Development of Pretreatment Protocols for Determination of Soybean β -Conglycinin in Processed Soybean Foods Using Commercial ELISA Kits

Tatsuya MORIYAMA^{1,2}, Erika YANO¹, Nobuhiro ZAIMA^{1,2}, Keiko MIYAZAKI³,
Kou SHIROTSUKI³, Aki SATO³ and Makoto SAWAGUCHI³

¹Department of Applied Biochemistry, Graduate School of Agriculture, Kindai University,
3327–204 Naka-machi, Nara, Nara 631–8505, Japan

²Agricultural Technology and Innovation Research Institute, Kindai University,
3327–204 Naka-machi, Nara, Nara 631–8505, Japan

³Misuzu Corporation, Department of Quality Technology, 1606 Wakasato,
Nagano, Nagano 380–0928, Japan

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Summary β -Conglycinin is the major storage protein in soybeans. Pre-clinical animal models and human clinical studies have demonstrated the triglyceride-lowering effect of this protein, suggesting that it could be put into practical use as a functional food material. To date, however, there are no accurate and simple assays for quantification of β -conglycinin. In this study, samples were pretreated by mixing them with rice flour powder prior to extraction of proteins. Then, we used commercially available ELISA kits for detection of allergens that could be present in any contaminating soybean residue. This enabled accurate and highly reproducible quantitation of β -conglycinin content in several processed soybean foods.

Key Words β -conglycinin, ELISA, assay, soybean, Kori-tofu, soymilk

Soybean is a vegetable protein food with a high protein content and an excellent amino acid balance. It has long been eaten in countries throughout Asia, including Japan, and contains many biologically active ingredients useful for prevention of chronic diseases (1, 2). Chief among the functional components with low molecular weight are soy isoflavone, lecithin, and saponin. Soy isoflavones have antioxidant and estrogenic effects and may attenuate bone density reduction in postmenopausal women (2–4). Saponins are also known to have anti-obesity effects (5, 6). In addition to the functionality of these small molecules, larger macromolecules such as soy proteins also exert physiological effects. For example, soy protein lowers serum cholesterol in experimental animals and humans (4, 7–12). The putative mechanism of action involves the generation of peptides during the digestion of soy protein (9–11). These peptides bind to bile acids and promote their excretion, thereby interfering with the enterohepatic circulation of bile acids and promoting the synthesis of bile acids from cholesterol in the liver (9–11). In addition, soy protein may also reduce the level of serum triglycerides (13). Specifically, the β -conglycinin (soybean 7S globulin) fraction of soy protein reduces the levels of both triglycerides and/or visceral fat (14–17). The functionality of this protein has been demonstrated both in experimental animals and humans, and has therefore been classified as a novel functional component of soy. β -Conglycinin consists of three subunits (α' , α , and β), with molecular weights of 75, 70 and 50 kDa, respectively. These three

subunits have similar primary structures, and all are glycoproteins. To date methods for the specific and commercially available quantification of these molecules are lacking. In 2005, an antigen-immobilization ELISA (direct ELISA) using mixed antibodies against three subunits of β -conglycinin was reported; this assay yielded quantitative values from various soybean processed foods and soybean cultivars (18). However, the antigen-immobilized ELISA (direct ELISA) approach is generally regarded as poorly quantitative and relatively non-specific. On the other hand, immunochemical assays with excellent specificity and quantitative properties can generally be achieved using sandwich ELISA methods. However, no sandwich ELISA for the components of β -conglycinin has been commercially available to date.

In 2015, the Morinaga Institute of Biological Science, Inc. used antibodies against β -conglycinin to develop and market a sandwich ELISA for the detection of contaminated soy protein residues in order to help people avoid allergen intake. However, this kit uses an antibody against β -conglycinin, and a soy extract protein is packaged as a standard, and the amount of soy protein mixed in is quantitatively detected with high sensitivity (<https://www.miobs.com/product/tokutei/faspek2/index.html>). Therefore, it is impossible to quantify the β -conglycinin amounts just using this kit. In addition, since this kit detects trace amounts of soy protein contaminant in processed foods with extremely high sensitivity, many samples must first be serially diluted before they can be assayed due to their high concentration of soy protein. These issues pose a problem with regard to obtaining accurate quantitative values.

E-mail: tmoriyama@nara.kindai.ac.jp

Table 1. List of materials used for premixing powders.

Material name	Source, product name, grade, etc.
Methylcellulose	Shin-Etsu Chemical Industry Co., Ltd.
Rice flour	Commercially available products
Wheat flour	Commercially available products
Silicon dioxide	FUJIFILM Wako Pure Chemical Corporation, reagent grade
Aluminum oxide	FUJIFILM Wako Pure Chemical Corporation, reagent grade
Calcium carbonate	Yoneyama Yakuhin Kogyo Co., Ltd., reagent grade

In this study, we implemented a sample pretreatment method and used commercially available ELISA kits to accurately quantify β -conglycinin with a high level of sensitivity using the coefficient for calculation determined in the present study.

MATERIALS AND METHODS

Materials. Commercial ELISA kits (FASPEK ELISA II Soybeans) for detection of contaminated soy protein residues containing allergens were purchased from Morinaga Institute of Biological Science, Inc. (Kanagawa). Methylcellulose, rice flour, wheat flour, silicon dioxide, aluminum oxide, calcium carbonate, and rice flour were used as candidate sample pretreatment materials, and were purchased from the places described in Table 1. Electrophoresis-related reagents, such as acrylamide solutions for SDS-PAGE, were obtained from Nacalai Tesque, Inc. (Kyoto). Various processed soybean foods were purchased commercially from local supermarkets. β -Conglycinin was purified from soybeans as described by Nagano et al. (19). For stirring the powder during pretreatment, plastic toy beads with a diameter of 6 mm were used after washing with ethanol. Western blot-related reagents were obtained from Bio-Rad Laboratories, Inc.

Electrophoresis (SDS-PAGE). To confirm the protein composition of the samples, SDS-PAGE electrophoresis (20) was performed. The electrophoresis apparatus used was a Bio-Rad Mini-Protein-3 system. Electrophoresis was carried out at 200 V constant for 35 min. After electrophoresis, gels were stained with CBB (Coomassie Brilliant Blue) R-350 (GE Healthcare).

Determination of β -conglycinin purity by SDS-PAGE. The purity of β -conglycinin extracted from soybeans was determined using the method of densitographic quantification after CBB staining. Briefly, SDS-PAGE and protein staining were followed by densitographic quantification of protein bands using AlphaEase™ software (Alpha Innotech, CA, USA).

Determination of the coefficient for calculation of β -conglycinin content. The ELISA kit used herein employs a calibration curve (prepared from a soy standard solution that accompanies the kit) in order to calculate the mass of soy protein contained in samples. In order to calculate the β -conglycinin content, a coefficient for cor-

rection must be determined. To this end, we attempted to calculate the coefficient by comparing the standard curves obtained with soybean standard supplied with the ELISA kit and with purified β -conglycinin.

Selection of materials during sample pretreatment. Since the sensitivity of this kit is extremely high, soy protein samples that contain β -conglycinin must be serially diluted before measurement. This leads to increased errors associated with measurements. To circumvent this problem, we aimed to develop a method in which the sample to be measured is instead first mixed with another powder that does not interfere with the quantitation. Kori-tofu was selected as the soy product to be measured, and we tested multiple powders (methylcellulose, rice flour, wheat flour, silicon dioxide, aluminum oxide, and calcium carbonate) as candidate 'diluent,' because they do not contain soy proteins that could interfere with the assay. The powders that mixed easily during stirring and that did not affect the final quantitative value were selected for further experiments.

As a method of selecting the pre-treated material, the variation of the measured value was evaluated from the absorbance value of the ELISA method for the pre-treated material alone and the result of measuring Kori-tofu as a sample using the pre-treated material. For the measurement of the ELISA absorbance value of the pre-treated material alone, 0.1 g of the pre-treated material to be measured was added to 19.9 mL of the extract liquid from a commercially available ELISA kit, and extraction was performed at 25°C overnight.

For pretreatment before extraction of Kori-tofu, 9.5 g of the pretreatment material and 0.5 g of the analytical sample (which had been defatted with diethyl ether in advance) were placed in a plastic container. Twelve grams of plastic beads were placed in a plastic container so that they could be uniformly mixed and agitated, tightly capped, and then manually shaken for 5 min; this mixture constituted the sample to be used in the ELISA. After accurately weighing 0.5 g of the premixed sample for the ELISA method, it was added to 19.5 mL of the extract buffer from the commercially available ELISA method kit, and extraction was performed overnight at 25°C. As a blank, extraction with only the sample pretreatment material was also performed. The extract was then centrifuged for 3,000 $\times g$ for 20 min and the intermediate layer was subjected to the ELISA method.

Subsequent analyses were performed according to the kit operating manual, and absorbance was measured using plate readers (Wallac ARVO 1420, Perkin Elmer).

Confirmation of the effect of pretreatment. In order to confirm the measurement accuracy following pretreatment, the following experiments were performed. First, cases where the soy product was measured with or without pretreatment were compared. Second, we determined the values obtained when different amounts of soy product were used during the extraction. Finally, we examined inter-laboratory variation by obtaining measurements from four different laboratories. The soy products used for the measurements were selected from commercial Kori-tofu (Misuzu Corporation).

Table 2. Selection of sample pretreatment materials.

Pretreated materials	Diluent absorbance Mean $\pm$ SD ($n=3$)	Mixing operability	Obtained ELISA data (β -conglycinin concentration of Kori-tofu)	
			β -Conglycinin concentration (mg/g)	Coefficient of variation (CV)
			Mean $\pm$ SD ($n=3$)	(%)
Methylcellulose	0.059 $\pm$ 0.007	$\triangle$	126.8 $\pm$ 40.9	32.3
Rice flour	0.041 $\pm$ 0.004	$\circ$	144.0 $\pm$ 8.0	5.6
Wheat flour	0.054 $\pm$ 0.007	$\triangle$	170.6 $\pm$ 30.2	17.7
Silicon dioxide	0.045 $\pm$ 0.013	$\circ$	192.5 $\pm$ 29.5	15.3
Aluminum oxide	0.042 $\pm$ 0.006	$\circ$	157.6 $\pm$ 9.3	5.9
Calcium carbonate	0.060 $\pm$ 0.009	$\circ$	157.2 $\pm$ 16.1	10.2

$\circ$: good, $\triangle$: somewhat difficult.

To confirm the effect of pretreatment, sample extraction following the ELISA method was carried out by adding 0.5 g of Kori-tofu powder directly to 19.5 mL of the extraction buffer without the pretreatment, and 0.5 g of powder obtained by premixing rice flour and Kori-tofu (19 : 1, v/v; 20-fold dilution) to 19.5 mL of the extraction buffer (with the pretreatment). Extraction was then carried out prior to measurement, and according to the procedures described in the ELISA kit manuals.

When comparing readings obtained with different amounts of soybeans, we adopted the following procedure. Powders were prepared by mixing rice flour and Kori-tofu at a ratio of 99 : 1 (Kori-tofu 0.1 g for 9.9 g of rice flour), 19 : 1 (Kori-tofu 0.5 g for 9.5 g of rice flour) and 9 : 1 (Kori-tofu 1.0 g for 9.0 g of rice flour). In each case, 0.5 g of these premixed powders was added to 19.5 mL of extraction solution before performing extraction and measurements as described in the ELISA kit manuals.

Inter-laboratory test. We evaluated inter-laboratory error by comparing measurements obtained from identical Kori-tofu sample and soybean flour specimens. The specimens had been defatted with diethyl ether in advance during the pre-treatment method at each laboratory. The experiments were performed at the Faculty of Agriculture, Kindai University (Lab. A), Research Institute of the Department of Quality Technology of Misuzu Corporation (Lab. B), Department of Food and Health Sciences, The University of Nagano (Lab. C) and the Food Technology Department of Nagano Prefecture General Industrial Technology Center (Lab. D). The researchers carried out the experiments three times. The equipment used by each laboratory is described as Table 2.

Calculation and detection of β -conglycinin contained in various soybean processed food products. We next attempted to calculate the amount of β -conglycinin in various commercially available soybean processed food products. In addition to Kori-tofu, commercial products of soybean flour, soymilk, and powdered natto were used. The Kori-tofu was powdered, soybean milk was freeze-dried, and the soybean flour and the powdered natto were subjected to defatting with diethyl ether. A

Fig. 1. SDS-PAGE of purified β -conglycinin. Purified β -conglycinin was subjected to SDS-PAGE and stained with CBB R-350 as described in "Materials and Methods." lane 1: blank, lane 2: 0.4 μ g, lane 3: 0.8 μ g, lane 4: 1.2 μ g, lane 5: 1.6 μ g of purified β -conglycinin.

sample was prepared by mixing 0.5 g of each powder and 9.5 g of rice flour by the above method, and 0.5 g of the mixed sample was added to 19.5 mL of the extraction buffer as described in the ELISA protocol prior to extraction and quantification.

Western blotting. To identify protein patterns in the extracts, each of the soybean products was electrophoresed by SDS-PAGE as described above, and then to Western blotting for β -conglycinin. Applied sample powder weights were indicated in figure legends. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore Immobilon-P™) using a semi-dry blotting method, blocked with a PBST containing skim milk, and then incubated with primary anti- β -con-

Fig. 2. Calibration curves of β -conglycinin and soybean protein standard equipped in the ELISA kit. Calibration curves were obtained with standard soybean protein supplied with the kit (○) and purified β -conglycinin (■).

glycinin antibody (rabbit polyclonal) produced previously (18) for 1 h. The membrane was washed with PBST, and incubated with HRP-labeled anti-rabbit IgG antibody for 1 h. Thereafter, the sample was washed again with PBST, and signals were detected by chemiluminescence using ECL-chemiluminescence Western blotting detecting reagents (GE-Healthcare) and X-ray films in a dark room.

RESULTS

Confirmation of β -conglycinin purity

Purified β -conglycinin was subjected to SDS-PAGE and purity was confirmed by CBB staining. As shown in Fig. 1, the three β -conglycinin subunits (α' , α , and β) were specifically detected, and virtually no other bands were present. Using densitometry software, β -conglycinin purity was calculated to be 85.0%.

Determination of the coefficient for calculation of β -conglycinin content

Purified β -conglycinin was measured in a commercial ELISA kit in three technical replicates; the absorbance was proportional to the soybean reference standard supplied (Fig. 2). By comparing the difference between the quantitative values of soybean reference standard and purified β -conglycinin, we considered a correction factor of 0.24 to be reasonable. This value was calculated by dividing the coefficient of β -conglycinin in the calibration curve by the coefficient of the soybean reference standard and multiplying by the purity (85.0%) of the purified β -conglycinin ($7.608/27.126 \times 0.85 = 0.24$).

Selection of sample pretreatment materials suitable for dilution of powder samples

In this study, we first considered the method of diluting powdered sample to be measured with another powder that does not interfere with quantitation. Kori-tofu was selected as a representative soybean product test sample, and the powders to be used as diluents were selected from methylcellulose, rice flour, wheat flour,

Table 3. Determination of β -conglycinin concentration of Kori-tofu with or without pretreatment.

	β -Conglycinin concentration (mg/g) Mean $\pm$ SD ($n=3$)	Coefficient of variation (CV) (%)
No pretreatment	179.2 $\pm$ 22.7	12.7
With pretreatment	147.8 $\pm$ 10.5	7.1

silicon dioxide, aluminum oxide, and calcium carbonate. These powders were chosen because they do not contain soybean proteins, they have particle sizes close to that of a powdery soybean product, they mix readily without sticking to containers when mixing, and they give a stable coloration in an ELISA assay. The powders that did not stick to the container wall surface when stirring, and did not affect the final quantitative value were selected for further experiments. The results are shown in Table 2. Using wheat flour, silicon dioxide, rice flour, and aluminium oxide, the blank absorbance values of the matrices were extremely low, and no color was observed on visual inspection. Methylcellulose and calcium carbonate had also low absorbances, although some color developed in the final solutions. Overall, the results confirmed that none of the pretreated materials had an adverse effect on the quantitative test. Methylcellulose and wheat flour had inferior mixing qualities than calcium carbonate, silicon dioxide, rice flour and aluminum oxide, since they adhered to the container wall during shaking. When methylcellulose was used as a pretreatment material, the coefficient of variation was as high as 32.3%, compared with the β -conglycinin concentration of 126.8 mg/g. The coefficients of variation for other materials were 17.7% for wheat flour, 15.3% for silicon dioxide, and 10.2% for calcium

Fig. 3. The dose-dependent measured values by adding Kori-tofu samples. Powdered Kori-tofu was defatted and pretreated with rice flour and subjected to ELISA assay as described in “Materials and Methods.” Data are shown as mean $\pm$ SD.

Table 4. Equipments used in each laboratory for Inter-laboratory evaluation.

Laboratories	Equipments used in each laboratory	
Lab. A	Shaker	Bio Shaker BR-23FP (TAITEC)
	Plate-reader	Wallac ArvoSX 1420 multilabel counter (Perkin Elmer)
Lab. B	Shaker	WaterBaths Shaker SB-220 (SHIMADZU)
	Plate-reader	iMark Microplate absorbance reader (BIO-RAD)
Lab. C	Shaker	Shaking Incubator SI-300 (AS ONE)
	Plate-reader	Microplate absorbance reader SH-1200Lab (Corona Electric)
Lab. D	Shaker	Bio-Shaker BR-43FM (TAITEC)
	Plate-reader	Varioskan Flash (ThermoFisher)

carbonate, while the coefficients of variation for rice flour and aluminum oxide were best at 5.6% and 5.9%, respectively. Based on these results we selected rice flour as the pretreatment mixing material for use in subsequent experiments.

Confirmation of measurement variation

We next used Kori-tofu as the sample to determine whether the pretreatment method could reduce variation in measurements. The data in Table 3 confirm that this was indeed the case. The SD and CV values obtained with pretreatment protocol were obviously reduced compared with those with no pretreatment.

Measurement of β -conglycinin content in Kori-tofu powder samples diluted in rice flour during pretreatment.

Kori-tofu was first powdered, defatted and mixed with rice flour. Then, samples of this mixture were subjected to extraction before being measured according to the Morinaga ELISA kit protocols. Figure 3 shows that the measured value increased proportionally (and in a linear manner) with the amount of Kori-tofu powder sample added.

Table 5. Measurements of β -conglycinin concentrations of Kori-tofu in four different laboratories.

Laboratories	β -Conglycinin concentration (mg/g) Mean $\pm$ SD (n = 3)	Coefficient of variation (CV) (%)
Lab. A	136.9 $\pm$ 0.8	0.6
Lab. B	135.7 $\pm$ 8.9	6.5
Lab. C	139.2 $\pm$ 5.4	3.9
Lab. D	133.9 $\pm$ 1.4	1.1
Average	136.3 $\pm$ 4.8	3.5

Table 6. Measurements of β -conglycinin concentrations of soybean flour in four different laboratories.

	β -Conglycinin concentration (mg/g) Mean $\pm$ SD (n = 3)	Coefficient of variation (CV) (%)
Lab. A	78.9 $\pm$ 7.3	3.1
Lab. B	71.0 $\pm$ 7.1	10.0
Lab. C	84.4 $\pm$ 0.8	1.0
Lab. D	72.9 $\pm$ 8.3	11.4
Average	76.8 $\pm$ 7.3	9.5

Inter-laboratory test

We next compared the measurements obtained using the pretreatment method in four different laboratories. The equipment used in each laboratory is shown in Table 4 and the results are shown in Table 5. The difference between the measured values for Kori-tofu was 136.3 mg/g on average, and the range of measured values between the laboratories was 133.9–139.2 mg/g. Based on these data, we conclude that there were no significant differences in measurements between laboratories.

In the case of soybean flour, as a result shown in the Table 6, the minimum and maximum laboratory measurements were 71.0 mg/g and 84.4 mg/g, respectively, compared with the average of 76.8 mg/g, respectively. Although there was some variation in comparison with the measured Kori-tofu values, there was no significant difference ($p > 0.05$, Dunnett test).

Determination of β -conglycinin content in various soybean processed foods

The β -conglycinin content in various processed soybean foods was quantified using the pretreatment method and commercial quantitative ELISA kits. First, in order to qualitatively confirm β -conglycinin in the processed soybean foods used for the measurements, we visualized the three β -conglycinin subunits following CBB-staining. All three subunits could be detected in the foods tested. An exception was natto; we were unable to confirm the presence of the α' subunit and levels of the β subunit were very low (Fig. 4).

Fig. 4. Protein patterns of processed soybean foods stained with CBB (A) and detection of β -conglycinin by Western blotting (B). Arrows indicate subunits of β -conglycinin. lane 1: soybean flour, lane 2: dried-soymilk, lane 3: Kori-tofu, lane 4: natto powder. Applied sample powder weights were 10 μ g/lane (A) and 0.25 μ g/lane (B).

Table 7. Determination of β -conglycinin contents in the various soybean processed foods.

	β -Conglycinin (mg/g defatted dry powder)	β -Conglycinin (mg/g raw product)
Soybean flour	118.8 $\pm$ 1.1	84.4 $\pm$ 0.8
Soymilk	128.7 $\pm$ 0.7	12.8 $\pm$ 0.1
Kori-tofu	199.5 $\pm$ 7.7	144.0 $\pm$ 8.0
Natto	14.8 $\pm$ 0.8	4.1 $\pm$ 0.2

The quantitative results obtained from foodstuffs in the defatted powder state and actual raw product form are shown in Table 7.

DISCUSSION

A potent triglyceride-lowering effect has been reported in a protein fraction called β -conglycinin (soybean 7S globulin) (14–17). The functionality of this protein has been demonstrated not only in animals but also in humans, and is expected to be a novel functional food component. Until now, the specific quantitation method of the present molecule has not been well established. Therefore, in this study, we tried to perform accurate quantitation with good reproducibility by devising pretreatment of samples using commercial ELISA kits for detection of contaminated soybeans residues containing allergens.

Here, we demonstrated that measurement of β -con-

glycinin content in food samples is more robust and reproducible if the samples are first diluted in another powdered material that is free from soybean contaminants, such as rice flour. In order to provide accurate quantification of β -conglycinin content, we determined that a correction coefficient must be applied when using commercial ELISA kits.

The ELISA kit we used this time is designed to calculate the mass of soybean protein from the calibration curve prepared with the attached soybean protein standard solution. In order to calculate the β -conglycinin content, it is necessary to fix a coefficient for correction. Comparison of the measured values of pure β -conglycinin with the soybean standard supplied with the ELISA kit resulted in a correction factor of 0.24. That is, by multiplying the value of the soy protein content calculated using this kit by this coefficient of 0.24, it was considered possible to quantify β -conglycinin. Glycinin and β -conglycinin have mainly been reported as the main composition of soy protein, but the ratio of β -conglycinin in soy protein is said to be 18% to 28% (21), and the correction factor of 0.24 can be judged to be within a reasonable range. Since this coefficient is the correction coefficient with the reference standard for commercial kits used this time, it is considered necessary to calculate the correction coefficient separately when performing the ELISA analysis by methods other than this kit.

Next, as a result of verifying whether or not the accuracy of the variation of the measured value is improved by the presence or absence of the pretreatment using Kori-tofu, which is a soy product, the standard deviation

was reduced by the pretreatment, and the improvement was observed. As a result of measuring the addition amount of Kori-tofu by varying the addition amount, the measured value increased linearly depending on the addition amount.

Various soybean products were subjected to SDS-PAGE electrophoresis and Western blotting, and the pretreatment method used in this study was used for measurements. As a result, α' , α , and β subunits were detected in soybean flour, soymilk, and Kori-tofu, and only β subunits were detected in natto. The presence of β -conglycinin in the soybean products tested in this study could be determined by Western blot analysis. As a result of measuring these soybean products by the ELISA method in this method, the measured value of β -conglycinin amount of each soybean product was obtained as shown in Table 7. Of these, soymilk has lower levels of β -conglycinin in its products compared to the strength of the SDS-PAGE bands. This is because soymilk beverages prepared by freeze-drying are subjected to a test, and the amount of β -conglycinin is calculated by the ELISA method and then converted into a beverage state. For soybean flour, Kori-tofu, and natto other than soybean milk, these values are measured according to the density of SDS-PAGE and Western blot bands.

In addition, the protein mass (per 100 g) of each product, quoted from the 2015 edition of Standard Tables of Food Composition in Japan (seven revisions) (<https://fooddb.mext.go.jp>), was 33.0 g for soybean flour, 3.6 g for soymilk, and 50.5 g for Kori-tofu. When the β -conglycinin content ratio was 18–28%, soybean flour, soymilk, and Kori-tofu all fell within the range of the values measured by the current ELISA methods. For natto, β -conglycinin might be degraded during fermentation. Similar results were reported previously (18).

In this study, we attempted to quantify β -conglycinin using commercially available ELISA kits for the detection of contaminated soybean residual proteins containing allergens constructed using antibodies against β -conglycinin. By applying the correction coefficient clarified current study and devising the pretreatment of the sample, it becomes possible to perform accurate quantitation with good reproducibility in a reasonable manner. In summary, our novel assay method facilitates the quantification of β -conglycinin containing at high concentrations, such as soybean processed foods. It can also be used for checking the content at the time of product sale or for mixed foodstuffs using ingredients other than soybean. However, more research is required in order to further reduce the chances of measurement errors due to slight contamination that may occur following adherence of the samples to test instruments, for example.

Authorship

Research conception and design: TM and MS; experiments: EY, KM, KS and AS; statistical analysis of the data: TM, EY, KM and MS; interpretation of the data: NZ, KS and AS; writing of the manuscript: TM.

Disclosure of state of COI

K.M., K.S., A.S. and M.S. are employees of Misuzu Corporation (Nagano). All the other authors declared no competing interests.

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