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Analytical Methods

A novel method using immuno-affinity chromatography for isolating β-conglycinin from soybean proteins

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ABSTRACT

A monoclonal antibody (Mab) $6G_4$ against soybean β-conglycinin has been prepared using a conjugate of chicken ovalbumin and a synthetic peptide that corresponded to one of the epitope sequences of β-conglycinin as the immunogen. An ELISA method for the quantification of β-conglycinin has also been developed. In the present study, we report a novel method for the purification of β-conglycinin by Mab $6G_4$-based immuno-affinity chromatography. β-Conglycinin with a purity of 92.9% was successfully isolated from soybean proteins. Western blot assay was used to further identify its characteristics and the results demonstrated that the purified β-conglycinin maintains its biological activities. Therefore, the Mab-based immuno-affinity chromatography is an available method for purification of β-conglycinin. It also provides a new opportunity for future study on the mechanism of food allergy responses using high purity β-conglycinin as the experimental material.

1. Introduction

Soybean is widely used as food ingredients because of its nutritional and functional properties (Astadi, Astuti, Santoso, & Nugraheni, 2008; Friedman & Brandon, 2001; Liu et al., 2008; Saito, Kohno, Tsumura, Kugimiya, & Kito, 2001). However, soybean is also considered as a major source of food allergies especially in industrialised countries (Guo, Piao, Cao, Ou, & Li, 2008; Herman, Helm, Jung, & Kinney, 2003; Huisman & Jansman, 1991). Soybean allergies are prevalently becoming "health hacker" in the world recently (Chandra, 2002; Song, Frias, Martinez-Villaluenga, Vidal-Valdeverde, & de Mejia, 2008; Zeiger, 2000).

As a major soybean allergen, β-conglycinin with α, α′ and β subunits plays important roles in hypersensitive responses for children (Xiang, Beardslee, Zeece, Marwell, & Sarath, 2002) and young animals such as piglets (Li et al., 1990), calves (Lallès & Dreau, 1996) and mice (Guo et al., 2008). However, study on β-conglycinin has been limited due to the difficulty of separating β-conglycinin from other soybean proteins. Most previous studies have mainly used soybeans or their extracts as the experimental materials but not pure β-conglycinin because of the lack of an available isolation approach. Although some methods such as ammonium sulphate precipitation (Thanh & Shibasaki, 1976), pH adjustment isolation (Lovati et al., 1992), ultrafiltration membrane separation (Wu, Murphy, Reuber, & Fratzke, 2000) and phytase treatment (Saito et al., 2001) can be conducted to isolate β-conglycinin from soybean proteins, it is difficult to obtain enough pure β-conglycinin for research purposes. Since monoclonal antibodies (Mabs) are powerful tools for many molecular immunology investigations (Nelson et al., 2000), they have frequently been applied in practical studies on recognising antigens and identifying new cell surface molecules. These binding concepts of allergen and antibody also provide a possibility for us to prepare an immuno-affinity column mediated by Mab and develop a new approach for purification of β-conglycinin which can be used for future research purposes.

2. Materials and methods

2.1. Preparation of monoclonal antibody against soybean β-conglycinin

The preparation of a stable murine-derived hybridoma cell line, named as $6G_4$, has been described previously (You et al., 2008). The Mab against soybean β-conglycinin produced by $6G_4$ is an IgG1 isotype. It shows high affinity for β-conglycinin with an association constant of $6.9 \times 10^9\ M^{-1}$ and can specifically bind to the α and α′ subunits of β-conglycinin (You et al., 2008).

2.2. Production and purification of antibodies

A large amount of Mab was produced *in vivo*. The hybridoma cells from $6G_4$ (1×10^6 cells/mouse) were injected

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intraperitoneally into BLAB/c mice pre-treated 7 days before with 0.5 mL mineral oil. About two weeks later, the ascitic fluids from mice inoculated by hybridoma cells were collected and centrifuged for 15 min at 10000×g to eliminate cells and dregs. The ascite supernatant was diluted with equal volume of 0.04 M barbital buffer (pH 7.0). An appropriate amount of SiO_2 powder was added into ascites dilution and gently stirred for 30 min. After centrifuging for 20 min at 2000×g, Mab $6G_4$ was primarily isolated from supernatant by ammonium sulphate, and a commercial Protein G Affinity Column (Amersham Biosciences, Uppsala, Sweden) chromatography was then performed to further purify IgG1 from ammonium sulfate precipitation.

2.3. Preparation of immuno-affinity column

The CNBr-activated Sepharose™ 4B (Amersham Biosciences, Uppsala, Sweden) medium was prepared according to its production instructions. Briefly, 2.3 g of CNBr-activated Sepharose™ 4B freeze-dried powder was suspended in 1 mM HCl (pH 2.5). After swelling, the medium was washed for 15 min with 1 mM HCl on a sintered glass filter to remove all additives.

Fifty-six mg of Mab $6G_4$ (IgG1) as ligand was dissolved in 8 mL of coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl) and mixed with prepared Sepharose 4B gel in a 10 mL test tube fitted with a stopper. Then, the tube was tied on the orbital shaker and the mixture was gently shaken for 1 h at 20–25°C. Excessive ligand was flushed away with at least five volumes of coupling buffer. Tris–HCl buffer (0.1 M, pH 8.0) was added to block any remaining active groups for 2 h. The gel was washed for at least three cycles of alternating pH using five volumes of each buffer. Each cycle consisted of a wash with 0.1 M acetate buffer (pH 4.0 containing 0.5 M NaCl) followed by a wash with 0.1 M Tris–HCl (pH 8.0 containing 0.5 M NaCl).

The gel coupled with IgG1 was loaded into a column (10 × 100 mm) which was washed with 20 mM sodium phosphate buffer (pH 7.0) at 4 °C. The residual space of the column was immediately filled with buffer, and the top of the column was connected to a pump. The pump was set to run at a flow rate of 1.3 mL/min. The elution was maintained for 10 bed volumes after a constant bed height was reached.

2.4. Extraction of total soybean proteins

Soybean seeds were ground with a Cyclotec Sample Mill (Model 1093, Tecator Inc., Herndon, VA, USA) to pass through a 60-mesh sieve. The soybean flour was defatted by *n*-hexane. The fat-free flour was then suspended in 0.03 M Tris–HCl buffer (pH 8.0 containing 0.01 M β-mercaptoethanol) (100 g flour per litre buffer). Proteins were extracted from the flour for 1.5 h by vortexing. 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). After being filtered through a 0.45 μm Millex GP filter (Millipore, Cork, Ireland), the supernatant was used as test sample solution and stored at −20 °C until analysis.

2.5. Immuno-affinity chromatography of β-conglycinin

The total proteins was loaded onto the immuno-affinity column. The outlet tubing of the column was connected to an HD21-1 detector and a protein auto-collector (Huxi instrument Co., Shanghai, China). Flow rate was maintained for 1.0 mL/min. The medium was washed with binding buffer until the base line was stable. Glycine–HCl elution buffer (0.1 M, pH 2.7) was used to elute the sample and β-conglycinin was collected using the auto-collector according to absorbance profile. The pH of collection (β-conglycinin) was regulated immediately to neutral with 100 μL of 1 M Tris–HCl (pH 9.0).

2.6. Identification of β-conglycinin by Western blot

The proteins isolated from the immuno-affinity column were analysed by 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the standard procedure (Laemmli, 1970). The separated proteins were then transferred electrophoretically from gel to a 0.45 μm nitrocellulose membrane in a mini Trans-Blot Cell Apparatus (BioRad) for 1.5 h at 100 V. The membrane was incubated in 3% bovine serum albumin (BSA) for 2 h at 37 °C. After a stringent wash with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST), the membrane was incubated with 0.1 μg/mL Mab $6G_4$ for 2 h at 37 °C. The unbound primary antibody was removed by washing with TBST. The horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG-HRP) was then added and the substrate 3,3′-diaminobenzidine (DAB) was used to show the specific protein bands.

The concentration of isolated β-conglycinin was determined with a Mab-based competitive ELISA method described previously (You et al., 2008) and the concentration of total proteins was tested by Bicinchoninic Acid Kit for Protein Determination (BCA™ Kit, Pierce, Rockford, IL, USA). The purity of β-conglycinin is expressed as a percentage as the content of β-conglycinin compared with that of total proteins.

2.7. Evaluation of chromatography efficiency in the case of different Mab/gel ratio

There is a close relationship between the ligand (Mab)/gel ratio and the chromatography efficiency. In order to enhance the chromatography efficiency, it is necessary to obtain an optimal ligand (Mab)/gel ratio. In this study, five grade concentrations of Mab, 2.5, 5.0, 7.5, 10.0, 12.5 mg/mL gel, were conducted to prepare five immuno-affinity columns with the same size. The efficiency of the purification of β-conglycinin under identical conditions, including soybean protein extraction and isolation procedures, was then evaluated.

The concentrations and purities of isolated β-conglycinin were determined and the results are reported as mean ± SEM. All data were the average values from five replicate determinations. Statistical analysis was done using the Stat-View program (SAS Institute, Cary, NC, USA). Differences between the concentrations (or purities) of β-conglycinin obtained from five immuno-affinity columns were examined for statistical significance using the Dunnett test. A difference was considered as significant at $P < 0.05$.

3. Results and discussion

Fig. 1 shows the SDS–PAGE profile (Left) and Gary Density profile (Right) of β-conglycinin purified from soybean proteins. Lane b represents total soybean proteins before purification and lane c represents β-conglycinin purified from total proteins. The purified β-conglycinin fraction contained mainly α, α′, and β subunits of β-conglycinin. Lane d is a grey density profile obtained from track c of electrophoresis. Direct observation clearly indicated that β-conglycinin was successfully isolated from soybean proteins. A Mab-based competitive ELISA method was used to further test its content. The result indicated that the purity of β-conglycinin was 92.9% (Data not shown).

In previous reports, the purity of β-conglycinin acquired from soybeans were 68% by ultrafiltration membrane separation method (Wu et al., 2000) and no more than 79% by ethanol extraction method (Rickert, Johnson, & Murphy, 2004). In the present study,