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Development of a monoclonal antibody-based competitive ELISA for detection of β-conglycinin, an allergen from soybean

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Abstract

Soybean β-conglycinin is one of the major food allergies for children and young animals. In order to detect immunoreactive β-conglycinin from soybean and soybean products, monoclonal antibodies against β-conglycinin were prepared using a conjugated chicken ovalbumin with a synthetic peptide that corresponded to one epitope sequence of β-conglycinin as the immunogen. The generated monoclonal antibodies, named as $6G_4$, $3B_7$, and $5F_{10}$, were identified as being of IgG_1 isotype and exhibited high specificity to β-conglycinin. A competitive enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibody $6G_4$ was established to determine β-conglycinin and showed an IC_{50} value of 4.7 ng/mL with a detection limit of 2.0 ng/mL. The recovery tests of β-conglycinin indicated that the monoclonal antibody $6G_4$-based competitive ELISA gave reliable reproducibility. Therefore, the produced monoclonal antibody $6G_4$ and the developed competitive ELISA could provide a valuable tool for sensitive determination of β-conglycinin and for future studies on food allergies related to soybean β-conglycinin.

Keywords: Soybean allergen; β-Conglycinin; Immunoassay; Monoclonal antibody

1. Introduction

Owing to its high nutritional value and good physicochemical properties, soybeans are widely utilized in diets fed to humans and animals (Easter & Kim, 1999; Friedman & Brandon, 2001; Hancock, Cao, Kim, & Li, 2000). However, food allergies have been recognized as a growing problem across the world (Chandra, 2002; Herman, Helm, Jung, & Kinney, 2003; Zeiger, 2000) and are increasingly concerned by many countries or organizations. A new allergy labeling law entitled "Food Allergen Labeling and Consumer Protection Act of 2004" was enacted by Food and Drug Administration in US, and in EU, by March 2005, a proposal of the new legislation was also under the consideration. Soybean is often cited as a major source of food allergies especially in industrialized countries (Herman et al., 2003; Huisman, 1989; Huisman & Jansman, 1991).

Soybean allergy has significant impact on sensitive people particularly infants and toddlers (Herian, Taylor, & Bush, 1990). The widespread use of soybean-based formulas exposes a large fraction of infants to soybean allergies and potential soybean sensitivity (Cantani & Lucenti, 1997; Heppell, Sissons, & Pedersen, 1987). Soybean allergies are also found in many animals that are fed soybean as a large component of their diets. Pigs and calves are among the farm animals with significant soybean sensitivity (Kilshaw & Sissons, 1979; Miller et al., 1984; Sissons, 1982).

At least 16 allergenic proteins in soy have been detected that bind to IgE of sera of soybean-sensitive patients. Among them β-conglycinin, which accounts for about 30% of the total soybean proteins, was shown to be the major soybean allergen (Maruyama et al., 2001). β-Conglycinin is well known as a major soybean storage protein, containing three different subunits, α, α′ and β

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with molecular weight of 58–77, 58–83, and 42–53 kDa, respectively (Mujoo, Dianne, & Ng, 2003; Perez, Mills, Lambert, Johnson, & Morgan, 2000; Shuttuck-Eidens & Beachy, 1985; Thanh & Shibasaki, 1977). At the same time, β-conglycinin plays important roles in hypersensitive responses. In children, hypersensitive responses to β-conglycinin occur frequently, especially in western countries (Xiang, Beardslee, Zeece, Marwell, & Sarath, 2002). When calves and piglets are fed β-conglycinin, transient hypersensitivity usually develops (Lallès & Dreau, 1996; Li et al., 1990). Therefore, the use of soybean and soybean products containing β-conglycinin bears certain risks in diets of human and animals.

In order to further investigate the content of β-conglycinin and its mechanism in hypersensitive responses, an effective tool for detection of β-conglycinin is necessary. However, most previous studies have mainly focused on the investigation of the structure and functional properties of β-conglycinin and their effects on gelling, foaming, and emulsification (Awazuhara et al., 2002; Ji, Cai, & Chang, 1999; Maruyama et al., 1998), but rare of its detection. It is well known that detection of allergens in food products is very difficult as they are often present in trace amounts and can be masked by the food matrix (Poms, Klein, & Anllam, 2004). Though quantification of β-conglycinin can be carried out by high performance liquid chromatography method (Mujoo, Trinh, & Ng, 2003), the method require time-consuming cleanup steps and expensive equipments. On the other hand, immunoassay method is extensively applied in broad areas due to its advantages in cost and operation step (relatively simple and can handle a lot of samples in a short of time). Some test kits have been commercially developed to determine the renatured soy proteins, kunitz trypsin inhibitor, and minimally processed soy flour protein in the food products. To date, however, an available approach to determine single β-conglycinin molecule is not established. This led us to develop a specific monoclonal antibody for the detection of β-conglycinin.

2. Materials and methods

2.1. Generation of hapten

The peptide, Arg-Pro-Gln-His-Pro-Glu-Arg (RPQHPER) is an epitope corresponding to the aminoacid residues 78–84 present in the α' subunit of β-conglycinin (Huang, Mills, Carter, & Morgan, 1998). This peptide was extended by adding one aminoacid to both the C- and N-terminal according to the natural aminoacid sequence of β-conglycinin, and therefore a nine-aminoacid peptide, Pro-Arg-Pro-Gln-His-Pro-Glu-Arg-Glu (PRPQHPERE) was synthesized using a Symphony 12-channel Multiple Peptide Synthesizer (Protein Technologies Inc., Tucson, AZ, USA). The synthetic peptide was used as the hapten.

2.2. Preparation of immunogen and plate-coating antigen

Because the bridging moiety affects the immunity of a crossed antigen, which is widely used to conquer unwanted cross-reactions among the compound antigens with the same hapten and improve immunoassay sensitivity (heterologous assays) (Beier, Ripley, Young, & Kasier, 2001; Greirson, Allen, Gare, & Watson, 1991), the immunogen and the plate-coating antigen were prepared by coupling the same hapten to two bridging moieties, ovalbumin and bovine serum albumin, respectively by two technical methods.

The immunogen was prepared by completing the linker arm of the synthetic peptide and conjugating the product to chicken ovalbumin (Sigma, St. Louis, MO, USA) according to the two-step glutaraldehyde method (Burgeon et al., 1991). The synthetic pathway is shown in Fig. 1. Briefly, 3 mg of ovalbumin was dissolved in 1 mL of 0.1 M carbonate buffer (containing 0.9% NaC1 and 0.1% sodium dodecyl sulfate, pH 9.0) and then mixed with 20 μL of 25% glutaraldehyde aqueous solution. The reaction was conducted for 1 h at 20–25 °C in the dark. The mixture was separated on Sephadex G-25 to eliminate excessive peptide. The ovalbumin bound with glutaraldehyde was collected and then incubated with 1.5 mg of the synthetic peptide for 16 h at 20–25 °C in the dark. Finally, the reaction was blocked by glycine (1 M, final concentration) for 16 h, and the solution was dialyzed against 0.01 M phosphate-buffered saline (pH 9.0).

The plate-coating antigen applied in enzyme-linked immunosorbent assay (ELISA) was prepared by the use of a conjugated bovine serum albumin (Sigma, St. Louis, MO, USA) with the synthetic peptide according to the method of Beier, Creemer, Ziprin, and Nisbet (2005). The synthetic pathway is shown in Fig. 2. Five milligrams of bovine serum albumin was dissolved in 1 mL 0.01 M sodium

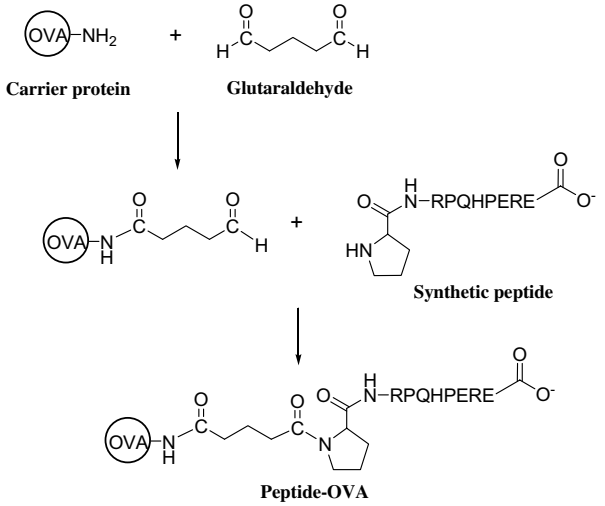

Fig. 1. Synthetic pathway for preparation of immunogen using ovalbumin (OVA) as carrier protein. In this reaction, glutaraldehyde plays a bridging role for coupling the synthetic peptide to the carrier protein.

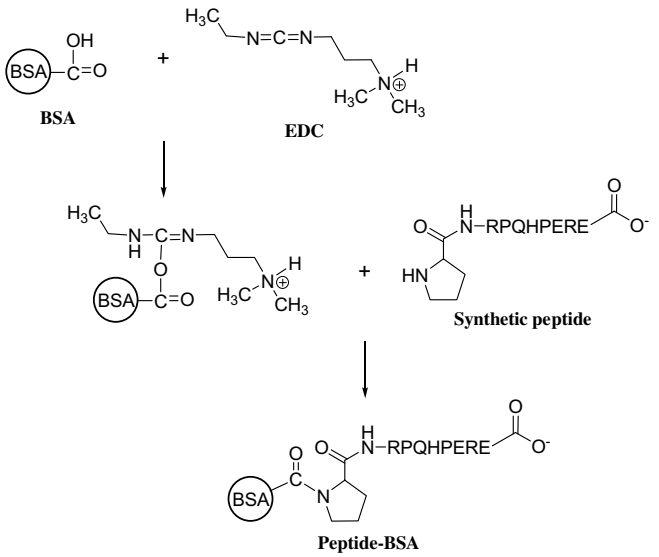

Fig. 2. Synthetic pathway for producing the plate coating antigen using bovine serum albumin (BSA) as carrier protein. The compound made up of BSA and 1-ethyl-3-(dimethyl-aminopropyl)carbodiimide-HCl (EDC), produced in the first step reaction, was an unstable amine-reactive intermediate.

borate buffer (pH 7.6) and 10 mg of 1-ethyl-3-(dimethyl-aminopropyl)carbodiimide-HCl (Sigma, St. Louis, MO, USA) was added to bovine serum albumin solution. The pH of mixture solution was adjusted to 4.5 with 0.1 M HCl at 20–25 °C. Ten minutes later, 1.5 mg of peptide was added to the solution above. The mixture was gently stirred for 16 h at 4 °C, and then extensively dialyzed against 0.01 M phosphate-buffered saline (phosphate-buffered saline, pH 7.6) to eliminate excessive peptide.

2.3. Preparation of soybean β-conglycinin and related substances

β-Conglycinin was isolated from defatted soy flour by the method of Thanh and Shibasaki (1976) and further purified through a 2.5 × 100 cm Sepharose CL-6B (Sigma, St. Louis, MO, USA) column. Glycinin was isolated by hydroxyapatite-Ultrogel chromatography of a cryo-precipitated soybean extract using the method of Eldridge and Wolf (1967). All the preparation were judged to be over 95% pure according to SDS-PAGE analysis. The commercial soybean agglutinin (Sigma, St. Louis, MO, USA) and trypsin inhibitor (Sigma, St. Louis, MO, USA) were also applied in this study.

2.4. Competitive ELISA

A competitive ELISA was developed to evaluate antibody specificity and determine immunoreactive β-conglycinin. Peptide-bovine serum albumin was used as plate-coating antigen. Ninety-six well immuno-module plates (JET, Tucker, Toronto, Canada) were coated with a solution (100 μL/well) containing the peptide-bovine serum albumin (2.5 μg/mL for supernatants of hybridoma cell culture test or 5.0 μg/mL for prepared monoclonal antibody test) in 0.05 M bicarbonate buffer (pH 9.6) and incubated for 1 h at 37 °C, followed by blocking the plate with 1% gelatin (150 μL/well) for 1 h at 37 °C. The plates were washed three times with wash buffer (0.01 M phosphate-buffered saline, containing 0.05% Tween-20, pH 7.4). Soybean β-conglycinin or its related competitor (synthetic peptide, soybean glycinin, 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 μL in continuous 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, goat anti-mouse IgG-HRP (Sigma, St. Louis, MO, USA) with 1:5000 dilutions in assay buffer, was added to each well and incubated for 1 h at 37 °C. Freshly made *o*-phenylenediamine (Sigma, St. Louis, MO, USA) substrate solution (0.4 mg/mL) was added to each well (100 μL/well). After incubating for 25 min at 37 °C, reaction was immediately terminated by 2 M H_2SO_4 solution (50 μL/well). The absorbance at 492 nm was then detected by using an immuno-Microplate Autoreader (Sunrise Tecan, Salzburg, Austria).

2.5. Noncompetitive ELISA

A noncompetitive 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, β-conglycinin and its related competitors.

2.6. Preparation and characterization of monoclonal antibodies

2.6.1. Immunization

Five, 60-day old, BALB/c female mice were subcutaneously immunized with 50 μg of peptide-ovalbumin emulsified in complete Freund's Adjuvant (Sigma, St. Louis, MO, USA). Four weeks later, immunization was performed with a boost every second week using the same dose of peptide-ovalbumin emulsified in incomplete Freund's Adjuvant (Sigma, St. Louis, MO, USA). Three days prior to the day of fusion, the mouse with the highest antibody titer of serum was selected to give an intraperitoneal injection with 50 μg of emulsified peptide-ovalbumin.

2.6.2. Fusing, screening, and cloning of hybridoma cells

The hybridoma cells were acquired by fusion of the spleen cells isolated from the immunized mouse and the mouse myeloma cells SP2/0 by PEG 2000 (Merk, Darmstadt, Germany) as the regular process described by Zeng et al. (2005). The antibody titer of the hybridoma culture

supernatants were measured by noncompetitive ELISA and confirmed by competitive ELISA. Those positive wells that exhibited both positive competition with its β-conglycinin and negative competition with analogues (i.e., glycinin) and related substances (i.e., trypsin inhibitor and agglutinin) were submitted to further subclone. The hybridoma cell line showed continuously positive signal in three subcloning was considered positive hybridoma cloning.

Large amount of monoclonal antibody was produced in vitro similar to the method of Beier et al. (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).

2.6.3. Characterization of monoclonal antibodies

2.6.3.1. Isotype and affinity. The isotypes of the purified monoclonal antibody were identified by a commercially available immunopure monoclonal antibody isotyping kit I (Pierce, Rockford, IL, USA). Relative affinity of the monoclonal antibody for β-conglycinin was measured by determining the 50% inhibition of control values (IC_{50}). The affinity constant (K_a) was calculated using the reported method (Beatty, Beatty, & Vlahos, 1987).

2.6.3.2. Specificity of antibody. Soybean β-conglycinin, analogue (i.e., soybean glycinin) and related substances (i.e., synthetic peptide, 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. Cross-reactivity was expressed as the concentration of β-conglycinin analogues or related substances required to produce 50% inhibition of antibody binding compared with β-conglycinin.

2.6.3.3. SDS-PAGE and Western blotting. In order to assess the specificity of the monoclonal antibody to β-conglycinin, the Western blot transfer technique, as described below, was performed. Soybean β-conglycinin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a minigel apparatus (BioRad Laboratories, Hertfordshire, UK) with 12.5% acrylamide separating gel and 4.5% acrylamide stacking gel according to the method of Laemmli (1970). The samples were dissolved in 1 M Tris–HCl buffer (containing 2.7 M glycerol, 0.15 M SDS and 0.15 mM bromophenol blue, pH 6.8). The sample load was 2 μg (protein) per well. Electrophoresis was performed at 100 V and 40 mA for 2.5 h.

The separated protein was transferred electrophoretically from gel to a 0.45 μm Nitrocellulose Membrane (Millipore, Bedford, MA, USA) in a mini Trans-Blot Cell Apparatus (BioRad) using the method of Nishizawa et al. (2003) with slight modification as follows. Wet blotting was performed at 360 mA for 60 min. The blots were probed with 0.1 μg/mL of primary antibody (monoclonal antibody). The goat anti-mouse IgG-HRP was used as the second antibody and KC™ General Western Blot Detection Kit (Roche Diagnostics Ltd., Lewes, UK) was used to detect the bound antibody.

2.7. Establishment of inhibition ELISA calibration curve for β-conglycinin

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

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

$OD_{\beta-conglycinin}$, absorbance measured by inhibition assay of a sample, in which the monoclonal antibody was pre-incubated with β-conglycinin; $OD_{control}$, 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 was pre-incubated with dilution buffer.

The tests for cross-reactivity of monoclonal antibody with β-conglycinin analogue (i.e., glycinin) and its related substances (i.e., trypsin inhibitor and agglutinin) were performed to evaluate the developed ELISA. The procedures were identical with that of cross-reaction analysis on antibody specificity in Section 2.6.3.

2.8. Recovery analysis of β-conglycinin spiked in soybean products by competitive ELISA

2.8.1. Preparation of samples and extraction of total soybean proteins

Soybean seeds and some soybean products (i.e., roasted full-fat soybean, extracted soybean meal, extruded soybean meal, fermented soybean paste, and soybean protein concentrate) were used in this study for evaluation of β-conglycinin recovery by competitive EILSA. Soybean seeds, roasted full-fat soybean, extracted soybean meal, extruded soybean meal, and fermented soybean paste were grounded with a cyclotec sample mill (Model 1093, Tecator Inc., Herndon, VA, USA) to pass through a 60-mesh sieve. The prepared flour were spiked with pure β-conglycinin at 80, 120, and 160 μg/g for soybean seeds, 20, 40, and 60 μg/g for roasted full-fat soybean, 10, 20, and 40 μg/g for both extracted soybean meal and extruded soybean meal, 5, 10, and 20 μg/g for fermented soybean paste, 1, 5, and 10 μg/g 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 liter buffer). Soybean seeds and roasted full-fat soybean were defatted by the use of n-Hexane before they entered the extraction process. Extracts

were centrifuged at 12,000*g* for 20 min at 4 °C using an Optima™ L-80 XP Ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatants were filtered through a 0.45 μm Millex GP filter (Millipore, Cork, Ireland), and then stored at −20 °C until analysis.

2.8.2. Detection of β-conglycinin

Each sample solution was diluted at its proper concentration before determination. Their pH was adjusted to 7.6 with 2 M HCL. The content of β-conglycinin from both soybean products and soybean products spiked with β-conglycinin were analyzed by the developed competitive ELISA.

2.9. Statistical analysis

All data were the average values from more than three replicate determinations. The results were expressed as mean values with a standard deviation (SD). The means procedure in SPSS 11.0 for windows (SPSS Inc., Chicago, USA) was used to analyze standard deviation.

3. Results and discussion

3.1. Preparation of hapten

As the peptide "RPQHPER" corresponding to the aminoacid residues 78–84 present in the α′ subunit of β-conglycinin is a nonimmunogenic small molecule by itself and lacking a functional group for coupling to proteins, the synthesis of haptens resembling the structure and electronic distribution of peptide itself as much as possible is a necessary and critical step in the preparation of high-specificity antibodies. Peptide fragments shorter than 10 residues seldom elicit protein-reactive antibodies (Chersi, Modugno, & Rosano, 1997) due to the possible reason: as the longer the peptide, the greater the probability that it contains the right number of aminoacids forming a β-turn (Krchknak, Mach, & Maly, 1987). Whereas long peptides (more than 15 aminoacid residues) tend to be less soluble, more difficult to synthesize, and more likely to assume structures unrelated to the native protein (van Regenmortel, Briand, Muller, & Plaue, 1988). In this study, the peptide "RPQHPER" was considered short and therefore the C- and N-terminal sequences corresponding to the native protein were extended and a nine-peptide "PRPQHPERE" was synthesized. The molecular mass of the synthetic peptide was approximately 1145 Da by mass spectrometry analysis with a purity of 97.8% by Reverse-phase HPLC analysis.

3.2. Production and characterization of monoclonal antibodies against β-conglycinin

To evaluate the suitability of the synthesized hapten to raise anti-β-conglycinin antibodies, five BALB/c mice were immunized with peptide-ovalbumin. One week following the cell fusion, growing hybridomas were observed in most of the culture wells. Supernatants of all wells were determined for anti-β-conglycinin activity using plates pre-coated with peptide-bovine serum albumin in ELISA. The hybridoma cultures from 20 wells showing high anti-β-conglycinin activity were selected for further investigation. The supernatants were titrated on plates pre-coated by peptide-bovine serum albumin and measured for competition against β-conglycinin, its analogue (glycinin) and related substances (i.e., trypsin inhibitor and agglutinin). Three hybridoma cell lines that exhibited positive competition with β-conglycinin but negative to glycinin, trypsin inhibitor and agglutinin were selected to be further subcloned. After diluting and determining three times, these monoclonal cultures and their corresponding Mabs were selected and named as $6G_4$, $3B_7$, and $5F_{10}$.

The isotypes of the three generated monoclonal antibodies, $6G_4$, $3B_7$, and $5F_{10}$, were found to be IgG_1 with *k* light chain which differed from the monoclonal antibody IgG_{2b} prepared using soybean β-conglycinin as the immunogen (Plumb et al., 1995). The monoclonal antibodies $6G_4$, $3B_7$, and $5F_{10}$ were evaluated for their specificity to β-conglycinin. The inhibition result for β-conglycinin with each monoclonal antibody was selected to be a 100% cross-reactivity value for that monoclonal antibody since β-conglycinin was the target substance that the monoclonal antibodies were detecting. Cross-reactivity of the monoclonal antibodies with other inhibitors was expressed as the concentration of inhibitors required to produce 50% inhibition of antibody binding compared with β-conglycinin. As shown in Table 1, the monoclonal antibodies $6G_4$ was most specific to β-conglycinin and showed no significant cross-reaction with β-conglycinin analogue (i.e., glycinin) and its related substances (i.e., trypsin inhibitor and agglutinin).

In addition, the specific binding of the monoclonal antibodies to β-conglycinin were also assessed by Western blotting. Fig. 3 demonstrated that all three monoclonal antibodies could recognize the α′ and α subunits of natural soybean β-conglycinin molecule. This may be due to the high homology (90.4%) in the core regions of the α′ and α subunits (Maruyama et al., 1998). This result indicated that the prepared monoclonal antibodies (IgG_1) were able to recognize the principal β-conglycinin molecule and could be employed for quantifying β-conglycinin.

3.3. Development of the competitive ELISA based on monoclonal antibody against β-conglycinin

The monoclonal antibody $6G_4$ with both strongest specificity and appropriate affinity was chosen to establish a calibration curve for β-conglycinin in competitive ELISA. As shown in Fig. 4, the concentration of β-conglycinin which inhibited 50% total binding of monoclonal antibody $6G_4$ (IC_{50}) was 4.7 ± 0.8 ng/mL, while the minimum detection limit which inhibited 20% total binding of monoclonal antibody $6G_4$ (IC_{20}) was 2.0 ± 0.6 ng/mL. The working ranges giving a 20–80% total binding of monoclonal anti-

Table 1
Cross-reactivity for various inhibitors (β-conglycinin and its analogues or related substances) in the Mabs disolution[a]

Inhibitors	Max concentration (ng/mL)	IC_{50}[b], mean ± SD (ng/mL) (cross-reactivity[c], %)		
		$6G_4$	$3B_7$	$5F_{10}$
β-Conglycinin	250	4.7 ± 0.8 (100)	7.1 ± 1.5 (100)	6.5 ± 1.7 (100)
Glycinin	5.0×10^5	6731.1 ± 1001.4 (<0.1)	5589.3 ± 1104.7 (0.1)	6212.6 ± 1423.8 (0.1)
Peptide (hapten)	1000	16.7 ± 3.1 (28.1)	27.3 ± 4.4 (26.0)	19.9 ± 3.9 (32.7)
Trypsin inhibitor	$>5.0 \times 10^6$	0[d]	0	0
Agglutinin	$>5.0 \times 10^6$	0	0	0

[a] The concentration of plate-coating antigen (peptide-BSA) and Mabs used for cELISA were 5.0 μg/mL and 25 ng/mL, respectively.
[b] The concentration of inhibitors required to produce 50% inhibition of antibody binding is represented by IC_{50}. Each IC_{50} value represents the mean from 11 replicates ($n = 11$).
[c] Cross-reactivity (CR) of inhibitors is expressed as a percentage as the IC_{50} value compared with β-conglycinin. It is calculated according to the formula: CR (%) = (IC_{50} of β-conglycinin)/(IC_{50} of certain inhibitor) × 100.
[d] Values of <0.01 are shown as 0.

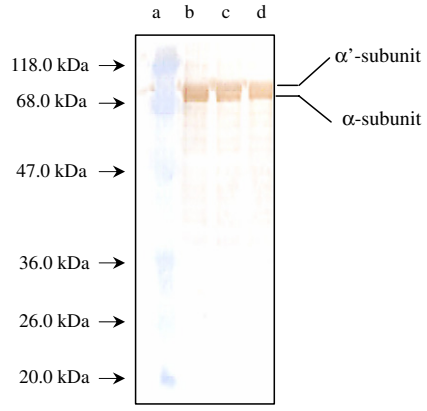

Fig. 3. Western blotting of Mabs with soybean β-conglycinin. (a) Pre-stained protein molecular weight marker (118.0 kDa, β-galactosidase from *E. coli*; 68.0 kDa, bovine serum albumin; 47.0, ovalbumin from chicken egg white; 36.0 kDa, lactate dehydrogenase from porcine muscle; 26.0 kDa, restriction edonuclease B sp 98I from *E. coli*; 20.0 kDa, β-lactoglobulin from bovine milk); (b) Mab $6G_4$; (c) Mab $3B_7$ and (d) Mab $5F_{10}$.

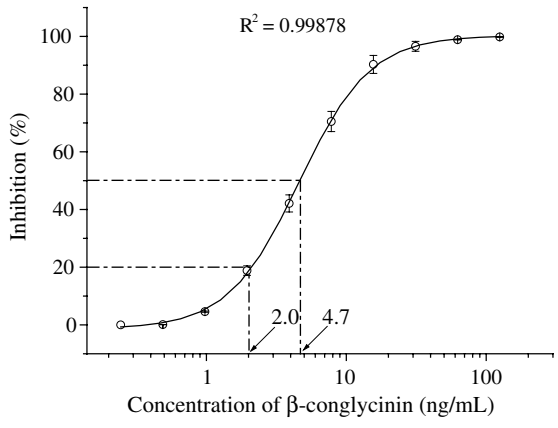

Fig. 4. Standard curve for β-conglycinin obtained with Mab $6G_4$ in peptide-BSA-coated format. Each point represents the mean ± standard deviation from eight determinations (○, $n = 8$) in cELISA. Each data point was calculated from the absorbance at 492 nm by use of the inhibition formula as described in Section 2. The optimal concentration of plate-coating antigen (peptide-BSA) and Mab $6G_4$ were 5.0 μg/mL and 25 ng/mL, respectively.

body $6G_4$ were 2.0–11.5 ng/mL. The tests of cross-reaction indicated that the cross-reactivity of monoclonal antibody $6G_4$ was lower than 0.5% with glycinin and not more than 0.05% with trypsin inhibitor and agglutinin (data not shown). These results showed that the monoclonal antibody $6G_4$ exhibited high sensitivity and specificity for the detection of β-conglycinin, which satisfied the pre-requisite of a specific antibody for a successful immunochemical assay.

3.4. Practical evaluation of the monoclonal antibody $6G_4$-based competitive ELISA for detection of β-conglycinin

In order to evaluate the validation and reliability of the monoclonal antibody $6G_4$-based competitive ELISA, the recovery tests and validation studies were performed for the determination of soybean and soybean products. Table 2 summarized the results of analyzing the content of β-conglycinin from some soybean samples. The average recoveries at three spiked levels were 100.4% (varying from 96.1% to 103.8%) for soybean seeds, 98.0% (varying from 94.4% to 102.2%) for roasted full-fat soybean, 100.2% (varying from 95.4% to 105.6%) for extracted soybean meal, 96.0% (varying from 89.4% to 102.5%) for extruded soybean meal, 97.7% (varying from 93.7% to 103.0%) for fermented soybean paste, 101.5% (varying from 91.9% to 109.5%) for soybean protein concentrate. These data indicated that the recoveries (average 89.4–109.5%) of β-conglycinin spiked in soybean samples were within an acceptable range with deviations from a mean also within an acceptable range.

The relative standard deviation (RSD) ranged from 5.1% to 9.9% for soybean seeds, 6.4% to 8.2% for roasted full-fat soybean, 4.5% to 9.0% for extracted soybean meal, 5.9% to 10.8% for extruded soybean meal, 5.5% to 8.3% for fermented soybean paste, 3.6% to 5.1% for soybean protein concentrate. The lower the spiked level, the larger the percent RSD for most samples. However, the largest RSD for soybean samples was no more than 11.0%. It can be concluded that the use of monoclonal antibody $6G_4$ in an optimized competitive ELISA format to analyze various

Table 2
Recovery analysis of β-conglycinin spiked in soybean products by competitive ELISA based on the monoclonal antibody $6G_4$

Samples	Mean[g] ± SD (μg/g)	Spiked level (μg/g)	Mean[h] ± SD (μg/g)	Recovery (%)	RSD (%)
Soybean seed[a]	131.2 ± 24.8	80.0	219.2 ± 21.7	103.8	9.9
		120.0	254.7 ± 21.9	101.4	8.6
		160.0	279.8 ± 14.3	96.1	5.1
Roasted full-fat soybean[b]	40.3 ± 6.2	20.0	58.7 ± 4.8	97.3	8.2
		40.0	75.8 ± 5.2	94.4	6.9
		60.0	102.5 ± 6.6	102.2	6.4
Extracted soybean meal[c]	36.1 ± 5.1	10.0	48.7 ± 4.4	105.6	9.0
		20.0	53.5 ± 3.3	95.4	6.1
		40.0	75.7 ± 3.4	99.5	4.5
Extruded soybean meal[d]	14.5 ± 3.2	10.0	25.1 ± 2.7	102.5	10.8
		20.0	33.2 ± 2.8	96.1	8.4
		40.0	48.7 ± 2.9	89.4	5.9
Fermented soybean paste[e]	8.4 ± 1.7	5.0	13.8 ± 1.1	103.0	8.3
		10.0	17.7 ± 1.5	96.0	8.2
		20.0	26.6 ± 1.5	93.7	5.5
Soybean protein concentrate[f]	0.0047 ± 0.0021	1.0	1.1 ± 0.06	109.5	5.1
		5.0	4.6 ± 0.2	91.9	4.7
		10.0	10.9 ± 0.4	103.0	3.6

[a] Jinong-09 variety obtained from Jilin Academy of Agriculture Science, Changchun, China, which contains 43.1% crude protein (analyzed value).
[b] Roasted for 15 min at 125 °C using a rotary drum dryer.
[c] A commercial solvent-extracted soybean meal (SBM) containing 44.7% crude protein (analyzed value).
[d] A commercial dehulled and extruded soybean meal containing 46.5% crude protein (analyzed value).
[e] A commercial microbes fermented soybean meal containing 50.4% crude protein (analyzed value).
[f] A commercial product containing 69.2% crude protein (analyzed value).
[g] Data represent means of seven replicate samples (only soybean products).
[h] Data represent means of three 5-replicate subsamples, which were spiked with pure β-conglycinin.

concentrations of β-conglycinin in soybean and soybean products demonstrated reliable reproducibility.

We also found that the immunoreactive β-conglycinin in unprocessed soybean seeds showed a highest content at 131.2 μg/g, while the immunoreactive β-conglycinin 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. The reason may be due to: the treatments such as heating, expanding, and aqueous alcohol extracting results in damage of the most β-conglycinin in soybean products. Especially for soybean protein concentrate, some soluble carbohydrates and antigenic factors were leached selectively and removed by the processing steps: cracked, dehulled, steam conditioned, flaked, and then aqueous alcohol extracted. In previous reports, the β-conglycinin content as total soybean proteins were 16.8–20.1% by the use of antiserum assay (Murphy & Resurreccion, 1984) and 21.8–35.1% (7S, mainly β-conglycinin) by the use of RP-HPLC (Mujoo et al., 2003), respectively. These data indicated that the β-conglycinin content as total soybean were about within 67.2–80.4 mg/g and 87.2–140.4 mg/g, respectively (DM basis, 12% moisture, the total protein content in soybean was assumed to be 40.0%). Our data for β-conglycinin content as soybean seeds are compatible with that obtained from RP-HPLC, but higher than that obtained from antiserum assay. As food allergen usually comprising of many potentially immunoreactive proteins, within a food allergen system, individual allergens also being affected differently by various processing methods such as thermal treatment, enzymatic hydrolysis, and chemical modification, it is difficult to anticipate the allergenicity or immunoreactive concentration of allergen. For instance, *Ara h1*, a major peanut allergen, was found to be stable and its IgE-bond concentration was unaffected by heating up to 140 °C for 15 min, although native *Ara h1* underwent a significant heat-induced denaturation on a molecular level (Koppelman, Bruijnzeel-Koomen, Hessing, & de Jonghi, 1999). Soy β-conglycinin showed similar characteristic to *Ara h1* when was heated up to 100 °C for 10 min (Huang et al., 1998). However, soy glycinin that heated at 90 °C for 10 min could be recognized more strongly by its specific antibody than native protein, but prolonged heating could cause decrease of its allergenicity (Huang et al., 1998). Therefore, the identity and processing history of the food allergen decide the allergenic potential. Moreover, the sensitization of determination techniques also affects the allergenicity or immunoreactive concentration of an allergen.

In previous study, the majority of methods developed to detect soybeans in food products are based on immunochemical assays (Boutten, Humbert, Chelbi, Durand, & Peyraud, 1999; Brandon, Bates, & Friedman, 1991; Hitchcock, Bailey, Crimes, Dean, & Davis, 1981; Yasumuto,

Sudo, & Suzuki, 1990). However, these methods have been considered to be lacking in sensitivity (Poms et al., 2004). Recently, three ELISA kits for soybean were developed and showed to be available commercially. The Tepnel BioSystems kit (Elisa Technologies, FL, USA) is used to detect renatured soy proteins, which is based on a competitive ELISA with a detection limit of <5000 ng/mg of food product. The second kit, named as ElisaSystems kit (ELISA Systems, Windsor, Australia), is based on a sandwich ELISA that aims at the soy Kunitz trypsin inhibitor in food product with a sensitivity of 1 ng/mg. The Veratrox for Soy Flour Allergen (Neogen Corp., MI, USA) with a detection limit in the range of 2.5–25 ng/mg is the third kit used for quantification of lightly processed soy flour protein in food products (cookies, crackers, and cereals). Other methods of detection of allergens such as immunohistochemical technique associated with image analysis and polymerase chain reaction (PCR) based on the amplification of specific DNA fragment have also been developed recently (L'Hocline & Boye, 2007). Comparing to the methods above, our technique for soy β-conglycinin showed higher sensitivity than the Tepnel BioSystems kit, similar efficiency with the ElisaSystems kit and Veratrox for Soy Flour Allergen, and simpler handling and lower cost of equipment than immunohistochemical and PCR techniques. However, it is still in the early stages of development. The practical evaluation of our method is only based on specific single foods rather than mixed products. Because the food allergens are usually complex mixtures of many potentially immunoreactive proteins, individual allergens within a food allergen system will also be affected differently by various processing methods. Therefore, it is necessary to prepare competitive ELISA test kit for β-conglycinin using the developed monoclonal antibody $6G_4$, and then perform the complete validation in our future work.

4. Conclusions

The goal of obtaining a high-affinity monoclonal antibody against β-conglycinin was achieved from mice immunized with a designed peptide-OVA conjugate. The developed competitive ELISA based on the monoclonal antibody $6G_4$ exhibited a low detection limit at 2.0 ng/mL of β-conglycinin. Therefore, the present study broadens the opportunity for sensitive detection of β-conglycinin from soybean and soybean products.

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