



Analytical Methods

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

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ABSTRACT

This study performs a sandwich ELISA for detection of trace amounts of glycinin in soybean products. We designed a soy-free mouse model to produce anti-glycinin monoclonal antibodies with high affinity and specificity. Using the monoclonal antibody as coating antibody, with the rabbit anti-glycinin polyclonal antibody as a detected antibody, the established sandwich ELISA showed high specificity for glycinin with minimum cross-reactions with other soy proteins. The practical working range of the determination was 3–200 ng/mL with detection limit of 1.63 ng/mL. The regaining of glycinin in spiked soybean samples were between 93.8% and 103.3% with relative standard deviation less than 8.3% (intra-day) and 10.5% (inter-day). The developed assay was used in analysing 469 soybean samples and five soybean products under different processing. The assay provides a specific and sensitive method for screening of glycinin and allows for further investigation into hypersensitive mechanisms to soybean proteins.

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1. Introduction

Soybean has been one of the most valuable sources of protein for both humans and animals. It has been well-used in food and feed industries (Friedman & Brandon, 2001). Several studies show that soybean-rich diets have positive health effects, such as lowering prevalence of high plasma cholesterol, cancer, diabetes mellitus, and obesity. However, soybean is among the “big 8” of the most allergenic foods. About 1–6% of infants are affected by soybean, while 2–4% of adults also suffer from soybean allergy with a variety of clinical symptoms (Herian, Taylor, & Bush, 1990; Tryphonas, Arvanitakis, Vavasour, & Bondy, 2003). Soybean antigenic proteins can cause allergic reactions in animals, which include decreasing digestion and absorption of nutrients, respiratory, cutaneous, and gastrointestinal symptoms, even resulting in death (Sampson, 2000).

Glycinin (11S) is a heterogeneous protein that accounts for about 40% of the total soybean proteins (Lallès et al., 1999). Each of the glycinin subunits can be dissociated under reducing conditions into acidic (A, 31–45 kDa) and basic (B, 18–20 kDa) polypeptide chains (Maruyama et al., 2004). Five major subunits of glycinin have been characterised, namely, A1aB2 (G1), A1bB1b (G2), A2B1a (G3), A3B4 (G4), and A5A4B3 (G5) (Ma, He, Sun, & Han, 2010). After infants and young animals were fed with diets consisting of soybean protein, most of the glycinin was digested and degraded

to peptides and amino acids, which served as nutrient sources. But, a few undigested glycinin can play a negative role in the intestine by depressing intestinal cell growth, damaging the cytoskeleton, and causing apoptosis in the piglet intestine or inducing allergic symptoms by entering the lymph and blood from gaps between intestinal epithelial cells (Chen et al., 2011; Walker-Smith, 1986). Soybean protein can cause normal IgE-mediated reactions and anaphylaxis, which is the most severe allergic reaction, characterised by a sudden onset of symptoms typical of IgE-mediated hypersensitivity (Wilson, Blaschek, & Mejia, 2005). Severe anaphylaxis can induce respiratory, cutaneous, cardiovascular, gastrointestinal symptoms, and even death (Sampson, 2000). Apart from other Soybean anti-nutritional factors, glycinin has thermal stability, which increases the difficulty of removing its antigenicity. Glycinin cannot be degraded by heating or extruding, which limits soybean application in food and feed industries (Li, 2003).

Since soybean products have been more and more widely used in the feed industry (Friedman & Brandon, 2001; Helm et al., 1998), the incident rate of soybean-induced allergies is expected to escalate. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Kim, Heugten, Ji, Lee, & Mateo, 2010), near-infrared reflectance spectroscopy (Cho, Iwamoto, & Saio, 1987), high performance liquid chromatography (HPLC) with ultraviolet or mass spectrometric detection and microfluidic lab-on-a-chip devices are common methods applied for detection of soybean proteins (Blazek & Caldwell, 2009; Castro-Rubio, Marina, & García, 2007; García, Heras, & Marina, 2007). However, most methods can only

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provide qualitative and semi-quantitative analysis, which cannot meet legal regulation requirements. SDS–PAGE is one of the most regular detection methods for proteins, but there are many factors influencing its sizing accuracy, and sometimes the protein may not migrate according to their molecular weight (Goetz et al., 2004). Thus, SDS–PAGE may generate false positive results, and the Coomassie Brilliant Blue quantitation method is not accurate. In addition to the weakness, methods like SDS–PAGE, HPLC and HPLC–MS/MS cannot detect the immuno-reactive glycinin. Compared with those methods, enzyme-linked immunosorbent assay (ELISA) possesses the advantage of technical simplicity and low requirement of equipment. Moreover, it can detect the immuno-reactive glycinin in soybean samples.

Immunoassay has been widely used in detection of globulins in soybean. There are reports about using competitive ELISA with polyclonal antibody (Pab) or monoclonal antibody (Mab) to determine glycinin content. Huang et al. defined two epitopes of these proteins and studied the thermal stability of the epitopes by using Mabs to glycinin (Huang, Mills, Carter, & Morgan, 1998). Ma, He, et al. (2010), Ma, Sun, et al. (2010) developed Mabs against glycinin using purified glycinin as the immunogen. After the Mabs were generated, a competitive ELISA was developed to measure glycinin. But such assay is not available when there is more than one epitope in detecting proteins. The most powerful ELISA assay format for the detection of protein is sandwich assay, in which the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody. The sandwich ELISA possesses several strengths in comparison with competitive ELISA.

There is another important aspect to take into consideration when characterising the normal immune response to glycinin and when using experimental animals for studying soya-specific immune reactions. In some of our previous studies, we found that pre-immune serum from normal experimental mice which were fed with commercial rodent feed containing soybean protein showed comparatively high “background” response when testing for antibodies in ELISA. And the components involved in the antigen-specific immune response are in some cases transmitted from mother to offspring both prenatally via the placenta and postnatally via maternal milk (Arvola et al., 2000; Bednar-Tantscher, Mudde, & Rot, 2001; Hanson et al., 2003). Brown Norway (BN) rat is a high IgE-responder and thus is widely used as an allergy model. Knippels, Penninks, and Houben (1998) investigated the influence of pro-exposure to soy proteins in dietary on oral sensitisation studies using BN rats. They found that soya-specific antibody was detected in the first offspring and one of the major factors leading the negatively results of many oral sensitisation studies was pre-exposure of antigen in test animals. In consideration of this aspect, we decided to use offspring from parents which were not exposed to soya protein. A sandwich ELISA for the detection of glycinin in soybean samples has not been reported so far.

The present study was aimed at developing and validating a sandwich ELISA for the determination of glycinin in soybean and soybean by-products. The mouse Mab and rabbit Pab against glycinin were produced based on purified glycinin, and more than 80 monoclonal antibodies have been screened to find out the most specific and sensitive one against glycinin. We developed a unique, double antibody sandwich ELISA. Apart from that, the method validation results showed that the detection limit, specificity, and linear range of sandwich ELISA were all better than those of previous method, such as competitive ELISA (Mansfield, Hagler, & Whitehouse, 2008; Redl, Husain, Bretbacher, Nemes, & Cichna-Markl, 2010; Zangar, Daly, & White, 2006). We also applied the novel assay in more than 400 soybean samples from different regions, as well as soybean by-products that were treated with different processing technique, which confirmed that sandwich ELISA is suitable for large-scale and high-output immuno-reactive

glycinin screening for accessing the processing techniques. This assay provides a specific and sensitive method for screening of glycinin and allows for further investigation into hypersensitive mechanisms to soybean proteins.

2. Experimental

2.1. Materials and apparatus

The immunisation experiment of this study was performed at China Agricultural University (Beijing, China). Freund's complete and incomplete adjuvants and 3,3' and 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 peroxidase (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 used here were analytical grade.

Each litre of phosphate buffered saline (0.01 M PBS) contained 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄, and adjusted to pH 7.4. Each litre of bicarbonate buffer contained 1.59 g Na₂CO₃ and 2.93 g NaHCO₃, and was adjusted to pH 9.6. Each litre of Tris-buffered saline contained 8.8 g NaCl, 0.2 g KCl, 3 g Tris base and 500 µL Tween-20, and 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). All animals used in this experiment were maintained and treated according to the guidelines of the China Agricultural University Animal Care and Use Ethics Committee (Referred to the Regulations of Laboratory Animal of China published in 1988) (Song, 1988). The soybean-free diet was made at the pilot mill of the Ministry of Agricultural Feed Industry Centre (Beijing, China). Soybean seeds were provided kindly 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).

The BALB/c mice were fed with soybean-free diet, and then gave birth to the next generation. The offspring generation mice, kept solely on the soya protein-free feed, were then used for producing monoclonal antibodies.

A Heraeus HERA Cell CO₂ incubator (Kendro Lab., Asheville, NC) was used for cell cultures. A high-speed refrigerated centrifuge (Sigma 3–30 K, Munich, Germany) was used to 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 well-wash machine for washing 96-well plates was purchased from Beijing Tuopu Analysis Instrument Company (Beijing, China).

2.2. Soy protein extraction

The soybean and soybean products were ground to fine powder and pass through the 60 mesh sieve. Kjeldahl analysis was used to

determine the crude protein content of each soybean sample (Thiex & Manson, 2002), and moisture content of each sample was determined by drying the sample at 103 °C for 4 h (AOAC, 2000). Before extraction, the sample was defatted with petroleum ether (Hei, Li, Ma, & He, 2012; Thiex, Anderson, & Gildemeister, 2003). Briefly, 0.2 g of finely ground soybean sample was placed into a 50 mL centrifuge tube with 10 mL of petroleum ether to marinate for 20 min. Then, the sample was centrifuged for 10 min at 1800 rpm, followed by decanting and discarding the supernatant. The residue was pulverised with glass rod, and stood at room temperature for 3 h for evaporation of the solvent and weighted each hour. The defatted soybean flour cannot be used to dissolve until the weight has not changed between 1 h. As glycinin is an abundant protein in the seed, for well extraction, 40–60 mg 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 supernatant was filtered through a 0.45 μ m Millex GP filter (Millipore, Cork, Ireland), and stored at –20 °C for further purification or ELISA analysis.

2.3. Preparation and purification of glycinin

According to previous report (Hu, Liu, Qiao, He, Ma, & Lu, 2013), glycinin was extracted and purified using salting fractionation and ionic precipitation method. Briefly, 0.025 M MgCl_2 was added into the soybean protein supernatant (the pH was adjusted to 6.4 with HCl). The precipitate was collected by refrigerated centrifuging at 10,000 rpm for 30 min at 4 °C. The precipitate was dissolved in 0.01 M PBS (pH 7.4, containing 0.01 M β -mercaptoethanol), then centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant was a crude-extract of glycinin, which was precipitated by ammonium sulphate, and purified with sepharose column.

2.4. SDS–PAGE

SDS–PAGE was applied to detect protein profiles in protein samples. The samples were dissolved in a 1 M Tris–HCl buffer. After that, supernatant was diluted with distilled water and mixed with SDS sample buffer, then loaded onto gradient gels containing 12% polyacrylamide. SDS–PAGE was performed in a vertical electrophoresis unit at 100 V constant voltages 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).

As for quantitation of glycinin by SDS–PAGE, the purified glycinin was collected for purification analysis using SDS–PAGE, and the concentration was determined by BCA™ protein kit according to previous report (Hu et al., 2013). Quantity One Software (Bio-Rad 1D) was applied to analyse the grey identity of each track on the SDS–PAGE gel.

2.5. Preparation of polyclonal antibodies

Two New Zealand White rabbits, fed and treated according to principles of the China Agricultural University Animal Care and Use Ethics Committee, were used to produce Pab. Blood samples (2 mL) were collected before immunisation as negative serum. Both rabbits were injected subcutaneously with 100 μ g purified glycinin during the six-week immunisation, after which several booster shots were conducted during the next 4 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 sulphate precipitation. The purified protein concentration was determined by BCA™ Protein Assay Kit, and the purity of the antibody was confirmed by SDS–PAGE.

2.6. Preparation of monoclonal antibodies

One adult female BALB/c mouse was fed a soybean-free diet and mated with a male one to produce offsprings. Three, second-generation female BALB/c mice were also fed with soybean-free diet until they were 60 days old. Then, the mice were subcutaneously immunised with 50 μ g purified glycinin emulsified in complete Freund's adjuvant (Sigma, St. Louis, MO). Four weeks later, the mice were re-immunised with 50 μ g of purified glycinin emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO). Booster injections were administrated bi-weekly for the next month. Three days before the day of fusion, the mice were bled and the antibody titer in their serum was determined using an Immuno-Microplate Auto-reader (Sunrise Tecan, Salzburg, Austria), which were measured the absorbance at 492 nm. The mouse with the highest antibody titer was selected to receive an intraperitoneal injection with 100 μ g purified glycinin. The mouse was killed 3 days later and sterilised with ethanol. The spleen tissue was removed from the opened peritoneal cavity and finely chopped with scissors into fragments, minced by syringe to single cells and washed twice with a solution containing 90% DMEM (HyClone, Logan UT) and 10% fetal bovine serum (Gibco, Grand Island, NY). After erythrocyte lysis, splenocytes were re-suspended in complete medium (90% DMEM supplemented with 10% fetal bovine serum). Hybridoma cells were produced by fusion of the spleen cells isolated from the immunised mouse and mouse myeloma cells SP2/0 purchased from the China Centre for Type Culture Collection (Wuhan City, China). Culture supernatants from individual hybridoma clones were screened first against glycinin by ELISA, then immunoblotting against soybean.

One week following cell fusion, growing hybridomas were observed in most of the culture wells. For the purpose of producing antibodies from different hybridoma clones, five stable antibody-producing clones were selected to be seeded and expanded in DMEM plus 10% low-IgG fetal bovine serum. The cells were injected intraperitoneally into the mouse to produce ascites. About 2 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.

2.7. Cross-reaction of antibody

Soybean glycinin, analogue like β -conglycinin and related substances like trypsin inhibitor and agglutinin were used in the cross-reaction study (Ma, He, et al., 2010; Ma, Sun, et al., 2010). Briefly, the inhibition effect for glycinin with each monoclonal antibody was set as the 100% cross-reactivity value. Cross-reactivity of the monoclonal antibodies with potential inhibitors was expressed as the concentration of inhibitor required to produce 50% inhibition of antibody binding compared with glycinin, which was represented by IC_{50} . Cross-reactivity (CR) of inhibitors is calculated as:

$$\text{CR}(\%) = (\text{IC}_{50} \text{ of glycinin}) / (\text{IC}_{50} \text{ of certain inhibitor}) \times 100.$$

2.8. Double antibody sandwich ELISA

A double antibody sandwich ELISA was performed to evaluate the antibody specificity and sensitivity, determine the immuno-reaction of glycinin and detect glycinin quantitatively. A 96-well microtiter plate was coated with 100 μ L/well of purified Mab (10 μ g/mL) 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 μ L/well of 5% skim milk for 1.5 h at 37 °C, after which the plate was washed. 100 μ L 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 μ L/well) 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 μ L/well) and incubated at 37 °C for 1 h. After washing the wells five times with PBS, peroxidase substrate TMB solution was added to each (100 μ L/well). Reaction was terminated by adding 4 M H_2SO_4 solution (50 μ L/well) after incubating for 15 min at room temperature. The optical density (OD) was measured at $\lambda = 450$ nm. Calibration curve was obtained by plotting the OD values against glycinin concentrations.

2.9. Recovery of glycinin spiked in soybean seeds measured by sandwich ELISA

Soybean seeds were used in this study to evaluate glycinin recovery by the newly developed sandwich ELISA according to the previous study (Ma, He, et al., 2010; Ma, Sun, et al., 2010). Briefly, the prepared soybean flours were spiked with pure glycinin at 50, 100 and 200 mg/g for soybean seeds. Proteins were extracted from the flour with 0.03 M Tris–HCl buffer for 1 h. The samples were defatted with petroleum ether. The defatted soybean flour was extracted and centrifuged. The supernatants were filtered through 0.45 μ m filter and stored for analysis.

Samples of each solution were diluted in such a manner that their concentrations fell in the linear portion of the curve. The content of glycinin from soybean seeds and soybean seeds spiked with glycinin were analysed by the sandwich ELISA developed above.

2.10. Western blot

Western blot was performed to study immuno-reactivity against the antibodies using purified glycinin. The remaining purified copies by SDS–PAGE were transferred onto a 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-glycinin anti-body (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 1 h. After three rinses, Super ECL Plus was added, and the luminescence produced was proportional to the amount of protein on the membrane. Photographic film was used to create an image of the antibodies bound to the blot.

3. Results and discussion

3.1. Characterisation of antigen purity

The quality of the purified soybean glycinin protein was tested by SDS–PAGE and BCA protein assay to guarantee the best quantitative determination of the purified glycinin. The purity of glycinin was estimated to be more than 80%, according to SDS–PAGE analysis (Fig. 1A, Lane 2), which meets the requirement of the ELISA experiments.

The isolation and purification of allergen proteins in soybean has always been a popular issue. Previously studies have been focused on developing powerful assays to solve this issue. For instance, several methods have been developed for the preparation of 11S fractions, including ultracentrifugation, fractionation, reversed-phase high performance liquid chromatography (García, Torre, Laborda, & Marina, 1997), acid and alkali separation (Hill &

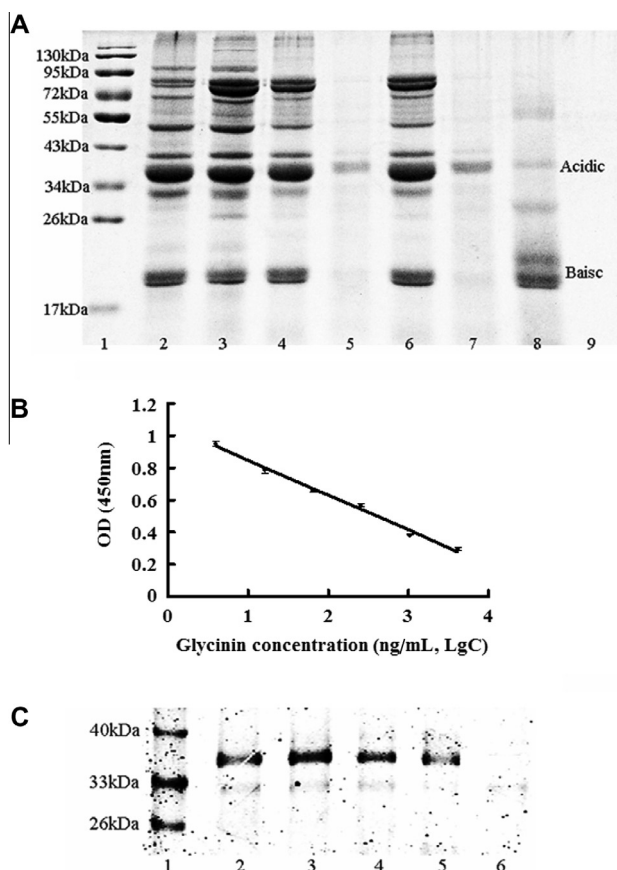


Fig. 1. (A) Protein profile of different soybean products determined by SDS–PAGE. Lane 1, pre-stained protein marker (kDa): 130, 95, 72, 55, 43, 34, 26, 17; Lane 2, primary purified glycinin; Lane 3, soybean protein; Lane 4, soybean meal; Lane 5, soybean protein isolate; Lane 6, extruded soybean; Lane 7, soybean protein concentrate; Lane 8, fermented soybean; Lane 9, blank control. Acidic and basic polypeptide chains of glycinin were shown in Right. (B) Standard calibration curve of competitive ELISA using polyclonal antibody from rabbit 2 (R2) as primary antibody. (C) Western blot analysis of Mab 6F2 with preliminary extracting glycinin protein and total soybean protein. Lane 1, protein marker (kDa): 40, 33, 26; Lane 2, Mab 6F2 reacted with 15 μ g preliminary extracting protein; Lane 3, 60 μ g preliminary extracting protein; Lane 4, 30 μ g preliminary extracting protein; Lane 5, Mab 6F2 reacted with 60 μ g total soybean protein; Lane 6, negative control without the primary antibody.

Breidenbach, 1974), pepsin decomposition of glycinin, and papain decomposition of glycinin subunits (Hou & Chang, 2004). These methods have been used widely. However, the purity of glycinin obtained with these methods cannot afford immunoassay applications. In our previous research, we developed a novel method, in which glycinin was extracted with salting out and ionic precipitation, and then purified with sepharose column (Hu et al., 2013). Another direction is to determine the concentration of glycinin properly; SDS–PAGE is still the most popular method so far; this method works particularly well when glycinin is the major component of the sample after purification.

3.2. Characterisation of antibodies

In comparison, the 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 showed that antibodies from rabbit No. 2 (R2) were better than those of the other rabbit. Thus R2 antibody was selected for further study. The titer of purified Pab from R2 was 1:20,000. Competitive

ELISA using Pab as primary antibody possessed a LOD of 1.63 ng/mL. The linear portion of the curve was 4–4000 ng/mL (Fig. 1B).

After fusion of the spleen cells isolated from an immunised mouse, hybridoma cells were successfully produced. 89 positive clones were obtained from the initial screening and, subsequently, 26 clones with good status and producing stable antibody were selected to repeat screening. Only 14 Mabs were selected for titer and cross-reaction screening. The results indicate that only Mabs 1B7, 2E2, 2A9, 4H6 and 6F2 had high positive reactions with glycinin. However, clones of 1B7, 2E2, 2A9 and 4H6 showed cross-reaction with glycinin (Table 1), and Mab 6F2 showed negligible cross-reaction with glycinin (<0.1%) and other proteins (<0.01%). Therefore, clones of 6F2 were selected for further study. The titer of Mab 6F2 was 1:2,000,000 and the concentration were confirmed to be 0.5 ng/mL.

SDS-PAGE and Western blot analysis were performed to determine the polypeptide chains of glycinin that reacted with the ELISA-positive clone 6F2. The glycinin samples were reacted with Mab 6F2 which were dissolved in Tris-HCl buffer at different concentrations together with total soybean protein and negative control (without primary antibody). The results show that there is a single band with an apparent molecular weight of approximately 36 kDa, corresponding to the acidic polypeptide chain of glycinin (Fig. 1C), which indicates that the Mab react with the acidic polypeptide chain of glycinin specifically and have no cross-reaction with other polypeptide chains of glycinin, as well as other soybean proteins. The acidic polypeptide chain possesses the best thermal stability in glycinin (Each subunit is compound with six acidic polypeptide chains and six basic polypeptide chains, and acidic polypeptide chains > basic polypeptide chains) (Liu, Wang, Cui, He, Wang, & Zeng, 2007; Mo, Zhong, & Wang, 2006); thus, quantitation of glycinin using the acidic polypeptide chains yields the most precise result. Therefore, Mab 6F2 was selected as the coating antibody for the quantitative determination of glycinin in spiked and real soybean samples.

3.3. Method optimisation and validation

As the ELISA plates were clear, high binding, polystyrene materials, plate coating is achieved through passive absorption of protein to the plastic of the assay microplate. This process occurs though hydrophobic interactions between the plastic and non-polar protein residues. Some proteins may require specific conditions or pretreatment for optimal binding. Two strategies were put forward to obtain optimal conditions for the sandwich ELISA assay. The first strategy was to utilise the anti-glycinin Pab which was immobilised on microtiter plate to capture protein, and anti-glycinin 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 indicated that the assay using Pab to capture and Mab to detect glycinin had high background noise. The second assay using Mab to capture protein showed very low background values and high sensitivity. After optimisation, the

optimal concentration of Mab 6F2 as a coating agent was 10 µg/mL and the concentration of Pab R2 as secondary antibody was 5 µg/mL, respectively.

Another factor was that the binding capacity of microplate wells was typically higher than the amount of protein coated in each well; so, the remaining surface area must be blocked to prevent antibodies or other proteins from absorbing non-specifically to the plate immediately after removing the coating solution. The selection of blocking agent was also critical to suppressing background noise and maintaining high sensitivity without altering or obscuring the epitope for antibody binding. Thus, after testing 1% BSA, gelatin and skim milk, 5% skim milk, which provided the lowest background noise, was picked as the blocking reagent.

The sandwich ELISA assay was applied to detect glycinin at various concentrations. The absorbance at $\lambda = 450$ nm showed linear relationship with the concentrations of glycinin in the range of 3–200 ng/mL (Fig. 2A). Then, soybean seeds diluted with protein-free diet were analysed to evaluate sensitivity of the method for real samples. The results showed that the LOD ($SN^{-1} = 3$) and the limit of quantification ($SN^{-1} = 10$) were 0.2 and 0.67 mg/g, respectively. The existence of fat ranging from 9% to 21% in soybean samples may interfere with the detection of glycinin in real samples. Soybean seeds were defatted using petroleum ether. The result showed that defatted soybean samples had better accuracy and reproducibility compared with full-fat soybean. The existence of fat in the sample decreased solubility of proteins and increased nonspecific binding of the anti-bodies. Therefore, all soybean samples were defatted prior to protein extraction. β -Mercaptoethanol was added to the extraction cocktail to enhance solubility of target

Table 1
Cross-reactivity of five selected Mabs to glycinin and related soybean allergens.^a

Allergens	Cross-reactivity ^b (%)				
	1B7	2E2	2A9	4H6	6F2
Glycinin	100	100	100	100	100
β -Conglycinin	79.6	1.2	3.9	15.5	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 and 25 ng/mL.

^b Cross-reactivity (CR) of competitors is expressed as the percentage calculated according to the formula: CR (%) = (IC_{50} of glycinin)/(IC_{50} of certain inhibitor) $\times$ 100.

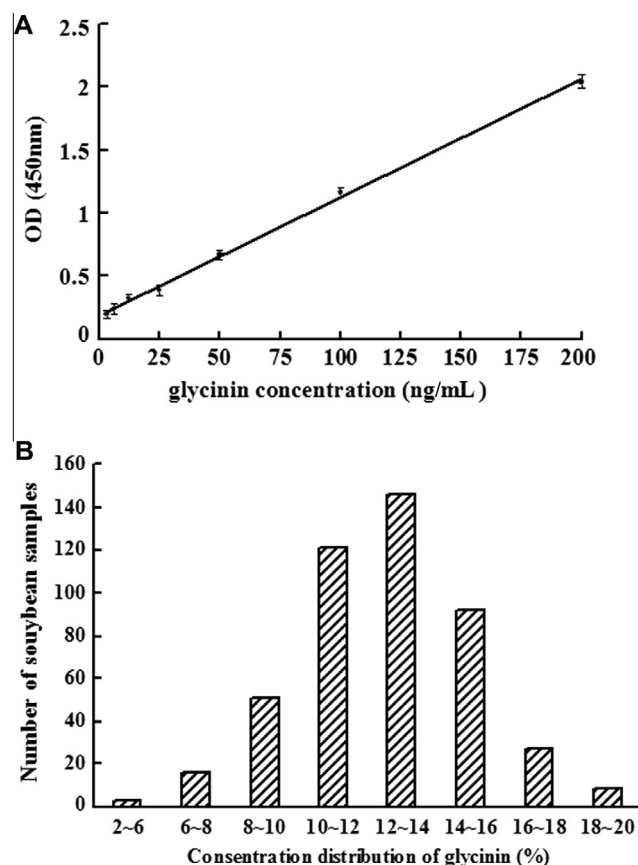


Fig. 2. (A) Standard calibration curve of the double antibody sandwich ELISA detection kit. Each data point represent mean of six measurements of the absorbance at 450 nm. (B) Concentration distribution of soybean glycinin (%) in different soybean cultivars.

Table 2
Recoveries of glycinin spiked in defatted soybean.

Concentration of glycinin in defatted soybean $\pm$ SD (mg/g, DM ^a)	Spiked level (mg/g)	Measured concentration $\pm$ SD (mg/g)	Mean recovery (%)	CV (%)
144.3 $\pm$ 14.9	50	182.3 $\pm$ 15.1	93.8	8.3
144.3 $\pm$ 14.9	100	252.4 $\pm$ 12.1	103.3	4.8
144.3 $\pm$ 14.9	200	334.3 $\pm$ 13.7	97.1	4.1

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

protein and prevent co-purification of host proteins, which may form disulphide bonds with target protein.

Recovery tests and validation studies were conducted using soybean and soybean by-products. Defatted soybean samples that were spiked with glycinin at 50, 100 and 200 mg/g were analysed to evaluate validity and reliability of the double antibody sandwich ELISA. Six replications were tested at each concentration. Recoveries of glycinin were calculated using calibration curve established with purified glycinin. The recoveries ranged from 93.8% to 103.3%, and coefficients of variations were less than 8.3% (Table 2). Intra-assay reproducibility was evaluated using defatted soybean spiked with glycinin at 50, 100, and 200 mg/g within a 6-day period. The results showed that intra-assay recoveries in those days ranged from 92.9% to 104.6%, and coefficient of variation (CV) was less than 10.5%. Thus, the ELISA assay developed here showed high reliability for the analysis spiked soybean samples.

We also measured the concentration of native glycinin in one defatted soybean sample by applying the developed ELISA assay. The concentration was 12.4% on total weight basis and 14.4% on dry matter basis. The data obtained from HPLC was compatible with the results obtained from ELISA. Therefore, the Mab 6F2/Pab double antibody sandwich ELISA can be used to accurately determine glycinin concentration in soybean samples.

3.4. Analysis of real samples

469 soybean samples from different origins and cultivars were collected and tested with the ELISA assay described above. Soy protein extracts were prepared by using 1% BSA diluting to a final concentration in the range of 12.5–100.0 ng/mL. In order to keep the concentration of the actual test solution within the linear range of the calibration curve, several trials were undertaken. After analysing a large quantity of the samples, the unknown sample can be determined with the right level of dilution. As there are some

Table 3
Average concentrations of glycinin in soybean samples from different origins.

Regions of origin	Number of samples	Mean glycinin concentration $\pm$ SD (mg/g, DM ^a)	Mean moisture $\pm$ SD (%)
North spring	105	118.4 $\pm$ 26.7	6.46 $\pm$ 0.52
Huanghuai summer	100	132.8 $\pm$ 20.5	6.34 $\pm$ 0.07
Changjiang spring–summer	79	122.1 $\pm$ 30.3	6.19 $\pm$ 0.44
Southeast spring–summer–autumn	63	124.9 $\pm$ 25.5	6.58 $\pm$ 0.39
South China four seasons	54	128.9 $\pm$ 26.7	6.46 $\pm$ 0.40
Overseas cultivars	68	131.5 $\pm$ 25.6	6.55 $\pm$ 0.40

^a DM, in a dry matter basis.

Table 4
ELISA and SDS–PAGE results of different soybean products.^a

Soybean products	Crude protein (%)	Glycinin concentration $\pm$ SD (mg/g, DM)	
		ELISA	SDS–PAGE
Soybean	43.1	124.1 $\pm$ 8.7	113.5 $\pm$ 5.9
Soybean meal	47.9	144.3 $\pm$ 7.8	132.0 $\pm$ 10.7
Soybean protein concentrat	66.4	21.4 $\pm$ 2.0	16.5 $\pm$ 1.7
Soybean protein isolate	44.8	13.2 $\pm$ 0.9	10.4 $\pm$ 1.0
Extruded soybean	36.9	108.7 $\pm$ 6.4	99.0 $\pm$ 5.4
Fermented soybean meal	43.6	6.4 $\pm$ 0.4	2.5 $\pm$ 0.3

^a Soybean data were obtained from the mean of 469 samples. Data for the other products were mean of 5 replications.

samples with extremely high or low concentration, more dilution is required. For instance, the samples were diluted by 10, 100 and 1000, such that at least one of the diluted samples falls into the linear range of the calibration curve. At the same time, standard glycinin solution was diluted with 1% BSA to 200, 100, 30, 10, 3, 1, 0.3, and 0.1 ng/mL. The samples were analysed in 6 replications. The results showed that the content of glycinin in soybean seeds ranged from 2.72% to 19.56%, while more than 20% of the samples in range of 12–14% (Fig. 2B). The concentration of glycinin varies for soybeans from different origins (Table 3). Samples from the Huanghuai region had the highest average glycinin content, and the North Spring had the lowest average glycinin content. According to our data, on average, samples from that region had a higher glycinin concentration, some individual samples from that group did have much lower glycinin concentrations (data not shown). So, the origin of the soybean samples does not have a direct relationship with the glycinin concentration in the sample. More interestingly, however, the moisture contents of samples varied very little between different regions (Table 3).

Several methods have been conducted to reduce soy protein allergenicity. Here, we evaluated the effectiveness of different processing methods in removing allergenic proteins from soy products with the developed ELISA assay. 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 4). Unprocessed soybean and soybean meal had the highest glycinin concentration at 124.1 and 144.3 mg/g, and glycinin concentration of extruded soybean was rather higher, while, glycinin in fermented soybean meal, soybean protein isolate and soybean protein concentrate showed much lower concentrations. These results are qualitatively consistent with previous literature reports and indicate that there are several effective processing techniques to remove glycinin from soy products, especially in fermented soybean meal; glycinin is nearly completely removed.

The above tests were also repeated using SDS–PAGE. For fermented soybean meal and soybean protein isolate, SDS–PAGE showed a very weak band from any acidic polypeptide chain of glycinin (Fig. 1A), while ELISA can still detect trace amount of glycinin in these samples (Table 4). For samples with higher glycinin concentration, SDS and ELISA showed similar results with the SDS–PAGE presenting slightly a lower value. So, compared with SDS–PAGE, the ELISA assay developed here has greater sensitivity to trace amount of glycinin.

4. Conclusion

In conclusion, a double antibody sandwich ELISA was developed to detect glycinin in soybean and soybean products. The assay is superior to other methods by showing high sensitivity towards glycinin with a LOD at 1.63 ng/mL, the assay also showed mini-

num cross-reactivity with other soybean proteins even in complex samples such as soybean meals. With the developed assay, we analysed 469 soybean samples from different origins, the results showed that the concentration of glycinin in most of the samples were in the 3–19% range. Another interesting finding is that the place of origin has limited influence on glycinin content of the soybean samples. Effective processing can remove most of the glycinin from soybean and soybean products; the developed assay provides a fast, convenient method to evaluate the effectiveness of these processing methods. Our results showed that in fermented soybean meal, the glycinin was nearly completely removed. Data from these real world samples provide a solid foundation for the evaluation of soybean, soybean products and soybean processing techniques. In the future, we will investigate the glycinin content of different cultivars of soybean and use genetic breeding to produce hypoallergenic soybeans.

Soybean protein has been so widely used in the food and feed industries that the identified allergenic proteins should be eradicated without the redux allergenic proteins in soybean. There exist a lot of opportunities and challenges to develop efficient methods to eradicate these immuno-dominant allergens while maintaining their nutritional value. Developing sensitive and convenient screening methods seems important as well. The double sandwich ELISA concept developed here with its high sensitive and specificity shows great benefit for the detection and screening of these allergenic proteins.

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References

- Association of Official Analytical Chemists (AOAC). 2000. Official methods of analysis (17th ed.). Method 930.15. Arlington.
- Arvola, M., Gustafsson, E., Svensson, L., Jansson, L., Holmdahl, R., Heyman, B., et al. (2000). Immunoglobulin-secreting cells of maternal origin can be detected in B cell-deficient mice. *Biology of Reproduction*, 63, 1817–1824.
- Bednar-Tantscher, E., Mudde, G. C., & Rot, A. (2001). Maternal antigen stimulation down regulates via mother's milk the specific immune responses in young mice. *International Archives of Allergy and Immunology*, 126, 300–308.
- Blazek, V., & Caldwell, R. A. (2009). Comparison of SDS gel capillary electrophoresis with microfluidic lab-on-a-chip technology to quantify relative amounts of 7S and 11S proteins from 20 soybean cultivars. *International Journal of Food Science and Technology*, 44, 2127–2134.
- Castro-Rubio, F., Marina, M. L., & García, M. C. (2007). Perfusion reversed-phase high-performance liquid chromatography/mass spectrometry analysis of intact soybean proteins for the characterization of soybean cultivars. *Journal of Chromatography A*, 1170, 34–43.
- Chen, F., Hao, Y., Piao, X. S., Ma, X., Wu, G. Y., & Qiao, S. Y. (2011). Soybean-derived β -conglycinin affects proteome expression in pig intestinal cells in vivo and in vitro. *Journal of Animal Science*, 89, 743–753.
- Cho, R. K., Iwamoto, M., & Saio, K. (1987). Determination of 7S and 11S globulins in ground whole soybeans by near infrared reflectance spectroscopic analysis. *Journal of the Japanese Society for Food Science and Technology*, 34, 666–672.
- Friedman, M., & Brandon, D. L. (2001). Nutritional and health benefits of soy proteins. *The Journal of Agricultural and Food Chemistry*, 49, 1069–1086.
- García, M. C., Heras, J. M., & Marina, M. L. (2007). Simple and rapid characterization of soybean cultivars by perfusion reversed-phase HPLC: Application to the estimation of the 11S and 7S globulin contents. *Journal of Separation Science*, 30, 475–482.
- García, M. C., Torre, M., Laborda, F., & Marina, M. L. (1997). Rapid separation of soybean globulins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A*, 758, 75–83.
- Goetz, H., Kuschel, M., Wulff, T., Sauber, C., Millerb, C., Fisherb, S., et al. (2004). Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination. *Journal of Biochemical and Biophysical Methods*, 60, 281–293.
- Hanson, L. A., Korotkova, M., Lundin, S., Haversen, L., Silfverdal, S. A., Mattsby-Baltzer, I., et al. (2003). The transfer of immunity from mother to child. *Annals of the New York Academy of Sciences*, 987, 199–206.
- Hei, W. J., Li, Z., Ma, X., & He, P. L. (2012). Determination of beta-conglycinin in soybean and soybean products using a sandwich enzyme-linked immunosorbent assay. *Analytica Chimica Acta*, 734, 62–68.
- Helm, R. M., Cockrell, G., Herman, E., Burks, A. W., Sampson, H. A., & Bannon, G. A. (1998). Cellular and molecular characterization of a major soybean allergen. *International Archives of Allergy and Applied Immunology*, 117, 29–37.
- Herian, A. M., Taylor, S. L., & Bush, R. K. (1990). Identification of soybean allergens by immunoblotting with sera from soy-allergic adults. *International Archives of Allergy and Applied Immunology*, 92, 193–198.
- Hill, J. E., & Breidenbach, R. W. (1974). Proteins of soybean seeds I. Isolation and characterization of the major components. *Plant Physiology*, 53, 742–746.
- Hou, H. J., & Chang, K. C. (2004). Structural characteristics of purified glycinin from soybeans stored under various conditions. *Journal of Agricultural and Food Chemistry*, 52, 3792–3800.
- Hu, S. D., Liu, H., Qiao, S. Y., He, P. L., Ma, X., & Lu, W. Q. (2013). Development of immuno affinity chromatographic method for isolating glycinin (11S) from soybean proteins. *Journal of Agricultural and Food Chemistry*, 61, 4406–4410.
- Huang, L., Mills, E. N., Carter, J. M., & Morgan, M. R. A. (1998). Analysis of thermal stability of soya globulins using monoclonal antibodies. *Biochimica et Biophysica Acta*, 1388, 215–226.
- Kim, S. W., Heugten, E. V., Ji, F., Lee, C. H., & Mateo, R. D. (2010). Fermented soybean meal as a vegetable protein source for nursery pigs: I. Effects on growth performance of nursery pigs. *Journal of Animal Science*, 88, 214–224.
- Knippels, L. M., Penninks, A. H., & Houben, G. F. (1998). Continued expression of anti-soy protein antibodies in rats bred on a soy protein-free diet for one generation: the importance of dietary control in oral sensitization research. *Journal of Allergy and Clinical Immunology*, 101, 815–820.
- Lallès, J. P., Tukur, H. M., Salgado, P., Mills, E. N., Morgan, M. R., Quillien, L., et al. (1999). Immunochemical studies on gastric and intestinal digestion of soybean glycinin and β -conglycinin in vivo. *Journal of Agricultural and Food Chemistry*, 47, 2797–2806.
- Li, D. F. (2003). *Soybean anti-nutrition factors*. Beijing: China Science and Technology Publishing House.
- Liu, C., Wang, H. L., Cui, Z. M., He, X. L., Wang, X. S., & Zeng, X. X. (2007). Optimization of extraction and isolation for 11S and 7S globulins of soybean seed storage protein. *Food Chemistry*, 102, 1310–1316.
- Ma, X., He, P. L., Sun, P., & Han, P. F. (2010a). Lipic acid: an immunomodulator that attenuates glycinin-induced anaphylactic reactions in a rat model. *Journal of Agricultural and Food Chemistry*, 58, 5086–5092.
- Ma, X., Sun, P., He, P. L., Han, P. F., Wang, J. J., Qiao, S. Y., et al. (2010b). Development of monoclonal antibodies and a competitive ELISA detection method for glycinin, an allergen in soybean. *Food Chemistry*, 121, 546–551.
- Mansfield, S., Hagler, J. R., & Whitehouse, M. E. A. (2008). A comparative study on the efficacy of a pest-specific and prey-marking enzyme-linked immunosorbent assay for detection of predation. *Entomologia Experimentalis et Applicata*, 127, 199–206.
- Maruyama, N., Prak, K., Motoyama, S., Choi, S. K., Yagasaki, K., Ishimoto, M., et al. (2004). Structure physicochemical function relationships of soybean glycinin at subunit levels assessed by using mutant lines. *Journal of Agricultural and Food Chemistry*, 52, 8197–8201.
- Mo, X. Q., Zhong, Z. K., & Wang, D. H. (2006). Soybean glycinin subunits: Characterization of physicochemical and adhesion properties. *Journal of Agricultural and Food Chemistry*, 54, 7589–7593.
- Redl, G., Husain, F. T., Bretbacher, I. E., Nemes, A., & Cichna-Markl, M. (2010). Development and validation of a sandwich ELISA for the determination of potentially allergenic sesame (*Sesamum indicum*) in food. *Analytical and Bioanalytical Chemistry*, 398, 1735–1745.
- Sampson, H. A. (2000). Food anaphylaxis. *British Medical Bulletin*, 56, 925–935.
- Song (1988). Regulations of laboratory animal of the People's Republic of China. *National Science and Technology Committee*, 1988, 2.
- Thiex, N. J., Anderson, S., & Gildemeister, B. (2003). Crude fat, diethyl ether extraction, in feed, cereal grain, and forage (Randall/Soxtec/Submersion Method): Inter laboratory study, ISO/IUPAC/AOAC harmonized protocol association of official analytical chemists [AOAC]. *Journal of AOAC International*, 86, 1–11.
- Thiex, N. J., & Manson, H. (2002). Determination of crude protein in animal feed, forage, grain, and oilseeds by using block digestion with a copper catalyst and steam distillation into boric acid: collaborative study. *Journal of AOAC International*, 85, 309–317.
- Tryphonas, H., Arvanitakis, G., Vavasour, E., & Bondy, G. (2003). Animal models to detect allergenicity to foods and genetically modified products: workshop summary. *Environmental Health Perspectives*, 111, 221–222.
- Walker-Smith, J. A. (1986). Food sensitive enteropathies. *Journal of Clinical Gastroenterology*, 15, 55–69.
- Wilson, S., Blaschek, K., & Mejia, E. G. (2005). Allergenic proteins in soybean: processing and reduction of P34 allergenicity. *Nutrition Reviews*, 63, 47–58.
- Zangar, R. C., Daly, D. S., & White, A. M. (2006). ELISA microarray technology as a high-throughput system for cancer biomarker validation. *Expert Review of Proteomics*, 3, 37–44.