disulfide bridges (*13*). In addition, the allergenicity is retained during

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10.1021/jf801550h CCC: $40.75
Published on Web 08/06/2008

heat processing and stable to digestion by the gastrointestinal tract (*15*). Accordingly, we considered that 2S albumin is suitable as a walnut marker protein for the determination of the walnut protein in processed foods possibly containing walnuts.

In the present study, we have developed the first highly specific and sensitive sandwich enzyme-linked immunosorbent assay (ELISA) to determine the walnut soluble protein in processed foods using the antibody to Jug r 1, walnut 2S albumin. We show that the detection method could be applicable to food-processed products and that the trace amount of walnut contained in commercial food products can be detected using the proposed sandwich ELISA.

MATERIALS AND METHODS

Food Materials. Walnut (Chandler, Haward, and Chinese walnut) and 10 kinds of tree nuts (almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan nut, peanut, pine nut, and mustard seed) were kindly provided by Tabata, Inc. (Chiba, Japan). Walnut (Miette) was purchased at Mitsuboshi Boeki Ltd. (Kobe, Japan). Walnut (Shinano walnut) was purchased at local supermarkets (Nagano, Japan) in 2006. All walnuts were roasted at 120 °C for 15 min using an oven (RCK-20BS2, Rinnnai Corp., Aichi, Japan). The other nuts were roasted using the oven (the roasting conditions are as follows, 155 °C for 15 min for almond, 120 °C for 5 min for cashew and macadamia, 120 °C for 15 min for pistachio, Brazil nut, and pecan nut, 155 °C for 15 min for hazelnut, and 165 °C for 20 min for peanut). Samples of the commercial processed foods and food ingredients were purchased at local supermarkets (Yokohama, Japan) in 2006.

Chemicals and Reagents. 2-Mercaptoethanol (2-ME), sodium dodecyl sulfate (SDS), polyoxyethylene-sorbitan monolaurate (Tween 20), and ammonium sulfate were supplied by Nakarai Tesque, Inc. (Kyoto, Japan) Albumin, from bovine serum (BSA), horseradish peroxidase (HRP), 3,3′5,5′-tetramethylbenzidine (TMB), and acetone were provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Toyobo Co., Ltd. (Osaka, Japan), Moss, Inc. (Maryland), and Kanto Chemical Co., Inc. (Tokyo, Japan). In addition, the following buffers were prepared for the experiment: 120 mM Tris-HCl (pH 7.4) containing 0.1% (w/v) BSA and 0.05% (v/v) Tween 20 (buffer A); and 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 0.05% (v/v) Tween 20 (buffer B).

Purification of Walnut 2S Albumin Protein (W2Sal). The walnuts (Chandler) were ground in a mill and defatted with acetone (1:10 w/v) with stirring for 2 h and then followed by paper filtration. The procedures of defatting and filtration were repeated three times before the walnuts were left to dry overnight. Two hundred and forty grams of defatted walnut flour was obtained from 957 g of raw walnuts. The defatted walnut flour was then extracted with 20 volumes of 20 mM Tris-HCl (pH 8.4) with stirring overnight followed by two centrifugations (first, 3000*g*, 20 min; second, 12000*g*, 20 min). Solid ammonium sulfate was added to the combined supernatants to 90% saturation (662 g of solid ammonium sulfate to 1 L of supernatant). The precipitated proteins were centrifuged and resuspended in 20 mM Tris-HCl (pH 8.4) buffer containing 2 M ammonium sulfate.

The resuspended walnut extract was subjected to hydrophobic chromatography on a HiTrap Phenyl Sepharose HP column (GE Healthcare U.K. Ltd., Buckinghamshire, U.K.) equilibrated with 20 mM Tris-HCl (pH 8.4) with 2 M ammonium sulfate and eluted with 0.8 M solid ammonium sulfate in 20 mM Tris-HCl (pH 8.4) buffer at the flow rate of 2 mL/min. The collected fractions containing W2Sal were desalted in 20 mM Tris-HCl (pH 8.4) buffer using a porous molecular membrane tubing (Centriplus YM-3, 3000 molecular weight, Millipore Corp., Billerica, MA). The desalted fraction containing W2Sal was loaded onto a cationic exchange column, which used the methacrylate polymer as a packing matrix (Fractogel EMD SO_3-650 (M), Merck, Darmstadt, Germany), equilibrated with 20 mM Tris-HCl (pH 8.4) and eluted with a salt gradient from 0 to 1 M NaCl, at the flow rate of 20 mL/min. The collected fractions were denatured by final concentration 1% (w/v) SDS and 4% (v/v) 2-ME. The denatured fractions were desalted using gel exclusive column chromatography (Sephadex G-25, GE Healthcare U.K. Ltd.) and PBS containing 1 mM EDTA and 1 mM 2-ME as the eluent. Sixty-two milligrams of W2Sal was purified from 233 g of defatted walnut (Chandler). All experiments were performed at 20−25 °C.

Preparation of the Antibody to Purified W2Sal. The rabbit antiserum against W2Sal was produced by immunization of Japanese white rabbits using purified W2Sal solution in Freund's complete adjuvant (containing 5 mg/mL, 2S albumin, and 1 mM 2-ME). The injections were repeated six times at the appropriate intervals (14 days). Whole blood was collected, and the serum was separated. The denatured 2S albumin specific polyclonal antibodies were purified from the serum using a 1 mL HiTrap NHS-activated column (GE Healthcare U.K., Ltd.), in which 10 mg of the denatured 2S albumin was fixed. The obtained antibodies were examined for the construction of the ELISA for detection of the walnut soluble protein.

SDS−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples were diluted 1:1 with sample buffer (4% w/v SDS, 28% v/v glycerol, 0.24 mg/mL bromphenol blue, with or without 20% v/v 2-ME). In reducing conditions, the samples were boiled for 5 min. Polyacrylamide gels and molecular weight standards used were Nupage Novex Bis-Tris Mini Gels (Invitrogen, Carlsbad, CA) and SeeBlue Plus2 Prestained Standard (Invitrogen). Electrophoresis was run at constant voltage (200 V) for 40 min using STC-808 (TEFCO, Tokyo, Japan) in reducing and nonreducing conditions. The gels were stained with Rapid CBB KANTO (Kanto Chemical Co., Inc., Tokyo, Japan).

Amino Acid Sequence. Purified W2Sal was subjected to SDS-PAGE and transferred onto a PVDF membrane (Amersham Hybond-P, GE Healthcare U.K., Ltd.) by a trans-blot sd semidry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). The N-terminal sequence was determined using blotted protein on a Procise 49X-HT protein sequencer (Applied Biosystems, Inc., Foster City, CA).

Sample Extraction. A Millser IFN-700G homogenizer (Iwatani International Corp., Osaka, Japan) was used to homogenize the food samples. The samples were treated with the Millser a few times for 30 s to homogeneity. Nineteen milliliters of buffer A containing 0.5% (w/v) SDS and 2% (v/v) 2-ME was added to 1 g of a homogenized sample. The sample was then shaken at 90−110 rpm over 12 h at 25 °C for extraction. After confirmation of sample pH at around 6.0−8.0 with pH paper, the sample was centrifuged at 3000*g* for 20 min, and then the supernatant was filtered through 5A filter paper (Advantec Toyo Kaisha, Ltd., Tokyo, Japan) to obtain the extract. As needed, the obtained extracts were analyzed with a 2D Quant Protein Assay Kit (GE Healthcare U.K. Ltd.). The sample extract diluted 20-fold with buffer A was used for ELISA, and in the case of more dilution, ab20-fold diluted sample extract was diluted with buffer A containing 0.025% (w/v) SDS and 0.1% (v/v) 2-ME.

Preparation of Calibration Standard Solution. For the determination of walnut protein using the ELISA, the preparation of a calibration standard solution is necessary. The walnuts (Chandler) were ground in a mill, and 0.4 g of walnuts was defatted with 10 mL of acetone with stirring for 1 min by vortex mixer. The solution was centrifuged at 10000*g* for 30 min, and then pellets were collected. The procedure of the defatting was repeated three times, and defatted walnut powder was dried for 24 h in the air. Twenty milliliters of buffer A containing 0.5% (w/v) SDS and 2% (v/v) 2-ME was added to 0.2 g of the defatted walnut powder. The mixture was then shaken at 90−110 rpm for 16 h at 25 °C 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 2D Quant Protein Assay Kit (GE Healthcare U.K. Ltd.). The initial extract was diluted to 50 ng/mL using dilution buffer A (120 mM Tris-HCl, pH 7.4, containing 0.1% w/v BSA and 0.05% v/v Tween 20) and stored as the calibration standard solution for the ELISA. The prepared calibration standard solutions were stored at 4 °C.

ELISA. A microtiter plate (F8 Maxisorp Nunc-Immuno module, Nunc Co.) was coated at 25 °C for 2 h with the prepared specific polyclonal antibody to anti-2S-albumin (100 μL of 2.5 μg/mL antibody protein solution in 50 mM sodium carbonate, pH 9.6), and the plate was blocked at 25 °C for 2 h with the blocking buffer (20 mM Tris,

Table 1. Recipes and Ingredients of Model Processed Foods

model processed food	content of walnut soluble protein	ingredients	cooking method
chicken meatballs	10 μg/g	chicken, lard, potato, starch, sugar	chicken meat was ground, added to the other materials, and kneaded
rice porridge	10 μg/g	rice	rice was cooked in a rice cooker
bread	10 μg/g	wheat flour, shortening, sugar, yeast, salt, skim milk	all materials were kneaded, leavened, and baked at 200 °C for 20 min
sponge cake	10 μg/g	wheat flour, egg, sugar, emulsifiable fat	all materials were kneaded and baked at 180 °C for 30 min
orange juice	10 μg/g	orange concentrate, sugar, citric acid	all materials were mixed, homogenized, and buffered to pH 4.5 with citric acid; the mixture was divided into cans and heated at 90 °C for 10 min
jelly	10 μg/g	sugar, agar, citric acid, sodium citrate, muscut flavaring	all material were mixed, heated at 90 °C for 10 min, and divided into cans
biscuit	10 μg/g	wheat flour, shortening, sugar, salt, bicarbonate, dihydroxysuccunic acid, lecithin, protease	all materials were kneaded and molded; the dough was baked at 240 °C for 8.5 min

pH 7.4, 150 mM NaCl, 0.05% v/v Tween 20, 0.1% w/v BSA) described in the previous study (*18*). The blocking buffer was removed, followed by drying of the plate. The diluted food sample and the walnut standard solution were added to the plate (100 μL/well) and incubated for 1 h at 25 °C. After six washings, the anti-2S-albumin antibody labeled with the HRP using the method described in the previous literature (*19*) as diluted with buffer B containing 1% (w/v) BSA was added to the ELISA plate at 100 μL/well and allowed to stand for 30 min for the secondary reaction. After six washings, TMB (100 μL/well) was added, and the enzyme reaction was allowed to run at 25 °C for exactly 10 min. The reaction was stopped by the addition of 100 μL/well of 0.5 M H_2SO_4. The absorbance was measured at 450 nm, the dominant wavelength, and at 620 nm, the subdominant wavelength. The amount of walnut protein was calculated using the calibration standard curve of walnut protein based on 2S albumin assay as a walnut marker protein. All experiments were performed in duplicate.

Preparation of Model Processed Foods. The defatted walnut powder was mixed with raw ingredients to prepare the processed food items containing the walnut protein at 10 μg/g (walnut soluble protein weight/food weight). Eighteen milligrams of the defatted walnut powder was added into 1 kg of food ingredient mixing sample. Chicken meatballs, rice porridge, bread, sponge cake, orange juice, jelly, and biscuits were prepared as model processed foods. **Table 1** shows the recipes and ingredients of model processed foods.

The chicken meatballs were made of white meat of chicken, lard, potato starch, sugar, and defatted walnut powder. Lard, potato starch, sugar, and defatted walnut powder were added to ground white meat of chicken and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into casings. These were stored at −20 °C before use.

The rice porridge was made of rice and water. The rice and defatted walnut powder were cooked in a rice cooker and stored at −20 °C before use.

The bread was made by the Oriental Yeast Co., Ltd. It was made of wheat flower, sugar, shortening, yeast, salt, skim milk, and water. All of the raw materials and the defatted walnut powder were kneaded, leavened, and baked at 200 °C for 20 min. These were stored at −40 °C before use.

The sponge cake was made by the Oriental Yeast Co., Ltd. It was made of wheat flower, sugar, whole egg, emulsifiable fat, and water. All of the raw materials and the defatted walnut powder were kneaded and baked at 180 °C for 30 min. These were stored at −40 °C before use.

The orange juice was made of orange concentrate, sugar, citric acid, and water. The orange concentrate, sugar, water and defatted walnut powder were mixed and homogenized. After the homogenate was buffered to pH 4.5 with citric acid, it was divided into cans and heated at 90 °C for 10 min. The cooked orange juice was stored at −40 °C before use.

The jelly was made of sugar, agar, citric acid, sodium citrate, muscat flavoring, and water. The raw materials and the defatted walnut powder were then mixed. The mixture was heated to 90 °C and divided into cans. After the canned jelly had been hardened at 15 °C for 3 h, it was stored at −40 °C before use.

The biscuits were made of wheat flower, sugar, shortening, bicarbonic acid, salt, bicarbonate, dihydroxysuccinic acid, lecithin, and proteinase. All of the raw materials and the defatted walnut powder were kneaded, molded, and baked at 240 °C for 8.5 min. These were stored at −40 °C before use.

Single Laboratory Validation. For the determination of the intra-assay precision, the mean coefficients of variation (CVs) in the seven kinds of model processed foods were based on six replicates of the extraction from the same model processed food. The interassay precision was determined as the mean CV on the basis of duplicate analyses on four different days. 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.

Statistical Analysis. Statistical comparisons were performed by using Student's *t* test or Scheffe's method after an analysis of variances (ANOVA). In all cases, probability (*P*) values below 0.05 were considered to be significant. 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 (*20*). Twelve test portions of each sample material were analyzed using the proposal method. The obtained concentrations of walnut soluble 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

Purification of W2Sal. W2Sal was purified from the defatted walnut powder (Chandler) using the method described under Materials and Methods. **Figure 1** shows the analysis of the purified W2Sal using the SDS-PAGE method in reduced or nonreduced conditions. In nonreduced conditions, only a single band with a molecular mass of 14 kDa was observed in lane 1. In reduced conditions, two bands with molecular masses of 7

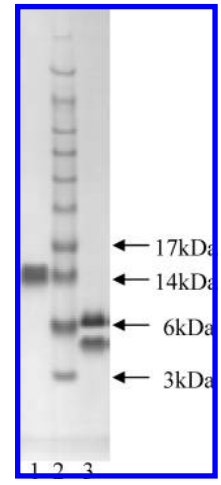

Figure 1. SDS-PAGE of the purified W2Sal. Lanes: 1, nonreduced purified W2Sal; 2, molecular weight marker; 3, reduced purified W2Sal. Purified W2Sal (1.7 μg) was applied to lanes 1 and 3. Arrowheads indicate the molecular weight of molecular weight markers.

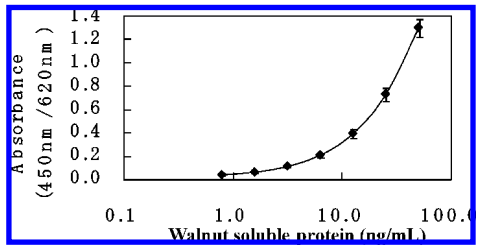

Figure 2. Representative calibration curve of the developed ELISA. The calibration curve shows the mean value of eight experiments and the standard deviation in each point of the curve. Concentration of standard solution = 0.78 ng/mL (0.31 μg/g), 1.56 ng/mL (0.62 μg/g), 3.13 ng/mL (1.25 μg/g), 6.25 ng/mL (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 2. Reactivity of Five Walnut Varieties Using the Developed ELISA[a]

walnut species	place of origin	protein (μg/g of walnut wt)	ratio to Chandler (raw) (%)
Chandler (raw)	California	134000	100
Chandler (roasted)	California	146000	109
Howard (raw)	California	144000	107
Howard (roasted)	California	168000	125
Miette (raw)	Grenoble, France	120000	90.0
Miette (roasted)	Grenoble, France	110000	82.1
Chinese walnut (raw)	China	115000	85.8
Chinese walnut (roasted)	China	121000	90.3
Shinano walnut (raw)	Nagano, Japan	138000	103
Shinano walnut (roasted)	Nagano, Japan	154000	115

[a] The walnut extracts were prepared according to the procedure given in Materials and Methods. The mean shows the average value (walnut soluble protein weight/walnut weight) of the walnut soluble proteins.

and 5 kDa were observed in lane 3. Two protein bands were sequenced as described under Materials and Methods. Only N-terminal amino acid sequencing of the 5 kDa protein was successful for analyzing the sequence of seven amino acids (PRRRGEG). The N-terminal amino acid sequence of the 5 kDa protein was determined and compared with the published sequences. The amino N-terminal amino acid sequences were consistent with that of English walnut (*J. regia*) 2S albumin (*14*). These results indicated that the purified walnut protein was W2Sal.

Construction of Sandwich ELISA. We established the ELISA for the detection of walnut soluble proteins, using anti-2S-albumin rabbit serum. **Figure 2** shows representative calibration curves of the developed sandwich ELISA for the determination of the walnut standard calibrators. All calibrators were measured in values of eight experiments. The dose response curves were obtained in the assay. The model that best describes the relationship between the absorbance and concentration of the antigen is a four-parameter logistic curve. The LOD and LOQ were found to be 0.39 and 0.78 ng/mL whole walnut proteins, equivalent to 0.156 and 0.312 μg/g in foods, respectively.

Reactivity of Five Walnut Varieties. We examined the walnut soluble protein amounts from raw and roasted walnuts using the developed ELISA for the values after four experiments. Consequently, walnut soluble protein amounts of both raw and roasted walnuts are not significantly different (P = 0.615). We consider that the results might be due to the extraction buffer containing SDS and 2-ME. As shown in **Table 2**, the various raw and roasted walnuts were examined to test the reactivity and specificity using the established ELISA. The activity of five raw walnut varieties ranged from 85.8 to 107% [ratio to Chandler (raw)]. On the other hand, the activity of the five roasted walnut varieties also showed a reactivity of 82.1−125% [ratio to Chandler (raw)]. These results suggest that the established ELISA has a reactivity similar to the major walnut varieties regardless of the roast processing or walnut variety.

Table 3. Cross-Reactivity of Various Tree Nuts Using the Developed ELISA[a]

nut sample	mean (μg/g)	nut sample	mean (μg/g)
pecan (raw)	100	cashew (raw)	<0.31
pecan (roasted)	100	cashew (roasted)	<0.31
hazelnut (raw)	1.0	pistachio (raw)	<0.31
hazelnut (roasted)	1.1	pistachio (roasted)	<0.31
peanut (raw)	<0.31	Brazil nut (raw)	<0.31
peanut (roasted)	<0.31	Brazil nut (roasted)	<0.31
macadamia nut (raw)	<0.31	pine nut (raw)	<0.31
macadamia nut (roasted)	<0.31	mustard seed (raw)	<0.31
almond (raw)	<0.31		
almond (roasted)	<0.31		

[a] The nut extracts were prepared according to the procedure given under Materials and Methods. Mean shows the average concentration (micrograms of walnut soluble protein weight per gram of food weight) of the walnut soluble proteins.

Cross-Reactivity to Various Tree Nuts and Food Ingredients. As shown in **Table 3**, the cross-reactivity to other tree nuts was investigated using 10 kinds of tree nuts. When we tested 1 g of tree nut samples and food ingredients, the values of both raw and roasted pecans, which belong to the walnut family, were 100 μg/g (corresponding to 0.07% of raw Chandler value, 134000 μg/g) and the values of hazelnut, raw and roasted, were 1.0 and 1.1 μg/g, respectively. We examined the soluble protein amounts from raw and roasted pecans using the developed ELISA for the values after three experiments. Consequently, soluble protein amounts of raw and roasted pecans are not significantly different (P = 0.581). Similar results are also obtained for the soluble protein amounts from raw and roasted hazelnuts (P = 0.101). The values of the other tree nuts were less than the LOQ (0.31 μg/g) of the developed ELISA. In addition, the values for 151 food ingredients, such as grains, crustaceans, eggs, fish, soybeans, milk, and sesame seeds, were less than the LOQ (0.31 μg/g) of the developed ELISA (data not shown). These results indicate that the developed ELISA has a high specificity, although the established ELISA has slight cross-reactivity to pecans and hazelnuts at levels greater than LOD.

Quantification and Validation of Walnut Soluble Protein in Model Processed Foods Using the Established ELISA. To examine the applicability of the established ELISA in processed foods, the walnut soluble proteins in seven model processed food samples were determined using the established ELISA. As shown in **Table 4**, the mean recoveries for all seven model processed food samples ranged from 83.4 to 123%. The interassay precision across all days was 0.7−8.8% CV for the seven model processed foods. The intra-assay precision for the seven model processed foods was 2.5−7.2% CV.

Application to Commercial Food Products. Forty-one various commercial food samples were analyzed by the established ELISA. Each commercial food was homogenized, and the extracts were prepared according to the extraction procedure described under Materials and Methods. As shown in **Table 5**, the 12 commercial foods with walnuts listed as ingredients were clearly detected. In contrast, 29 products without any walnut labeling on the ingredients list were detected to be <0.3 μg/g (μg of walnut soluble protein/g of food sample weight); however, three commercial foods labeled as having pecans on the ingredients list showed very low levels (2.9−8.2 μg of walnut soluble protein/g, walnut soluble protein), although the composition of pecans appears to be major in those commercial

Table 4. Quantification of Walnut Proteins in Model Processed Foods Using the Established Sandwich ELISA[a]

model processed food	mean (μg/g)	recovery (%)	intra-assay (CV %)	interassay (CV %)
rice porridge	11.5	115	4.3	3.1
chicken meatballs	12.0	120	8.8	7.2
bread	12.3	123	0.7	3.2
sponge cake	10.0	100	6.1	2.8
biscuits	8.3	83	4.8	4.7
jelly	10.2	102	6.7	2.5
orange juice	10.1	101	4.3	5.0

[a] The intra-assay precision was calculated from six replicates of extraction from the same model processed food. The interassay precision was calculated by duplicate analysis on four different days. The model processed foods contained 0 μg/g of walnut soluble proteins. Mean = concentration of walnut soluble proteins. Recovery = mean/10 μg/g $\times$ 100. CV % = coefficient of variation.

foods. There were three positives in the no-declaration samples on the ingredients list and no false negatives from the declaration samples analyzed in this study. These results suggest that the established ELISA could be applicable for the determination of walnut proteins in processed foods.

DISCUSSION

We established the sandwich ELISA for the detection of walnut proteins. It is an important examination to verify the utility of the developed ELISA regardless of the source of walnut variety. In **Table 2** we have shown that the developed ELISA has a similar reactivity to the major walnut varieties regardless of the roasting processing or walnut variety.

It is also important that there is no cross-reactivity of the detection method because cross-reactivity to food materials can cause a false-positive result. The reactivity and specificity of the established ELISA were examined using extracts from various raw tree nuts, roasted tree nuts, and 151 food ingredients. As shown in **Table 3**, the developed ELISA had no cross-reactivity to any of the examined tree nuts or 151 food ingredients except for pecans and hazelnuts. The developed ELISA had slight cross-reactivity to pecans and hazelnuts at a level greater than the LOD. These results suggest that this method could be specific to the walnut protein.

To evaluate the established ELISA for the determination of the walnut soluble proteins in processed foods, a recovery study and intra- and interassays were examined using seven model processed food samples. The LOD of the established ELISA for the sample solution was 0.39 ng/mL, equivalent to 0.156 μg of walnut soluble protein/g of food sample weight. In Japan, the threshold of food allergens in processed foods for labeling has been set at levels of 10 μg/g (the corresponding allergenic ingredient soluble protein weight/food sample weight) (*21*). Therefore, it is necessary for the detection method to be able to accurately detect the corresponding allergenic walnut protein ingredient at this level. The present results showed that the established ELISA is sensitive and has good accuracy and precision. The Japanese government, Ministry of Health, Labor and Welfare of Japan (MHLW), has also established the interlaboratory validation protocol in the official guidelines published in 2006 (*20*). 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 AOAC (*22*). The immunoassay has to be satisfactory for the recoveries (50−150%) using the Japanese validation criteria. The present results show that the proposed ELISA is suitable for detection in the presence of hidden walnut soluble protein in processed foods because of satisfactory recoveries and specificity according to the Japanese validation criteria, although the present validation studies were conducted in-house. Further studies of the interlaboratory validation using the processed food models are now in progress.

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

commercial food	declaration on label	mean (μg/g)
caramel nuts	walnut	14000
banana crunch	walnut	14700
fruit cake	walnut	299
tart	walnut	22200
butter cake with walnut	walnut	21300
biscuit with nuts	walnut, almond	4920
biscuit with caramel nut 1	walnut, hazelnut	28800
biscuit with caramel nut 2	walnut	38900
bread with walnut 1	walnut	12700
bread with walnut 2	walnut	9520
bread with walnut 3	walnut	15600
walnut butter	walnut	>20
stick chocolate 1	pecan	4.2
caramel	pecan	8.2
pecan crunch	pecan	2.9
chocolate spread	hazelnut	<0.31
stick biscuit with chocolate	hazelnut, almond	<0.31
chocolate with almond product	almond	<0.31
chocolate balls with whole almond 1	almond	<0.31
chocolate balls with whole almond 2	almond	<0.31
chocolate balls with whole almond 3	almond	<0.31
almond crunch 1	almond	<0.31
almond crunch 2	almond	<0.31
stick biscuit with chocolate	almond, macadamia nut	<0.31
stick biscuit with chocolate	almond, hazelnut	<0.31
stick chocolate 2	almond	<0.31
stick chocolate 3	almond, hazelnut	<0.31
cheese	almond	<0.31
pretzel with chocolate 1	almond	<0.31
breakfast cereal 1	almond, coconut	<0.31
chocolate balls with whole peanut	peanut	<0.31
peanut butter	peanut	<0.31
bread with peanut cream	peanut	<0.31
chocolate with whole macadamia	macadamia nut	<0.31
chocolate balls with macadamia1	macadamia nut	<0.31
chocolate balls with macadamia2	macadamia nut	<0.31
chocolate with cashew and almond	cashew, peanut	<0.31
chocolate with cashew	cashew	<0.31
caramel	Brazil nut	<0.31
pretzel with chocolate 2	coconut	<0.31
breakfast cereal 2	coconut	<0.31

[a] Detection of walnut soluble proteins in 41 kinds of commercial foods. Declaration of label = label of package. Mean is the average concentration (walnut soluble protein wt/food wt) of the walnut soluble proteins.

Furthermore, we examined 41 commercial processed foods labeled as containing tree nuts using the developed ELISA. The developed ELISA could detect walnut protein at >20 μg/g in the 12 commercial foods in accordance with the labeling of walnuts on the ingredients list and detected the walnut protein at <0.3 μg/kg in the 29 commercial foods without the labeling of walnuts on the ingredients list. These results demonstrate that the developed method would be applicable for processed foods to ensure the validity of the labeling of walnuts. We are considering future studies to assess the application of the processed foods containing a high percentage of fat and milk proteins, such as ice cream and salad dressing.

In conclusion, we have developed a rapid, specific, and sensitive immunoassay system to detect walnut soluble proteins in foods processed by heating and pressurization. This ELISA is shown to have acceptable accuracy and precision and few

false positives or false negatives. This method has been demonstrated to be suitable for the quantitative measurement of specific walnut proteins in processed foods.

ACKNOWLEDGMENT

We thank Dr. N. Sato and Dr. Y. Nakao (Oriental Yeast Co., Ltd.) for providing the model processed foods (bread and sponge cake).

LITERATURE CITED

(1) Jansen, J. J.; Kardinaal, A. F.; Huijbers, G.; Vlieg-Boerstra, B. J.; Martens, B. P.; Ockhuizen, T. Prevalence of food allergy and intolerance in the adult Dutch population. *J. Allergy Clin. Immunol.* **1994**, *93*, 446–456.

(2) Bousquest, J.; Björkstén, B.; Bruijnzeel-Koomen, C.; Hugget, A.; Ortolani, C.; Warner, J. O.; Smith, M. Scientific criteria and the selection of allergenic foods for product labelling. *Allergy* **1998**, *53*, 3–21.

(3) Ebisawa, M.; Ikematsu, K.; Imai, T.; Tachimoto, H. Food allergy in Japan. *J. World Allergy Org.* **2003**, *15*, 214–217.

(4) Bock, S. A.; Munoz-Furlong, A.; Sampson, H. A. Fatalities due to anaphylaxis reaction to foods. *J. Allergy Clin. Immunol.* **2001**, *107*, 191–193.

(5) Pumphrey, R. S. H. Lessons for management of anaphylaxis from a study of fatal reaction. *Clin. Exp. Allergy* **2000**, *30*, 1144–1150.

(6) Pumphrey, R. S. H.; Roberts, I. S. D. Postmortem findings after fatal anaphylactic reactions. *J. Allergy Clin. Pathol.* **2000**, *53*, 273–276.

(7) Clark, A. T.; Ewan, P. W. Interpretation of tests for nut allergy in one thousand patients, in relation to allergy or tolerance. *Clin. Exp. Allergy* **2003**, *33* (8), 1041–1045.

(8) Food and Agriculture Organization of the United Nations, World Health Organization (FAO), Report of FAO technical consultation on food allergens; Rome, Italy, Nov 13−14, 1995.

(9) Hefle, S. L.; Nordlee, J. A.; Taylor, S. L. Allergenic foods. *Crit. Rev. Food Sci. Nutr.* **1996**, *36* (suppl.), 69–89.

(10) Su, M.; Venkatachalam, M.; Teuber, S. S.; Roux, K. H.; Sathe, S. K. Impact of γ-irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins. *J. Sci. Food Agric.* **2004**, *84*, 1119–1125.

(11) Brezna, B.; Hudecova, L.; Kuchta, T. A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food. *Eur. Food Res. Technol.* **2006**, *223*, 373–377.

(12) Yano, T.; Sakai, Y.; Uchida, K.; Nakao, Y.; Ishihata, K.; Nakano, S.; Yamada, T.; Sakai, S.; Urisu, A.; Akiyama, H.; Maitani, T. Detection of walnut residues in processed foods by polymerase chain reaction. *Biosci., Biotechnol., Biochem.* **2007**, *71* (7), 1793–1796.

(13) Robotham, J. M.; Teuber, S. S.; Sathe, S. K.; Roux, K. H. Linear IgE epitope mapping of the English walnut (*Juglans regia*) major food allergen, Jug r 1. *J. Allergy Clin. Immunol.* **2002**, *109* (1), 143–149.

(14) Crespo, J. F.; James, J. M.; Fernandez-Rodriguez, C.; Rodriguez, J. Food allergy: nuts and tree nuts. *Br. J. Nutr.* **2006**, *96*, 95–102.

(15) Teuber, S. S.; Dandekar, A. M.; Peterson, W. R.; Sellers, C. L. Cloning and sequencing of a gene encording a 2S albumin seed storage protein precausor from English walnut (*Juglans regia*)—a food allergen. *J. Allergy Clin. Immunol.* **1998**, *101*, 807–814.

(16) Youle, R. J.; Huang, A. H. C. Occurrence of low molecular weight and high cysteine containing albumin storage proteins in oilseeds of diverse species. *Am. J. Bot.* **1981**, *68*, 44–48.

(17) Bock, S. A.; Atkins, F. M. The natural history of peanut allergy. *J. Allergy Clin. Immunol.* **1989**, *83*, 1074–1078.

(18) Watanabe, Y.; Aburatani, K.; Mizumura, T.; Sakai, M.; Muraoka, S.; Mamegoshi, S.; Honjoh, T. Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent. *J. Immunol. Methods* **2005**, *300*, 115–123.

(19) Yoshitake, S.; Imagawa, M.; Ishikawa, E.; Niitsu, Y.; Urushizaki, I.; Nishimura, M.; Kanazawa, R.; Kurosaki, H.; Tachibana, S.; Nakazawa, N. Mild and efficient conjugation of rabbit Fab′ and horseradish peroxidase using a maleimide compound and its use for enzyme immunoassay. *J. Biochem.* **1982**, *92* (5), 1413.

(20) Thompson, M.; Wood, R. International harmonaized protocol for proficiency testing of analytical laboratories. *J. AOAC Int.* **1993**, *76*, 926–940.

(21) Ministry of Health, Labor and Welfare of Japan, Notification 106001, Nov 2002.

(22) AOAC Int. Guidelines for collaborative study procedures to validate characteristics of a method of analysis. Appendix D. *Official Methods of Analysis of AOAC International*, 18th ed.; AOAC International: Washington, DC, 2005.

Received for review May 19, 2008. Revised manuscript received July 11, 2008. Accepted July 11, 2008. This study was supported by Health and Labor Sciences Research Grants for Research from the Ministry of Health, Labor, and Welfare of Japan.

JF801550H