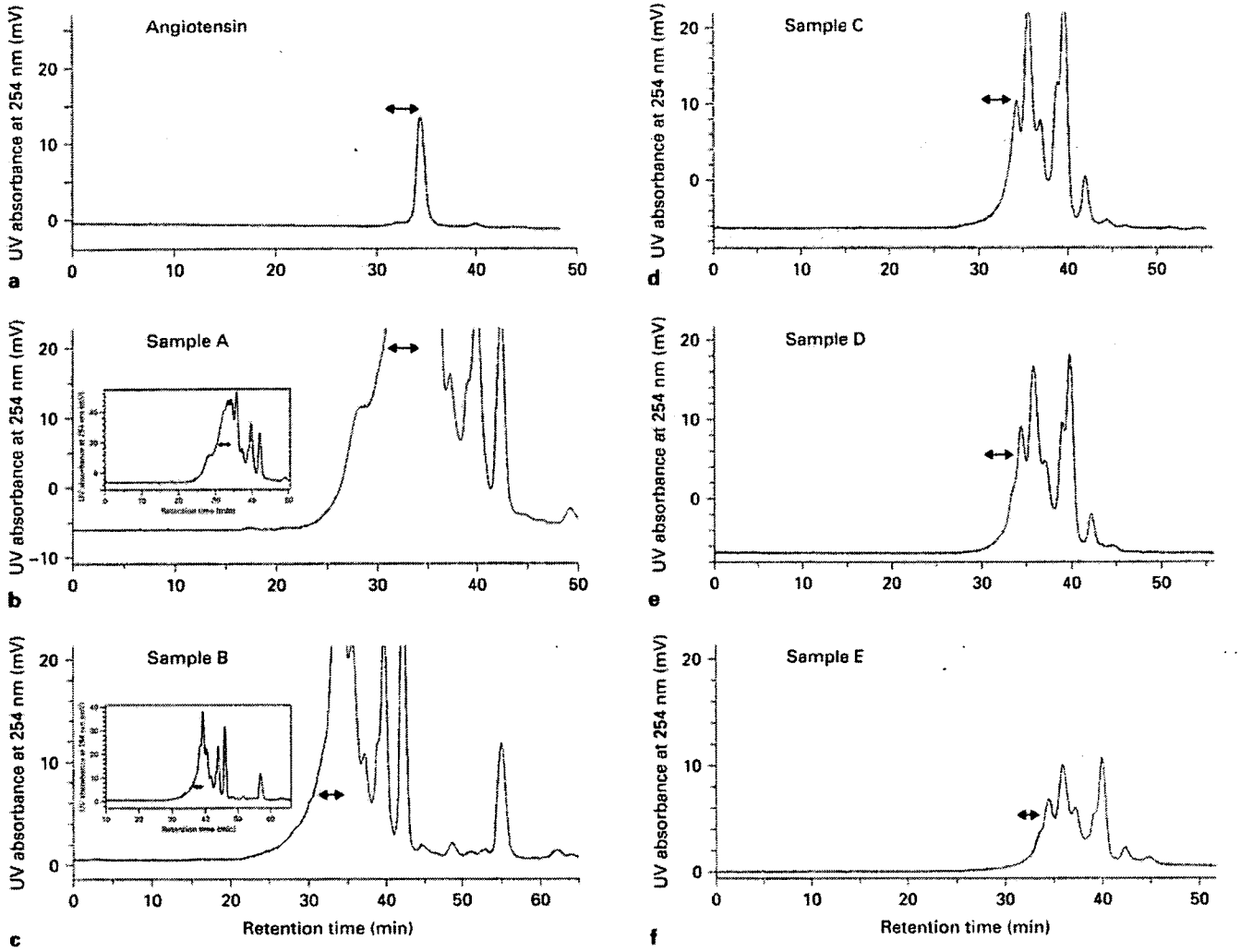
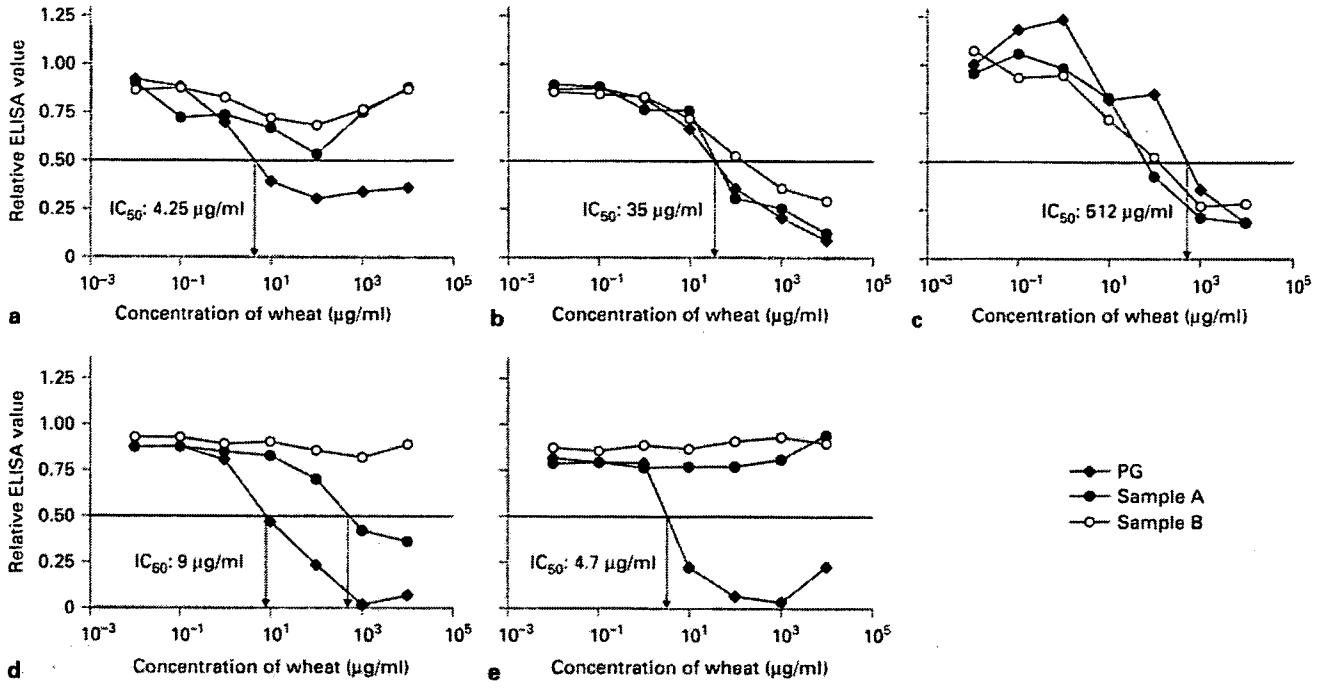


Fig. 2. Chromatograms from SEC of samples A–E. Angiotensin (50 μg/ml) (**a**), enzymatic hydrolysates (**b**, **c**), acidic hydrolysates (**d**–**f**). Two-sided arrows represent the range between the retention time of angiotensin (1,050 Da) and the retention time of bacitracin (1,450 Da). SEC was performed using the Superdex™ Peptide HR 10/30 (10 mm ID × 310 mm; Amersham Pharmacia Biotech AB). The mobile phase was PBS with a flow rate of 0.5 ml/min. Angiotensin was eluted at a retention time of 35 min.

wheat protein (fig. 3, 4). With 0.1 mg/ml of a pancreatic hydrolysate of wheat gluten as the positive control [12, 13], the ELISA inhibition of the pancreatic hydrolysate of the wheat gluten reached about 60–70% with 1,000 μg/ml using the three patient sera (fig. 3). The concentrations of the pancreatic hydrolysates of the wheat gluten for the IC_{50} were 4.25 μg/ml (serum I), 35 μg/ml (serum II), 512 μg/ml (serum III), 7.8 μg/ml (serum IV) and 2.4 μg/ml (serum V), respectively. These results suggest that the five patient sera should be judged to be appropriate for the IgE antibody to wheat in this assay. As shown in figure 3, the wheat protein enzymatic hydrolysates clearly inhibited the binding ability between the IgE antibodies of the sera II–IV and wheat proteins, though they did not clearly inhibit the binding ability of those in sera I and V. On the contrary, as shown in figure 4, only sample D of the wheat protein acid hydrolysates appeared to inhibit the binding ability between the IgE antibodies in the patient sera and wheat proteins, when 10,000 μg/ml of wheat gluten acid hydrolysates were incubated with



3

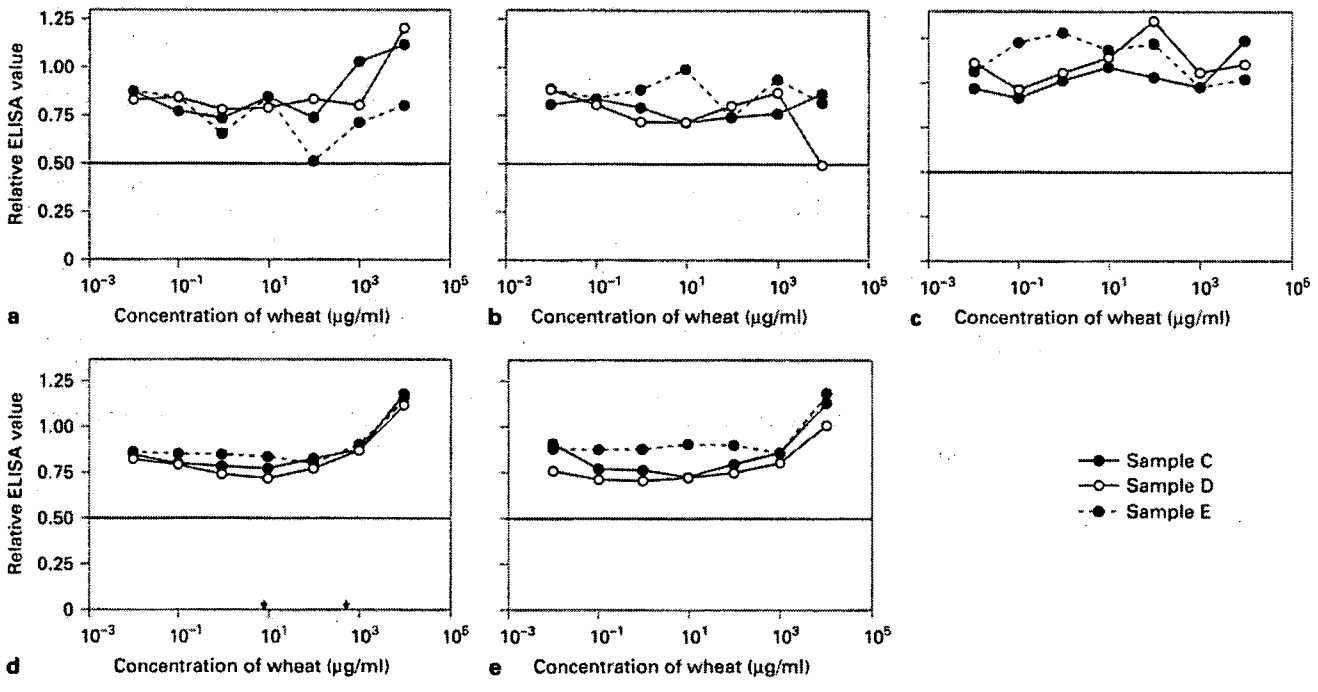


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Fig. 3. Inhibition ELISA of wheat protein acidic hydrolysate using five patient sera: serum I (**a**), serum II (**b**), serum III (**c**), serum IV (**d**) and serum V (**e**). The relative ELISA value was calculated as A/B, where A is the ELISA absorbance value of the preincubated test peptide sample solution and B is that of the control. PG = Pancreatic hydrolysate of wheat gluten.

Fig. 4. Inhibition ELISA of wheat protein enzymatic hydrolysate using five patient sera: serum I (**a**), serum II (**b**), serum III (**c**), serum IV (**d**) and serum V (**e**). The relative ELISA value was calculated as A/B, where A is the ELISA absorbance value of the preincubated test peptide sample solution and B is that of the control.

patient serum II. These results suggested that wheat protein enzymatic hydrolysates still retain a binding ability to the patient IgE antibody and could cause food allergies.

Discussion

Considering the profile analyses and the assessment of allergenicity of the wheat protein hydrolysates in this study, we presumed that the wheat protein enzymatic hydrolysates containing a high concentration of peptides with a mass greater than 1,050 Da could cause some food allergic reactions after their consumption because of the IgE binding ability. However, we also considered that the wheat protein acid hydrolysates might cause some food allergic reactions because of the presence of the binding ability to the patient IgE with very high concentrations of these hydrolysates in terms of the immediate type allergy, though the possibility of this occurrence appears to be extremely low.

A number of studies have suggested that peptide fragmentation generated from proteolytic digestion or hydrolysis of food allergens can elicit IgE responses in already sensitized individuals [14–16]. Maynard et al. [14] showed that 8 out of 19 exhibited a specific IgE response to different tryptic peptides among the sera from patients with cow's milk allergy. Peptide fragments resulting from the proteolysis of ovomucoid and ovalbumin were also reported to react with IgE from people allergic to eggs [15, 16]. However, it has been reported that there seems to be a size limitation on the ability of peptides to react with IgE of already sensitized individuals. Van Beresteijn et al. [17] tested the immunological properties of the hydrolysates of a whey protein concentrate and found that the minimal molecular mass to elicit immunogenicity and allergenicity appeared to be between 3,000 and 5,000 Da. The minimum size for a peptide to bridge two IgE molecules on the surface of mast cells to initiate degranulation was thought to be at least 3,000 Da [18]. Considering the binding properties of one IgE antibody and peptide, these studies appear to be consistent with our present findings.

To prevent allergenic reactions, allergic individuals should strictly avoid the consumption of allergenic foods. Therefore, the Joint FAO/WHO Codex Alimentary Commission Session recommended the labeling of eight kinds of foods including wheat. In Japan, the labeling system for allergenic food materials has already been enforced since April 2002. In the Japanese labeling system, the labeling for five food materials including wheat has been mandatory, and for twenty other food materials, it has been recommended. Foods containing wheat protein hydrolysates should also be labeled. However, some patients have been confused by the labeling of some processed foods, because they had been able to eat such foods previously. The present study suggests that if the wheat protein acid hydrolysates are degraded into fragments less than approximately 1,050 Da, they are less likely to elicit food allergic reactions related to the IgE mediated activation of the mast cells.

In conclusion, we demonstrated that the uptake of wheat protein enzymatic hydrolysates might still possibly cause food allergic reactions in patients allergic to wheat, and the processed foods containing them should be labeled. However, we have to consider whether the processed foods containing wheat protein acid hydrolysates should be labeled.

Acknowledgments

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References

1 Sampson HA: Update on food allergy. J Allergy Clin Immunol 2004;113: 805–819.

2 Maruyama N, Ichise K, Katsube T, Kishimoto T, Kawase S, Matsumura Y, Takeuchi Y, Sawada T, Utsumi S: Identification of major wheat allergens by means of the *Escherichia coli* expression system. Eur J Biochem 1998; 255:739–745.

3 Palosuo K, Alenius H, Varjonen E, Koivuluhta M, Mikkola J, Keskinen H, Kalkkinen N, Reunala T: A novel wheat gliadin as a cause of exercise-induced anaphylaxis. J Allergy Clin Immunol 1999;103:912–917.

4 Shewry PR, Tatham A S: The prolamin storage proteins of cereal seeds: structure and evolution. Biochem J 1990;267:1–12.

5 Tanabe S, Arai S, Watanabe M: Modification of wheat flour with bromelain and baking hypoallergenic bread with added ingredients. Biosci Biotechnol Biochem 1996;60:1269–1272.

6 Ikezawa Z, Tsubaki K, Yokota S: Effect of hypoallergenic wheat (HAW-A1) on atopic dermatitis (AD) with wheat allergy, and its antigenic analysis using sera from patients with AD. Jpn J Allergol 1994;43:679–688.

7 Watanabe M, Watanabe J, Sonoyama K, Tanabe S: Novel method for producing hypoallergenic wheat flour by enzymatic fragmentation of the constituent allergens and its application to food processing. Biosci Biotechnol Biochem 2000;64:2663–2667.

8 Yamada K, Urisu A, Kakami M, Koyama H, Tokuda R, Wada E, Kondo Y, Ando H, Morita Y, Torii S: IgE-binding activity to enzyme-digested ovomucoid distinguishes between patients with contact urticaria to egg with and without overt symptoms on ingestion. Allergy 2000;55:565–569.

9 Host A, Halken S: Hypoallergenic formulas – when, to whom and how long: after more than 15 years we know the right indication! Allergy 2004;59(suppl 78):45–52.

10 Schlichtherle-Cerny H, Amado R: Analysis of taste-active compounds in an enzymatic hydrolysate of deamidated wheat gluten. J Agric Food Chem 2002;50:1515–1522.

11 Tanabe S, Watanabe J, Oyama K, Fukushi E, Kawabata J, Arai S, Nakajima T, Watanabe M: Isolation and characterization of a novel polysaccharide as a possible allergen occurring in wheat flour. Biosci Biotechnol Biochem 2000; 64:1675–1680.

12 Tanabe S, Arai S, Yanagihara Y, Mita H, Takahashi K, Watanabe M: A major wheat allergen has a Gln-Gln-Gln-Pro-Pro motif identified as an IgE-binding epitope. Biochem Biophys Res Commun 1996;219:290–293.

13 Tanabe S, Tesaki S, Yanagihara Y, Mita H, Takahashi K, Arai S, Watanabe M: Inhibition of basophil histamine release by a haptenic peptide mixture prepared by chymotryptic hydrolysis of wheat flour. Biochem Biophys Res Commun 1996;223:492–495.

14 Maynard F, Jost R, Wal JM: Human IgE binding capacity of tryptic peptides from bovine alpha-lactalbumin. Int Arch Allergy Immunol 1997;113:478–488.

15 Kurisake J, Konishi Y, Kaminogawa S, Yamuchi K: Studies on the allergenic structure of hen ovomucoid by chemical and enzymic fragmentation. Agric Biol Chem 1981;45:879.

16 Elsayed S, Hammer AS, Kalvenes MB: Antigenic and allergenic determinants of ovalbumin. 1. Peptide mapping, cleavage at the methionyl peptide bonds and enzymic hydrolysis of native and carboxymethyl OA. Int Arch Allergy Appl Immunol 1986;79:101–107.

17 van Beresteijn EC, Peeters RA, Kaper J: Molecular mass distribution, immunological properties and nutritive value of whey protein hydrolysates. J Food Prot 1994;57:619–625.

18 Huby RDJ, Dearman RJ, Kimber I: Why are some proteins allergen? Toxicol Sci 2000;55: 235–246.

FOOD COMPOSITION AND ADDITIVES

Interlaboratory Evaluation of Two Enzyme-Linked Immunosorbent Assay Kits for the Detection of Egg, Milk, Wheat, Buckwheat, and Peanut in Foods

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The labeling of 5 major allergenic ingredients (egg, milk, wheat, buckwheat, and peanut) is mandatory in Japan, and 2 series of enzyme-linked immunosorbent assay (ELISA) kits have been established as official screening methods. However, these official methods have not provided the necessary sensitivity, due in part to poor extraction efficiency. To address this need, 2 novel ELISA kits have been developed: the FASTKIT ELISA Ver. II Series and the FASPEK Allergenic Substances Detection Kit. The new kit systems use an improved extraction buffer that can extract insoluble proteins produced by processing and feature new antibodies that bind to the denatured proteins extracted with the new extraction buffer. The analytical performances of the 2 new ELISA kit series were evaluated in an interlaboratory study. Ten laboratories participated in the study and determined the major allergenic ingredients contained in 5 types of model processed food. The 2 ELISAs displayed fairly good reproducibility and sufficient recovery.

The number of patients with food allergies in Japan continues to rise (1). The reason appears to be due to drastic changes in dietary habits in Japan. The most effective means of preventing allergic reactions to food is to avoid foods that contain allergens; it is therefore essential that patients with food allergies be able to obtain accurate information on food allergens contained in processed foods. The Japanese Ministry of Health, Labor and Welfare (MHLW) organized a Labeling Study Group to discuss an appropriate food labeling system. The study group submitted a report on their discussions in 2000, in response to which the MHLW decided that processed foods containing 5 major allergic ingredients (egg, milk, wheat, buckwheat, and peanut) should be labeled (2).

The MHLW has developed detection methods for the 5 major allergic ingredients mentioned above and has established them as the official Japanese methods. These official methods consist of 2 kinds of enzyme-linked immunosorbent assay (ELISA) kits as the screening methods (3, 4), the Western blot method for egg and milk, and the polymerase chain reaction (PCR) method for wheat, buckwheat, and peanut as the confirmation method.

Unfortunately, the official ELISA kits were not adequate in some areas. They could not detect the allergic ingredients in highly processed foods in spite of being able to detect those added to extracts of processed foods. One of the reasons for this low sensitivity was the low extraction efficiency of proteins from processed foods (5–9). Similarly, the antibody used could not recognize proteins that had been denatured during food processing. To remedy these defects, we developed a novel extraction buffer for extracting the insoluble proteins produced by heat and pressure processing, and with new antibodies that recognize the denatured proteins extracted using the new buffer (10). We have established 2 ELISA kits for detecting the 5 major allergic ingredients. Both use the same extraction buffer, leading to reduced

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Table 1. Allergenic foods spiked to the test materials

Test material	Allergenic ingredients
Sausage	Egg, milk, wheat, buckwheat, peanut
Boiled beef	Egg, milk, wheat, buckwheat, peanut
Tomato sauce	Wheat, buckwheat, peanut
Cookie	Egg, milk, buckwheat
Orange juice	Egg, milk, wheat, buckwheat, peanut
Strawberry jam	Egg, milk, wheat, peanut

differences between the results from the kits arising from the sample extraction, and both are less labor-intensive.

This report describes the results of the interlaboratory study on the performance of these improved ELISA kits.

Experimental

Materials and Methods

Test materials.—Six model processed foods (sausage, boiled beef, tomato sauce, cookie, orange juice, and strawberry jam) containing major allergenic ingredients were prepared and used as the test materials. Table 1 shows the allergic ingredients spiked to the model processed foods. Each ingredient was spiked to 5 types of processed foods. The allergic ingredients were spiked at the ingredient stage before processing to obtain a final level of 10 μg/g protein.

Preparation of spiking powders or solutions of the allergic ingredients.—Egg powder for spiking was prepared from the eggs of White Leghorn hens by freeze-drying. Milk from Holstein Friesian cows was also freeze-dried. An equivalent mixture of 14 brands of whole wheat flour, an equivalent mixture of whole buckwheat flours produced in China and Japan, and Virginia peanuts produced in Chiba Prefecture were used to prepare the standard bulk powders. The content of protein in each spiking powder was assayed using a 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden). The amount of protein present in 1 g spiking powder was about 450 mg for egg, 260 mg for milk, 100 mg for wheat, 70 mg for buckwheat, and 90 mg for peanuts. The amount of each standard bulk powder to obtain a final protein concentration of 10 μg/g was calculated, taking into account the protein content and the change in weight of the model processed foods during preparation. The spiking solution of the allergic ingredient was prepared by dissolving or suspending the bulk powder in purified water.

Preparation of model processed foods.—All the model processed foods were prepared following the usual procedures used by the manufacturers. The prepared model processed foods were homogenized with a food processor (sausage, boiled beef, and tomato sauce) or a homogenizer (cookie, orange juice, and jam), and sent to the participants.

Sausage was made of pork leg meat (minus bones, sinews, blood vessels, and fat), vegetable oil, salt, sugar, ice water, and the spiking solutions. Vegetable oil, salt, sugar, ice water, and the spiking solutions were added to the meat and mixed thoroughly. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into sausage casings. These were heated at 80°C for 20 min, cooled in flowing water for 5 min, and kept in a refrigerator at 5°C overnight.

Boiled beef was made of beef shoulder meat (minus bones, sinews, blood vessels, and fat), agar solution, salt, sugar, and the spiking solutions. The meat was immersed in the agar solution containing salt, sugar, and the spiking solutions, and the mixture was kept in a refrigerator at 5°C for 60 min. The mixture was then placed in an aluminum pouch, heated at 121°C for 1 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

Tomato sauce was made of tomato purée, sugar, Worcestershire sauce, apple vinegar, salt, potato starch, water, and the spiking solutions. The measured raw materials were mixed thoroughly and placed in an aluminum pouch. The sauce was heated at 90°C for 30 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

Cookies were made of wheat, sugar, shortening, salt, raising agents, lecithin, water, and the standard bulk powders. The raw materials were homogeneously mixed and kneaded for 20 min. The dough was then rolled out, cut with a cookie cutter, and baked at 240°C for 7 min.

Orange juice was made of water, concentrated juice, sugar, citric acid, and the standard bulk powders. The raw materials were mixed homogeneously. Each 190 mL sample of the mixture was canned and heated at 90°C for 10 min.

Strawberry jam was made of strawberries, saccharides, pectin, citric acid, and the standard bulk powders. The raw materials were mixed thoroughly and heated at 94°C for 4 min.

Homogeneity tests of samples.—The homogeneity of the samples was verified following the procedure laid out in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (11), except that the number of test samples was 6. Twelve test portions were analyzed using the ELISA kits. Sausage, boiled beef, and tomato sauce were analyzed with FASTKIT (Nippon Meat Packers, Osaka, Japan), and cookie, orange juice, and jam were analyzed with FASPEK (Morinaga Institute of Biological Science, Yokohama, Japan). The obtained concentrations of allergic protein were submitted to one-way analysis of variance (ANOVA). Table 2 shows the averaged concentration, the relative standard deviation (RSD) values calculated from s_s (SD of sampling) and s_a (SD of analysis), and the F-ratios. The F-ratios for all samples were below the critical F value.

ELISA Kits

FASTKIT ELISA Ver. II Series (FASTKIT).—The detection procedure was prescribed by the manufacturer. Diluted standard solutions and sample solutions were added to an antibody-coated plate and incubated for 1 h at room temperature. The plate was washed with washing buffer, and then a solution of biotinylated polyclonal antibodies that recognizes multiple antigens was added and the mixture was

Table 2. Homogeneity test results of the test materials

	Mean	RSD%[a]	*n*	*F*-ratio	Fcrit[b]
Sausages					
Egg	5.3	4.6	6	0.67	4.39
Milk	5.2	10.2	6	0.70	4.39
Wheat	11.3	4.0	6	1.52	4.39
Buckwheat	5.9	5.3	6	0.41	4.39
Peanuts	8.0	3.6	6	0.51	4.39
Boiled beef					
Egg	7.4	5.4	6	0.15	4.39
Milk	5.2	3.1	6	1.32	4.39
Wheat	12.1	5.0	6	0.27	4.39
Buckwheat	6.1	4.0	6	1.32	4.39
Peanuts	7.4	3.4	6	0.28	4.39
Tomato sauce					
Wheat	13.0	14.0	6	3.70	4.39
Buckwheat	15.7	10.3	6	4.10	4.39
Peanuts	14.0	10.6	6	2.82	4.39
Cookie					
Egg	4.6	2.7	6	0.90	4.39
Milk	8.1	5.8	6	0.05	4.39
Buckwheat	15.4	6.4	6	1.70	4.39
Orange juice					
Egg	7.6	2.9	6	1.46	4.39
Milk	7.5	3.0	6	0.85	4.39
Wheat	13.2	4.7	6	1.75	4.39
Buckwheat	14.0	12.5	6	0.32	4.39
Peanuts	11.8	2.3	6	0.57	4.39
Strawberry jam					
Egg	6.8	4.7	6	0.59	4.39
Milk	11.8	1.8	6	0.85	4.39
Wheat	1.1	7.8	6	3.37	4.39
Peanuts	5.5	3.9	6	0.82	4.39

[a] RSD% calculated from s_e (SD of sampling) and s_a (SD of analysis).
[b] Fcrit = Critical *F* value.

incubated for 1 h at room temperature. The plate was again washed with the washing buffer, after which the streptavidin–peroxidase reagent was added, and the mixture was incubated for 30 min at room temperature. The plate was once more washed, and a solution of 3,3′,5,5′-tetramethylbenzidine, the substrate for the enzyme, was added and incubated for 20 min at room temperature. The reaction was stopped with 0.5 N sulfuric acid, and the absorbances were measured at 450 nm, with 630 nm as the reference wavelength.

FASPEK allergenic substances detection kit (FASPEK).—The polyclonal antibodies used in FASPEK recognized ovalbumin (egg), casein (milk), gliadin (wheat), soluble buckwheat protein (buckwheat), and soluble peanut protein (peanut). The detection procedure as prescribed by the manufacturer was followed. Diluted standard solutions and sample solutions were added to an antibody-coated module and incubated for 1 h at room temperature. After washing the module, a solution of the antibody, labeled with peroxidase, was added and allowed to stand for 30 min. After the second washing, a solution of 3,3′,5,5′-tetramethylbenzidine was added and the module was allowed to stand at 25°C for exactly 10 min. The reaction was stopped by addition of 0.1 N sulfuric acid, and the absorbances were measured at 450 nm, with 630 nm as the reference wavelength.

Extraction

The extraction procedure was common to both ELISA series. A 1 g portion of the sample was extracted with 19 mL extraction buffer containing sodium dodecyl sulfate (SDS) and mercaptoethanol. The extraction buffer was also common to both ELISA series. The mixture was shaken horizontally overnight (>12 h) at room temperature, and then centrifuged at 3000 × *g* for 20 min after adjustment of the pH to 6. The supernatant was filtered or centrifuged, if necessary, diluted 20 times, and subjected to ELISA.

Calibration Standard Solutions

The calibration standard solutions were common to both ELISA kits. The ingredients for the calibration solutions were same as those of the bulk standards powder for spiking to the samples: *Egg.*—Fresh eggs of White Leghorn hen, homogenized and freeze-dried; *Milk.*—Fresh milk of Holstein cows, freeze-dried after defatting by churning; *Wheat.*—A mixture of 14 species of wheat, pulverized; *Buckwheat.*—A mixture of buckwheats from Ibaraki Prefecture (Japan) and from China, pulverized; *Peanuts.*—Virginia species from Chiba Prefecture (Japan), ground in a mortar.

The ingredients were extracted with 20 mL extraction solution containing 0.5% SDS and 2% mercaptoethanol by shaking overnight. The protein content of the initial extract was assayed using a 2-D Quant Kit (Amersham Biosciences).

The initial extract was diluted to make up the calibration standard solution (50 ng/mL extracted protein). The calibration standard solutions of egg and milk were provided by Nippon Gene Co. Ltd. (Toyama, Japan) and those of wheat, buckwheat, and peanut were provided by Oriental Yeast Co. Ltd. (Nagahama, Japan).

Interlaboratory Study

Ten laboratories participated in the interlaboratory study, coordinated by the National Institute of Health Sciences (Tokyo, Japan). The coordinator sent the 6 test materials (3 g each) and 10 ELISA kits plus the extraction solution and the calibration standard solutions. The participants took 2 portions from each test material, extracted the protein using the extraction procedure, and assayed each extract with the ELISA kits. The calibration standard solution was diluted and assayed simultaneously with the sample extracts. The averaged absorbance of 3 wells was used for the calculation. The obtained absorbance data of calibration solutions and test samples were reported to the coordinator.

The coordinator calculated the 4-parameter logistic calibration curve from the absorbance data of the calibration

Table 3. Results of interlaboratory study for FASPEK: protein recovery content, %

Lab	Sausage, μg/g		Boiled beef, μg/g		Cookie, μg/g		Orange juice, μg/g		Jam, μg/g	
					Egg					
A	6.8	68	7.2	72	4.7	47	8.1	81	8.1	81
B	8.7	87	8.2	82	5.7	57	8.8	88	9.3	93
C	8.2	82	8.2	82	5.1	51	8.3	83	8.4	84
D	7.2	72	8.0	80	5.6	56	9.0	90	9.0	90
E	4.8	48	2.7[a]	27[a]	4.0	40	5.4[a]	54[a]	2.8[a]	28[a]
F	8.4	84	8.4	84	6.0	60	9.7	97	9.9	99
G	7.0	70	7.1	71	4.4	44	7.3	73	7.2	72
H	6.5	65	7.2	72	5.2	52	8.3	83	8.9	89
I	6.0	60	7.2	72	4.8	48	8.3	83	8.6	86
J	6.6	66	6.9	69	4.9	49	8.0	80	8.6	86
					Milk					
A	10.6	106	11.8	118	10.1	101	9.1	91	13.1	131
B	11.3	113	11.5	115	9.9	99	10.0	100	14.4	144
C	13.7	137	13.4	134	10.6	106	9.7	97	14.0	140
D	11.4	114	11.2	112	10.0	100	9.3	93	14.4	144
E	9.2	92	9.7	97	6.1	61	6.3	63	9.8	98
F	13.0	130	12.5	125	11.5	115	9.6	96	14.2	142
G	10.8	108	9.3	93	7.6	76	7.5	75	12.2	122
H	8.6	86	12.4	124	11.1	111	9.5	95	15.0	150
I	9.6	96	11.1	111	9.4	94	9.3	93	15.3	153
J	11.2	112	12.3	123	10.4	104	8.8	88	14.6	146
					Wheat					
A	9.3	93	12.4	124	12.3	123	12.6	126	1.6	16
B	11.8	118	14.0	140	11.8	118	11.8	118	1.3	13
C	11.2	112	12.1	121	14.1	141	11.2	112	1.1	11
D	11.0	110	11.0	110	10.7	107	12.0	120	1.8	18
E	11.4	114	13.4	134	12.0	120	12.5	125	1.1	11
F	11.6	116	12.1	121	12.0	120	12.1	121	1.7	17
G	10.6	106	11.7	117	11.2	112	11.3	113	1.7	17
H	1.0[b]	10[b]	12.1	121	14.3	143	13.0	130	2.1	21
I	8.3	83	10.1	101	11.8	118	11.3	113	1.2	12
J	10.9	109	11.7	117	12.2	122	11.9	119	2.6	26
					Buckwheat					
A	10.4	104	12.8	128	15.9	159	14.8	148	15.6	156
B	9.7	97	11.3	113	16.4	164	15.2	152	13.8	138
C	11.5	115	14.3	143	14.9	149	16.9	169	14.8	148
D	9.8	98	12.1	121	15.0	150	15.7	157	17.2	172
E	5.7[b]	57[b]	6.4	64	10.0	100	11.0	110	9.4	94
F	10.1	101	12.2	122	13.7	137	14.8	148	16.4	164
G	10.4	104	13.6	136	15.1	151	15.6	156	14.7	147
H	10.3	103	15.2	152	17.5	175	17.4	174	15.9	159
I	10.0	100	12.8	128	14.8	148	13.9	139	14.1	141
J	8.8	88	11.1	111	12.7	127	13.6	136	13.9	139
					Peanuts					
A	27.4	274	23.4	234	19.8	198	17.7	177	12.7	127
B	20.9	209	15.2	152	18.7	187	17.2	172	11.8	118
C	19.8	198	14.0	140	16.1	161	14.5	145	13.1	131
D	16.6	166	15.5	155	15.4	154	15.3	153	12.4	124
E	3.8	38	3.6	36	5.8[b]	58[b]	8.7	87	4.6[b]	46[b]
F	23.0	230	16.8	168	16.3	163	15.0	150	11.3	113
G	17.6	176	15.2	152	16.9	169	15.3	153	11.1	111
H	5.6	56	15.4	154	17.6	176	15.6	156	11.9	119
I	17.7	177	17.1	171	14.3	143	13.5	135	11.8	118
J	20.5	205	16.2	162	15.1	151	13.5	135	10.3	103

[a] Values removed after the Cochran test.

[b] Values removed after the Grubbs test.

Table 4. Results of interlaboratory study for FASTKIT: protein recovery content, %

Lab	Sausage, µg/g		Boiled beef, µg/g		Cookie, µg/g		Orange juice, µg/g		Jam, µg/g	
					Egg					
A	7.2	72	8.1	81	7.2	72	10.5	105	9.9	99
B	8.1	81	8.0	80	6.8	68	9.4	94	9.4	94
C	7.8	78	8.0	80	6.2	62	9.3	93	9.4	94
D	7.2	72	8.6	86	6.8	68	10.2	102	10.2	102
E	5.9	59	6.7	67	7.0	70	9.7	97	10.0	100
F	7.1	71	7.6	76	6.1	61	9.6	96	9.2	92
G	6.4	64	7.8	78	6.0	60	9.2	92	8.5	85
H	6.9	69	7.9	79	7.8	78	11.1	111	10.2	102
I	5.4	54	7.3	73	30.5[a]	305[a]	9.6	96	9.3	93
J	5.3	53	6.2	62	5.6	56	9.2	92	9.2	92
					Milk					
A	7.3	73	6.9	69	5.4	54	8.9	89	9.0	90
B	6.4	64	6.0	60	4.6	46	8.1	81	8.9	89
C	6.4	64	6.1	61	4.4	44	7.9	79	8.5	85
D	7.4	74	6.8	68	5.1	51	8.5	85	9.4	94
E	6.0	60	5.7	57	5.3	53	8.8	88	9.6	96
F	6.4	64	6.2	62	4.6	46	8.2	82	8.4	84
G	7.0	70	6.4	64	4.5	45	7.2	72	7.2	72
H	4.7	47	7.0	70	5.9	59	9.3	93	9.3	93
I	5.6	56	6.2	62	4.5	45	8.6	86	8.8	88
J	5.3	53	6.2	62	5.1	51	9.4	94	10.0	100
					Wheat					
A	13.4	134	13.2	132	12.5	125	13.7	137	2.5	25
B	12.9	129	13.0	130	13.5	135	13.7	137	3.7	37
C	11.5	115	11.4	114	14.7[b]	147[b]	11.2	112	3.5	35
D	11.3	113	12.2	122	11.3	113	12.7	127	2.6	26
E	12.2[b]	122[b]	9.6	96	11.9	119	13.3	133	2.9	29
F	11.1	111	10.5	105	9.7	97	11.9	119	2.5	25
G	11.2	112	11.2	112	10.3	103	11.0	110	1.9	19
H	0.5[a]	5[a]	11.0	110	13.2	132	11.6	116	3.3	33
I	8.7	87	10.7	107	10.4	104	11.2	112	2.4	24
J	9.9	99	10.6	106	11.7	117	12.4	124	3.2	32
					Buckwheat					
A	8.6	86	6.7	67	14.7	147	9.3	93	14.8	148
B	6.9	69	5.1	51	14.0	140	9.9	99	13.3	133
C	6.8	68	5.7	57	11.9	119	8.6	86	13.0	130
D	5.5	55	5.9	59	12.7	127	8.2	82	15.5	155
E	6.0	60	5.1	51	12.3	123	8.7	87	13.6	136
F	7.9	79	6.0	60	11.5	115	8.4	84	11.9	119
G	4.8	48	5.3	53	10.8	108	7.2	72	12.6	126
H	3.9	39	5.7	57	12.9	129	9.4	94	14.2	142
I	8.8	88	10.2[a]	102[a]	13.3	133	8.8	88	14.7	147
J	6.9	69	6.6	66	10.9	109	9.2	92	12.1	121
					Peanuts					
A	16.7	167	12.8[a]	128[a]	12.3	123	10.2	102	9.7	97
B	10.6	106	8.1	81	13.3	133	12.3	123	10.5	105
C	8.7	87	7.8	78	11.4	114	10.5	105	12.2	122
D	6.7	67	8.1	81	10.6	106	10.9	109	11.3	113
E	8.7	87	7.0	70	9.5	95	9.9	99	10.7	107
F	12.6	126	9.2	92	11.4	114	10.4	104	10.2	102
G	7.1	71	7.6	76	10.9	109	9.6	96	9.1	91
H	5.6	56	8.6	86	12.6	126	11.7	117	10.5	105
I	10.0	100	9.3	93	12.3	123	11.1	111	11.5	115
J	8.8	88	8.5	85	11.7	117	10.4	104	10.5	105

[a] Values removed after the Grubbs test.

[b] Values removed after the Cochran test.

standard solution and calculated the concentration of allergen protein in the sample using the calibration curve. The average of the 3 results was treated as the data for 1 portion.

Statistical Analysis

The reported values from the participants are summarized in Tables 3 and 4. Twenty data items, as 2 portions from 10 laboratories, were fed into the calculation. The Cochran and Grubbs tests were used to remove outlying data (P = 2.5%). The removed values are also shown in Tables 3 and 4. Recovery, repeatability, and reproducibility were calculated by one-way ANOVA using the remaining data after removal of outliers.

Results and Discussion

Sample Homogeneity

The resultant F-ratios of the homogeneity test of sausage, boiled beef, cookie, orange juice, and jam were <2. The critical value of F was 4.69, and the homogeneity of the samples was sufficient. The F-ratios from tomato sauce were higher than the others, but lower than the critical F. For most samples, the RSD values among portions were <10% and smaller than the expected RSD_R values.

Recovery

The recovery, repeatability (RSD_r), and reproducibility (RSD_R) values calculated using ANOVA are shown in Table 5 with the number of remaining laboratories after removing outliers.

The recoveries of egg and milk proteins from 5 types of test materials were >50%, with one exception. The FASTKIT for milk gave a low recovery of milk protein in the cookies. The recoveries of milk protein using FASPEK were higher than those using the FASTKIT for all the samples, whereas recoveries of egg proteins were comparable between the kits.

The recoveries of wheat proteins from sausage, boiled beef, tomato sauce, and orange juice were almost 100% for both kits. Both kits gave low recoveries of <30% for wheat protein in jam. There were large differences between the recoveries of buckwheat between the kits. FASPEK showed recoveries of 100–140% for boiled beef, tomato sauce, cookie, and orange juice, whereas the recoveries using the FASTKIT were 50–100% and lower than those with FASPEK for all the test materials. The recoveries of peanut protein showed similar patterns to those of buckwheat. The recoveries of peanut protein using FASPEK were >100% for all the test materials, whereas FASTKIT gave recoveries >100%.

There were discrepancies in the recoveries of some proteins between the 2 kits. Because the extracts from each test material and the calibration standards were shared between the tests, these discrepancies were due to differences in reactivity to the denatured proteins between the antibodies used. The results from orange juice prepared using short heating showed comparable recovery between the 2 kits.

Repeatability

Repeatability is a measure of the variance arising from the extraction and the determination procedure in a laboratory. In most cases, RSD_r values were <10%. The RSD_r values of FASPEK for wheat protein from tomato sauce and those for buckwheat protein from tomato sauce and jam were >10%. The results of a homogeneity test of tomato sauce and orange juice (Table 2) showed large s_a values for wheat and buckwheat, suggesting that the extraction efficiency of wheat or buckwheat protein from an acidic matrix is susceptible to small variations in extraction conditions. The difference in variability in the extraction efficiency may be attributable to the difference in types of antibodies used.

Reproducibility

Reproducibilities, expressed by RSD_R values, were <20% for egg and milk in all the test materials. The low recovery of wheat protein from jam resulted in high RSD_R values. The large RSD_r of FASPEK for wheat protein in tomato sauce was reflected in the RSD_R values but the value of 19% demonstrated acceptable reproducibility. RSD_R values of wheat protein in other samples were satisfactory. The results for buckwheat and peanuts also gave good RSD_R values, except for sausage. As shown in Table 2, the homogeneity of sausage samples was guaranteed and the RSD_R values for egg or milk protein in sausage were similar to other test materials. The reason for the large RSD_R values of buckwheat and peanut proteins in sausage can be attributed to the variation in extraction efficiency of buckwheat protein and peanut protein.

Sensitivity

Figure 1 shows the calibration curves of the 2 ELISAs. All the curves are almost straight between 5 and 25 ng/mL, and give sufficiently high absorbance at 25 ng/mL that corresponds to 10 μg/g allergen protein in the sample.

Specificity

The cross-reactivity of FASTKIT and FASPEK was investigated by the manufacturers. The cross-reactivity of FASTKIT with 120 kinds of commodities was surveyed. The kit for egg and the kit for buckwheat did not display any cross-reactivity. The kit for milk displayed cross-reactivity with goat and sheep milk, and the kit for wheat reacted with rye, oats, and barley. The kit for peanuts displayed cross-reactivity with macadamia nuts and kelp (2).

The cross-reactivity of the FASPEK kit with 140 kinds of commodities was surveyed. The FASPEK for egg displayed cross-reactivity with quail and duck eggs. The FASPEK for milk reacted with goat and sheep milk. The FASPEK for wheat displayed cross-reactivity with many species of grain, including rye, oats, and barley, and other commodities such as toasted almonds, poppy seeds, and coriander. The FASPEK for peanuts reacted with macadamia nuts. The FASPEK for buckwheat did not cross-react with any of the foods investigated but reacted with many Polygonum plants (2).

Table 5. Recoveries, repeatabilities (RSD_r), and reproducibilities (RSD_R) of the FASPEK and FASTKIT for egg, milk, wheat, buckwheat, and peanut

Sample	No. of laboratories		Mean, μg/g		s_r, μg/g		s_R, μg/g		Recovery, %		RSD_r, %		RSD_R, %	
	FASPEK	FASTKIT	FASPEK	FASTKIT	FASPEK	FASTKIT	FASPEK	FASTKIT	FASPEK	FASTKIT	FASPEK	FASTKIT	FASPEK	FASTKIT
Egg														
Sausage	10	10	7.0	6.7	0.3	0.3	1.2	1.0	70	67	5	4	17	15
Boiled beef	9	10	7.6	7.6	0.3	0.2	0.6	0.7	76	76	4	2	8	10
Cookie	9	9	5.2	6.6	0.2	0.3	0.6	0.7	52	66	4	5	11	11
Orange juice	10	10	8.1	9.8	0.3	0.2	1.2	0.7	81	98	4	2	14	7
Jam	9	10	8.7	9.5	0.4	0.3	0.8	0.6	87	95	5	3	9	6
Milk														
Sausage	10	10	10.9	6.3	0.6	0.3	1.6	0.9	109	63	5	5	15	14
Boiled beef	10	10	11.5	6.4	0.86	0.3	1.4	0.5	115	64	7	4	12	7
Cookie	10	10	9.7	4.9	0.5	0.2	1.7	0.5	97	49	5	4	17	10
Orange juice	10	10	8.9	8.5	0.4	0.3	1.2	0.7	89	85	5	4	13	8
Jam	10	10	13.7	8.9	0.4	0.2	1.7	0.8	137	89	3	2	12	9
Wheat														
Sausage	9	8	10.7	11.3	0.8	0.6	1.3	1.6	107	113	7	5	12	14
Boiled beef	10	10	12.1	11.4	0.9	0.7	1.3	13	121	114	7	7	11	11
Tomato sauce	10	9	12.2	11.6	2.3	1.1	2.3	1.5	122	116	19	9	19	13
Orange juice	10	10	12.0	12.3	0.3	0.5	0.6	1.2	120	123	3	4	5	9
Jam	10	10	1.6	2.8	0.3	0.3	0.5	0.7	16	28	18	18	33	22
Buckwheat														
Sausage	9	10	10.1	6.6	0.5	0.5	0.8	1.7	101	66	5	8	8	25
Boiled beef	10	9	12.2	5.8	0.8	0.4	2.5	0.7	122	58	7	6	20	11
Tomato sauce	10	10	14.6	12.5	2.1	0.8	2.6	1.4	146	125	15	7	18	11
Cookie	10	10	14.9	8.8	1.3	1.1	2.0	1.1	149	88	8	13	13	12
Orange juice	10	10	14.6	13.6	1.8	1.0	2.5	1.4	146	136	12	7	17	10
Peanut														
Sausage	10	10	17.3	9.5	2.1	0.4	7.5	3.2	173	95	5	4	45	34
Boiled beef	10	9	15.2	8.2	1.0	0.6	4.9	0.9	152	82	5	7	11	10
Tomato sauce	9	10	16.7	11.6	0.9	0.5	1.9	1.2	167	116	5	4	15	10
Cookie	10	10	14.6	10.7	0.9	0.5	2.6	0.9	146	107	6	5	14	9
Orange juice	9	10	11.8	10.6	0.9	0.6	1.1	1.0	118	106	8	5	11	9

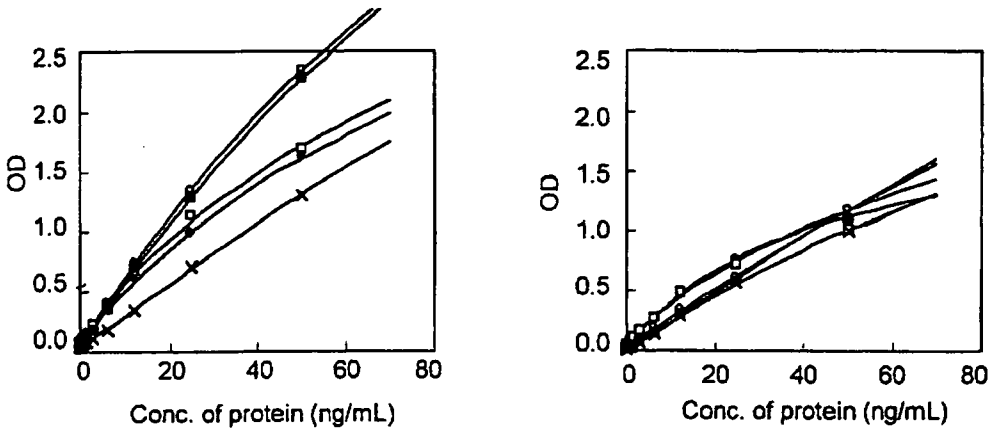

Figure 1. Calibration curves of FASTKIT (left) and FASPEK (right); O = egg; ● = milk; □ = wheat; ■ = buckwheat; × = peanuts.

Conclusions

In conclusion, the results from the interlaboratory study suggest that the 2 test kits, FASPEK and FASTKIT, correctly determined egg and milk protein. Wheat, buckwheat, and peanut, even if contained in highly processed foods such as sausage or cookie, were determined by the 2 kits, although the interlaboratory variations were higher than those for egg and milk. Neither kit could determine wheat protein contained in jam. The interlaboratory study was performed using highly processed model foods rather than the standard reference materials that contained less processed protein to ensure that the ELISA kits could detect the allergic substances under actual conditions. The results demonstrated that the kits would detect the allergic ingredients contained in processed foods and support the food labeling system.

The notable feature of these kits is the unified extraction solution. A unified extract from a sample can be used for determination with both kits, and the variation of results between the 2 kits can be significantly reduced. Furthermore, the standard calibration solutions were also unified. This should make it possible to compare the results of the 2 kits using a common measure and make the results traceable to a defined amount of protein.

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References

(1) Ebisawa, M. (2003) *Report to the Grant from the Ministry of Health, Labor and Welfare of Japan*, Tokyo, Japan, pp 160–162

(2) Ministry of Health, Labor and Welfare of Japan (November 6, 2002) *Notification No. 106001*

(3) Takahata, Y. (2002) *J. Food Hyg. Soc. Jpn.* 43, J275–J277

(4) Mamegoshi, S. (2002) *J. Food Hyg. Soc. Jpn.* 43, J277–J279

(5) Akiyama, H., Isuzugawa, K., Harikai, N., Watanabe, H., Iijima, K., Yamakawa, H., Mizuguchi, Y., Yoshikawa, R., Yamamoto, M., Sato, H., Watai, M., Arakawa, F., Ogasawara, T., Nishihara, R., Kato, H., Yamauchi, A., Takahata, Y., Morimatsu, F., Mamegoshi, S., Muraoka, S., Honjoh, T., Watanabe, T., Wakui, C., Imamura, T., Toyoda, M., & Maitani, T. (2003) *J. Food Hyg. Soc. Jpn.* 44, 213–219

(6) Akiyama, H., Isuzugawa, K., Harikai, N., Watanabe, H., Iijima, K., Yamakawa, H., Mizuguchi, Y., Yoshikawa, R., Yamamoto, M., Sato, H., Watai, M., Arakawa, F., Ogasawara, T., Nishihara, R., Kato, H., Yamauchi, A., Takahata, Y., Morimatsu, F., Mamegoshi, S., Muraoka, S., Honjoh, T., Watanabe, T., Sakata, K., Imamura, T., Toyoda, M., Matsuda, R., & Maitani, T. (2004) *J. Food Hyg. Soc. Jpn.* 45, 120–127

(7) Akiyama, H., Isuzugawa, K., Harikai, N., Watanabe, H., Iijima, K., Yamakawa, H., Mizuguchi, Y., Yoshikawa, R., Yamamoto, M., Sato, H., Watai, M., Arakawa, F., Ogasawara, T., Nishihara, R., Kato, H., Yamauchi, A., Takahata, Y., Morimatsu, F., Mamegoshi, S., Muraoka, S., Honjoh, T., Watanabe, T., Sakata, K., Imamura, T., Toyoda, M., Matsuda, R., & Maitani, T. (2004) *J. Food Hyg. Soc. Jpn.* 45, 128–134

(8) Akiyama, H., Nakamura, K., Harikai, N., Watanabe, H., Iijima, K., Yamakawa, H., Mizuguchi, Y., Yoshikawa, R., Yamamoto, M., Sato, H., Watai, M., Arakawa, F., Ogasawara, T., Nishihara, R., Kato, H., Yamauchi, A., Takahata, Y., Morimatsu, F., Mamegoshi, S., Muraoka, S., Honjoh, T.,

Watanabe, T., Sakata, K., Imamura, T., Toyoda, M., Matsuda, R., & Maitani, T. (2004) *J. Food Hyg. Soc. Jpn.* 45, 313–318

(9) Akiyama, H., Nakamura, K., Harikai, N., Watanabe, H., Iijima, K., Yamakawa, H., Mizuguchi, Y., Yoshikawa, R., Yamamoto, M., Sato, H., Watai, M., Arakawa, F., Ogasawara, T., Nishihara, R., Kato, H., Yamauchi, A., Takahata, Y., Morimatsu, F., Mamegoshi, S., Muraoka, S., Honjoh, T., Watanabe, T., Sakata, K., Imamura, T., Toyoda, M., Matsuda, R., & Maitani, T. (2004) *J. Food Hyg. Soc. Jpn.* 45, 325–331

(10) Watanabe, Y., Aburatani, K., Mizumura, T., Sakai, M., Muraoka, S., Mamegosi, S., & Honjoh, T. (2005) *J. Immunol. Methods* 300, 115–123

(11) Thompson, M., & Wood, R. (1993) *J. AOAC Int.* 76, 926–940

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A Reliable and Sensitive Immunoassay for the Determination of Crustacean Protein in Processed Foods

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Among food allergens, crustacea such as shrimps, crabs, and lobsters are a frequent cause of adverse food reactions in allergic patients. The major allergen has been identified as a muscular protein, tropomyosin. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of crustacean protein in processed foods was developed using the sample dilution buffer that is added to porcine tropomyosin. The sandwich ELISA method was highly specific for the Decapoda group, apart from minor cross-reactivities to other crustacea and mollusks. The recovery ranged from 85 to 141%, while the intra- and interassay coefficients of variation were less than 2.8 and 8.4%, respectively.

KEYWORDS: Crustacea; food allergy; enzyme immunoassay; ELISA; tropomyosin

INTRODUCTION

In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy (*1*, *2*). Burks et al. (*3*) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan, the number of patients with food allergies, especially among young children, is increasing.

To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. However, various studies have shown that severe allergenic reactions can be caused by the accidental intake of food products containing allergenic materials (*4*, *5*). Therefore, sufficient information regarding potentially allergenic ingredients in food products is necessary. The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations, such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food Agriculture Organization (Codex 1998). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to label eight kinds of foods that contain ingredients known to be allergens, including soybeans (FAO 1995, *6*). In Japan, the Ministry of Health, Labor, and Welfare (MLHW) has enforced a labeling system for allergenic food material since April 2002 to provide information about these foods to the allergic consumer. In this system, labeling for five food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory and is recommended for 20 other food materials, such as soybeans and shrimp. In Japan, it became clear, based on epidemiological investigations, that the number of patients with a crustacean allergy such as to shrimp or crab has increased (*7*, *8*).

In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea and because the number of patients with an allergy to Crustacea has been increasing, although the crustacean allergy is still less prevalent than the peanut allergy in the food-allergic population (*9*, *10*). Crustacean allergic reactions may occur due to trace amounts of the crustacean protein, and anaphylaxis to Crustacea has been reported (*11*, *12*).

In the present study, we developed a reliable sandwich enzyme-linked immunosorbent assay (ELISA) method with a high sensitivity for Crustacea. We showed that this detection method could be applicable to food-processing products and that the trace amount of Crustacea contained in commercial food products can be detected using the proposed sandwich ELISA method.

MATERIALS AND METHODS

Food Samples. The black tiger prawn (*Penaeus monodon*) was purchased from Intergrated Aquaculture Specialist, Inc. (Cebu, Philippines). The common Crustacea and mollusks, namely, northern shrimp (*Pandalus borealis*), Japanese spiny lobster (*Panulirus japonicus*),

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Caribbean spiny lobster (*Panulirus argus*), red king crab (*Paralithodes camtschatica*), swimming crab (*Portunus trituberculatus*), Japanese mantis shrimp (*Squilla oratoria*), euphausia (*Euphausia similis*), opossum shrimp (*Neomysis japonica*), acorn barnacle (*Balanus rostratus*), goose barnacle (*Pollicipes mitella*), common octopus (*Octopus vulgare*), giant octopus (*Paroctopus dofleini*), ocellated octopus (*Octopus ocellatus*), Japanese common squid (*Todardes pacificus*), spear squid (*Loligo kobiensis*), cuttlefish (*Sepia subaculeata*), common scallop (*Patinopecten yessoensis*), Japanese oyster (*Crassostrea gigas*), bloody clam (*Scapharca broughtonii*), blue mussel (*Mytilus edulis*), short-neck clam (*Tapes japonica*), common freshwater clam (*Corbicula leane*), Japanese hard clam (*Meretix lusoria*), Sakhalin surf clam (*Spisula sachalinensis*), horned turban (*Turbo cornutus*), Japanese abalone (*Haliotis discus*), and whelk (*Babylonia japonica*), and other ingredients and commercial processed foods were purchased at local stores in Japan.

Preparation of Model Processed Foods. The model processed foods were prepared according to a previously reported cooking procedure (*13*). Specifically, the freeze-dried black tiger prawn muscle powder was mixed with raw foods and then cooked to prepare the processed food items containing the shrimp protein at 10 μg/g as the final concentration. The following were prepared as the model processed foods.

Fish meat sausages were made of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and the spiking powder. Lard, salt, sugar, ice water, and the spiking powder were added to the minced fish flesh and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into the fish sausage casings. These were then heated at 121 °C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5 °C overnight.

The freeze-dried egg soup was made of egg, potato starch, milk sugar (lactose), salt, and the spiking powder. Eggs, potato starch, milk sugar, salt, and the spiking powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep freezer at −80 °C, and then lyophilized for 20 h.

The chicken meatball was made of white meat of chicken, lard, potato starch, sugar, and the spiking powder. Lard, potato starch, sugar, and the spiking 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 then preserved in a deep freezer at −20 °C.

Preparation of Black Tiger Prawn Protein Standards. A 0.1 g sample of the freeze-dried black tiger muscle powder was added to 20 mL of phosphate-buffered saline [10 mM Na-phosphate, 154 mM NaCl (pH 7.4)] containing 5 g/L sodium dodecyl sulfate (SDS), 20 mL/L β-mercaptoethanol, 10 μL/mL protease inhibitor cocktail, and 10 μL/mL 0.5 M ethylenediaminetetraacetic acid (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL). The mixture was then shaken for 15 h at room temperature for extraction. After the extraction, the sample was centrifuged at 10000*g* for 30 min and the supernatant was filtered through a 0.8 μm microfilter paper (DISMIC-25cs; Advantec, Tokyo, Japan) to obtain the extract. The extract was then heated at 100 °C for 10 min. The obtained extracts were analyzed using a 2D Quant Protein Assay Kit (GE Healthcare UK Ltd. NA, England).

Purification of Black Tiger Prawn Tropomyosin (BTTM), Red King Crab, Swimming Crab, Japanese Oyster, Common Scallop, Japanese Common Squid, and Porcine Tropomyosin (PTM). The purification of the BTTM was carried out according to the methods reported by Nagpal et al. (*14*), Ishikawa et al. (*15*), and Miegel et al. (*16*), respectively. The black tiger prawn muscles (100 g) were homogenized with 200 mL of a solution containing 20 mM KCl, 1 mM $KHCO_3$, 0.1 mM $CaCl_2$, and 0.1 mM dithiothreitol (DTT). After centrifugation (3000*g* for 5 min at 4 °C), 200 mL of acetone was added to the precipitant. The suspension was filtered through cheesecloth, and the residue was then washed three times with 200 mL of acetone. Finally, the residue was allowed to dry at room temperature for 2–3 h. The dried powder was extracted overnight at room temperature with 200 mL of 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 1 mM DTT. After filtration through cheesecloth, the residue was once more extracted with 200 mL of 1 M KCl. The extracts were combined and cooled to 4 °C. Ammonium sulfate was added to produce an approximate 30% saturation. After 2 h, the solution was centrifuged (18000*g* for 60 min at 4 °C) and ammonium sulfate was then added to the supernatant (60% saturation). After 2 h, the solution was centrifuged and the precipitant was dissolved in 20 mL of 5 mM Tris-HCl (pH 7.5) containing 0.1 mM $CaCl_2$, and 0.1 mM DTT and was dialyzed overnight against 6 L of the same solution. The pH was then adjusted to 4.6 by the addition of HCl, and the tropomyosin precipitate was removed by centrifugation. The precipitate was dissolved in 25 mM Tris-HCl (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 0.1 mM DTT and then chromatographed on a HiLoad Superdex 200 pg column (Φ26 mm × 600 mm; GE Healthcare UK Ltd.) equilibrated with the same buffer. Fractions of 5 mL were collected at a flow rate of 2.5 mL/min. The SDS-polyacrylamide gel electrophoresis analyses for all fractions were performed, and the fractions with the band corresponding to 37 kDa were combined (*17*). The combined fraction was then diluted with an equal volume of 0.2% trifluoroacetic acid and applied to reverse-phase high-performance liquid chromatography on a Wakosil-II 5C18 AR prep column (Φ10 mm × 250 mm; Wako Chemicals, Japan). The column was eluted at a constant flow rate of 2.5 mL/min by a gradient of acetonitrile in 0.1% trifluoroacetic acid. The tropomyosin-containing fractions were collected and lyophilized. The red king crab, swimming crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins were obtained according to the purification procedure of BTTM. Tropomyosin derived from the porcine skeletal muscle (PTM) was obtained using the purification procedure of Greaser et al. (*18*) and Bailey et al. (*19*).

Production of Monoclonal Antibodies and Rabbit Polyclonal Antibodies to BTTM. The anti-BTTM monoclonal antibodies were generated at Nippon Biotest Laboratories, Inc. (Tokyo, Japan). For the production of the monoclonal antibodies against BTTM, female BALB/c mice were immunized with the purified BTTM. Fusion of the spleen cells was performed according to the method of Kohler and Milstein (*20*). The cell culture supernatants were screened for specific anti-BTTM antibodies by a direct ELISA with purified BTTM on a solid phase. The positive hybridomas were cloned and subcloned by limiting dilution. The positive hybridoma cells were intraperitoneally administered into BALB/c mice to induce the ascite tumors. The antibody was purified from the ascite fluid using a HyperD Protein A column (Bio Sepra Inc., Marlborough, MA). The specificity of the monoclonal antibodies was demonstrated by a direct ELISA method with purified black tiger prawn, red king crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins. The polyclonal antibodies were generated at Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). The rabbit antiserum against BTTM was produced by immunization of New Zealand rabbits with purified BTTM in Freund's adjuvant. Injections were repeated six times at appropriate intervals (7 days). Whole blood was collected, and the serum was separated. The polyclonal antibodies were purified from the serum using a HiTrap Protein A HP column (GE Healthcare UK, Ltd.). The polyclonal antibodies were immunoabsorbed against Japanese common squid purified tropomyosin. The immunoabsorption was performed using the Japanese common squid tropomyosin-coupled column to removed further antibodies to molluskan protein. The specificity of the absorbed polyclonal antibodies was demonstrated by direct ELISA using the various purified tropomyosins.

Preparation of Sample Solution. The samples were treated with the Ace AM-4 homogenizer (Nissei, Tokyo, Japan) a few times for 30 s for homogeneity. Nineteen milliliters of 120 mM Tris-HCl (pH 7.4) containing 1 g/L bovine serum albumin (BSA), 0.5 mL/L Tween 20, 5 g/L SDS, and 20 mL/L β-mercaptoethanol (*21*) was added to 1 g of a homogenized sample, which was then shaken for 12 h at room temperature for extraction. After the extraction, the sample was centrifuged at 3000*g* for 20 min, and the supernatant was filtered through 5AB paper (Advantec) to obtain the extract.

Procedure of the Direct ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international, Rochester, NY) were coated overnight at 4 °C with 100 μL of purified tropomyosin (0.5 μg/mL) in coating buffer (50 mmol/L sodium carbonate, pH 9.6). The plates were then washed three times with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.4, containing 154 mmol/L NaCl). The plates were blocked

for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950 (Supelco, Bellefonte, PA). After the plates were washed six times with TBS containing 0.5 mL/L Tween 20 (TBS-T), diluted monoclonal antibodies or polyclonal antibodies were added to the wells and incubated at 25 °C for 1 h. After the wells were washed with TBS-T, 100 μL of horseradish peroxidase-labeled goat antimouse or antirabbit IgG serum was added to each well. After washing, 100 μL of the substrate solution containing 3,3′,5,5′-tetramethylbenzidine (SureBlue TMB Microwell Peroxidase Substrate; KPL, Gaithersburg, MD) was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read on a SPECTRAmax 250 microplate reader (Molecular Devices Corp., Menlo Park, CA) at the wavelength of 450 nm.

Procedure of the Sandwich ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international) were coated with 100 μL/well monoclonal antibodies (Mab #32, 10 μg/mL; and Mab #54, 20 μg/mL in 50 mmol/L sodium carbonate, pH 9.6) for 18 h at 4 °C. After they were washed three times with TBS, the plates were blocked for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950. After the blocking buffer had been aspirated, the plates were dried in an incubator for 2.5 h at 30 °C, sealed in an aluminum-coated pack with drying agent (I.D. Sheet Desiccant; I.D., Tokyo, Japan), and stored at 4 °C until used. The food sample extracts were diluted 1:20 with the sample dilution buffer [TBS containing 2 g/L BSA, 0.02 g/L PTM, 0.5 mL/L Tween 20, and 0.5 mL/L ProClin 950]. The diluted sample or calibrator (100 μL) was added in triplicate to the coated wells, and the plates were then incubated for 90 min at 25 °C. After the plate had been washed four times with TBS-T, horseradish peroxidase-conjugated absorbed polyclonal antibodies (100 μL) were added to each well, and the plate was then incubated for 90 min at 25 °C. After another four washes with TBS-T, 100 μL of 3,3′,5,5′-tetramethylbenzidine solution (SureBlue Reserve TMB Microwell Peroxidase Substrate, KPL) as a substrate was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read using a SPECTRAmax 250 microplate reader at a wavelength of 450 nm. Standard curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration.

Eleven crustacean protein extracts and two crustacean purified tropomyosins were tested in the concentration range from 0 ng/mL to 90 μg/mL. The concentration–response curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration, and the curves were fitted to a four-parameter logistic equation, $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the concentration of the analyte giving 50% responses (RC_{50}), and D is the minimum absorbance for no analyte. The reactivity values were calculated as follows: reactivity % = [RC_{50} of black tiger prawn protein (or tropomyosin)/RC_{50} of target crustacean protein (or tropomyosin)]. Seventeen molluskan sample extracts were diluted 1: 20 with the sample dilution buffer containing PTM and analyzed using the sandwich ELISA method.

Evaluation of Assay Variation. For determination of the intra-assay precision, the mean coefficients of variation (CVs) were based on 10 replicates. The interassay precision was determined as the mean CVs on the basis of triplicate analyses on 10 different days. The limit of detection (LOD) for the sandwich ELISA was calculated as three times the standard deviation (SD) of the buffer blank mean value after 25 experiments. The limit of quantification (LOQ) was calculated as 10 times the SD of the buffer blank mean values after 25 experiments.

RESULTS

Construction of Sandwich ELISA. To evaluate the characteristics of the absorbed polyclonal antibodies and monoclonal antibodies to the BTTM, we tested the reactivity using a direct ELISA assay. We showed that the absorbed polyclonal antibody could be clearly detected for the crustacean tropomyosin but not for the molluskan tropomyosin. For the preparation of monoclonal antibodies to BTTM, nine monoclonal antibodies were obtained. Of these monoclonal antibodies, Mab #32 and #54 gave a satisfactory specificity and reactivity. In the examination of the different antibody combinations, using Mab #32 and #54 as the capture antibody and the HRP-conjugated absorbed polyclonal antibody as the detected antibody for the sandwich ELISA was found to provide the best results in terms of sensitivity and specificity to determine the total crustacean protein. However, as shown in **Figure 1**, a satisfactory dilution linearity could not be obtained when the dilution tests were performed using the tentatively constructed sandwich ELISA method and the three model processed foods. These results suggest that the food matrix could affect the dilution linearity in the tentatively constructed ELISA method. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. As shown in **Figure 2**, the dilution linearity was satisfactorily improved by the addition of the PTM to the sample dilution buffer (r^2 = 0.996–0.999), confirming parallelism between the calibrators and the food samples. We statistically compared the two correlation coefficients of the dilution curves obtained using a sample dilution buffer containing PTM and those obtained without PTM for the assay of the model processed foods. A statistical test between the two correlation coefficients was performed using the Z-transformation test. P values of less than 0.05 were considered statistically significant. In the case of the chicken meatball, there was a statistically significant difference between the two correlation coefficients of the dilution curve (P = 0.036). In the case of

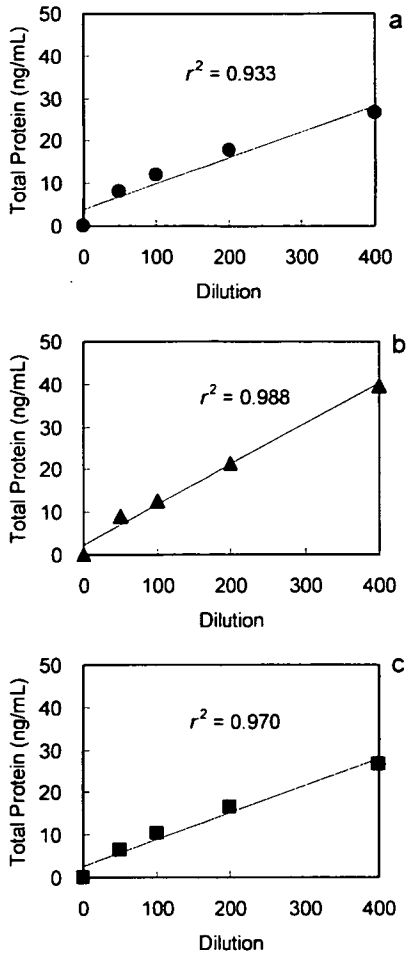

Figure 1. Linearity of dilution curves for model processed foods using the sample dilution buffer without PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

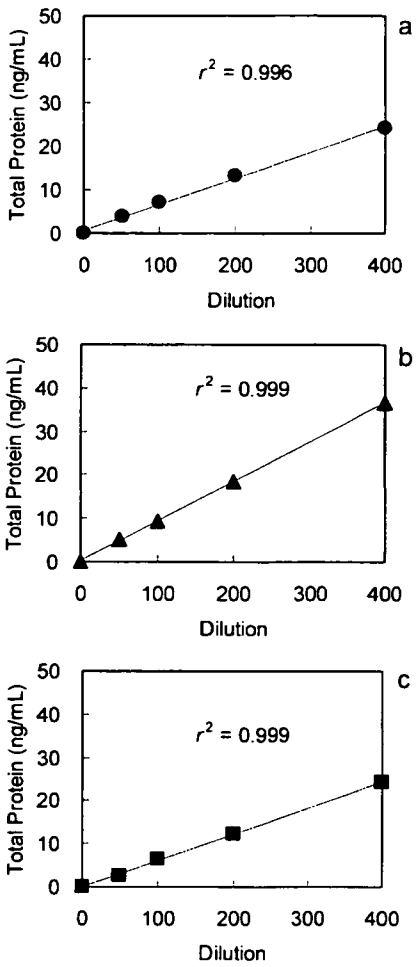

Figure 2. Linearity of dilution curves for model processed foods using the sample dilution buffer with PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

the fish meat sausage, a dilution curve using a sample dilution buffer to which was added PTM tended to show a good linearity when compared with using a tentative sample dilution buffer ($P = 0.081$). Meanwhile, there was no statistically significant difference in the freeze-dried egg soup ($P = 0.127$). These results suggested that the addition of PTM to the sample dilution buffer significantly improved the dilution linearity. Consequently, we established the sandwich ELISA method using a sample dilution buffer with PTM to minimize the food matrix effects.

Reactivity and Specificity Test. Various crustacean proteins, molluskan protein samples, and two crustacean purified tropomyosins (black tiger prawn and swimming crab) were examined to test the reactivity and specificity using the sandwich ELISA method. As shown in **Table 1**, the reactivities of the Decapoda group, which includes prawns and lobsters, are greater than 65.8%, and those of the crabs range between 28.5 and 38.5%. In contrast, the reactivities of the other Crustacea, such as the Japanese mantis shrimp, euphausia, and acorn barnacle, are less than 11.3%. The swimming crab purified tropomyosin demonstrated a reactivity of 154% as compared to the reactivity of black tiger purified tropomyosin. When all of the molluskan samples were tested, all of the levels were determined by the sandwich ELISA method to be less than 1.0 mg/kg (**Table 2**). These results suggest that the sandwich ELISA method has a specific reactivity to the Decapoda group, which includes prawns, shrimps, lobsters, and crabs.

Table 1. Reactivity Levels of Various Crustacean Samples in the Sandwich ELISA Method[a]

sample	RC_{50} (ng/mL)	reactivity (%)
Decapoda group		
black tiger prawn	9.5	
northern shrimp	14.4	65.8
Japanese spiny lobster	8.4	114.3
Caribbean spiny lobster	9.0	105.6
red king crab	24.6	38.5
swimming crab	33.4	28.5
other varieties of Crustacea		
Japanese mantis shrimp	124.4	7.6
euphausia	799.3	1.2
opossum shrimp	8060.4	0.1
acorn barnacle	83.8	11.3
goose barnacle	166.7	5.7

[a] RC_{50} is the concentration of analyte giving a 50% OD_{max} response. Reactivity % = (RC_{50} of black tiger prawn protein/RC_{50} of target crustacean protein).

Table 2. Cross-Reactivity of Various Molluskan Samples in the Sandwich ELISA Method

sample	cross-reactivity in ELISA (mg/kg)
Cephalopoda group	
common octopus	<1.0
giant octopus	<1.0
ocellated octopus	<1.0
Japanese common squid	<1.0
spear squid	<1.0
cuttlefish	<1.0
Bivalvia group	
common scallop	<1.0
Japanese oyster	<1.0
bloody clam	<1.0
blue mussel	<1.0
short-neck clam	<1.0
common freshwater clam	<1.0
Japanese hard clam	<1.0
Sakhalin surf clam	<1.0
Gastropoda group	
horned turban	<1.0
Japanese abalone	<1.0
whelk	<1.0

Limit of Detection and Limit of Quantification. The best model that describes the relationship between the absorbance and the antigen concentration is a four-parameter logistic curve (**Figure 3**). The LOD of the ELISA method determined using the standard proteins is 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and LOQ is 2.25 ng/mL, equivalent to a 0.9 mg/kg sample. Consequently, the practical determination range lies between 1.56 and 50 ng/mL. For the final evaluation of the validation data for the sandwich ELISA and its application, the LOQ for routine analysis was set to 1.0 mg/kg sample. This level was considered to give a safety margin to the majority of consumers with an allergy to peanuts (*22*).

Quantification of Crustacean Protein in Model Processed Foods Using the Sandwich ELISA. To test the applicability of the sandwich ELISA in processed foods, the crustacean protein in three model processed food samples was determined using the sandwich ELISA. As shown in **Table 3**, the mean recoveries for all three model processed food samples ranged from 85 to 141%. The precision data from the three model processed foods are shown in **Table 4**. The interassay precision across all days was 5.3, 6.2, and 8.4% CV for the three model processed foods. The intra-assay precision for the three model processed foods was 2.8, 2.3, and 2.8% CV, respectively.

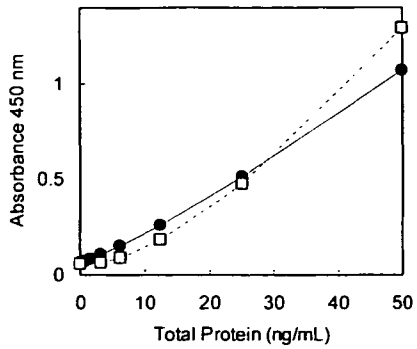

Figure 3. Representative standard curve using the shrimp protein standard in the sandwich ELISA method. The sample diluted buffer with PTM (solid circle); without PTM (open square).

Table 3. Recoveries of Crustacean Protein from Three Model Processed Foods

sample	concentration (mg/kg)	recovery (%)
fish meat sausage	25.0	96
	12.5	107
	6.3	114
	3.1	120
freeze-dried egg soup	29.8	124
	14.9	124
	7.4	125
	3.7	141
chicken meatball	25.0	97
	12.5	100
	6.3	105
	3.1	85

Table 4. Intra- and Interassay Variances in the Sandwich ELISA Method Using Three Model Processed Foods[a]

sample	concentration (mg/kg)	intra-assay	interassay
fish meat sausage	10	2.8	5.3
freeze-dried egg soup	11.9	2.3	6.2
chicken meatball	10	2.8	8.4

[a] The intra-assay variances were calculated from 10 replicates of the same extract, and the interassay variances were calculated from triplicate analysis of the same extract on 10 different days.

Application to the Commercial Food Products. Thirty-two different commercial food samples were analyzed by the sandwich ELISA method. Each commercial food was homogenized, and the extracts were obtained according to the extraction procedure described in the Materials and Methods section. As shown in **Table 5**, 15 commercial foods with a label of shrimp or crab on the ingredients list were clearly detected. In contrast, the levels in products without a label of shrimp or crab on the ingredients list were detected to be less than 1.0 mg/kg. There were no false positives from the no-declaration samples and no false negatives from the declaration samples analyzed in this study. When commercial food products containing shrimp or crab were serially diluted and assayed, each sample gave results close to linearity (r^2 = 0.993–1.000), confirming parallelism between the calibrators and the food samples. These results show that the sandwich ELISA method could appropriately determine the crustacean protein in the processed foods.

DISCUSSION

We established the sandwich ELISA method for the detection of crustacean protein that has a specific reactivity to the

Table 5. Analysis of Various Commercial Food Samples for Using the Sandwich ELISA Method

sample	declaration	substance	quantitative (mg/kg)	regression (r^2)
bean jammed	+	crab	264	0.998
seafood curry	+	shrimp	1780	0.999
beef curry	−		<1.0	
base of pilaf	+	crab	1100	1.000
cream pasta source	−		<1.0	
croquette	+	crab	404	1.000
croquette	−		<1.0	
croquette	−		<1.0	
dumpling	+	shrimp	77000	0.995
dumpling	+	crab	1040	1.000
dumpling	−		<1.0	
base of fried rice	+	shrimp	653	0.993
base of risotto	+	crab	36.7	0.998
spray-dried soup	−		<1.0	
gratin	+	shrimp	22400	0.995
gratin	−		<1.0	
snack	+	shrimp	100	0.998
cookie	−		<1.0	
Japanese rice cookie	−		<1.0	
fried food (prawn)	+	shrimp	282000	0.995
fried food (chicken)	−		<1.0	
fried food (poke)	−		<1.0	
fried food (oyster)	−		<1.0	
fried food (squid)	−		<1.0	
noodle	+	shrimp	145000	0.998
noodle	−		<1.0	
Japanese wheat noodle	−		<1.0	
steamed fish paste	+	crab	176	0.999
steamed fish paste	−		<1.0	
fried fish paste	+	shrimp	46.4	0.995
terrine	+	shrimp	1560	0.997
fish sausage	−		<1.0	

Decapoda group in Crustacea and applied this method to processed food. Jeoung et al. (*23*) already reported a determination method for tropomyosin. However, the cross-reactivity to mollusks and the application to processed foods have not yet been sufficiently clarified. Therefore, the reactivity and specificity of the sandwich ELISA method were tested using extracts from various Crustacea, mollusks, and commercial foods. In the test of all of the molluskan sample extracts, the reactivity levels were extremely low. The house dust mite was reported to cross-react with crustacean allergens (*24*). However, the monoclonal antibodies as the capture antibody do not cross-react with the house dust mite in the Western blot analysis (data not shown). These results suggest that this method would be specific to the Crustacea protein. However, the possibility of a cross-reaction with other less commonly used mollusks or other ingredients, such as crustacean extractants as seasonings, cannot be excluded and remains to be examined. It will be necessary to clarify the applicability of the present method.

The reactivities of lobster and prawn are similar to those of the black tiger prawn. Those of the crab group appear to be lower than those of the black tiger prawn. However, the purified swimming crab tropomyosin showed a high reactivity (154%). These results suggest that the variety of reactivities among the Decapoda group may be involved in the difference of the tropomyosin contents in the sample extracts.

Furthermore, we found that the addition of porcine skeletal tropomyosin to the sample dilution buffer in the sandwich ELISA method can appropriately determine the crustacean protein in processed foods without any food matrix effects.

As described in the Results section, the sample extracts of the model processed foods were serially diluted and assayed using the tentatively constructed sandwich ELISA method, and

a good linearity could not be observed (r^2 = 0.936–0.995). We considered that this result would be due to food matrix effects. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. Consequently, the dilution linearity for model processed food was significantly improved by the addition of the PTM to the sample dilution buffer (r^2 = 0.996–0.999). The addition of troponin or actin failed to improve the dilution linearity (data not shown). These results suggest that tropomyosin may be involved in the food matrix effects, although the food matrix effect mechanism remains unclear. This method offers a new perspective for the determination of various proteins in processed food and is expected to be extremely useful in other protein-measuring methods using ELISA.

To evaluate the sandwich ELISA method for the determination of crustacean protein in processed foods, a recovery study and intra- and interassays were tested using model processed foods. The results of the analysis show that this method has a good accuracy and precision. The sandwich ELISA method's sensitivity was 0.71 ng/mL, corresponding to the 0.29 μg crustacean protein/g food sample weight. This result indicates that the sandwich ELISA method is suitable for detection in the presence of hidden crustacean protein in processed foods.

In conclusion, this sandwich ELISA method is shown to have an acceptable accuracy and precision and no false positive or false negative. This method has been demonstrated to be suitable for the quantitative measurement of the specific crustacean protein in processed foods without food matrix effects.

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.; Bjorksten, B.; Bruijnzeel-Koomen, C. A.; Hugget, A.; Ortolani, C.; Warner, J. O.; Smith, M. Scientific criteria and the selection of allergenic foods for product labeling. *Allergy* **1998**, *53*, 37–21.

(3) Burks, W.; Bannon, G. A.; Sicherer, S.; Sampson, H. A. Peanut-induced anaphylactic reactions. *Int. Arch. Allergy Immunol.* **1999**, *119*, 165–172.

(4) Bock, S. A.; Atkins, F. M. The natural history of peanut allergy. *J. Allergy Clin. Immunol.* **1989**, *83*, 900–904.

(5) Furlong, T. J.; DeSimone, J.; Sicherer, S. H. Peanut and tree nut allergic reactions in restaurants and other food establishments. *J. Allergy Clin. Immunol.* **2001**, *108*, 867–870.

(6) Hefle, S. L.; Nordlee, J. A.; Taylor, S. L. Allergenic foods. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, S69–89.

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

(8) Ebisawa, M. Epidemiology of food allergy (its comparison between Japan and other countries). *Arerugi* **2007**, *56*, 10–18.

(9) Bock, S. A.; Atkins, F. M. Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. *J. Pediatr.* **1990**, *117*, 561–567.

(10) Sicherer, S. H.; Furlong, T. J.; Maes, H. H.; Desnick, R. J.; Sampson, H. A.; Gelb, B. D. Genetics of peanut allergy: A twin study. *J. Allergy Clin. Immunol.* **2000**, *106*, 53–56.

(11) Sampson, H. A.; Metcalfe, D. D. Food allergies. *J. Am. Med. Assoc.* **1992**, *268*, 2840–2844.

(12) Foucard, T.; Malmheden, Y. I. A study on severe food reactions in Sweden—Is soy protein an underestimated cause of food anaphylaxis? *Allergy* **1999**, *54*, 261–265.

(13) Matsuda, R.; Yoshioka, Y.; Akiyama, H.; Aburatani, K.; Watanabe, Y.; Matsumoto, T.; Morishita, N.; Sato, H.; Mishima, T.; Gamo, R.; Kihira, Y.; Maitani, T. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *AOAC Int.* **2006**, *89*, 1600–1608.

(14) Nagpal, S.; Rajappa, L.; Metcalfe, D. D.; Subba Rao, P. V. Isolation and characterization of heat-stable allergens from shrimp (*Panaeus indicus*). *J. Allergy Clin. Immunol.* **1989**, *83*, 26–36.

(15) Ishikawa, M.; Shimakura, K.; Nagashima, Y.; Shiomi, K. Isolation and Properties of allergenic proteins in the Oyster *Crassostrea gigas*. *Fish. Sci.* **1997**, *64*, 610–614.

(16) Miegel, A.; Kobayashi, T.; Maeda, Y. Isolation, purification and partial characterization of tropomyosin and troponin subunits from the lobster tail muscle. *J. Muscle Res. Cell Motil.* **1992**, *13*, 608–618.

(17) Suma, Y.; Ishizaki, S.; Nagashima, Y.; Lu, Y.; Ushio, H.; Shiomi, K. Comparative analysis of barnacle tropomyosin: divergence from decapod tropomyosins and role as a potential allergen. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2007**, *147*, 230–236.

(18) Greaser, M. L.; Gergely, J. Reconstitution of troponin activity from three protein components. *J. Biol. Chem.* **1971**, *246*, 4226–4233.

(19) Bailey, K. Tropomyosin: A new asymmetric protein component of the muscle fibril. *Biochemistry* **1948**, *43*, 271–279.

(20) Kohler, G.; Milstein, C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **1976**, *6*, 511–519.

(21) Watanabe, Y.; Aburatani, K.; Mizumura, T.; Sakai, M.; Muraoka, S.; Mamegosi, 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.

(22) Hourihain, J. O. B.; Kilburn, S. A.; Nordlee, J. A.; Hefle, S. L.; Taylor, S. L.; Warner, J. O. An evaluation of the sensitivity of subjects with peanut allergy to very low dose of peanut protein: A randomized, double-blind, placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* **1997**, *100*, 596–600.

(23) Jeoung, B.; Reese, G.; Hauck, P.; Oliver, J. B.; Daul, C. B.; Leher, S. B. Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibody-based sandwich ELISA. *J. Allergy Clin. Immunol.* **1997**, *100*, 229–234.

(24) Witteman, A. M.; Akkerdass, J. H.; Leeuwen, J.; Zee, J. S.; Aalberse, R. C. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. *Int. Arch. Allergy Immunol.* **1994**, *105*, 56–61.

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Molecular Cloning of Tropomyosins Identified as Allergens in Six Species of Crustaceans

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Although tropomyosin is known to be a major allergen of crustaceans, its structural information is limited to only five species. In this study, tropomyosin was confirmed to be a major allergen in six species of crustaceans (black tiger prawn, kuruma prawn, pink shrimp, king crab, snow crab, and horsehair crab) by immunoblotting. Then, the amino acid sequences of tropomyosins from these crustaceans were elucidated by a cDNA cloning technique. Sequence data for crustacean tropomyosins including the obtained results reveal that fast tropomyosins are contained in shrimps (or prawns) and lobsters, slow tropomyosins in crabs, and both tropomyosins in crayfishes and hermit crabs. Although fast and slow tropomyosins share a high sequence identity (about 90%) with each other, significant differences are observed in specific regions between both tropomyosins.

KEYWORDS: Allergen; cDNA cloning; crab; cross-reactivity; crustacean; prawn; shrimp; tropomyosin

INTRODUCTION

Crustaceans are widely consumed as delicious foods throughout the world. However, they are simultaneously recognized as one of the most common causes of immunoglobulin E (IgE)-mediated food allergy. Following their ingestion, hypersensitive reactions, such as urticaria, asthma, diarrhea, and anaphylaxis, are immediately induced in allergic individuals. Most edible crustaceans are members of the order Decapoda composed of two suborders, Dendrobranchiata (including shrimps or prawns) and Pleocyemata (including shrimps or prawns, crayfishes, lobsters, hermit crabs, and crabs), and hence previous studies on crustacean allergens have been performed with decapods. So far, the major allergen of crustaceans has been identified at the molecular level as tropomyosin, a 35−38 kDa protein constituting thin myofilaments together with actin and troponin, in the following six species: Indian white shrimp, *Penaeus indicus* (*1*); brown shrimp, *Peanaeus aztecus* (*2*); sand shrimp, *Metapenaeus ensis* (*3*); American lobster, *Homarus americanus* (*4*); spiny lobster, *Panulinus stimpsoni* (*4*); and red crab, *Charybdis feriatus* (*5*). The three species of shrimps belong to the suborder Dendrobranchiata and the rest to the suborder Pleocyemata. Although tropomyosin is assumed to be a major allergen in common with decapod crustaceans, no experimental data are available on allergens in shrimps belonging to the suborder Pleocyemata and hermit crabs including commercially important species such as king crab.

Crustacean tropomyosins show IgE cross-reactivity with one another and also with those from various invertebrates including mollusks, house dust mites, and cockroaches (*6*−*12*). Obviously, the first step toward molecular understanding of the cross-reactivity among tropomyosins from various sources is the elucidation of their amino acid sequences. As for crustacean tropomyosins, those from brown shrimp (*13*), sand shrimp (*3*), American lobster (*14*, *15*), spiny lobster (*4*), and red crab (*5*) have already been clarified for their amino acid sequences. The tropomyosins from two species of shrimps, American lobster and spiny lobster, share an extremely high sequence identity (>98%) with one another. In accordance with this, the eight IgE-binding epitopes proposed for brown shrimp tropomyosin (Pen a 1) (*10*, *16*) are completely conserved in the tropomyosins from the other three species, except that the spiny lobster tropomyosin has one alteration in one epitope. On the other hand, the red crab tropomyosin bears a somewhat different amino acid sequence, and its sequence identity with those of the above four species is about 90%. Importantly, the red crab tropomyosin has as many as six alterations in the region 44−55 corresponding to one of the Pen a 1 epitopes. At present, however, it is unknown whether the amino acid sequence features of the red crab tropomyosin are common to crab tropomyosins.

In view of the circumstances described above, this study was initiated to obtain further evidence that tropomyosin is a major allergen in decapod crustaceans using the following six species widely consumed in Japan: two species of the suborder Dendrobranchiata, black tiger prawn (*Penaeus monodon*) and kuruma prawn (*Penaeus japonicus*), and four species of the suborder Pleocyemata, pink shrimp (*Pandalus eous*), king crab (*Paralithodes camtschaticus*) (a kind of hermit crabs), snow crab (*Chionoecetes opilio*), and horsehair crab (*Erimacrus isenbekii*).

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