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Determination of beta-conglycinin in soybean and soybean products using a sandwich enzyme-linked immunosorbent assay

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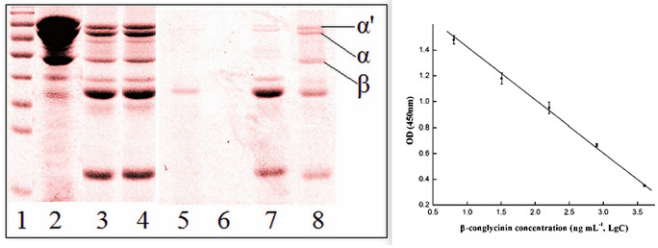
HIGHLIGHTS

- A double antibody sandwich ELISA was developed to measure β-conglycinin in soybean and soybean products.
- The assay had a practical working range between 3 and 100 ng mL^{-1}, and showed no cross-reactivity with other proteins.
- The assay was used to analyze 469 soybean seeds and five soybean products treated with different processing techniques.

GRAPHICAL ABSTRACT



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ABSTRACT

Soybean protein has long been recognized as a source of dietary allergens for humans and animals with β-conglycinin being the major allergen. This paper presents a sandwich enzyme-linked immunosorbent assay (ELISA) that allows for the detection of trace amount of β-conglycinin in soybean and soybean products. In the sandwich ELISA, mouse anti-β-conglycinin monoclonal antibody (Mab 5C5) was used as coating antibody, and rabbit anti-β-conglycinin polyclonal antibody (Pab) was used as secondary antibody. The assay showed high specificity for β-conglycinin with minimum cross-reactions with other soy proteins. The practical working range for the determination of β-conglycinin using the developed assay was 3–100 ng mL^{-1} and the limit of determination (LOD) was 1.63 ng mL^{-1}. The recoveries of β-conglycinin in spiked soybean samples were between 88.1% and 106.6% with relative standard deviation less than 8.9% (intra-day) and 13.1% (inter-day). The developed method was used to analyze 469 soybean seed samples from different sources as well as five soybean products treated with different processing techniques. The data showed that the concentration of β-conglycinin decreased significantly after processing, especially for soybean protein isolation, where the concentration of β-conglycinin dropped to nearly zero. The assay provides a specific and sensitive method for the screening of β-conglycinin and allows for further investigation into hypersensitive mechanisms of soybean proteins and development of soybean processing techniques to reduce their negative effects.

1. Introduction

Soybeans have a high nutritional value and are one of the major vegetable protein sources used in food and feed industries [1]. There is evidence that individuals who consume soybean-rich diets exhibit lower prevalence of high plasma cholesterol, cancer, diabetes mellitus, and obesity [2]. Clinical trials have also shown reductions in triglycerides and total and low-density-lipoprotein cholesterol when soybean protein is substituted for animal protein [3].

Unfortunately, soybean ranks among the "big 8" of the most allergenic foods. Up to 1–6% of young children and 2–4% of adults

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suffer from clinical symptoms of soybean allergy [4,5]. Allergenic reactions are mainly caused by antigenic proteins in soybean, which can interfere with the digestion and absorption of nutrients, disturb normal metabolism, cause adverse physiological responses, and result in hypersensitivity and even death [4,6,7].

Beta-conglycinin has been shown to be the major soybean allergen [8]. It accounts for about 30% of the total soybean proteins and contains three different subunits, α, α′ and β, with molecular weights of 58–77, 58–83, and 42–53 kDa, respectively [9,10]. When infants or young animals were fed with diets containing soybean protein, the majority of the β-conglycinin is digested and degraded to peptides and amino acids. However, undigested β-conglycinin can directly cause intestinal damage by depressing intestinal cell growth, damaging the cytoskeleton, and causing apoptosis in the piglet intestine or induce allergic symptoms by entering the lymph and blood from gaps between intestinal epithelial cells [11,12]. Food anaphylaxis can induce respiratory, cutaneous, cardiovascular and gastrointestinal symptoms and even cause death [4,13]. Moreover, β-conglycinin shows strong thermal stability [14], the antigen activity cannot be destroyed using conventional processing techniques, such as heating to 100 °C. So the direct use of soybean and soybean products in food and feed industries in greatly limited by their allergenic properties.

With the increasing consumption of soybean products, the incidence of soybean-induced allergies is expected to escalate [15]. Detection methods currently in use include, SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) [16], near-infrared reflectance spectroscopy [17], HPLC (high performance liquid chromatography) with ultraviolet or mass spectrometric detection [18,19], and microfluidic lab-on-a-chip devices [20]. However, these methods suffer from low sensitivity and specificity. Most of the methods can only provide qualitative and semi-quantitative analysis, and cannot meet legal regulation requirements. For example, nowadays the most popular detection method is SDS-PAGE, there are many factors affecting the sizing accuracy of SDS-PAGE, some protein molecules may not migrate according to their molecular weight [21], thus, this method generate false positive results frequently, and quantitation based on staining with Coomassie Brilliant Blue is not accurate at all. Moreover, some methods, such as SDS-PAGE, HPLC, and HPLC–MS/MS, cannot detect the content of immunoreactive β-conglycinin. It is very important to detect the immunoreactive β-conglycinin since only immunoreactive β-conglycinin can cause allergic reactions. However, enzyme-linked immunosorbent assay (ELISA) with the advantages of technical simplicity and low requirement of equipment, can detect the immunoreactive β-conglycinin in soybean samples.

In previous reports, competitive ELISAs with polyclonal antibody (Pab) or monoclonal antibody (Mab) have been developed to determine β-conglycinin. Moriyama et al. prepared a rabbit anti-β-conglycinin polyclonal antibody specifically reactive to the subunits of β-conglycinin. With that antibody a competitive ELISA for the quantification of β-conglycinin in processed foods and seeds was developed [22]. You et al. developed monoclonal antibodies against β-conglycinin using a conjugated chicken ovalbumin with a synthetic peptide that corresponded to one epitope sequence of β-conglycinin as the immunogen. A competitive ELISA based on the monoclonal antibody was established to determine β-conglycinin [23]. Such assay is not suitable for the detection of proteins with more than one epitope. The most powerful ELISA assay format for the detection of protein is the sandwich assay, in which the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody. The sandwich ELISAs offer several advantages compared with competitive ELISAs. Firstly, the ELISA microarray is a highly stringent assay with exceptional specificity because it requires two antibodies for the detection of each analyte [24]. Secondly, the limit of detection of sandwich ELISAs is generally lower since it is more precise to measure a large signal against a low background signal (sandwich ELISA) than to measure the difference between two large signals (competitive ELISA) [25]. Thirdly, sandwich ELISAs show less interfering matrix effects than competitive ELISAs [26]. Here, the matrix effects include significant amounts of proteins, fat, fiber, minerals and other ingredients in samples that can interfere with detection.

To the best of our knowledge, a sandwich ELISA for the detection of β-conglycinin in soybean samples has not been reported in peer reviewed journal so far. Thus, the present study aimed at developing and validating a sandwich ELISA for the determination of β-conglycinin in soybean and soybean products. This assay offers several unique features. Firstly, although ELISA is a mature analytical method, major breakthrough recently in the field of ELSIA is the development of new, sensitive and specific antibodies. So this study focused on the development of rabbit polyclonal antibody and mouse monoclonal antibody. The mouse Mab and rabbit Pab against β-conglycinin were prepared based on purified β-conglycinin. More than 100 monoclonal antibodies have been screened to find out one this most specific and sensitive to β-conglycinin, and based on these, a double antibody sandwich ELISA was developed. Secondly, the method validation results show that the detection limit, specificity, and linear range of sandwich ELISA are all better than these of previous competitive ELISA. Thus, the developed sandwich ELISA is suitable for large-scale and high-throughput immunoreactive β-conglycinin screening for accessing the breeding programs and processing techniques. With the developed assay, we measured β-conglycinin content in more than 400 soybean samples from different sources as well as soybean products that were treated with different processing techniques.

2. Experimental

2.1. Materials and apparatus

Freund's complete and incomplete adjuvants, and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma Company (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) was obtained from BRL-Gibco (Grand Island, NY). Goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidae (HRP) was obtained from Jackson Immuno-Research Laboratories (West Grove, PA). BCA™ Protein Assay Kit was obtained from Pierce (Rockford, IL). Protein G agarose column was obtained from Upstate Biotechnology (Placid, NY). ELISA plates (96 wells) and other cell culture plastic wares were obtained from Costar (Cambridge, MA). Super ECL Plus was obtained from Beijing's Pulitzer Gene Technology Company (Beijing, China). All other chemicals were analytical grade.

Each liter of phosphate buffered saline contained 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 and was adjusted to pH 7.4. Each liter of bicarbonate buffer contained 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$, and was adjusted to pH 9.6. Each liter of tris-buffered saline contained 8.8 g NaCl, 0.2 g KCl, 3 g Tris base, 500 μL Tween-20 and was adjusted to pH 7.4. All water used was Milli-Q water (>18.2 MΩ, Millipore, Billerica, MA).

BALB/c mice and New Zealand White rabbits were purchased from the Institute of Genetics and Developmental Biology Chinese Academy of Sciences (Beijing, China). The soybean-free diet was made at the pilot mill of the Ministry of Agricultural Feed Industry Centre (Beijing, China). Soybean seeds were kindly provided by the Institute of Crop Science at the Chinese Academy of Agricultural Sciences (Beijing, China). Soybean products were obtained from the

Ministry of Agriculture Feed Safety and Bio-availability Evaluation Center (Beijing, China).

A Heraeus HERA Cell CO_2 incubator (Kendro Lab., Asheville, NC) was used for cell cultures. A high speed refrigerated centrifuge (Sigma 3–30 K, Sigma, Munich, Germany) was used to for sample pretreatments. Foss Kjeldahl™ 2100 (Beijing Tuopu Analysis Instrument Company, Beijing, China) was used to determine crude protein. Absorbance values were read with an automatic microplate reader (Biotek, Winooski, VT). SDS-PAGE electrophoresis slot and Western blot electrophoresis transfer slot were purchased from BioRad Laboratories (Hertfordshire, England). An automatic wellwash machine for washing 96-well plates was purchased from Beijing Tuopu Analysis Instrument Company (Beijing, China).

2.2. Soy protein extraction

Soybeans and soybean products were ground to fine powder to pass through a 60-mesh sieve. The crude protein content of each soybean sample was determined by Kjeldahl analysis [27], and moisture content of each sample was determined by drying the sample at 103 °C for 4 h [28]. Before extraction, the sample was defatted with petroleum ether. Briefly, weigh 0.2 g finely ground soybean sample into 50 mL centrifuge tube and add 10 mL petroleum ether to marinate for 20 min. Centrifuge 10 min at 1800 rpm. Decant and discard supernatant. Pulverize residue with glass rod. Then defatted soybean flour was extracted with 20 mL 0.03 M Tris–HCl buffer (pH 8.0, with 0.01 M β-mercaptoethanol) under continuous agitation for 1.5 h, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatants were filtered through 0.45 μm filter, and stored at −20 °C for further purification or ELISA analysis.

2.3. Preparation and purification of β-conglycinin

Extraction and purification of β-conglycinin were performed as described by Guo [29]. Briefly, supernatant from soybean extract was adjusted to pH 6.4 with HCl followed by refrigerated centrifuging at 10,000 rpm for 30 min at 4 °C. The remaining supernatant was adjusted to pH 4.8, and centrifuged at 15,000 rpm for 30 min at 4 °C. After the second centrifugation, the precipitate was dissolved in 0.03 M Tris–HCl (pH 8.0, 0.01 M β-mercaptoethanol) and adjusted to pH 6.2, then centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant obtained after the third centrifugation was crude-extract of β-conglycinin, the pH of the supernatant was adjusted to 7.6. The extract was precipitated by ammonium sulfate, and purified with sepharose column. Then the purified β-conglycinin was analyzed the purification using SDS-PAGE, and determined the concentration by BCA™ protein kit.

2.4. Preparation of polyclonal antibodies

Two New Zealand White rabbits were used to produce Pab. The animals were fed according to principles of the China Agricultural University Animal Care and Use Committee. Blood samples (2 mL) were collected before immunization as negative serum. Both rabbits were immunized subcutaneously with 100 μg purified β-conglycinin during the six-week immunization. After the initial dose, several booster shots were administered during the next four weeks. Three days after the last injection, blood samples were collected directly from the heart and centrifuged at 4000 rpm for 20 min. The antibody was purified from the antiserum by ammonium sulfate precipitation. The purified protein concentration was determined by BCA™ Protein Assay Kit and the purity of the antibody was confirmed by SDS-PAGE.

2.5. Preparation of monoclonal antibodies

One adult female BALB/c mouse was fed with soybean-free diet and mated to produce offspring. Three second generation female BALB/c mice were also fed with soybean-free diet and prepared to be subcutaneously immunized with 50 μg purified β-conglycinin at the age of 60 days. After two weeks, the mouse that produced the highest titer and showed the best sensitivity toward β-conglycinin was used for fusion experiments. Spleen from that mouse was removed, and splenocytes were fused with mouse myeloma cell SP2/0. Culture supernatant from individual hybridoma clones were screened first against β-conglycinin by ELISA, then immunoblotting against soybean.

To produce antibodies from different hybridoma clones, five selected stable antibody-producing clones were seeded and expanded in DMEM plus 10% low-IgG fetal bovine serum. The cells were injected intraperitoneally into the mouse to produce ascites. About two weeks later, ascites were obtained to extract antibodies. The crude-extracted antibody was further purified with Protein G affinity column. The concentration of purified IgG was determined by BCA™ protein kit.

2.6. Double antibody sandwich ELISA

A double antibody sandwich ELISA was developed to evaluate antibody specificity and sensitivity, determine immunoreaction of β-conglycinin and detect β-conglycinin quantitatively. A 96-well microtiter plates was coated with 100 μL well^{-1} of purified Mab (10 μg mL^{-1}) in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were washed three times with 0.01 M PBS (pH 7.4) to remove unbound Mab. Excess binding sites were blocked with 200 μL well^{-1} of 5% skim milk for 1.5 h at 37 °C. The plate was washed, and 100 μL of samples dissolved in 1% BSA at various concentrations were added into each well and incubated for 1.5 h at 37 °C. After washing the plate three times with PBS, purified Pab dissolved in 5% skim milk was added (100 μL well^{-1}) and incubated for 1 h at 37 °C. The reaction was terminated by washing the wells four times with PBS. Goat anti-rabbit IgG-HRP conjugate, diluted to 1:2000 using 5% skim milk was added (100 μL well^{-1}) and incubated at 37 °C for 1 h. Followed by five times washes with PBS, peroxidase substrate TMB solution was added to each well (100 μL well^{-1}). After incubating for 15 min at room temperature, reaction was terminated by adding 4 M H_2SO4 solution (50 μL well^{-1}). The optical density (OD) was measured at λ = 450 nm. Calibration curve was obtained by plotting the OD values against β-conglycinin concentrations.

2.7. SDS-PAGE and Western blot

SDS-PAGE was performed to detect protein profiles of the protein samples. The supernatant was diluted with distilled water and mixed with SDS sample buffer, then loaded onto gradient gels containing 5–12% polyacrylamide. SDS-PAGE was performed in a vertical electrophoresis unit at 100 V constant voltage until the tracking dye migrated to the bottom edge of the gel (about 2.5 h). The gel was stained with Coomassie Brilliant Blue R-250 (0.05%, w/v).

Purified β-conglycinin was used to study immunoreactivity against the antibodies by Western blot. Proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane for 1 h at 100 V. The membrane with transferred polypeptides was immersed in 5% skim milk in TBST at room temperature for 2 h. After one rinse with TBST, the proteins were probed with anti-β-conglycinin antibody (1:3000) at room temperature for 1 h. After three rinses with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:4000) at room temperature for

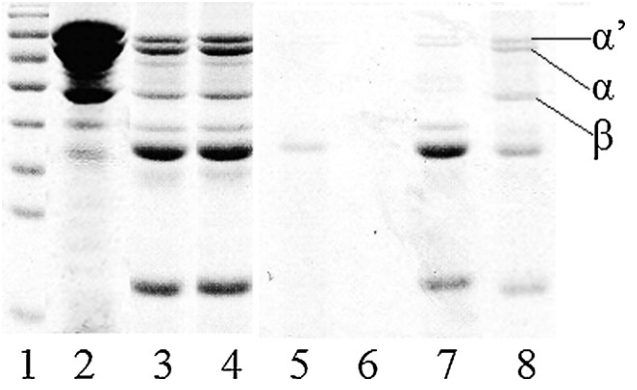

Fig. 1. Protein profile of different soybean products determined by SDS-PAGE (5–12% gradient gel). Lane 1, prestained protein marker (kDa): 170, 130, 95, 72, 55, 43, 34, 26, 17; Lane 2, purified β-conglycinin; Lane 3, soybean protein; Lane 4, soybean meal; Lane 5, soybean protein concentrate; Lane 6, soybean protein isolate; Lane 7, extruded soybean; Lane 8, fermented soybean. α, α′ and β represent 3 subunits of β-conglycinin.

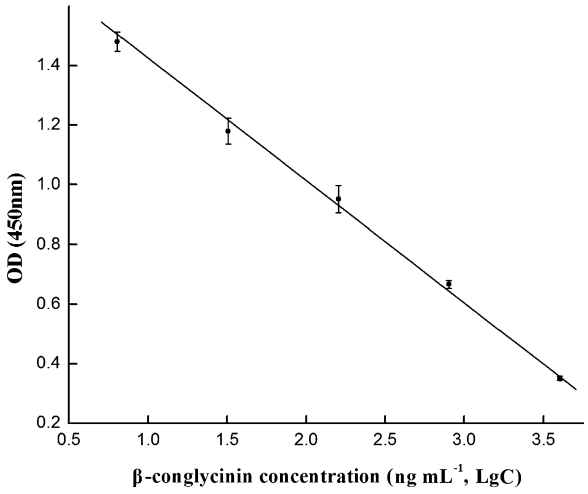

Fig. 2. Standard calibration curve of competitive ELISA using polyclonal antibody from rabbit 1 (R1) as primary antibody.

1 h. After three rinses, Super ECL Plus was added, the luminescence produced is proportional to the amount of protein on the membrane. Photographic film was used to create image of the antibodies bound to the blot.

3. Results and discussion

3.1. Characterization of antigen purity

The isolation and purification of allergen proteins in soybean has always been a worldwide problem. In the past several decades, considerable efforts have been focused on developing powerful techniques to solve this issue. For example, several methods have been developed for the isolation of β-conglycinin, such as, acid and alkali separation [30], phytic acid enzyme degradation [31]; pepsin decomposition of glycinin, papain decomposition of β-conglycinin subunits [32] and liquid chromatography [33]. These methods have been used widely in industry. However, the purity of β-conglycinin obtained with these methods is too low for immunoassay applications. So, in this study, we referred to the patent of Professor Shuntang Guo of China Agricultural University, in which β-conglycinin was extracted with salting out and ionic precipitation, and then purified with sepharose column. Another issue with purified β-conglycinin is to determine the concentration of β-conglycinin properly; to date, SDS-PAGE is still the most popular method, this method works particularly well when β-conglycinin is the major component of the sample after purification. So we used SDS-PAGE and BCA protein assay to determine the quality of the purified protein to guarantee the best quantitative determination of the purified β-conglycinin. The purity of β-conglycinin was estimated to be more than 85% according to SDS-PAGE analysis (Fig. 1, Lane 2). Such purity is good enough for the ELISA experiments.

3.2. Characterization of antibodies

Based on titer and IC_{50} (the half maximal inhibitory concentration is interpolated as the amount of inhibitor that produces 50% of inhibition.) of Pab obtained from the two rabbits, we found that antibody from rabbit No. 1 (R1) was better than those of the other rabbit, therefore R1 antibody was selected for further study. The titer of purified Pab from R1 was 1:10,000. Competitive ELISA using Pab as primary antibody possessed a LOD of 6.4 ng mL^{-1}, the linear portion of the curve was 6.4–4000 ng mL^{-1} (Fig. 2).

For Mab against β-conglycinin, 134 positive clones were obtained from the initial screening and subsequently 32 clones were selected to repeat screening. Only 20 Mabs were left for titer and cross-reaction screening. The results indicate that only Mabs 2A9, 3C8, 4E5, 4F1, and 5C5 had the high positive reactions with β-conglycinin. However, clones of 2A9, 3C8, 4E5 and 4F1 showed significant cross-reaction with glycinin (>57%) (Table 1), while Mab 5C5 showed negligible cross-reaction with glycinin (<0.1%) and other proteins (<0.01%). Thus, based on titer and cross-reaction results, clones of 5C5 were selected for further study. The titer of Mab 5C5 was 1:2,000,000 and the concentration was confirmed to be 0.5 ng mL^{-1}.

Western blot analysis was used to determine the subunit of β-conglycinin that reacts with the ELISA-positive clone 5C5. Beta-conglycinin at different concentrations together with total soybean protein and negative control (without primary antibody) were reacted with Mab 5C5. The results show that there is a single band with an apparent molecular weight of approximately 49 kDa, corresponding to the β subunit of β-conglycinin (Fig. 3). The Western blot results indicate that the Mab react with the β subunit of β-conglycinin specifically and have no cross-reaction with other subunits of β-conglycinin as well as other soybean proteins. Among the three subunits of β-conglycinin, the β subunit exhibits the best thermal stability (β > α′ > α) [34,35], thus quantitation of β-conglycinin using the β subunits yields the most precise result. Therefore, Mab 5C5 was selected as the coating antibody for the quantitative determination of β-conglycinin in spiked and real soy samples.

Table 1
Cross-reactivity of five selected Mab to β-conglycinin and related soybean allergens.[a]

Allergens	Cross-reactivity[b] (%)				
	2A9	3C8	4E5	4F1	5C5
β-Conglycinin	100	100	100	100	100
Glycinin	89.1	57.2	89.0	57.7	0.1
Trypsin inhibitor	<0.01	<0.01	<0.01	<0.01	<0.01
Agglutinin	<0.01	<0.01	<0.01	<0.01	<0.01

[a] The concentrations of coating antigen and Mab were 5.0 μg mL^{-1} and 25 ng mL^{-1}.

[b] Cross-reactivity (CR) of competitors is expressed as the percentage calculated according to the formula: CR (%) = (IC_{50} of β-conglycinin)/(IC_{50} of certain inhibitor) × 100.

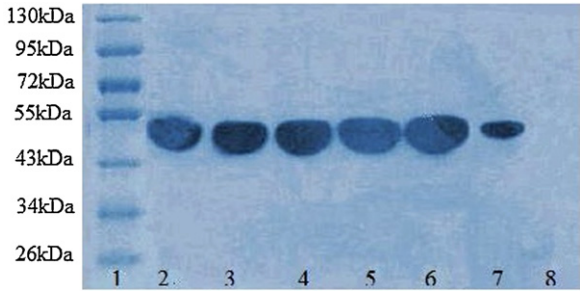

Fig. 3. Western blot analysis of Mab 5C5 with purified β-conglycinin and total soybean protein. Lane 1, prestained protein marker (kDa): 170, 130, 95, 72, 55, 43, 34, 26; Lane 2, Mab 5C5 reacted with 15 μg β-conglycinin; Lane 3, 18.75 μg β-conglycinin; Lane 4, 22.5 μg β-conglycinin; Lane 5, 26.25 μg β-conglycinin; Lane 6, 30 μg β-conglycinin; Lane 7, Mab 5C5 reacted with 58 μg total soybean protein; Lane 8, negative control without the primary antibody.

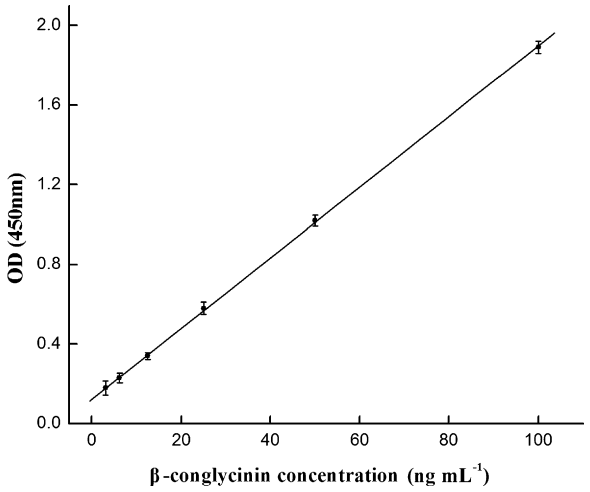

Fig. 4. Standard calibration curve of the double antibody sandwich ELISA detection kit. Each data point represents mean of six measurements of the absorbance at 450 nm.

3.3. Method optimization and validation

The ELISA plates are clear, high binding, polystyrene materials, plate coating is achieved through passive adsorption of protein to the plastic of the assay microplate. This process occurs though hydrophobic interactions between the plastic and non-polar protein residues. Individual proteins may require specific conditions or pretreatment for optimal binding. To obtain optimal conditions for the sandwich ELISA assay, two strategies were introduced. Firstly anti-β-conglycinin Pab was immobilized on microtiter plate to capture protein, and anti-β-conglycinin Mab was used as secondary antibody. Secondly, Mab was coated on microtiter plate to capture protein, and Pab served as the detection agent. The results showed that the assay using Pab to capture and Mab to detect β-conglycinin had high background noise. On the contrary, the assay using Mab to capture protein showed very low background values and high sensitivity. After optimization, the optimal concentration of Mab 5C5 as coating agent was 10 $\mu g\,mL^{-1}$ and the concentration of Pab R1 as secondary antibody was 5 $\mu g\,mL^{-1}$, respectively.

Since the binding capacity of microplate wells is typically higher than the amount of protein coated in each well, after removing the coating solution, the remaining surface area must be blocked to prevent antibodies or other proteins from adsorbing non-specifically to the plate during subsequent steps. The selection of blocking agent is also very critical to suppress background noise and maintain high sensitivity, without altering or obscuring the epitope for antibody binding. Thus, after tested 1% BSA, gelatin and skim milk, 5% skim milk was chosen as the blocking reagent because it provided the lowest background noise. Also, samples were dissolved in 1% BSA instead of skim milk as in most other ELISA experiments. Because foam produced by skim milk cause data inconsistence for parallel experiments.

The sandwich ELISA assay was used to analyze β-conglycinin at various concentrations. The absorbance at $\lambda = 450$ nm showed linear relationship with the concentrations of β-conglycinin in the range of 3–100 $ng\,mL^{-1}$ (Fig. 4). Then, soybean seeds diluted with protein-free diet were analyzed to evaluate sensitivity of the method for real samples. The results showed that the LOD ($S\,N^{-1} = 3$) and the limit of quantification ($S\,N^{-1} = 10$) were 0.2 $mg\,g^{-1}$ and 0.67 $mg\,g^{-1}$, respectively. Soybean contains 19% oil; the existence of fat in the sample may interference with the detection of β-conglycinin in real samples. Soybean seeds were defatted using petroleum ether. The result shows that defatted soybean samples had better accuracy and reproducibility compared with full-fat soybean. The existence of fat in the sample decreases solubility of proteins and increases nonspecific binding of the antibodies. Therefore, all the soybean samples were defatted prior to protein extraction. β-Mercaptoethanol was added to the extraction cocktail to enhance solubility of target protein and prevent copurification of host proteins which may form disulfide bonds with target protein.

Defatted soybean samples spiked with β-conglycinin at 50, 100, and 200 $mg\,g^{-1}$ were analyzed to evaluate validity and reliability of the double antibody sandwich ELISA. Six replications were tested at each concentration. Recoveries of β-conglycinin were calculated using calibration curve obtained with purified β-conglycinin. The recoveries ranged from 88.1% to 106.6% and coefficients of variations were less than 8.9% (Table 2). Intra-assay reproducibility was evaluated using defatted soybean spiked with β-conglycinin at 50, 100, and 200 $mg\,g^{-1}$ within a 6-day period. The results showed that intra-assay recoveries in those days ranged from 97.7% to 105.1%, and coefficient of variation (CV) was less than 13.1%. Thus, the ELISA assay developed here showed good reliability for the analysis spiked soybean samples.

Using the developed ELISA assay, we measured the concentration of native β-conglycinin in one defatted soybean sample. The concentration was 10.5% on total weight basis and 11.8% on dry matter basis. The result is consistent with data obtained from HPLC [8]. Therefore, the Mab 5C5/Pab double antibody sandwich ELISA can be used to accurately determine β-conglycinin concentration in soybean samples.

3.4. Analysis of real samples

469 samples soybean samples of different origin and breed were collected and tested with the ELISA assay describe above. Before ELISA analysis, soy protein extracts were further diluted with 1% BSA to a final concentration in the range of 25–100 $ng\,mL^{-1}$. In order to keep the concentration of the actual test solution within the linear range of the calibration curve, the experience is also very important. After analyzed large quantity of samples, the unknown sample can be determined with the right level of dilution one time from the sample information. Of course, for samples with extremely high or low concentration, a few more dilution series is required. For example, the samples were diluted by 10, 100 and 1000 times, such that at least one of the diluted samples falls in to the linear range of the calibration curve. At the same time, standard β-conglycinin solution was diluted with 1% BSA to 100, 30, 10, 3, 1, 0.3, 0.1, and 0.03 $ng\,mL^{-1}$. The samples were analyzed in 6 replications. The results showed that the concentration of β-conglycinin in soybean seeds ranged from 3.99% to 14.78%, with more than 60% of the samples in range of 8–11% (Fig. 5). The

Table 2
Recoveries of β-conglycinin spiked in defatted soybean.

Concentration of β-conglycinin in defatted soybean (mg g^{-1}, DM[a])	Spiked level (mg g^{-1})	Measured concentration (mg g^{-1})	Mean recovery (%)	CV (%)
118.0	50	171.3	106.6	8.9
118.0	100	213.6	95.6	6.9
118.0	200	294.2	88.1	5.4

[a] DM, in a dry matter basis. Mean recovery and CV were calculated with 6 replicates.

Table 3
Average concentrations of β-conglycinin in soybean samples from different origins.[a]

Regions of origin	Number of samples	Average β-conglycinin concentration (mg g^{-1})	Mean moisture ± SD (%)
North spring	105	91.7 ± 18.0	6.46 ± 0.52
Huanghuai summer	100	98.3 ± 15.5	6.34 ± 0.07
Changjiang spring–summer	79	85.1 ± 16.7	6.19 ± 0.44
Southeast spring–summer–autumn	63	88.9 ± 16.0	6.58 ± 0.39
South China four seasons	54	91.4 ± 20.1	6.46 ± 0.40
Overseas cultivars	68	95.5 ± 19.1	6.55 ± 0.40

[a] Average β-conglycinin concentrations are on a dry matter base.

Table 4
Concentrations of β-conglycinin in selected soybean samples from Huanghuai region.

No.	Code	β-conglycinin concentration (mg g^{-1})	No.	Code	β-conglycinin concentration (mg g^{-1})	No.	Code	β-conglycinin concentration (mg g^{-1})
1	ZDD01612	107.7	18	ZDD03026	103.5	35	ZDD08728	116.8
2	ZDD01683	68.5	19	ZDD03106	57.3	36	ZDD08928	79.6
3	ZDD01720	83.4	20	ZDD03191	51.5	37	ZDD10100	116.3
4	ZDD01983	95.2	21	ZDD03222	69.4	38	ZDD18524	77.2
5	ZDD02114	70.8	22	ZDD03237	56.0	39	ZDD18529	100.4
6	ZDD02159	58.8	23	ZDD03458	101.7	40	ZDD18558	79.0
7	ZDD02315	61.5	24	ZDD03540	102.0	41	ZDD18630	99.1
8	ZDD02400	87.3	25	ZDD03570	98.1	42	ZDD18632	89.0
9	ZDD02626	82.2	26	ZDD08190	110.7	43	ZDD18771	101.9
10	ZDD02764	80.3	27	ZDD08228	103.3	44	ZDD18835	75.1
11	ZDD02864	95.7	28	ZDD08238	111.3	45	ZDD18870	97.8
12	ZDD02866	100.3	29	ZDD08251	93.5	46	ZDD19027	96.3
13	ZDD02892	82.9	30	ZDD08352	108.3	47	ZDD19144	94.7
14	ZDD02913	51.1	31	ZDD08472	119.5	48	ZDD19381	97.2
15	ZDD02921	51.5	32	ZDD08690	113.9	49	ZDD19409	86.3
16	ZDD02940	52.5	33	ZDD08697	83.4	50	ZDD19410	82.4
17	ZDD02990	88.3	34	ZDD08705	116.4			

concentration of β-conglycinin varies for soybeans from different origins (Table 3). Samples from the Huanghuai region had the highest average β-conglycinin content; the 100 samples from that region had an average β-conglycinin concentration of 98.3 mg g^{-1}, while the 79 samples from the Changjiang region had the lowest average concentration of β-conglycinin at 85.1 mg g^{-1}. Table 4 lists detailed β-conglycinin concentration of 50 selected soybean samples from the Huanghuai region. It can be seen that even though on average, samples from that region had a higher β-conglycinin concentration; some individual samples from that group did have much lower β-conglycinin concentrations. For example, sample ZDD02921, ZDD02940 and ZDD03191 all had less than 5.3% β-conglycinin. So the origin of the soybean samples does not have a direct relationship with the β-conglycinin concentration in the sample. Another interesting finding in this experiment is that the moisture contents of the samples are very similar regardless the place of origin.

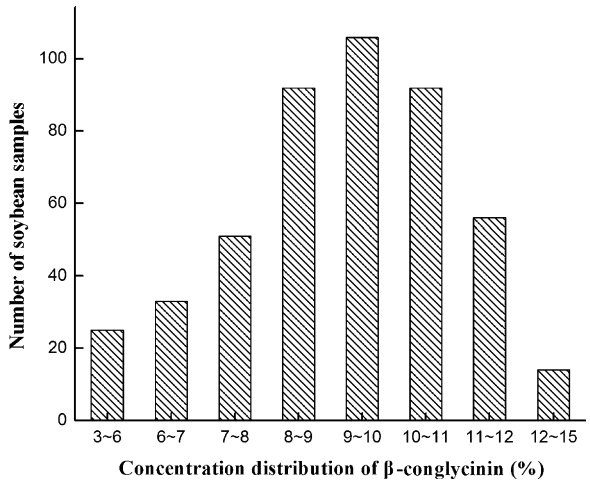

Fig. 5. Concentration distribution of soybean β-conglycinin (%) in different soybean breeds.

Various processing methods have been developed to reduce soy protein allergenicity. Here, with the developed ELISA assay, we evaluated the effectiveness of different processing methods in removing allergenic proteins from soy products. Five soybean products including soybean meal, soybean protein concentrate, soybean protein isolate, extruded soybean and fermented soybean were collected and tested by the double antibody sandwich ELISA (Table 5). Unprocessed soybean and soybean meal had the highest β-conglycinin concentration at 104.7 mg g^{-1} and 118.0 mg g^{-1}, while, β-conglycinin in fermented soybean meal, extruded

Table 5
ELISA and SDS-PAGE results of different soybean products.[a]

Soybean products	Crude protein (%)	β-Conglycinin concentration (mg g^{-1}, DM)	
		ELISA	SDS-PAGE
Soybean	43.1	104.7	95.3
Soybean meal	48.2	118.0	107.6
Soybean protein concentrate	67.9	4.8	0
Soybean protein isolate	45.3	<0.2	0
Extruded soybean	37.7	7.6	6.0
Fermented soybean meal	52.4	19.2	14.8

[a] Soybean data were obtained from the mean of 469 samples. Data for the other products were mean of five replications.

soybean, soybean protein concentrate and soybean protein isolate showed much lower concentrations. These results are qualitatively consistent with previous literature reports [18,36,37] and indicate there are several effective processing techniques to remove β-conglycinin from soy products, especially, in soybean protein isolate, β-conglycinin is nearly completely removed.

The above samples were also tested with SDS-PAGE. For soybean protein concentrate and soybean protein isolate, SDS-PAGE showed no band from any subunit of β-conglycinin (Fig. 1), while ELISA can still detect trace amount of β-conglycinin in these samples (Table 5). For samples with higher β-conglycinin concentration, SDS and ELISA showed similar results with the SDS-PAGE present slightly lower value. So, compared with SDS-PAGE, the ELISA assay developed here has better sensitivity for trace amount of β-conglycinin.

4. Conclusion

In summary, we designed a double antibody sandwich ELISA to detect β-conglycinin in soybean and soybean products. The assay showed high sensitivity towards β-conglycinin with a LOD at 1.63 ng mL^{-1}, exceeding most other detection techniques reported recently. The assay also showed minimum cross-reactivity with other soybean proteins even in complex samples such as soybean meals. With the developed assay, we analyzed 469 soybean samples from different origins, the results showed that the concentration of β-conglycinin in most of the samples were in the range of 8–11% range. Another interesting finding is that the place of origin has very limited influence on β-conglycinin content of the soybean samples. Effective processing can remove most of the β-conglycinin from soy and soy products; the developed assay provides a fast, convenient method to evaluate the effectiveness of these processing methods. Our results showed that in soy protein isolate, the β-conglycinin was nearly completely removed. Data from these real world samples provide a solid foundation for the evaluation of soybean, soy products and soybean processing techniques. Future work is granted to investigate the β-conglycinin content of different breeds of soybean and use genetic breeding to produce hypoallergenic soybeans.

There are over 20 identified allergenic proteins in soybean, so there exists a lot opportunity and challenge to develop efficient methods to eradicate these immunodominant allergens while maintain their nutritional value, as well as develop sensitive and convenient screening methods. The double sandwich ELSIA concept developed here with its high sensitive and specificity is a very promising technique for the detection and screening of these allergenic proteins.

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