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Development of Immunoaffinity Chromatographic Method for Isolating Glycinin (11S) from Soybean Proteins

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ABSTRACT: A monoclonal antibody (Mab), 4B2, against soybean glycinin was prepared using the preliminary extracted natural glycinin as the immunogen in our previous study. Herein, we established a novel method for the purification of glycinin by Mab 4B2-based immunoaffinity chromatography. The characteristics of the purified glycinin were identified by SDS-PAGE, Western blot, and histamine release assay. Glycinin was successfully isolated from soybeans with a yield of 16.8% and a purity of 93.8%, which were significantly higher than those produced using other traditional procedures. The acidic polypeptides of the purified glycinin can be recognized by the Mab 4B2, but not the basic polypeptides. In addition, the histamine release ratio of the purified glycinin was similar to that of natural glycinin, which indicated that the purified glycinin maintained its biological activities. Further study revealed that the Mab/gel ratios ranging from 6.0 to 12.0 mg/mL were suitable for the isolation of glycinin using immunoaffinity chromatography. Taken together, this new method based on immunoaffinity chromatography could be used for high-yield and high-purity natural glycinin production and would facilitate future study on the mechanism of soybean-induced food allergy.

KEYWORDS: *glycinin, monoclonal antibody, immunoaffinity chromatography, purification, soybean*

INTRODUCTION

Soybean and its byproducts are one of the most important sources of dietary protein for both humans and animals.[1,2] It provides high-quality proteins with a well-balanced supply of amino acids. However, soybean protein can also be a source of dietary allergens for human infants and young animals such as piglets and calves, causing intestinal anaphylactic reactions and diarrhea.[3,4]

Accumulated studies have shown that there are at least 21 allergenic proteins in soybean.[5] Among them, glycinin is one of the important soybean allergens, with a molecular mass of 300−380 kDa, and accounts for about 19.5−23.1% of the total soybean proteins.[6] Glycinin is composed of acidic and basic polypeptides connected to each other. The acidic (A1a, A1b, A2, A3, A4, A5) and basic (B1a, B1b, B2, B3, B4) polypeptides are linked by a disulfide bond, with molecular weights of 34−44 and 20 kDa, respectively.[7] It has been reported that the acidic chain of the G1 subunit has an immunoglobulin E-binding region,[8] which had been validated to increase serum glycinin-specific immunoglobulin E (IgE) antibody and induce anaphylaxis. [9]

However, there have been limited studies on glycinin-induced anaphylaxis, due to the difficulty of separating glycinin from other soybean proteins. In those cases, soybeans or preliminary separated glycinin, but not highly purified glycinin, has been used as the experimental material.[9,10] Although there are many attempts including ammonium sulfate precipitation, pH adjustment isolation, ultrafiltration membrane separation, and phytase treatment in the isolation of glycinin from soybean proteins, it is difficult to obtain enough pure glycinin for research purposes.[11−14]

Monoclonal antibodies (Mabs) are powerful tools for recognizing antigens and identifying new cell surface molecules,[15] which can be used to prepare Mab-mediated immunoaffinity columns[16] and develop a new approach for purification of glycinin.

In our previous study, monoclonal antibodies against glycinin were prepared using purified glycinin as the immunogen. The generated Mabs, named 4B2, were identified as being IgG2a isotypes and exhibited high specificity to glycinin.[17] Herein, we developed a novel method for the purification of glycinin by immunoaffinity chromatography based on Mab 4B2.

MATERIALS AND METHODS

Reagents. A commercial Protein G affinity column and CNBr-activated Sepharose 4B medium were provided by Amersham Biosciences (Uppsala, Sweden). The horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG-HRP) was purchased from ZSGB-Bio Company (Beijing, China). The BCA protein assay kit was obtained from Pierce (Rockford, IL, USA). Nitrocellulose membranes (Hybond, ECL) were provided by Amersham Pharmacia (London, UK).

Preliminary Extraction of Glycinin from Soybean. Glycinin was preliminarily extracted from soybean according to the previous reports.[18,19] Briefly, soybean seed, obtained from the Chinese Academy of Agricultural Sciences (Beijing, China), was milled using a Cyclotec sample mill (model 1093, Tecator Inc., Herndon, VA, USA). The soybean powder was defatted with *n*-hexane and was suspended in 0.03 M Tris-HCl buffer. The soybean proteins were extracted in the turbid liquid. The total extract was centrifuged at 12000*g* for 20 min at 4 °C using an Optima L-80 XP Ultracentrifuge (Beckman Coulter, CA, USA). The supernatant was filtered through a 0.45 μm Millex GP filter (Millipore, Cork, Ireland). Then 0.025 M

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$MgCl_2$ was adding into the supernatant (adjusting the pH to 4.5 by HCl).[20,21] The precipitate was collected for the immunoaffinity purification.

Production and Purification of Antibodies against Soybean Glycinin. A stable murine-derived monoclonal antibody against the soybean glycinin hybridoma cell line, named 4B2, was obtained and identified as being an IgG2a isotype, which exhibited a high specific binding to the acidic chains of glycinin.[17]

To prepare the glycinin-specific immunoaffinity column, a large amount of monoclonal antibodies was produced in vivo according to the procedures of You et al.[16] Briefly, BLAB/c mice were pretreated 7 days before with 0.5 mL of mineral oil and then were injected intraperitoneally with the hybridoma cells from 4B2 (2×10^6 cells/mouse). Two weeks later, the ascitic fluids (about 1.5−2 mL/mouse) inoculated by hybridoma cells from mice were collected and centrifuged for 15 min at 10000*g*. The ascite supernatant was mixed with an equal volume of 0.04 M barbital buffer (pH 7.0). SiO_2 powder was added into the ascites dilution (0.05 g/mL), and the solution was gently stirred for 30 min and then centrifuged for 20 min at 2000*g*. The supernatant was collected and added with ammonium sulfate to primarily isolate Mab 4B2.

Preparation of the Immunoaffinity Column. The CNBr-activated Sepharose 4B medium was prepared following the manufacturer's instructions. Briefly, 2.0 g of CNBr-activated Sepharose 4B agarose gel freeze-dried powder was dissolved in 1 mM HCl (pH 2.5). Following swelling, the total medium was washed for 15 min in 1 mM HCl and filtered with sintered glass to remove protective additives. In addition, 60 mg of monoclonal antibody 4B2 (IgG2a) was dissolved in 8 mL of coupling buffer (containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) and then mixed with Sepharose 4B agarose gel in a 10 mL tube (with a stopper). The mixture was gently shaken for 1 h at room temperature in an orbital shaker and then purged with six volumes of coupling buffer to remove the superfluous monoclonal antibody. Aminoethyl alcohol buffer (1 M, pH 8.0) was added for 2 h to protect the active epitopes of the monoclonal antibody. The mixed gel was then washed alternately with buffer (containing 0.1 M acetate and 0.5 M NaCl, pH 4.0) and 0.1 M Tris-HCl buffer (pH 8.0), with each buffer used at least three times.[16]

The prepared agarose gel (coupled with monoclonal antibody 4B2) was gently loaded into a chromatographic column (10 × 100 mm), filled with 20 mM PBS (pH 7.0) and precooled at 4 °C for 2 h. After loading the gel, the remaining space in the column was immediately filled with buffer, and the top of the column was linked with the pump, which was working at a flow rate of 1.5 mL/min. The elution was maintained for 10 bed volumes after a constant bed height was reached.[16]

Immunoaffinity Chromatography of Glycinin. The preliminarily extracted glycinin was loaded onto the immunoaffinity column. The outlet tubing of the column was connected to an HD21-1 detector and a protein autocollector (Huxi Instrument Company, Shanghai, China). The flow rate was maintained at 1.5 mL/min. The media was washed with 20 mM PBS (pH 7.0) until the baseline was stable. Glycine-HCl elution buffer (0.1 M, pH 2.7) was used to elute the sample. The glycinin solution was collected when the protein with the highest peak reached the detector of the protein autocollector. The pH value of the glycinin solution was regulated immediately to neutral with 100 μL of 1 M Tris-HCl (pH 9.0).

Identification of Glycinin by SDS-PAGE and Western Blot. SDS-PAGE and Western blot analysis were performed in order to assess the purity and the specificity of glycinin. The protein solution purified by the immunoaffinity column was measured using a BCA protein assay kit using bovine serum albumin as the standard. Two copies of identical samples (30 μg of total protein per well) were separated with 12.5% acrylamide separating gel according to standard procedures using a Minigel Apparatus (BioRad Laboratories, Hertfordshire, CA, USA). One copy was stained with Coomassie Brilliant Blue R250 for 30 min, washed in washing buffer (45% methanol, 10% acetic acid, 45% H_2O, v/v) for 30 min, and analyzed.

The remaining copy was transferred electrophoretically to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 3% bovine serum albumin for 2 h at room temperature. The membranes were then incubated with 0.1 μg/mL of the corresponding 4B2 monoclonal antibody for 2 h at 37 °C. The unbound primary antibody was removed by washing with TBS-T. The membranes were incubated with secondary antibody (the horseradish peroxidase-conjugated goat anti-mouse IgG) in the dark for 1 h at room temperature. Following another three washes with TBS-T, the membranes were treated with 3,3′-diaminobenzidine and hydrogen peroxide to show the specific protein bands.

Evaluation of Chromatography Efficiency. It is reported that the ligand (Mab)/gel ratio can affect the chromatography efficiency.[16] In order to optimize the chromatography efficiency, five concentrations of Mab, 3.0, 6.0, 9.0, 12.0, and 15.0 mg/mL gel, were used to prepare corresponding immunoaffinity columns with the same size. Under identical conditions (including soybean protein extraction, isolation procedures, and so on), the yield and purity of glycinin were recorded to evaluate the efficiency of the purification.

Animals and Sensitization Protocol. All animals used in this experiment were maintained according to the guidelines of the China Agricultural University Animal Care and Use Ethics Committee. The rats were sensitized by means of subcutaneous injection with 0.5 mg of glycinin according to the previous report.[9] On the last day, all rats were sacrificed. The blood was collected, the sensitized serum was isolated, and a sample of jejunum was quickly obtained from the control rats for histamine release analysis.

Measurement of Histamine Release Ratio. Intestinal mast cells were isolated from the sample of jejunum, and the histamine release testing was conducted as previously described.[9] Briefly, purified mast cells were resuspended in complete Hank's balanced salt solution (1×10^6 cell/mL) and incubated in medium that was supplemented with the sensitized serum for 2 h. Then, both soybean protein and immunoaffinity-purified glycinin were added to the suspension (final concentration of 50 μg/mL) and cultured for another 1 h, respectively. The histamine content of both the cell pellet and the supernatant was measured. The histamine release ratio (%) was calculated as

$$\{\text{supernatant histamine}/(\text{supernatant histamine} + \text{cell pellet histamine})\} \times 100$$

Statistical Analysis. The concentrations and purities of isolated glycinin were reported as mean ± SEM and analyzed using the analysis of variance (ANOVA) procedure of the SAS system (version 8.2, SAS Institute, NC, USA). A p value less than 0.05 was considered statistically significant.

RESULTS

Characterization of Immunoaffinity Purified Glycinin. As shown in Figure 1, on the left is an SDS-PAGE profile of glycinin purified from preliminarily extracting glycinin, and on the right is a gray density profile. Lane B represents total preliminarily extracted glycinin before purification, lane C represents glycinin purified from total proteins, and lane D is the negative control. The purified glycinin fraction contained mainly acidic (34−44 kDa) and basic (about 20 kDa) chains of glycinin. Lane E is a gray density profile obtained from track c of electrophoresis. Direct observation clearly indicated that glycinin was successfully isolated from soybean proteins. A Mab-based competitive ELISA method was used to further test its content.[17] The purity of glycinin was 93.8%.

Specific Binding Ability of Purified Glycinin to Mab. Bioactivity to the natural glycinin (soybean proteins before purification) was an important index to evaluate the feasibility of the immunoaffinity column, which was assessed by the specific binding of glycinin to Mab using Western blot assay. As shown in Figure 2, the Mab 4B2 could recognize the acidic chains of the glycinin molecule. The result indicated that the

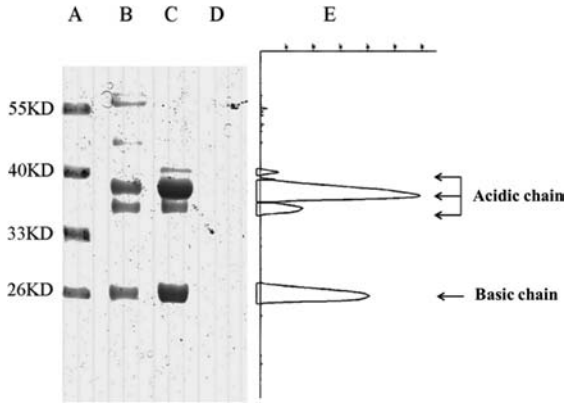

Figure 1. SDS-PAGE profile (left) and gray density profile (right) of glycinin purified by monoclonal antibody 4B2 based immunoaffinity chromatography. Proteins were separated by a 12.5% SDS-PAGE and stained with Coomassie Blue. Lane A: protein molecular weight markers; B: total array of preliminarily extracted glycinin before purification; C: glycinin separated from preliminarily extracted glycinin using the immunoaffinity column; D: blank control lane; E: gray density profile obtained from track c of electrophoresis using Syngene Analysis Software (version 3.03.03).

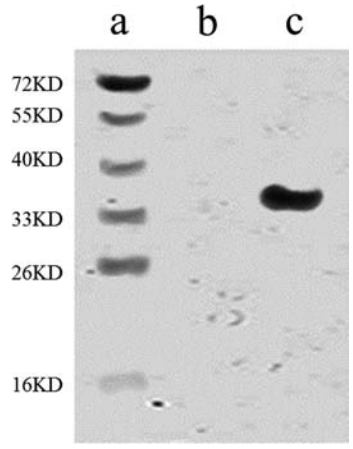

Figure 2. Western blot of glycinin purified by monoclonal antibody 4B2 based immunoaffinity chromatography. The blots were probed with primary antibody (monoclonal antibody 4B2). The goat anti-mouse IgG-HRP was used as the second antibody, and 3,3′-diaminobenzidine was used to detect the bound antibody. Lane a: protein molecular weight markers; b: negative control (dissolved buffer); c: glycinin purified by the immunoaffinity column.

purified glycinin maintained the Mab 4B2-specific epitopes and associated natural bioactivity of the protein molecule after immunoaffinity chromatography.

Histamine Release Ratio of Purified Glycinin. Compared with the negative control, the soybean protein (containing natural glycinin) significantly increased ($p < 0.05$) the release of histamine from mast cells, as indicated by a higher release ratio (Figure 3). The histamine release ratio for the immunoaffinity-purified glycinin did not differ from that of the natural glycinin group, which indicated that the purified glycinin maintained the allergenic bioactivity of the protein molecule.

Effects of Mab/Gel Ratio on the Efficiency of Immunoaffinity Chromatography. It is reported that the coupling ratio of Mab and gel is one major factor impacting the isolation efficiency during the preparation of the immunoaffinity column.[16] Herein, the effect of Mab/gel ratio on isolation efficiency was assessed. As shown in Table 1, different concentrations of Mab induced marked changes in yield and purity of isolated glycinin. When the concentration of Mab coupled in Sepharose 4B gel was 6.0, 9.0, and 12.0 mg/mL, the glycinin yields were significantly higher than those in the 3.0 and 15.0 mg/mL treatments ($p < 0.05$). The difference in the yield was not significant within 6.0, 9.0, and 12.0 mg/mL treatments ($p > 0.05$, 11.1−16.8 g/kg soybean). On the other hand, no significant difference in terms of purity of isolated glycinin was observed when the ratio of Mab coupled in Sepharose gel ranged from 6.0 to 15.0 mg/mL, whereas the purity in the 3.0 mg/mL treatments was statistically inferior to the other treatments ($p < 0.05$).

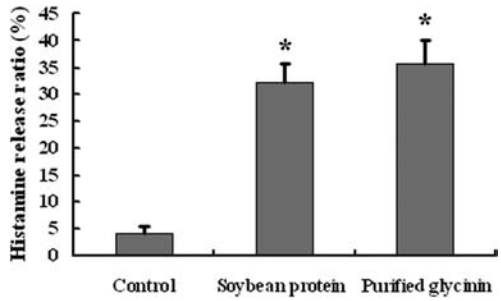

Figure 3. Detection of histamine release ratio of soybean protein and the purified glycinin. The rats were sensitized by glycinin. Intestinal mast cells were isolated from the sample of jejunum and incubated with the sensitized serum. Both soybean protein and immunoaffinity purified glycinin were added to the suspension (final concentration of 50 μg/mL) and cultured for another 1 h, respectively, with casein as the control. Asterisk represents $p < 0.05$ when compared with the control.

In addition, the immunoaffinity chromatographic column had been reused four times. The purity of 93.8% and yield of 16.8% were obtained for the first time, and the purity of 91.4% and yield of 9.6% for the fourth time. The results indicated that the immobilized monoclonal antibody column was capable of multiple purification processes for economic purposes, with constant purity and decreased yield.

DISCUSSION

In the study, we developed a novel method for isolating glycinin from soybean by using immunoaffinity chromatography based on the monoclonal antibody 4B2, which was prepared in our lab. The monoclonal antibody 4B2 prepared using purified glycinin as the immunogen in soybean-free-diet mice exhibited higher specificity to glycinin than the antibodies reported before.[17] Therefore, the efficiency of our developed purification method is improved, such as higher purity and yield than before. According to the results from Western blot and histamine release ratio experiments, our developed method can maintain the bioactivity of natural glycinin better than other chemical isolating methods. Iwabuchi et al. (1987) indicated that some chemical or physical purification methods may cause denaturing of glycinin protein, since the antigenicity of glycinin is more conformation dependent than β-conglycinin.[29] Additionally, the study relatively simplified the purification steps and saved much time, at the same time guaranteeing a high purity and natural protein bioactivity.

In our previous study, Mabs against glycinin were prepared using natural soybean glycinin as the immunogen. The

Table 1. Effects of Monoclonal Antibody (Mab)/Gel Ratio on the Efficiency of Immunoaffinity Chromatography for the Isolation of Soybean Glycinin

	concentration of Mab coupled in Sepharose 4B gel (mg/mL)[a]				
	3.0	6.0	9.0	12.0	15.0
yield in soybean (g/kg)	11.1 ± 1.5 b	15.2 ± 1.2 a	16.8 ± 0.9 a	16.4 ± 1.1 a	12.3 ± 0.8 b
purity of isolated glycinin (%)	85.7 ± 1.3 b	93.8 ± 2.3 a	92.9 ± 2.0 a	92.2 ± 0.9 a	90.0 ± 1.6 a

[a]The purity of isolated glycinin is expressed as a percentage of the content of glycinin compared with the total glycinin protein. All data are means ± SEM (n = 5). Means within the same row that have no common superscript letters are significantly different ($p < 0.05$).

generated Mab 4B2 exhibited high specificity to glycinin.[17] Herein, an immunoaffinity chromatography method based on Mab 4B2 was developed, and glycinin was successfully isolated from soybean proteins. The yield (16.8%) of glycinin was significantly higher than the yields of 11.1, 12.1, 9.7, and 14.4% for differential scanning calorimtry (DSC) and dynamic viscoelastic measurements,[13] a pilot-plant scale membrane filtration system,[14] ultrafiltration membrane separation,[22] and ethanol extraction,[22] respectively. This improved yield is most likely due to the strong protein−antibody binding, which ensures that more protein binds with the immunoaffinity chromatography column.[16] The attachment is so firm that it prevents glycinin from being washed away by the sodium phosphate buffer. The more glycinin bound to the chromatography column, the less production waste.

Except for the yield, the purity (93.8%) from our modified Mabs-based immunoaffinity chromatography method was significantly higher than those produced using other traditional procedures (the purity of glycinin ranged from 62.6 to 80.8%).[13,14,22,23] This may be due to the following reasons. First, highly specific Mabs with moderate affinity in the immunochemical approach were used in our study. This binding ability between the high-quality Mabs and the target protein relies only on the specific recognition of the antibody with the antigenic determinant, which is completely unrelated to the extraction environment (pH, ethanol concentration, temperature, and water-to-flake ratio) and other properties.[23−25] After a long-term flush, it is ensured that the nonspecific binding proteins were washed out, which increased the purity of glycinin. Second, the convenient and rapid isolation procedure used in our study can protect the target protein from degrading and denaturing. It is well known that a complex and time-consuming isolation procedure has an increased probability of degradation or denaturation of the target protein.[26] The previous methods all took more than an hour for isolation.[13,14,22−26] In comparison, our purification procedure is very convenient, and the duration of a processing cycle is 5−10 min.[16] Third, CNBr-activated Sepharose 4B was selected as the isolating medium, which is stable under a wide range of pH and temperatures. The regeneration ability of the medium can also improve the efficiency of the extraction enormously.[16] Furthermore, Thanh et al. determined the purifying efficiency of glycinin, which was isolated in several different ways by an immunological method.[29] Some isolation methods, such as sodium dodecyl sulfate gel electrophoresis, denature the protein conformation of glycinin and synchronously change the antigenicity. Thus, our method avoids this protein denaturing.

As the structure and characteristics of glycinin have an important influence on its functional and nutritional properties,[27,28] the physicochemical characteristics and bioactivity of purified glycinin are also important indexes to evaluate the feasibility of the immunoaffinity column. Similar to our previous report,[17] the present study demonstrated that the Mab 4B2 could recognize the acidic chains of the purified glycinin molecule, mediated by the Mab 4B2-specific epitopes, suggesting that the isolated glycinin maintains the natural bioactivity of the integral protein molecule after immunoaffinity chromatography.[27] In addition, it is reported that the natural glycinin is sufficient to increase serum-specific IgE antibody and induce anaphylaxis.[9] Our data showed that similar to the natural glycinin in soybean proteins, the purified glycinin through immunoaffinity chromatography could increase the release of histamine from mast cells, as indicated by a higher release ratio, indicating that the purified glycinin maintained the allergenic bioactivity of the protein molecule.[9]

It is well documented that different concentrations of Mab coupled in Sepharose 4B gel can influence the yields and purities of protein.[16] Our data revealed that when the Mab/gel ratio ranged from 6.0 to 12.0 mg/mL, the yield and the purity of separated glycinin were much higher than those of the other concentrations of Mabs coupled in Sepharose 4B gel, which illuminated that either a low or high Mab/gel ratio inhibited the efficiency of glycinin isolation.

In conclusion, a Mab-based immunoaffinity chromatography has been developed with improved yields and purity, which provided a novel method for the isolation of glycinin. Under the optimized conditions, this technique can be used to obtain glycinin with high purity. The high-purity glycinin is useful for future studies to reveal how glycinin functions in anaphylaxis.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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