

Enzyme-Linked Immunosorbent Assay Kit for the Determination of Soybean Protein in Processed Foods: Interlaboratory Evaluation

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The labeling of foods containing ingredients derived from soybean is recommended in Japan because of an increasing number of patients who are allergic to soybeans. To ensure proper labeling, a novel sandwich ELISA kit for the determination of soybean protein in processed foods (FASTKIT Ver. II, "Soybean," Nippon Meat Packers, Inc.; "soy kit") has been developed. Five types of incurred samples (model processed foods: rice gruel, sausage, sweet adzuki bean soup, sweet potato cake, and tomato sauce) containing 10 µg soybean soluble protein/g food were prepared for use in interlaboratory evaluations of the soy kit. The soy kit displayed a sufficient RSD_R value (interlaboratory precision: 9.3–13.4% RSD_R) and a high level of recovery (97–114%) for all the incurred samples. The RSD_r value for the incurred samples was mostly <4.8%. The results of this interlaboratory evaluation suggest that the soy kit can be used as a precise and reliable tool for the determination of soybean proteins in processed foods.

Epidemiological studies suggest that between 6 and 8% of children in industrialized countries exhibit some form of IgE-mediated hypersensitivity manifesting as asthma, atopic dermatitis, or allergic rhinitis (1–3). Among these hypersensitivities, allergic reactions to foods are an important worldwide health problem. Burks et al. (4) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan, as well, the number of patients with food allergies, especially among young children, is increasing (5). The reason appears to be

related to the drastic changes in dietary habits that have occurred 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. Nevertheless, various studies have shown that severe allergic reactions can be induced by the accidental intake of food products containing allergenic materials (6, 7). Therefore, 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 and Agriculture Organization (FAO). In 1999, the Joint FAO/WHO Codex Alimentarius Commission Session agreed to label eight kinds of foods containing known allergens, including soybean (8, 9). Soybean is one of the "big eight" allergenic foods that are believed to be responsible for 90% of all food allergies (10). Subsequently, various countries or regions have considered the labeling list established by the Codex guidelines and have developed their own lists of the most commonly allergenic foods.

Since April 2002, the Ministry of Health, Labor and Welfare (MHLW) of Japan has enforced a labeling system for allergenic food materials to ensure that information on these foods is available to allergic consumers. According to Japanese regulations, the labeling of food products containing eggs, milk, wheat, buckwheat, peanuts, shrimp, and crab is mandatory, and recommended for 18 other food materials, including soybean, in light of the number of allergic patients and the degree of seriousness of the allergic reactions. In a ministry notification published in 2002 (11), the MHLW announced the official Japanese methods for detecting allergens requiring mandatory labeling and the threshold

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Table 1. Homogeneity test results of the incurred samples

Sample	Mean, $\mu\text{g/g}$	RSD, % ^a	<i>n</i>	<i>F</i> -ratio	<i>F</i> _{crit} ^b
Rice gruel	10.4	2.2	6	1.0	4.4
Sausage	8.4	4.6	6	0.6	4.4
Sweet adzuki bean soup	10.9	3.6	6	1.3	4.4
Sweet potato cake	9.9	3.3	6	1.1	4.4
Tomato sauce	9.4	3.3	6	0.5	4.4

^a RSD% calculated from s_s (SD of sampling) and s_a (SD of analysis).

^b *F*_{crit} = Critical *F* value.

value for labeling (10 $\mu\text{g/g}$), the allergenic ingredient soluble protein weight/food weight. The MHLW also described the criteria for interlaboratory validation protocols in their official guidelines, which were published in 2006 (12). Briefly, interlaboratory validation protocols must meet the following criteria: number of laboratories ≥ 8 , number of incurred samples ≥ 5 , number of dose levels ≥ 1 (including 10 $\mu\text{g/g}$, the allergenic ingredient soluble protein weight/food weight), recovery of 50–150%, $\text{RSD}_R \leq 25\%$. The reference material, the initial extract solution, and the allergen extraction procedure were also specified and standardized in the guidelines.

Epidemiological investigations in Japan have shown that the number of patients with soybean allergy has increased (13). In recommendations regarding labeling, soybean labeling may be particularly important because of the vast number of food products containing some form of soybean and because the number of patients with allergies to soybean has been increasing, although soybean allergy is still less prevalent than, for example, milk allergy within the food-allergic population (14, 15). Soybean allergic reactions can be elicited by the intake of trace amounts of soybean proteins, and anaphylaxis caused by soybean has been reported (16, 17). Therefore, a reliable method of detecting soybean protein is necessary for monitoring to ensure accurate labeling. We have developed a highly sensitive ELISA kit capable of detecting soybean protein, the FASTKIT Ver. II, “Soybean,” hereafter referred to as the “soy kit” (18). In the present study, we describe the results of an interlaboratory evaluation of the performance of this ELISA kit.

Experimental

Materials and Methods

Preparation of defatted soybean powder.—Equal amounts of several varieties of soybeans (*Glycine max* var.), including *Enrei*, *Haruyutaka*, *Nattosoryu*, and *Toyomusume*, were homogenized in a food processor (IFM-700G; Iwatani International Co., Osaka, Japan), and the mixed soybean powder was collected through a 0.3 mm mesh. After fats were removed with acetone and *n*-hexane, the defatted soybean powder was air-dried for 48 h. Soybean soluble protein (SP) was then extracted from the defatted soybean powder using

20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.5% sodium dodecyl sulfate (SDS), and 2% 2-mercaptoethanol (2-ME). The SP content was then calculated using a 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK). The amount of SP per 1 g defatted soybean powder was ca 225 mg.

Preparation of incurred samples.—To obtain a final soybean protein concentration of 10 $\mu\text{g/g}$ (SP weight/sample weight) in the incurred samples, the amount of defatted soybean powder to be spiked in the incurred samples at the ingredient stage was calculated, taking into account the protein content of the defatted soybean powder and the change in weight of the incurred samples during preparation. Because the protein amount per 1 g of the defatted soybean powder was ca 225 mg, 44.4 mg of the defatted soybean powder was spiked to make 1 kg of each incurred sample [the final concentration was calculated for 10 $\mu\text{g/g}$ (SP weight/sample weight)]. The ratio of the SP weight to the defatted soybean powder weight was estimated to be 22.5%.

All the incurred samples were prepared according to the general procedures of food manufacturers. The detailed procedures were as follows:

The rice gruel sample consisted of rice, sugar, and water. Rice, sugar, water, and the defatted soybean powder were mixed in a blender (MX-X6; Panasonic Co., Osaka, Japan) and homogenized. The homogenate was packed in a retort bag (SP-1000K; Mita Rika Kogyo Co., Ltd, Osaka, Japan) and cooked at 121°C for 10 min. The cooked rice gruel was then cooled in flowing water and stored at –20°C.

The sausage sample consisted of pork, salt, sugar, and water. These ingredients plus the defatted soybean powder were mixed and homogenized in a food processor. The homogenate was packed in a retort bag and cooked at 80°C for 20 min. The cooked sausage was then cooled in flowing water and stored at –20°C.

The sweet adzuki bean soup sample consisted of adzuki beans, sugar, and water. The adzuki beans were precooked in boiling water for 40 min, then filtered through a 0.3 mm mesh. The cooked adzuki beans, sugar, water, and the defatted soybean powder were mixed in a blender and homogenized. The homogenate was packed in a retort bag and cooked at 100°C for 10 min. The cooked sweet adzuki bean soup was then cooled in flowing water and stored at –20°C.

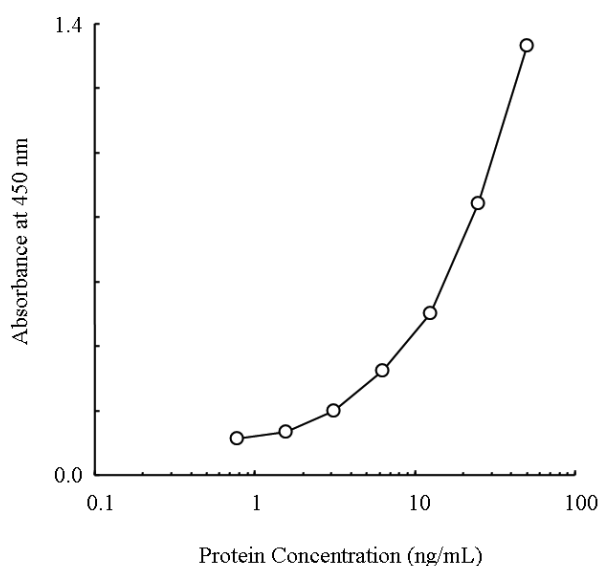


Figure 1. Soybean protein calibration curve for the soy kit. The calibration curve was obtained from the mean value after eight experiments. Concentration of calibration standard solution (calculated SP weight/food weight) = 0.78 ng/mL (0.31 μ g/g), 1.56 ng/mL (0.62 μ g/g), 3.13 ng/mL (1.25 μ g/g), 6.25 ng/mL (2.5 μ g/g), 12.5 ng/mL (5 μ g/g), 25 ng/mL (10 μ g/g), and 50 ng/mL (20.0 μ g/g). The correlation coefficient between the protein concentration and the optical density was found to be >0.999.

The sweet potato cake sample consisted of sweet potato, sugar, and water. The sweet potato was precooked for 5 min in a microwave oven (RE-S240; Sharp Co., Osaka, Japan). The cooked sweet potato, sugar, water, and the defatted soybean powder were mixed and homogenized in a food processor. The homogenate was packed in the retort bag and cooked at 100°C for 15 min. The cooked sweet potato cake was then cooled in flowing water and stored at -20°C.

The tomato sauce sample consisted of tomato puree, salt, sugar, and water. Tomato puree, salt, sugar, water, and the defatted soybean powder were mixed in a blender and homogenized. The homogenate was packed in the retort bag and cooked at 100°C for 20 min. The cooked tomato sauce was then cooled in flowing water and stored at -20°C.

Each type of incurred sample was also prepared as a blank sample that did not contain the defatted soybean powder to examine potential contamination, false positives, and matrix effects. All the values for the blank samples of the model processed foods that were determined using the soy kit were less than the LOD values determined by the in-house study (data not shown).

Homogeneity tests of incurred samples.—The homogeneity of the incurred samples was verified by the coordinator prior to distribution, following the procedure described in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (19); the only modification to this procedure was that the number of test materials was six.

Twelve test portions of each incurred sample were analyzed using the soy kit. The resulting SP concentrations were then determined using a one-way analysis of variance (ANOVA).

Test materials.—Five incurred samples (model processed foods: rice gruel, sausage, sweet adzuki bean soup, sweet potato cake, and tomato sauce) containing SP were prepared and used as test materials. The defatted soybean powder was spiked at the ingredient stage before processing to establish a final dose level of 10 μ g/g (SP weight/sample weight). The prepared incurred samples were homogenized with a food processor (BLIXER-5 plus; Robot Coupe USA Inc., Jackson, MS) and sent to the participating laboratories as test materials.

ELISA Kit [FASTKIT ELISA Ver. II "Soybean" (soy kit; 18)]

Polyclonal antibodies to p34, the allergenic protein in soybean (also known as Gly m Bd 30K), were used in the soy kit, and the SP solution was used for calibration. The outline of the procedure was as follows: Diluted standard solutions and sample solutions were added to a polyclonal antibody-coated module and incubated for 1 h at room temperature. After the module was washed, a solution containing the biotin-labeled polyclonal antibody to p34 was added and allowed to stand for 1 h at room temperature. After the module was washed, a solution of horseradish peroxidase-conjugated streptavidin was added and allowed to stand for 30 min at room temperature. After the final washing, a solution of 3,3',5,5'-tetramethylbenzidine was added, and the module was allowed to stand at 25°C for exactly 20 min. The reaction was stopped by the addition of 0.25 M sulfuric acid, and the absorbances were measured at 450 nm, with 650 nm as the reference wavelength.

Extraction

The incurred samples were homogenized in a food processor. A 1.0 g portion of the test material was extracted using 19 mL of the extraction buffer [120 mM Tris-HCl, pH 7.4; 0.1% bovine serum albumin (BSA); 0.05% Tween 20; 2% SDS; and 2% 2-ME]. The mixture was shaken horizontally overnight (16 h) at room temperature, then centrifuged at 3000 $\times$ g for 20 min after the pH was adjusted to 6.0–8.0. The supernatant was filtered, if necessary, diluted 20 times using diluting buffer (0.1 M phosphate-buffered salt, pH 7.4; 0.1% BSA; 0.1% Tween 20; 0.1% SDS; and 0.1% 2-ME), and subjected to ELISA.

Calibration Standard Solution

The calibration standard solution was prepared according to the official guideline in Japan and the previous reports (11, 20, 21). The initial extract for the calibration standard solution was prepared from the defatted soybean powder as follows: a 0.3 g sample of the defatted soybean powder was added to a 20 mL mixture of 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.5% SDS, and 2% 2-ME. The mixture was then shaken for 16 h at room temperature prior to extraction. After the extraction, the sample was centrifuged at 10 000 $\times$ g for 30 min, and the supernatant was filtered through a 0.8 μ m

Table 2. Results of the interlaboratory study for the soy kit: protein recovery content^a

Lab	Rice gruel		Sausage		Sweet adzuki bean soup		Sweet potato cake		Tomato sauce	
	Mean, $\mu\text{g/g}^b$	Recovery, % ^c	Mean, $\mu\text{g/g}$	Recovery, %	Mean, $\mu\text{g/g}$	Recovery, %	Mean, $\mu\text{g/g}$	Recovery, %	Mean, $\mu\text{g/g}$	Recovery, %
A	9.2	92	9.3	93	10.3	103	9.4	94	9.0	90
B	9.8	98	9.0	90	13.7	137	9.1	91	8.7	87
C	11.4	114	10.5	105	11.0	110	10.1	101	10.1	101
D	10.9	109	10.3	103	11.6	116	10.2	102	9.9	99
E	9.7	97	9.3	93	10.9	109	9.2	92	9.1	91
F	10.1	101	9.9	99	10.5	105	8.6	86	9.1	91
G	11.8	118	12.5	125	14.3	143	13.0	130	12.7	127
H	9.9	99	10.0	100	10.5	105	9.6	96	9.4	94
I	10.2	102	10.5	105	11.8	118	11.3	113	10.5	105
J	8.8	88	8.5	85	9.3	93	8.5	85	8.0	80
K	10.1	101	9.9	99	11.0	110	10.2	102	9.8	98

^a The incurred samples contained SP of approximately 10 $\mu\text{g/g}$.

^b Mean = average concentration of SP (SP weight/food weight).

^c Recovery = mean/10 $\mu\text{g/g}$ (%).

micro-filter paper (DISMIC 25cs; Advantec, Tokyo, Japan) to obtain the extract. The protein content of the initial extract was assayed using a 2-D Quant Kit (GE Healthcare). The initial extract was diluted with 0.1 M phosphate-buffered salt (pH 7.4), 0.1% SDS, 0.1% 2-ME, 0.1% BSA, and 0.1% Tween 20 to compose the calibration standard solution (50 ng/mL extracted protein) for the soy kit. The calibration standard solutions for SP were provided by Oriental Yeast Co., Ltd (Nagahama, Japan).

Interlaboratory Study

Eleven laboratories participated in the interlaboratory evaluation, which was organized by the National Institute of Health Sciences (Tokyo, Japan). The participants included manufacturing companies, public research institutes, local public inspection institutes, and private inspection institutes. The organizer sent each laboratory the five test materials (3 g each) and the ELISA kit plus the extraction solution and the calibration standard solution. The participants took two portions from each test material, extracted the protein using the extraction procedure, and assayed the extract using the ELISA kit. The calibration standard solution was diluted and assayed simultaneously with the incurred sample extracts. Each sample extract was analyzed in triplicate (three wells/sample extraction), and the average absorbance of three wells was used for the calculation. The resulting absorbance data for the calibration solutions and test materials were reported to the coordinator.

The organizer calculated a 4-parameter logistic calibration curve based on the absorbance data of the calibration standard solution and calculated the concentrations of SP in the incurred samples using the calibration curve. Each set of

samples was extrapolated from the standard curve run in the same plate.

This study was performed between July and August 2007.

Statistical Analysis

Twenty-two data items (two portions evaluated at 11 laboratories) were used in the calculations. The Cochran's test and the Grubbs' test were used to remove outlying data ($P = 2.5\%$). The Cochran's test was used to remove data from laboratories reporting a significantly large variability between the results of the two portions taken from the test material. The Grubbs' test was used to remove data from laboratories reporting a mean of the results for the two portions that was significantly different from those of the other laboratories. The use of statistical tests to identify outliers was in accordance with the AOAC protocol (22). Recovery, repeatability, and reproducibility were calculated with data generated by one-way ANOVA applied to data after the removal of outliers.

Results and Discussion

Homogeneity of the Test Materials

Table 1 shows the average concentration, the RSD percentages calculated from s_s (SD of sampling) and s_a (SD of analysis), and the F -ratios. The values of s_s and s_a were calculated with data generated by one-way ANOVA. The F -ratios for all the incurred samples were below the critical F value. The resultant F -ratios of the homogeneity tests for the rice gruel, sausage, sweet adzuki bean soup, sweet potato cake, and tomato sauce samples were <1.3 . As the critical value of F was 4.4, the homogeneity of the test materials was regarded as sufficient for the interlaboratory evaluation. For

Table 3. Recoveries, RSD_r, and RSD_R of the soy kit for SP

Sample	No. of laboratories	Mean, µg/g	s _r , µg/g	s _R , µg/g	Recovery, %	RSD _r , %	RSD _R , %
Rice gruel	11	10.2	0.5	0.9	102	4.8	9.3
Sausage	11	10.0	0.3	1.1	100	3.5	10.8
Sweet adzuki bean soup	11	11.4	0.3	1.5	114	2.5	12.9
Sweet potato cake	11	9.9	0.4	1.3	99	3.9	13.4
Tomato sauce	11	9.7	0.5	1.3	97	4.8	13.2

most of the test materials, the RSD values between the portions were <4.6%, which was smaller than the required RSD_R values (≤25%).

Calibration Curve

Figure 1 shows the calibration curve for the determination of SP using the soy kit. The calibration curve was obtained using the 4-parameter logistic model. This graph showed an excellent correlation between the protein concentration and the optical density in the range of 0.78–50 ng/mL (correlation coefficient >0.999), and 25 ng/mL, which corresponds to an SP dose level of 10 µg/g in the test materials, falls within the dynamic range of the curve. We determined the LOD and LOQ according to the guidelines issued by the International Standards Organization (ISO) and the International Union of Pure and Applied Chemistry (23) by the in-house study. The LOD was calculated as 3 times the SD of the mean value of the dilution buffer after eight experiments. The LOQ was calculated as 10 times the SD of the mean value of the dilution buffer after eight experiments. The LOD and the LOQ of the soy kit, as determined using the dilution buffer, were 0.47 ng/mL (equivalent to 0.19 µg/g of food sample) and 0.78 ng/mL (equivalent to 0.31 µg/g of food sample), respectively. Consequently, the practical determination range was between 0.78 and 50 ng/mL.

Recovery, Repeatability, and Reproducibility

The values reported by the participants are summarized in Table 2. Two kinds of statistical analyses, the Cochran's test and the Grubbs' test, found no outliers in these values. The recovery, RSD_r, and RSD_R values calculated using an ANOVA are shown in Table 3.

As shown in Table 3, the recovery of SP from the five types of test materials using the soy kit ranged from 97 to 114%. These values indicate a high degree of recovery for all the test materials, especially for the sausage and sweet potato cake samples (100 and 99%, respectively). The recovery of SP from the sweet adzuki bean soup sample was comparatively higher than those of the other four incurred samples. Since we confirmed that this kit does not cross-react with adzuki beans in a previous article (18), this result is clearly not caused by the adzuki beans, which were one of the ingredients in the incurred sample. As shown in Table 2, laboratories B and G reported high recoveries of SP (137 and 143%, respectively).

Because of the difficulty in sampling suspended foods, such as adzuki bean soup, the amounts sampled by these two laboratories might have been incorrect. In any event, these recovery values were not too high and satisfied the criteria (50–150%), and no outlier existed statistically.

Repeatability is a measure of the variance arising from the entire analytical procedure in a particular laboratory. In the intralaboratory evaluations, all the RSD_r values were found to be <4.8%. According to Horwitz's theory, the RSD_r value is generally likely to be <2/3 of the RSD_R value (24). In this study, all the RSD_r values for the soy kit were <2/3 of the corresponding RSD_R value. Thus, these satisfactory RSD_r values were considered to guarantee the reliability of this analytical method.

The reproducibilities, expressed by the RSD_R values of SP from the five types of test materials using the soy kit, ranged between 9.3 and 13.4%. Thus, the soy kit displayed a relatively high level of reproducibility in the interlaboratory evaluation results. Therefore, we concluded that this kit would be very useful as a general method for monitoring allergen ingredients to ensure correct labeling.

We have already reported the interlaboratory studies of 12 ELISA kits for the determination of egg, milk, wheat, buckwheat, peanut, and crustacean protein in processed foods (20, 21). In the case of one buckwheat kit, the results of recovery, repeatability, and reproducibility in the detection of buckwheat proteins in the five model processed foods were 58–136%, 6–13% RSD_r, and 10–25% RSD_R, respectively. In addition, the results of recovery of one milk kit were 89–137%, and the results of reproducibility of one crustacean kit were 18–21% RSD_R. The results of the interlaboratory validation of the soy kit were fairly excellent compared with those of previous interlaboratory studies examining other ELISA kits on the basis of recovery, repeatability, and reproducibility.

The Japanese government MHLW has established the interlaboratory validation protocol in the official guidelines published in 2006 (12). Briefly, the following criteria for the interlaboratory validation protocol were defined: number of laboratories ≥8; number of incurred samples ≥5; number of dose levels ≥1, including 10 µg/g (allergenic ingredient soluble protein weight/food weight); recovery of 50–150%; and RSD_R ≤25%. These criteria were based on the ISO 5725 (JIS Z8402) guidelines, which are mostly the same as those of

AOAC (22). In the official guidelines, the initial extract solution and the extraction procedure from allergen were specified and standardized.

The present study suggests that the soy kit is a reliable and precise method of determining the SP content, and that the performance of the soy kit satisfies the validation criteria described in the official guidelines published by the Japanese government. Because food allergies can induce severe disease, the accuracy of the method is crucial. The data obtained in the interlaboratory evaluation clearly shows that the soy kit can quantify the SP content of the incurred samples very accurately. Moreover, the soy kit produces good repeatability and reproducibility measures because of the high precision of the assay performance. The present interlaboratory evaluation was performed using five incurred samples, including highly processed foods such as rice gruel, sausage, and tomato sauce, as it is necessary to ensure that the kit is able to detect SP in commercial foods to guarantee accurate labeling. The present results demonstrate that the ELISA soy kit is capable of detecting SP contained in processed foods and supporting the food labeling system in accordance with Japanese regulations.

Conclusions

In conclusion, the present ELISA kit is a rapid, precise, and reliable tool for the analysis of SP in processed foods. The proposed system is capable of accurately monitoring labeling systems in a reliable manner and may be useful for inspections performed in accordance with Japanese regulations.

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