

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.

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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 8.0) 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.

3. Results and discussion

3.1. Characterization of the IAC column

3.1.1. Coupling efficiency and pAb density

The coupling efficiency related with the preparation of the affinity medium, the capacity for specific absorption, and the steric hindrance as well [24]. The medium and the ligand protein are two of the most important factors that influence the coupling efficiency of immunoaffinity column [25]. The primary amine of pAb could combine with the activated CNBr-activated Sepharose 4B medium with covalent bonds in the buffer [26]. As shown in Table 1, with the increase of the concentration of pAb (1.0–3.0 mg/mL), the density of the immunoaffinity medium increased. While the concentration of pAb was 3.0 mg/mL, the density of the immunoaffinity medium reached maximum 18.1 mg pAb/g of medium (dry weight) as well as 3.62 mg pAb/mL of Sepharose gel and the corresponding coupling efficiency was higher than 90%. This result suggested that most of the pAb had been adsorbed on the medium with a high efficiency. Herein, the high coupling efficiency suggested that the suitable concentration of the antibody (3.0 mg/mL) and affinity medium (pAb/gel 18.1 mg/g or 3.62 mg/mL) were chosen.

3.2. Capacity of the IAC column

Column capacity is one of the important indicators to evaluate the efficacy of the IAC column [25], characterizing how much target proteins the IAC could adsorb. In Fig. 1, the curve of amount of bound Ara h 2 against loaded protein were presented. When the loaded Ara h 2 ranged from 0.1 to 0.8 mg, the quantity of Ara h 2 adsorbed by the IAC column increased linearly, until the bound Ara h 2 reached 0.56 mg which is the column capacity. In another words, the column could bind 0.75 mg Ara h 2 per mL column medium. Comparing IAC column prepared in the same method to extract sidestream smoke [27], the column capacity was higher for Ara h 2. The main reasons might be that the Ara h 2 antibody has a better combining capacity with CNBr and a high antibody/medium density.

3.3. Recovery of the IAC column

To examine the extraction efficiency of the column for Ara h 2, 1 mL of Ara h 2 solutions at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL were loaded on IAC column respectively. The recoveries of the IAC column for different concentrations of pure Ara h 2 are shown in Table 2. The recoveries of Ara h 2 ranged from 93 to 100% for all concentrations. The high recovery was most likely due to high purity and activity of the pAb [25].

3.4. Reusability of the IAC column

Excessive Ara h 2 was continuously loaded on the IAC column to test its stability. It was observed with 5 repeat usages, there was no obvious decrease of the amount of extracted Ara h 2 comparing to the first time. As shown in Fig. 2, the capacity of the IAC column decreased to about 92% in the 9th usage, and about 88% in the 11th

Table 1

Coupling efficiencies and pAb density of the IAC column made from 0.15 g medium (dry weight).

Concentration of PAb (mg/ml)	1.0	1.5	2.0	2.5	3.0
Coupling efficiencies (%)	98	98	98	92	90
pAb/gel (mg/g)	6.5	9.8	13.0	15.4	18.1

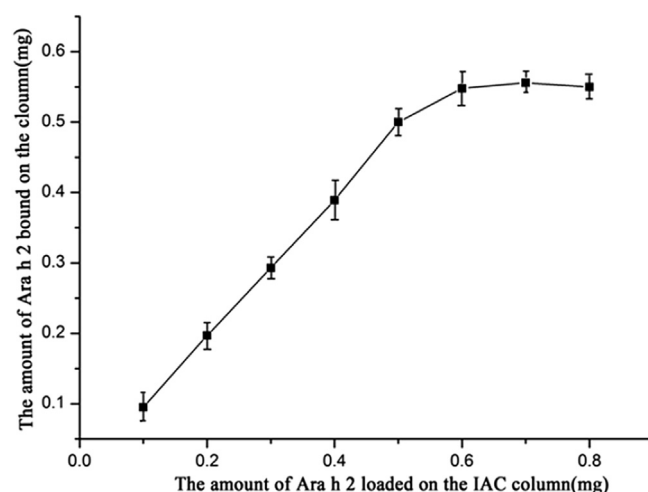


Fig. 1. The capacity of the IAC column. This IAC column was made from 0.75 mL of agarose gel and 1 mL of the polyclonal antibody (3.0 mg/mL).

Table 2

Recoveries of the IAC column for different concentrations of pure Ara h 2 solutions (n = 3). The IAC column was made from 0.75 mL of agarose gel and 1 mL of the polyclonal antibody (3.0 mg/mL).

Ara h 2 added (mg)	Ara h 2 bound (mg)	Recovery (%)	RSD (%)
0.10	0.10	100	3.4
0.20	0.19	95	2.9
0.30	0.28	93	2.3
0.40	0.38	95	4.7
0.50	0.49	98	3.1

usage.

3.5. Ara h 2 was purified by IAC column chromatography

The column could separate the protein in two fractions: a flow through fraction and a fraction eluted during Glycine-HCl elution buffer (0.1 M, pH 2.7), as the elution profiles measured at 280 nm of UV wavelengths shown in Fig. 3. The PBS washed out unbound protein of nonspecific adsorption, then Gly-HCl (pH 2.7) solution decreased pH, desorbing the antigen from antibody, and Ara h 2 flowed out with the eluate. Typically, the specific elution peak lasted about 2–3 min, and the nonspecific one lasted about 8 min, the baseline would last 5 min or less, so it could take 15 min or less for the target protein to be purified from peanut protein extract.

The second elution peak on the curve washed by Gly-HCl buffer was detected by SDS-PAGE and Western blotting. SDS-PAGE analysis was shown in Fig. 4 (left). There were two adjacent protein bands near 17 kDa (lane of RE, BE), which can be identified as two isoforms of Ara h 2 [4–28]. Another protein band could be seen in the vicinity of 15 kDa which is band of Ara h 6 (14.5 kDa) [4–29]. As reported, there is 59% amino acid similarity between Ara h 2 and Ara h 6 [23], and they both can be cross-reactive with each other according to the IgE-immunoblotting test [3]. So it was not strange that Ara h 6 could be also captured by the pAb of Ara h 2 in the IAC column. Analyzed by Quantity One software, the purities of Ara h 2 were 87% and 80% for RE and BE respectively. If taking into account the effects of Ara 6, the purity of 2S protein (Ara h 2/6) from BE and RE were more than 90%. Although there was a little Ara h 6, it doesn't affect the Ara h 2 analysis largely, considering their amino acid similarity. Western blotting result proved that the purified proteins were our target proteins, Ara h 2 (about 17 kDa) and Ara h

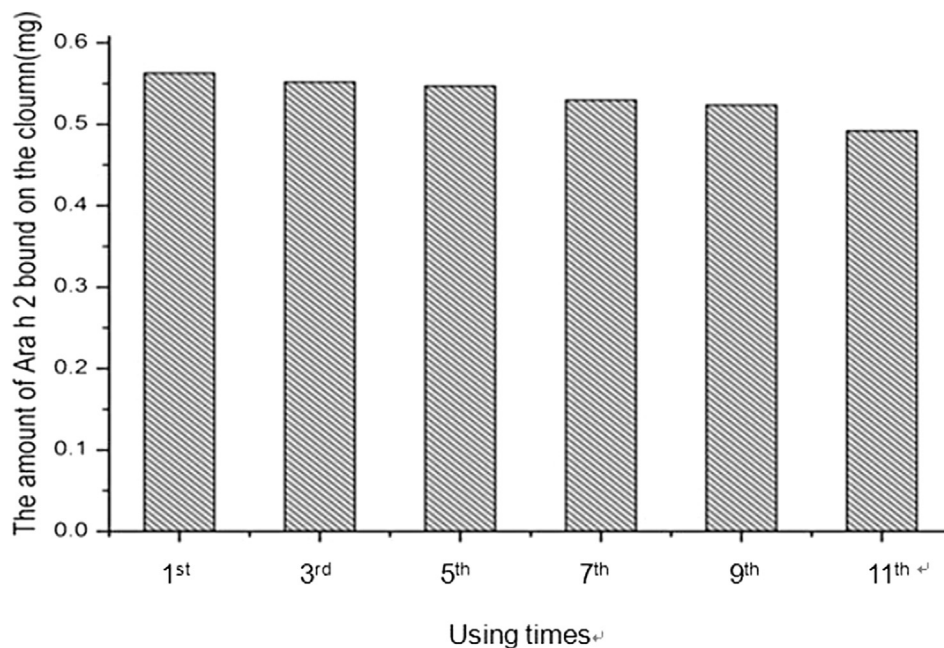


Fig. 2. The variation among binding capacities of the IAC column vs. reuse. The IAC column was made from 0.75 mL of agarose gel and 1 mL of the polyclonal antibody (3.0 mg/mL).

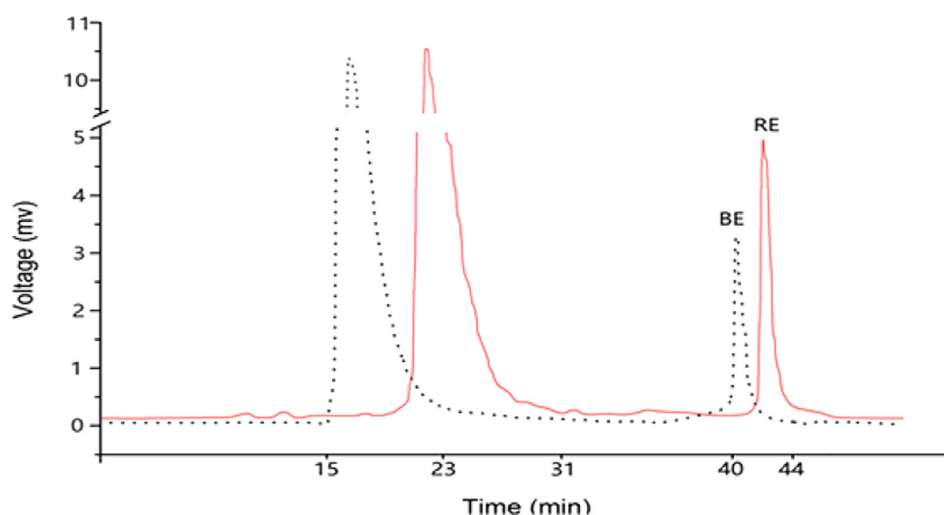


Fig. 3. Elution curves of Ara h 2 purified from different samples with the IAC column at 280 nm of UV wavelengths. The IAC column was made from 0.75 mL of agarose gel and 1 mL of the polyclonal antibody (3.0 mg/mL). Samples loaded were boiled peanut protein extract (BE black dot line) and raw peanut protein extract (RE, red solid line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6 (about 15 kDa), which could react with anti-Ara h 2 sera. It is worth noting that a band of about 34 kDa from BE was recognized by the sera in Fig. 4 (right). This band was speculated to be dimer of Ara h 2, as reported that protein polymers were produced by heat treatment [11]. This result indicated that the IAC column could not only separate the Ara h 2 itself, but also its processed products. The latter provided possibility of separating Ara h 2 processed products by IAC method.

The yields were quantified as $87 \pm 4.6\%$ and $80 \pm 9.3\%$ for RE and BE respectively by ELISA with standardized curve method (Data not shown). Less Ara h 2 was purified from boiled peanut protein than that from raw peanut. For the avoidance of column jam, the boiled sample was filtered, during which the Ara h 2 might be lost. In the

elution profiles, the both peaks of RE were higher than BE, the reason might be the heat treatment caused a decrease of soluble protein, including Ara h 2 [30]. On the other hand, the ion exchange chromatography could extract only $37 \pm 5.4\%$ Ara h 2 from BE, and $51 \pm 6.2\%$ from RE (data not shown). That meant the home-made IAC column has much higher purification rate than traditional method, and was less time consuming.

4. Conclusions

In this study, an IAC method was developed for the extraction of Ara h 2. By coupling pAb on CNBr-activated Sepharose 4B, the capacity and recovery of the IAC column were good. The home-made

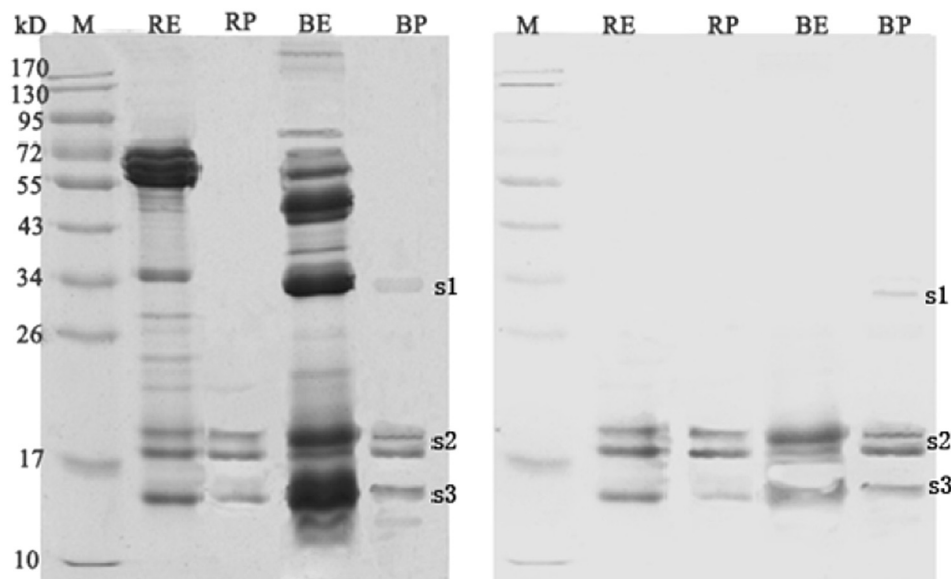


Fig. 4. SDS-PAGE (Left) and western blotting (Right) profiles of different samples. M: marker; RE: raw peanut protein extract; RP: Products separated from RE by polyclonal antibody based immunoaffinity chromatography column; BE: boiled peanut protein extract; BP: Products separated from BE by polyclonal antibody based immunoaffinity chromatography column. s1: dimer of Ara h 2; s2: Ara h 2; s3: Ara h 6. The IAC column was made from 0.75 mL of agarose gel and 1 mL of the polyclonal antibody (3.0 mg/mL).

IAC column could separate Ara h 2 with higher purification rate, higher yield and faster than traditional. Also the column could capture the derivative of Ara h 2, which made IAC column feasible to extract derivative of protein after processing.

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