



Analytical Methods

Development of monoclonal antibodies and a competitive ELISA detection method for glycinin, an allergen in soybean

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ABSTRACT

Soybean glycinin is a major food allergen causing anaphylaxis. A sensitive detection method for glycinin is needed to evaluate soybean allergies in food and feed products. In the present study, monoclonal antibodies (Mabs) against glycinin were prepared using purified glycinin as the immunogen. The generated Mabs, named 3B2 and 4B2, were identified as being IgG_{2b} and IgG_{2a} iso-types respectively, and exhibited high specificity to glycinin. Then we developed a competitive ELISA based on Mab 4B2 to measure glycinin which showed an IC₅₀ value of 1.7 ng/mL with a detection limit of 0.3 ng/mL, and the linear portion of the curve was 0.3–11.2 ng/mL. Recovery tests indicated that the competitive ELISA based on Mab 4B2 gave reliable reproducibility. The produced Mab 4B2 and the developed ELISA could provide a valuable tool for sensitive determination of glycinin and for future studies conducted to reveal the mechanism of how glycinin functions in anaphylaxis.

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1. Introduction

Soybeans and soybean by-products are widely used in the food and feed industries because of their high nutritional value (Hancock, Cao, Kim, & Li, 2000) and good physiochemical properties. However, soybean protein has long been recognised as a source of dietary allergens for humans (Foucard & Yman, 1999). In addition, with the increased use of soybean protein in the feed industry, there is increased interest in its allergenic properties because of the potential to cause hypersensitivity reactions in young animals such as piglets (Sun, Li, Li, Dong, & Wang, 2008b) and calves (Lallès & Dreau, 1996).

At least 21 allergenic proteins have been detected in soybean (Wilson, Blaschek, & de Mejia, 2005). Among them is glycinin (most of 11S components, accounted for about 19.5–23.1% of the total soybean proteins), which has been identified as a major soybean allergen with a molecular mass of 300–380 kDa (Mujoo, Trinh, & Ng, 2003). It is reported that glycinin causes damage to intestinal morphology, disorders of immune function, growth depression and diarrhea (Liu, Feng, Xu, Wang, & Liu, 2008). The severity of the immune reactions depends on the dose of glycinin with higher doses causing more severe symptoms (Sun et al., 2008b).

It is reported that glycinin was composed with acidic and basic polypeptides handed over with each other respectively, the acidic (A1a, A1b, A2, A3, A4, A5) and basic (B1a, B1b, B2, B3, B4) polypeptides were linked by a S–S disulfide bond, with molecular weight of 34–44 kDa and 20 kDa respectively (Maruyama et al., 2004). Although among different species glycinin exhibited polymorphism, all the composite polypeptides could be the products of more than one gene even though they were homologous in the regions sequenced respectively (Hou & Chang, 2004; Staswick, Hermanson, & Nielsen, 1981), which determined that they shared some antigenic determinants. On the other hand, previous studies have shown that the acidic chain of the G1 subunit has an immunoglobulin E-binding region (Beardslee, Zeece, Sarath, & Markwell, 2000), which had been proved to increase serum glycinin-specific immunoglobulin E (IgE) antibody and induce the anaphylaxis (Liu et al., 2008).

Most glycinin studies conducted to date have focused on the structural and functional properties of glycinin, as well as its effects on gelling, foaming, and emulsification (Awazuhara et al., 2002). However, little research has been conducted on the development of methods for its detection. The detection of allergens in food products is very difficult as allergens are often present in trace amounts and can be masked by the food matrix (Poms, Klein, & Anllam, 2004). Although quantification of glycinin can be carried out by high performance liquid chromatography (Mujoo et al., 2003), the method requires time-consuming cleanup steps and expensive equipment.

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Immunoassay methods have been extensively applied in a broad number of areas because they involve the use of relatively inexpensive equipment and are suitable for high throughput screening (Burks, Brooks, & Sampson, 1988; You et al., 2008). Some commercial test kits have been developed to determine other antigenic proteins in soybean such as β -conglycinin and Kunitz trypsin inhibitor (Brandon, Bates, & Friedman, 1989; Hitchcock, Bailey, Crimes, Dean, & Davis, 1981; You et al., 2008) but at present, there is no immunological method available for the detection of glycinin. In the present study, we established and evaluated a monoclonal antibody based competitive ELISA for the detection of glycinin in soybean and soybean by-products.

2. Materials and methods

2.1. Preparation of soybean glycinin and related substances

Crude-extracts of glycinin and β -conglycinin, kindly provided by Professor Shuntang Guo of China Agricultural University, were extracted with salting out and ionic precipitation methods. The extracts were purified using a 2.5×100 cm Sepharose CL-6B followed by a DEAE-52 column (Sigma, St. Louis, MO) and analysed using SDS-PAGE (For details see Section 2.4.4). The glycinin was estimated to be more than 92% pure (Fig. 1B). The remaining 8% were mainly salts (sodium chloride et al.) and the peptides from soybean protein hydrolysates (SPH), as well as a spot of other soybean proteins (β -conglycinin et al.). A commercially available soybean agglutinin and trypsin inhibitor (Sigma, St. Louis, MO) were also used in this study.

2.2. Preparation of monoclonal antibodies

2.2.1. Animals and immunisation

The immunisation part of this study was conducted at China Agricultural University (Beijing, China). Ten, second-generation (F2), female BALB/c mice, born at China Agricultural University, were fed a soybean-free diet. The animals were maintained and treated according to the principles of the China Agricultural University Animal Care and Use Committee. When they were 60 days old, the mice were subcutaneously immunised with 50 μ g of 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 given

bi-weekly for the next month. Three days prior to the day of fusion, the mice were bled and the antibody titer in their serum was determined using an Immuno-Microplate Autoreader (Sunrise Tecan, Salzburg, Austria) and measuring 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 three days later by cervical dislocation and sterilised with ethanol. The peritoneal cavity was opened and the spleen was removed. The spleen tissue was 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).

2.2.2. Cell fusion

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). The cells were fused by exposure to polyethylene glycol (Merck & Company, Darmstadt, Germany) according to the protocol described by Zeng et al. (2005). Hybridoma cells were plated onto four identical 96-well microtiter plates (Canda Jet Bio-Chemicals Int., Guangzhou, China) in proliferation medium containing 90% DMEM and 10% fetal bovine serum. The cells were incubated at 37 °C and 5% CO₂ in a standard cell culture incubator (Thermo Scientific, Waltham, MA).

2.2.3. Screening and cloning of hybridoma cell lines

One week following cell fusion, growing hybridomas were observed in most of the culture wells. The supernatant culture fluid from all wells was tested for anti-glycinin activity by non-competitive ELISA as described in Section 2.3. Cell lines with a high affinity for glycinin were selected and the antibody titer and specificity of their culture supernatants was confirmed by competitive ELISA as described in Section 2.3. Then cell lines that exhibited both positive competition with its glycinin and negative competition with analogues (i.e. β -conglycinin) and related substances (i.e. trypsin inhibitor and agglutinin) were submitted to further subclone by the Limiting Dilution method (Köhler & Milstein, 1975).

The above process was repeated three times to produce monoclonal clones for every positive subclone present, and the subclones which affinity for glycinin turned weak were deleted. The hybridoma cell lines showed continuously positive signal with glycinin but negative signal to β -conglycinin, trypsin inhibitor and agglutinin in three subcloning were selected to perform further determination and investigation.

2.3. Competitive ELISA and non-competitive ELISA

A competitive ELISA was carried out to screen the hybridoma cell lines for anti-glycinin activity. Purified glycinin was used as the plate-coating antigen in order to develop a competitive ELISA for immuno-reactive glycinin. A total of five 96-well microtiter plates (Canda Jet Bio-Chemicals Int., Guangzhou, China) were coated with a solution (100 μ L/well) containing purified glycinin (10 μ g/mL) in 0.05 M bicarbonate buffer (pH 9.6) and incubated for 1 h at 37 °C. The plates were then blocked by the addition of 1% gelatin (150 μ L/well) for 1 h at 37 °C. All plates were subsequently washed three times with washing buffer (0.01 M phosphate-buffered saline containing 0.05% Tween-20, pH 7.4).

Soybean glycinin or its related competitor (soybean β -conglycinin, trypsin inhibitor, or agglutinin) was dissolved in assay buffer (0.01 M phosphate-buffered saline, containing 0.001% Tween-20 and 0.1% gelatin, pH 7.4) and added into each well by 50 μ L in con-

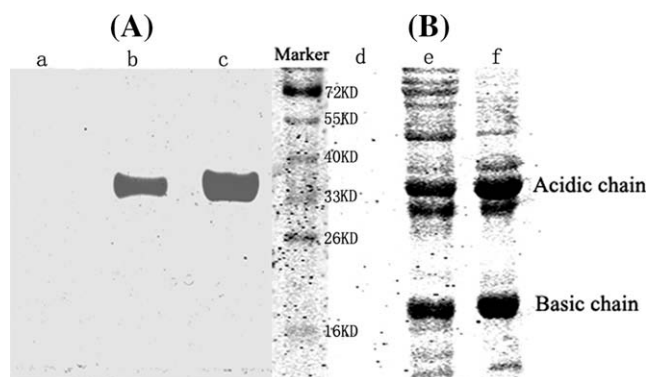


Fig. 1. Samples of soybean seed and purified glycinin were analysed using SDS-PAGE and Western Blot techniques. (A) Western Blot analysis of monoclonal antibody 4B2 aimed at soybean glycinin. (B) Corresponding SDS-PAGE analysis. Line a and d, negative control (dissolved buffer); line b and e (soybean proteins; line c and f (purified glycinin). Experiments were performed in triplicate with similar results obtained each time. Only the graph from one replicate is presented.

tinuous series 2-fold dilution to prepare a concentration gradient of competitors. Hybridoma culture supernatants or prepared monoclonal antibodies were then added into each well by same volume, which was incubated for 1 h at 37 °C, followed by three washes.

The secondary antibody was goat anti-mouse IgG-HRP (Sigma, St. Louis, MO) and was diluted 1:5000 in the assay buffer and added to each well and then incubated for 1 h at 37 °C. Colour was developed by adding freshly made *o*-phenylenediamine (Sigma, St. Louis, MO) substrate solution (0.4 mg/mL) to each well (100 µL/well). After incubating for 25 min at 37 °C, the reaction was immediately terminated by the addition of 2 M H₂SO₄ solution (50 µL/well). The absorbance was then measured at 492 nm using an Immuno-Microplate Autoreader (Sunrise Tecan, Salzburg, Austria). A hybridoma cell line which showed a continuously positive signal with OD value >1.0 in three consecutive subcloning was considered a positive cloning.

A non-competitive ELISA was used to determine the antibody titers of mouse sera or cell culture supernatants. The procedures were identical with that of competitive ELISA except for the detection of the antigen, glycinin and its related competitors.

2.4. Production and characterisation of monoclonal antibodies

2.4.1. Large-amount production and purification of monoclonal antibodies of selected positive clones

The clones with good status and producing stable antibody in Section 2.2.3 were selected and were expanded again. Large amount of monoclonal antibody was produced in vitro similar to the method of Beier, Ripley, Young, and Kasier (2001), culturing and expanding hybridoma cells, collecting the culture supernatants, and then precipitated by ammonium sulfate. The crude-extracted antibody was further purified with a Protein G affinity column (Amersham Biosciences, Uppsala, Sweden) and concentrated by Vacufuge® 5301 Vacuum Concentrator (Eppendorf international, Hamburg, Germany). The purified antibodies were used for further investigation.

2.4.2. Affinity and isotype

The affinity and specificity of the produced antibodies were confirmed by competitive ELISA again. Relative affinity of the monoclonal antibodies for glycinin was measured by determining the 50% inhibition of control values (IC₅₀). The iso-types of the purified monoclonal antibodies were identified by a commercially available immunopure monoclonal antibody isotyping kit I (Pierce, Rockford, IL, USA).

2.4.3. Cross-reaction of antibody

Soybean glycinin, analogue (i.e. soybean β-conglycinin) and related substances (i.e. trypsin inhibitor and agglutinin) were used in the cross-reaction study. Each compound above was dissolved in an assay buffer and examined as a potential inhibitor by competitive ELISA. In order to compare cross-reactivity, the inhibition effect for glycinin with each monoclonal antibody was set as the 100% cross-reactivity value for that monoclonal antibody since glycinin was the target substance that the monoclonal antibodies were detecting. Cross-reactivity of the monoclonal antibodies with the inhibitors β-conglycinin, trypsin inhibitor or agglutinin was expressed as the concentration of inhibitor required to produce 50% inhibition of antibody binding compared with glycinin, which was represented by IC₅₀. Cross-reactivity (CR) of inhibitors is calculated according to the following formula:

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

2.4.4. SDS-PAGE and western blotting

SDS-PAGE and Western Blot analysis were performed in order to assess the purity of glycinin and the specificity of the monoclonal antibody. The samples were dissolved in 1 M Tris-HCl buffer (containing 2.7 M glycerol, 0.15 M sodium dodecyl sulfate and 0.15 mM bromophenol blue, pH 6.8) and measured using a BCA Protein Assay kit (Pierce, Rockford, IL) using bovine serum albumin as the standard. Two copy of identical samples (30 µg total protein per well) were separated with 12.5% acrylamide separating gel using a Minigel Apparatus (BioRad Laboratories, Hertfordshire, UK). One copy was stained with Coomassie Brilliant Blue R250 for 30 min, washed in washing buffer (45% methanol, 10% acetic acid, 45% H₂O, v/v) for 30 min and analysed.

The remaining copy was transferred to nitrocellulose membranes (Hybond™, ECL™, Amersham Pharmacia, UK). The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% non-fat milk for 2 h at room temperature. The membranes were then incubated with 25 ng/mL of the corresponding primary monoclonal antibody overnight at 4 °C. The membranes were washed with TBS-T buffer three times for 10 min and then incubated with secondary antibody (IRD Fluor 780-labelled goat anti-mouse IgG) in the dark for 1 h at room temperature. Following another three washes with TBS-T for 10 min, the IR-fluorophores on the membrane were excited at 780 nm and the emission at 820 nm was quantified using channel 800 of the LI-COR Infrared Imaging System (Odyssey, Lincoln, NE) and analysed with Odyssey software (Odyssey, Lincoln, NE).

2.5. Establishment of inhibition ELISA calibration curve for glycinin

A competitive ELISA based on a prepared monoclonal antibody was conducted to establish an inhibition calibration curve for glycinin. The inhibition for glycinin in the competitive ELISA was calculated using the following formula:

$$\text{Inhibition } (\%) = \left(1 - \frac{OD_{\text{glycinin}} - OD_{\text{control}}}{OD_{\text{dilution buffer}} - OD_{\text{control}}} \right) \times 100$$

OD_{glycinin}: Absorbance measured by inhibition assay of a sample, in which the monoclonal antibody 4B2 was pre-incubated with glycinin.

OD_{control}: Absorbance measured by inhibition assay of a negative control antibody (1 µg/mL of normal mouse IgG).

OD_{dilution buffer}: Absorbance measured by inhibition assay of a sample, in which the monoclonal antibody 4B2 was pre-incubated with dilution buffer.

Tests for cross-reactivity of the monoclonal antibody with glycinin analogue (i.e. β-conglycinin) and its related substances (i.e. trypsin inhibitor and agglutinin) were performed to evaluate the developed ELISA. The procedures were identical with those of the cross-reaction analysis conducted for antibody specificity presented in Section 2.4.3.

2.6. Recovery of glycinin spiked in soybean products measured by competitive ELISA

2.6.1. Preparation of samples and extraction of soybean proteins

Soybean seeds and some soybean products were used in this study to evaluate glycinin recovery by the newly developed competitive ELISA. Soybean seeds, roasted full-fat soybean, extracted soybean meal, extruded soybean meal, and fermented soybean paste were ground with a Cyclotec Sample Mill (Model 1093, Tecator Inc., Herndon, VA) to pass through a 60-mesh sieve. The prepared flours were spiked with pure glycinin at 50, 100 and

200 µg/mg for soybean seeds; 10, 20 and 40 µg/mg for roasted full-fat soybean, extracted soybean meal, extruded soybean meal and fermented soybean paste, and finally 1.0, 2.0, and 4.0 µg/mg for soybean protein concentrate. Proteins were extracted from the flour with 0.03 M Tris–HCl buffer (pH 8.0, containing 0.01 M β-Mercaptoethanol) for 1 h with vortexing (100 g flour per litre buffer). Soybean seeds and roasted full-fat soybean were defatted using n-Hexane before they entered the extraction process. Extracts were centrifuged at 12,000g for 20 min at 4 °C using an Optima™ L-80 XP Ultracentrifuge (Beckman Coulter, Inc, Palo Alto, CA). The supernatants were filtered through a 0.45 µm Millex GP filter (Millipore, Cork, Ireland), and then stored at –20 °C until analysis.

2.6.2. Detection of glycinin

Samples of each solution were diluted in such a manner that their concentrations fell in the linear portion of the curve. The pH of the solution was adjusted to 7.6 with 2 M HCl. The content of glycinin from both soybean products and soybean products spiked with glycinin were analysed by the competitive ELISA developed above.

2.7. Statistical analysis

All data were obtained from three independent experiments. Our experimental data were analysed according to the method of the previous study (You et al., 2008). Briefly, the data were presented as the mean and standard deviation (SD), which were analysed with the “Descriptive Statistics” procedure in SPSS 11.0 (SPSS Inc., Chicago, IL). The inhibition ELISA standard curve for glycinin (Fig. 2) was analysed with the “Harris Model” in Curve Expert 1.3 software.

3. Results

3.1. Production and characterisation of monoclonal antibodies against purified glycinin

Hybridoma cells were produced by fusion of the spleen cells isolated from a mouse immunised against purified glycinin and cells from a mouse myeloma cell line. One week following cell fusion, growing hybridomas were observed in most of the culture wells. The supernatants of all the wells were tested by non-competitive ELISA. The hybridoma cultures from the 32 wells exhibiting the highest anti-glycinin activity were selected for further

diluted, subcloned and determined as above three times. The subclones which affinity for glycinin turned weak were deleted. The hybridoma cell line showed continuously positive signal in three subcloning was considered positive hybridoma cloning. At last the results of the competitive ELISA validated that two hybridoma cell lines exhibited strongly positive competition with glycinin but were negative to β-conglycinin, trypsin inhibitor and agglutinin, and these two cell lines were selected and named as 3B2 and 4B2. The iso-types of the two generated monoclonal antibodies, 3B2 and 4B2, were identified to be the IgG_{2b} and IgG_{2a} iso-types respectively, with *k* light chain.

The monoclonal antibodies 3B2 and 4B2 were evaluated for their specificity to glycinin using a cross-reaction assay. As shown in Table 1, the monoclonal antibodies 3B2 and 4B2 showed inhibitory concentration (IC₅₀) values for glycinin of 3.3 ng/mL and 1.7 ng/mL, respectively, which was significantly lower than the values for β-conglycinin, trypsin inhibitor and agglutinin (*P* < 0.01), indicating that the monoclonal antibodies 3B2 and 4B2 were very specific to glycinin and showed no significant cross-reaction with β-conglycinin, trypsin inhibitor or agglutinin.

The specificity of the monoclonal antibody 4B2 to glycinin was assessed by Western blotting. As shown in Fig. 1A, monoclonal antibody 4B2 could recognise the acidic polypeptides of natural soybean glycinin molecule, but not the basic polypeptides.

3.2. Development of the competitive ELISA based on monoclonal antibody 4B2 against glycinin

The monoclonal antibody 4B₂ was chosen to establish a calibration curve for glycinin in the competitive ELISA because it had both strong specificity and appropriate affinity. The optimal concentration of glycinin and monoclonal antibody 4B2 were determined to be 10.0 µg/mL and 25 ng/mL, respectively. As shown in Fig. 2, the concentration of glycinin which inhibited 50% of the total binding of monoclonal antibody 4B2 (IC₅₀) was 1.7 ± 0.4 ng/mL, while the minimum detection limit which inhibited 20% total binding of monoclonal antibody 4B2 (IC₂₀) was 0.3 ± 0.1 ng/mL. The linear portion of the curve, with a working range producing 20–80% total binding of monoclonal antibody 4B2, was 0.3–11.2 ng/mL.

3.3. Practical evaluation of the monoclonal antibody 4B2-based competitive ELISA for detection of glycinin

Recovery tests and validation studies were performed using soybean and soybean products. The content of glycinin measured in six soybean products is shown in Table 2. The immuno-reactive glycinin in unprocessed soybean seeds was the highest at

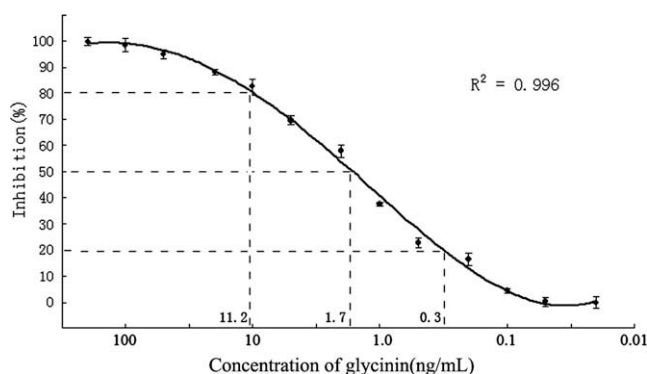


Fig. 2. Inhibition ELISA standard curve for glycinin obtained with monoclonal antibody 4B2 in glycinin-coated format. Each point represents the inhibition rate with the mean ± standard deviation from 12 determinations (*n* = 12) in the newly developed cELISA. The inhibition rate for glycinin in the competitive ELISA was calculated as described in Section 2. The optimal concentrations of glycinin and monoclonal antibody 4B2 were 10.0 µg/mL and 25 ng/mL, respectively.

Table 1

Cross-reactivity for various inhibitors or analogues of glycinin in the monoclonal antibody solution^a.

Inhibitors	Max concentration (ng/mL)	IC ₅₀ ^b , mean ± SD (ng/mL) CR (cross-reactivity, %)	
		3B2	4B2
Glycinin	1.0 × 10 ³	3.3 ± 0.8 (100.0)	1.7 ± 0.4 (100.0)
β-Conglycinin	1.0 × 10 ⁶	1884.6 ± 2.2 (0.18)	3597 ± 10.8 (0.05)
Trypsin inhibitor	>5.0 × 10 ⁶	0 (<0.01)	0 (<0.01)
Agglutinin	>5.0 × 10 ⁶	0 (<0.01)	0 (<0.01)

^a The optimal concentration of purified glycinin and monoclonal antibodies used for cELISA were 10.0 µg/mL and 25 ng/mL, respectively.

^b The values of IC₅₀ and CR% were calculated as described in Section 2. Each IC₅₀ value represents the mean from 12 replicates (*n* = 12).

Table 2Recovery of glycinin spiked in soybean products determined by cELISA using monoclonal antibody 4B2^a.

Samples	Mean $\pm$ SD ($\mu\text{g}/\text{mg}$)	Spiked level ($\mu\text{g}/\text{mg}$)	Mean $\pm$ SD ($\mu\text{g}/\text{mg}$)	Recovery (%)	RSD (%)
Soybean seed ^b	185.4 $\pm$ 15.1	50.0	239.4 $\pm$ 18.2	101.7	5.6
		100.0	292.9 $\pm$ 22.4	102.6	7.8
		200.0	394.7 $\pm$ 26.1	102.4	6.2
Roasted full-fat soybean ^c	48.6 $\pm$ 5.9	10.0	57.1 $\pm$ 3.8	97.4	4.4
		20.0	70.2 $\pm$ 3.3	102.3	5.9
		40.0	85.9 $\pm$ 4.3	97.0	7.5
Extracted soybean meal ^d	39.7 $\pm$ 3.3	10.0	47.9 $\pm$ 5.1	96.4	6.2
		20.0	59.1 $\pm$ 3.6	99.0	3.7
		40.0	78.0 $\pm$ 4.9	97.9	4.2
Extruded soybean meal ^e	22.3 $\pm$ 4.9	10.0	31.5 $\pm$ 2.2	97.5	6.8
		20.0	43.6 $\pm$ 3.1	103.1	7.0
		40.0	63.9 $\pm$ 4.5	102.6	7.7
Fermented soybean paste ^f	12.5 $\pm$ 8.7	10.0	21.9 $\pm$ 2.4	97.3	6.2
		20.0	33.0 $\pm$ 1.7	101.5	4.6
		40.0	54.8 $\pm$ 2.0	104.4	7.4
Soybean protein concentrate ^g	0.18 $\pm$ 0.03	1.0	1.12 $\pm$ 0.15	94.9	9.5
		2.0	2.09 $\pm$ 0.11	95.9	4.6
		4.0	4.30 $\pm$ 0.20	102.9	7.1

^a Data represent the means of 12-replicate subsamples.^b Heinong-38 variety obtained from Jilin Academy of Agriculture Science, Changchun, China, which contains 40.8% crude protein (analysed value).^c Roasted for 15 min at 125 °C using a rotary drum dryer.^d A commercial solvent-extracted soybean meal (SBM) containing 47.6% crude protein (analysed value).^e A commercial dehulled and extruded soybean meal containing 44.2% crude protein (analysed value).^f A commercial microbial fermented soybean meal containing 39.9% crude protein (analysed value).^g A commercial product containing 67.3% crude protein (analysed value).

185.4 $\pm$ 15.1 $\mu\text{g}/\text{mg}$, while the immuno-reactive glycinin in the soybean products such as roasted full-fat soybean, extracted soybean meal, extruded soybean meal, fermented soybean paste, and soybean protein concentrate was much lower. The lowest glycinin level was 0.18 $\pm$ 0.03 $\mu\text{g}/\text{mg}$ in soybean protein concentrate.

When spiked with pure glycinin at three different levels, the average recoveries varied from 94.9% to 102.9% in soybean and the soybean products, which are within an acceptable range. In addition, the relative standard deviation (RSD) ranged from 3.7% to 9.5% which indicated that the monoclonal antibody 4B2-based competitive ELISA gave sensitive and reliable reproducibility.

4. Discussion

Glycinin has been identified as a major allergen for infants and adults (Nowak-Węgrzyn, 2003), as well as young animals (Lallès and Dreau, 1996; Sun et al., 2008b). It is reported that glycinin causes damage to intestinal morphology, disorders of immune function, growth depression and diarrhea (Liu et al., 2008). The severity of the immune reactions depends on the dose of glycinin with higher doses causing more severe symptoms (Sun et al., 2008b).

A sensitive detection method for glycinin is needed in order to prevent soybean allergies in food and feed products. In addition, the development of an effective detection method for glycinin could be valuable as a tool to reveal the mechanism through which glycinin functions in hypersensitive responses and find effective therapeutic agents to reduce its impact (Sun et al., 2008a).

Although accurate quantification of glycinin can be carried out by high performance liquid chromatography (Mujoo et al., 2003), the method requires time-consuming cleanup steps and expensive equipment (Lallès et al., 1999). Other methods for the detection of allergens such as immunohistochemical techniques associated with image analysis and polymerase chain reactions (PCR) based on the amplification of specific DNA fragments have also been developed recently (L'Hocline and Boye, 2007), but these are also time consuming and expensive.

There have been several previous attempts to develop methods for the detection of allergens in soybean which require relatively inexpensive equipment and are suitable for high throughput

screening (Boutten, Humbert, Chelbi, Durand, & Peyraud, 1999; Burks et al., 1988). The majority of the methods which have been developed to detect soybeans in food products are based on immunochemical assays (Burks et al., 1988; Hitchcock et al., 1981). However, most of these methods are based on polyclonal antiserum and are considered to be lacking in sensitivity (Poms et al., 2004). Using polyclonal anti-sera, it takes a long time to purify the antigen in order to produce sufficient quantities of antibody, the antigen obtained may still be contaminated by trace amounts of other proteins and the anti-sera produced may not have strong specificity for the antigen (You et al., 2008). These shortcomings can be overcome by using the monoclonal hybridoma technique (Huang, Mills, Carter, & Morgan, 1998).

In the only other study that we are aware of which attempted to characterise and produce monoclonal antibodies against glycinin, rats were immunised with a synthetic peptide that corresponded to residues 86–104 of the acidic polypeptide of glycinin as the immunogen and not purified glycinin (Jane et al., 1991). It is reported that the regions recognised by IFRN 0025 corresponded to surface loops, which are flexible in nature and easily subjected to conformational changes via thermal and proteolytic processing (Huang et al., 1998). Therefore, the monoclonal antibody IFRN 0025 was not considered suitable for accurate quantitative analysis.

The key requirement for successful immunochemical assay is the availability of antibodies with high specificity and desired affinity (Singh, Srivastava, Raghava, & Varshney, 2006). In the present study, monoclonal antibodies prepared by two selected clones (3B2 and 4B2) were identified as having particularly high affinity for glycinin. The iso-types of the two generated monoclonal antibodies, 3B2 and 4B2, were identified to be the IgG_{2b} and IgG_{2a} iso-types respectively, with κ light chain. The monoclonal antibody 4B2 showed an higher affinity to glycinin with IC₅₀ value of 1.7 ng/mL and scarcely any cross-reactivity with its analogue (soybean β -conglycinin) and related substances (trypsin inhibitor and agglutinin), so the monoclonal antibody 4B2 was the most sensitive to glycinin.

Our study identified that monoclonal antibody 4B2 recognised the epitope regions in the acidic chain of glycinin, and the acidic chain of the G1 subunit has an immunoglobulin E-binding region

(Beardslee et al., 2000), which had been shown to increase serum glycinin-specific immunoglobulin E (IgE) antibody and induce anaphylaxis (Sun et al., 2008b). Therefore the content of glycinin detected by the monoclonal antibody 4B2-based ELISA might represent the IgE-induced immunoreactivity of glycinin (Hou & Chang, 2004).

Based on monoclonal antibody 4B2, we developed a competitive ELISA for the detection of glycinin. The assay showed an inhibitory concentration (IC_{50}) value of 1.7 ng/mL with a detection limit of 0.3 ng/mL. The linear portion of the curve, with working ranges producing 20–80% total binding of monoclonal antibody 4B2, were 0.3–11.2 ng/mL.

In order to evaluate the validation and reliability of the monoclonal antibody 4B2-based competitive ELISA, recovery tests and validation studies were performed for the determination of soybean and soybean products (You et al., 2008). The results indicated that the competitive ELISA gave sensitive and reliable reproducibility. Comparatively, our data for glycinin content in soybean seeds by means of competitive ELISA were compatible with the data obtained from the HPLC method.

Our data revealed that the immuno-reactive glycinin of soybean products (i.e. roasted full-fat soybean, extracted soybean meal, extruded soybean meal, fermented soybean paste, and soybean protein concentrate) were much lower than that of soybean seeds, which was similar to the previous researches (Song, Frias, Martinez-Villaluenga, Vidal-Valverde, & Gonzalez de Mejia, 2008). The reason may be due to the fact that treatments such as heating, expanding, and aqueous alcohol extracting result in primary structure damage of the glycinin molecule in soybean products (You et al., 2008).

Because food allergens are usually complex mixtures of many potentially immuno-reactive proteins, individual allergens within a food allergen system will be affected differently by interactions and various processing methods such as thermal treatment, enzymatic hydrolysis, and chemical modification (Hou & Chang, 2004; Plumb et al., 1994). In the future, we will focus on further practical evaluation of our competitive ELISA method based on mixed products (Lallès et al., 1999).

In conclusion, a high-affinity monoclonal antibody against glycinin was obtained by means of mice immunised with purified glycinin. Based on the monoclonal antibody 4B2 the developed competitive ELISA exhibited high-sensitivity with a low detection limit at 0.3 ng/mL of glycinin, which provide a valuable tool for sensitive determination of glycinin and for future studies on food allergies related to soybean glycinin.

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