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Development of immunoaffinity chromatographic method for Ara h 2 isolation

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ABSTRACT

Ara h 2 is considered a major allergen in peanut. Due to the difficulty of separation, Ara h 2 had not been fully studied. Immunoaffinity chromatography (IAC) column can separate target protein with high selectivity, which made it possible to purify Ara h 2 from different samples. In this study, IAC method was developed to purify Ara h 2 and its effect was evaluated. By coupling polyclonal antibody (pAb) on CNBr-activated Sepharose 4B, the column for specific extraction was constructed. The coupling efficiency of the IAC column was higher than 90%, which made the capacity of column reached 0.56 mg per 0.15 g medium (dry weight). The recovery of Ara h 2 ranged from 93% to 100% for different concentrations of pure Ara h 2 solutions in 15 min. After using a column 10 times, about 88% of the column capacity remained. When applied to extract Ara h 2 from raw peanut protein extract and boiled peanut protein extract, the IAC column could recovery 94% and 88% target protein from the mixture. SDS-PAGE and Western blotting analysis confirmed the purified protein was Ara h 2, its purity reached about 90%. Significantly, the IAC column could capture dimer of Ara h 2, which made it feasible to prepared derivative of protein after processing.

1. Introduction

Peanut (*Arachis hypogaea*) and its products are important sources of dietary protein for humans and animals, but peanut is listed among eight major food allergens by FAO [1,2]. To date, 13 allergens in peanut protein are officially registered at the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies. The purification of these peanut allergens had gained a considerable amount of attention in recent years [3–5]. Ara h 2 was considered a major allergen [6], which could be recognized by serum immunoglobulin E (IgE) from over 90% of peanut-allergic patients [7,8]. However, there have been limited studies on Ara h 2 induced anaphylaxis due to the difficulty of separating Ara h 2 from different materials [9–11]. Although ion exchange chromatography could extract high purity Ara h 2 from raw peanut [12], it was difficult to obtain Ara h 2 and its derivative from processed protein.

Affinity purification has been used as a tool to develop products for diagnostics and therapeutics [13,14]. Immunoaffinity chromatography (IAC) is a type of liquid chromatography in which the stationary phase consists of an antibody or antibody-related reagent, providing better sample cleanup and higher selectivity [15,16]. Traditional immunoaffinity supports have been based on carbohydrate-related media, and Sepharose 4B has proved to be suitable for routine IAC [17]. When the purified antibody was available, it can be covalently immobilized to CNBr-activated agarose. This would not affect the activity of the binding sites or the accessibility of these sites to their target proteins too much [18].

In the presented work, the pAb against Ara h 2 purified from rabbit serum was immobilized to CNBr-activated Sepharose 4B medium to prepare an IAC column, and then the IAC-based method for purification of Ara h 2 was established during the column characterizing.

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2. Materials and methods

2.1. Reagents

A commercial CNBr-activated Sepharose 4B medium were provided by Amersham Biosciences (Uppsala, Sweden). The horseradish peroxidase-conjugated secondary antibody (HRP -Goat anti-rabbit IgG), Tween 20, *o*-phenylenediamine, and electrophoresis chemicals were all obtained from Sigma (St Louis, MO, USA). Nitrocellulose membranes (Hybond, ECL) were obtained from Millipore (Darmstadt, Germany). All the other chemicals used in the present study were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Polyclonal antibody against Ara h 2 and peanut samples

Peanut seeds were purchased from a local supplier (Nanchang, China). The raw peanut protein extract (RE) were extracted from defatted raw peanut powder by Tris-HCl buffer (50 mM, pH 8.0) as previously described by Wu Z et al. [19]. Ara h 2 was isolated from RE using anion exchange chromatography according to the previous report [12]. Antiserum against Ara h 2 was produced from New Zealand male rabbits following Luo C et al. [20]. The polyclonal antibody (pAb) against Ara h 2 was purified from antiserum by affinity chromatography on a CNBr-activated Sepharose 4B column with 15.7 mg Ara h 2 per gram CNBr-activated Sepharose (dry weight). It required about 0.4 mL antiserum for 1 mg pAb could be purified. After it was tested by indirect ELISA and Western blotting, the pAb was stored at −80 °C until use.

The boiled peanut protein extract (BE) was prepared by boiling raw peanut protein extract (RE) at 100 °C for 30 min.

2.3. Preparation and characterization of the Immunoaffinity Chromatography (IAC) column

2.3.1. Preparation of the IAC column

IAC column was prepared following the manufacturer's instructions. Briefly, 0.15 g of CNBr reactivated Sepharose 4B agarose gel freeze-dried powder was dissolved in 1 mM HCl (pH 2.5). Following swelling, the Sepharose 4B agarose gel was washed for 15 min in 1 mM HCl and filtered with sintered glass to remove protective additive, this resulted in 0.75 mL of agarose gel left. Then different quantities of home-made pAb (1.0, 1.5, 2.0, 2.5, 3.0 mg) were dissolved in 1 mL of coupling buffer (containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) and mixed with these 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 washed again with 5 mL coupling buffer. The amount of initially loaded antibody (T) and antibody found in eluate (R) were estimated by the Bradford assay [21]. The difference between them was regarded as antibody binding to the column. So the coupling efficiency (E) could be calculated as E (%) = 100% (T − R)/T, while the density of the immunoaffinity medium pAb/gel (mg/g) was calculated as antibody/medium ratio, namely, (T−R)/0.15 here. The antibody Sepharose gel was mixed with enough blocking buffer (containing 0.1 M Tris-HCl, PH 0.8) for 2 h in an orbital shaker before loading.

The 0.75 mL prepared Sepharose gel (coupled with pAb) was gently loaded into a chromatographic column (10 × 100 mm), 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, three times each and equilibrated with 20 mM PBS (pH 7.4) precooled at 4 °C for 2 h. The elution was maintained for 10 bed volumes after a constant bed height was reached. In the case of long-term storage, 0.01% (w/v) sodium azide was added to the column as a bacteriostatic agent.

2.4. Column capacity of the IAC column

The Ara h 2-binding capacity was determined by breakthrough volume test [22]. Ara h 2 solution (1 mL) containing 0.2, 0.4, 0.6, 0.8 and 1.0 mg protein were loaded on the IAC column. The outlet tubing of the column was connected to an HD21-1 detector and a protein collector (Jingke, Nanchang, China). The flow rate was maintained at 1.5 mL/min. The media was washed with 20 mM PBS (pH 7.4) until the baseline was stable. Glycine-HCl elution buffer (0.1 M, pH 2.7) was used to elute the sample with the flow rate of 1 mL/min. The Ara h 2 solution was collected during specific elution, typically last 2–3 min. The pH value of the Ara h 2 solution was adjusted immediately to pH 7 with 100 μL of 1 M Tris-HCl pH 9.0. Quantification of Ara h 2 in the elution volume was performed by the Bradford assay.

2.5. Recovery of the IAC column

1 mL Ara h 2 solutions at the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 mg/mL were loaded on the IAC column. The IAC column was then washed with 15 mL of PBS and eluted with 10 mL Gly-HCl buffer. The eluate in the eluting step was collected and the quantification of the protein in solution was assessed by the Bradford assay. The same recovery processes were repeated three times.

2.6. Reusability of the IAC column

The capacity of the immunosorbents to withstand repeated uses was evaluated with Ara h 2 column. Recoveries were determined after loading the column with 1 mL of a solution containing 1 mg of Ara h 2, then washed with PBS until the baseline appeared, and finally eluted with Gly-HCl buffer. Gels were regenerated with 10 mL PBS and re-used for a new cycle. The process was repeated 11 times, and after each cycle the concentrations of Ara h 2 in the loading, washing, and eluate were measured by the Bradford assay.

2.7. Purification of Ara h 2 from peanut samples

2.7.1. Protein separation

The peanut samples were loaded on IAC column and washed with PBS buffer until the baseline appeared. Then the column was washed with Gly-HCl buffer, and eluate was collected. The amount of Ara h 2 in eluate was measured by the Bradford assay.

2.8. Identification of Ara h 2

The protein solutions purified by the immunoaffinity column from RE and BE were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition (2.5% β-mercaptoethanol) as described by *Laemmli* [23].

After electrophoresis, western blotting was performed as following. Protein was transferred to NC membranes (Immobilon Millipore, Polylabo) for 60 min at 100 V using a MiniProtean III system (Bio-Rad). The membranes were saturated with TBST supplemented with 5% milk powder, before being incubated overnight at 4 °C in rabbit antiserum. Membranes were then washed, and incubated in the horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature. After several times of washing, the membranes were treated with 3, 3-diaminobenzidine and hydrogen peroxide to show the specific protein bands.