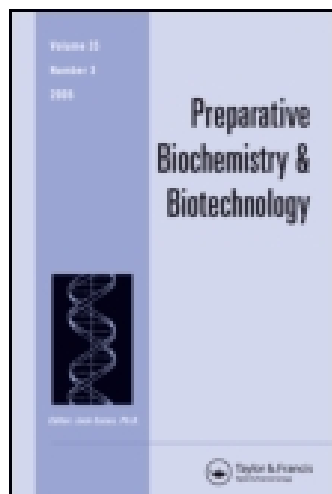




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Purification and Recombinant Expression of Major Peanut Allergen Ara h 1

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Purification and Recombinant Expression of Major Peanut Allergen Ara h 1

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Reaction to peanut, as one of the major food allergens, has become an increasingly common life-threatening disorder. Although peanut allergens have been extensively identified, Ara h 1 is still too expensive to be applied in food safety or clinical utility. In this study, the purification, expression, and immunological analyses of Ara h 1 are investigated. It was shown that a high purity (>95%) of Ara h 1 could be prepared by either purification or expression. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and mass spectroscopy were used to identify the Ara h 1, and it was found that natural Ara h 1 (nAra h 1) and expressed Ara h 1 (rAra h 1) have the same properties, including amino acid sequence. In particular, rAra h 1 reacted positively

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with anti-nAra h 1 serum, showing their similar immunological property. Thus, by either purification or expression, Ara h 1 could be prepared with low cost, as performed in the present work. SDS-PAGE, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), and immunological analysis confirmed that both forms of Ara h 1 had the same properties.

Keywords *Ara h 1, expression, immunological analysis, MALDI-TOF MS, peanut, purification*

INTRODUCTION

Peanut (*Arachis hypogaea*) is listed among eight major food allergens by the Food and Agriculture Organization of the United Nations (FAO),^[1] and peanut allergy became an increasingly common life-threatening disorder,^[2] attracting more and more interest.^[3–6] Currently, 11 proteins, namely, Ara h 1–Ara h 11, have been identified to be peanut allergens.^[7] As recognized by serum immunoglobulin E (IgE) from more than 90% of peanut-allergic patients, Ara h 1 is regarded as a major allergen of peanut.^[8,9] The structural and immunologic properties of Ara h 1 have been well characterized.^[10] Besides their structure and other properties, the applications of an allergen in food safety and in diagnosis and treatment for peanut allergy patients is developing,^[11,12] requiring more natural or recombinant allergen with high purity. Actually, many attempts have been made to prepare pure allergens, for example, peanut allergen Ara h 1 and Ara h 2.^[10,13]

However, there is no suitable way to prepare of Ara h 1 at low cost, so that the price of Ara h 1 is too high to be in used outside of scientific research. For better understanding of this allergen and its utility in food safety or medicine, a more inexpensive Ara h 1 is needed. The two origins of allergen are isolation of natural allergen^[14] or recombinant expression of protein in a microorganism.^[15] And the properties, especially immunological properties, of between the native allergen and recombinant one could be the same, which would make it possible to use recombinant allergen for immunological or clinical studies.^[16]

Our previous work on Ara h 2 and Ara h 6 has been reported elsewhere, in which these two allergens were purified or/and recombinantly expressed.^[17,18] In the present work, the preparation of peanut allergen Ara h 1, both a natural one and a soluble recombinant one, is described. Immunological analysis and mass spectroscopic identification were performed to define the properties of Ara h 1.

MATERIALS AND METHODS

Purification of Natural Ara h 1

Whole peanut protein extract (WPPE) was prepared following Luo et al.^[19] Raw peanuts (*Arachis hypogaea*) were purchased from a local supplier (Nanchang, China) and were stored at -20°C until use. After shelling, the skins were removed, and the kernels were ground in liquid nitrogen using a mortar and pestle. The meal was then defatted by stirring with 5 volumes of precooled acetone for 1 hr at 4°C , and it was recovered by centrifuging at $6,000 \times g$ for 20 min at 4°C . After being defatted 3 times, the meal was allowed to dry in the air and suspended in 5 volumes of 50 mM Tris-HCl (pH 7.2), followed by stirring for 12 hr at 4°C . Then the

suspension was centrifuged at $6,000 \times g$ for 20 min at 4°C , and the supernatant was collected as WPPE.

Natural Ara h 1 (nAra h 1) was purified from WPPE through three steps of chromatography. First, with anion-exchange chromatography, extraction was performed in a DEAE-Sepharose Fast Flow column (GE Healthcare, USA), washing with gradient elution at a flow rate of 1.5 mL/min, and the concentration of NaCl increased from 0 M to 0.4 M in Tris-HCl buffer (50 mM, pH 8.0) within 400 min. Fractions were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to find Ara h1 concentrated in the fourth peak. Then Ara h 1 was separated from collection of the fourth peak by Superdex 200 gel filtration chromatography (GE Healthcare, USA), washing with Tris-HCl buffer (50 mM, pH 8.0), 0.2 mL/min, and Ara h1 was found in peak 1. At last, ConA Sepharose 4B affinity chromatography (GE Healthcare, USA) was used to purify the Ara h 1, washing with Tris-HCl buffer (50 mM, pH 8.0) at 1 mL/min, and then Tris-HCl buffer (50 mM, pH 8.0) with 0.2M NaCl at 1 mL/min.

Production of Ara h 1 Polyclonal Antibody

Polyclonal antibody against Ara h 1 was produced from New Zealand male rabbits from the Institute of Occupational Medicine of Jiangxi (permission number SYXK (Gan) 2009-003). All animal experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

Before immunizing, 1 mL of blood from a noninjected rabbit was collected by the ear bleeding as a negative serum blank. Two rabbits were immunized by subcutaneously injecting into their shaved backs 0.2 mg of purified Ara h 1 in Freund's complete adjuvant (Sigma, USA). Subsequent injections of Ara h 1 in incomplete Freund's adjuvant (Sigma, USA) were performed three times at 2-week intervals, and the immune responses of rabbits were monitored by indirect enzyme-linked immunosorbent assay (ELISA). When the serum titer reached 1:100,000, the rabbits were finally bled and each antiserum was separated by centrifuging at $4,000 \times g$ for 10 min at 4°C . All sera were stored at -80°C until use.

Recombinant Expression of Ara h 1

Total RNA of peanut was extracted from the peanut seeds using the SV Total RNA Isolation System Kit (Promega, USA). Then the DNA fragments of Ara h 1 were amplified by reverse-transcription polymerase chain reaction (RT-PCR) cDNA Synthesis Kit (Tiangen, China), with primers 5'-CGGGGTACCAAGTCACCTTAC CGGAAAACA-3' and 5'-CCC AAGCTTTCAGTTAAAAGCCTTCAAATTGA-3'. To facilitate the downstream purification, six histidines ($6 \times \text{His tag}$) were added at the C-terminus. The RT-PCR product was cloned into a pMD18-T-simple vector for amplifying and analysis. After sequence confirmation, the target gene obtained from pMD18-T-Ara h 1 was cloned into a pET-32a expression vector and double digested with *KpnI* and *HindIII*. The recombinant plasmids pET-32a-Ara h 1 were transformed into *Escherichia coli* BL21(DE3)plysS using the Gene Pulser MXcell Electroporation system (Bio-Rad). The recombinant proteins were produced by incubating the host cell at 24°C with

0.3 mM IPTG induction and 160 rpm shaking. An Ni-NTA superflow column (Qiagen, USA) was used for Ara h1 purification.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed essentially according to Laemmli^[20] using the Mini Protean system (BioRad, USA). The 5% stacking gels and 15% separating gels were used in reducing SDS-PAGE analysis. Samples were mixed (1:1, v/v) with sample buffer (20 mM Tris, pH 6.7, 2 mM ethylenediamine tetraacetic acid [EDTA], 2% (w/v) SDS, 25% (v/v) glycerol, 0.02% (w/v) bromophenol blue), boiled for 5 min, followed by centrifuging at $4,000 \times g$ for 5 min, and the supernatants were loaded onto the gel. Gels were stained with Coomassie brilliant blue R-250 and destained in a destaining solution (7.5% HAc (v/v), 5% methanol (v/v) in water). After destaining, gels were scanned, followed by calculating the band intensity with Quantity One software (Bio-Rad). The separated proteins were transferred to polyvinylidene difluoride membranes (PVDF, Millipore, USA) by applying a constant current of 50 mA for 2 hr at room temperature. The membranes were blocked with 5% skim milk (w/v) in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 1% Tween-20 (TBST) for 1 hr at room temperature, followed by overnight incubation with the rabbit anti-Ara h 6 serum (1:5,000 dilutions in TBST) at 4°C. Then the membrane was washed 3 times in TBS and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000 dilutions in TBST, Sigma, USA) for 2 hr at room temperature. After washing for another 3 times, the peroxidase-positive images were developed by incubating the membrane in 20 mL TBS containing 12 mg 4-chloro-1-naphthol, 4 mL ethanol, and 12 μ L 30% H₂O₂ at room temperature for 10 min.

Mass Spectrometry

SDS-PAGE-purified Ara h 1 as described earlier was cut according to bands, and sealed in sterile distilled water before identification. Protein identification was carried out using tandem matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry^[21] with the ABI 4800 plus mass spectrometer (ABI, USA) in the Institute of Life and Health Engineering, Jinan University, Guangzhou, China.

RESULTS AND DISCUSSION

After three-step chromatography, Ara h 1 could be isolated. As shown in Figure 1, the DEAE-Sepharose Fast Flow column could separate the whole peanut protein extract into more than nine parts, among which Ara h 1 concentrated in peak 4. As indicated in the insert in Figure 1, only lane 4, corresponding peak 4, and a band of molecular mass ~ 64 kD appeared. Therefore, this peak was collected for further purification via Superdex 200 gel filtration chromatography. Only two peaks appeared during washing for 10 hr (Figure 2A), and the main peak formed at 300 min, containing Ara h 1 and some mixed protein (Figure 2B). Moreover, ConA Sepharose 4B affinity chromatography was used to clean the contaminating protein. After affinity purification, the purity of Ara h 1 reached as high as 95%, according to analysis of Quantity One, and the band

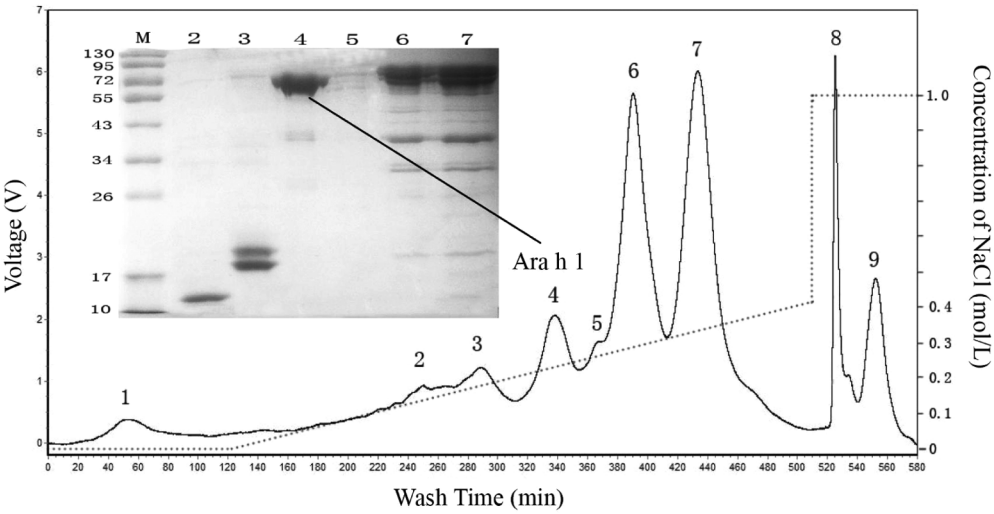


FIGURE 1 Fragments of whole peanut protein extract separated by anion-exchange chromatography, detected by SDS-PAGE (insert).

of 33 kD did not decrease (Figure 2C). It was concluded that this 33-kD protein is fragment of Ara h 1,^[22] which had also been proven by our MS analysis (data not shown).

After purification, we can get about 40 mg Ara h 1 from 10 g peanut seeds with pretty low cost. The purity of protein is more than 95%, while the only contamination is a band of about

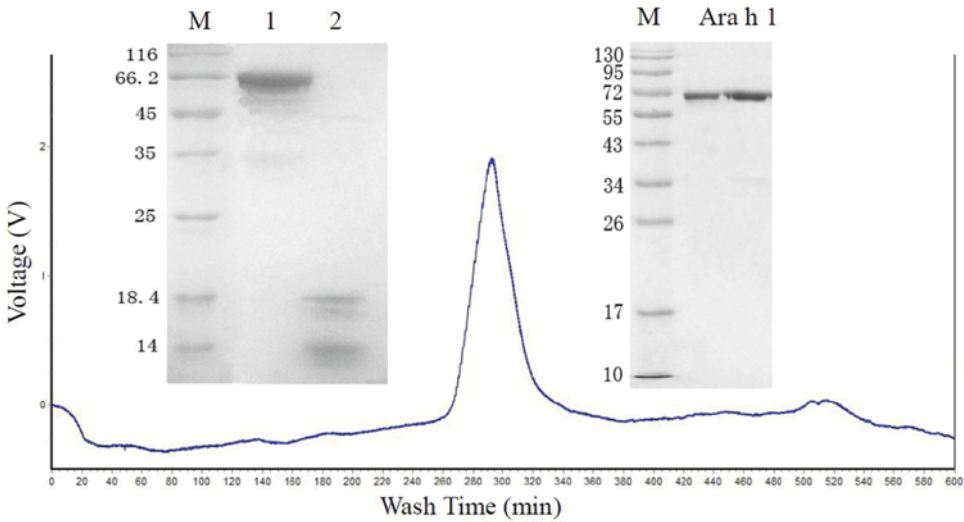


FIGURE 2 Fragments of peanut allergen Ara h 1 separated by ConA Sepharose 4B (A), and SDS-PAGE detection before (B) and after (C) the chromatography.

33 kD, which was reported previously.^[23] In native PAGE, Ara h 1 focused as a band near 200 kD, indicating its trimmer form, the natural status of Ara h 1.^[24] It takes less than 24 hr for a round of purification, and this is available in a common laboratory. Moreover, the cost is much lower than commercially available Ara h 1, while the purity and quantity are better.

Rabbit antiserum against Ara h 1 was collected and the titer was measured by indirect ELISA. The immunoblotting indicated high specificity of antiserum, and ELISA data showed a titer of 1:200,000. According to Western blotting shown in Figure 3, the specificity of antibody is satisfied; no other peanut protein reacts with the antisera.

For recombinant expression of Ara h 1, the cDNA fragment of 1786 bp was finally amplified by PCR with primers referred to earlier. Database sequence homology analyses of nucleotide sequence with published Ara h 1 were performed using the BLAST program. Multiple sequence alignment showed that the cDNA shared high identity (99%) with material described in a previous publication.^[25] The cDNA fragment was recombined with the expression vector pET-32a, and expressed in *E. coli* BL21(DE3)-plysS after IPTG induction. Then the collection of recombinant bacteria and the ultrasonic extraction of His-Ara h 1 fusion protein were performed. Finally the soluble recombinant protein was purified by Ni-NTA affinity chromatography. Although Ara h 1 had been reached by recombinant expression in 1995,^[25] the purification of the rAra h 1 without a tag is not easy. But it is not difficult to purify the protein with a His tag. The purity of rAra h 1 could be higher than 95%.

Before purification, Western blotting was performed to clarify the existence of rAra h 1 in total protein expression. The result (Figure 4, left) showed that a band between 72 kD and 90 kD could react with anti-Ara h 1 antibody. However, for expression without IPTG induction, no band was recognized by this antibody. This confirmed that IPTG induced Ara h 1 expressing in the *E. coli* expression system. In the SDS-PAGE results, the expressed His tagged fusion protein exhibited a higher molecular mass than its native form because of the tag. In Figure 4,

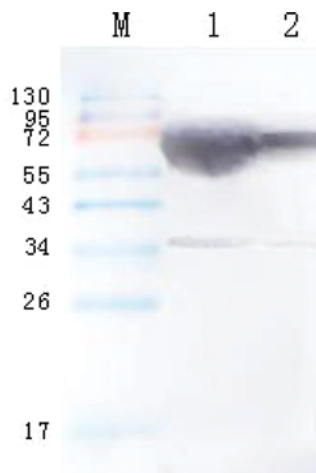


FIGURE 3 Western blotting analysis by the rabbit antisera. M: prestained protein marker; 1: whole peanut protein extract; 2: purified Ara h 1.

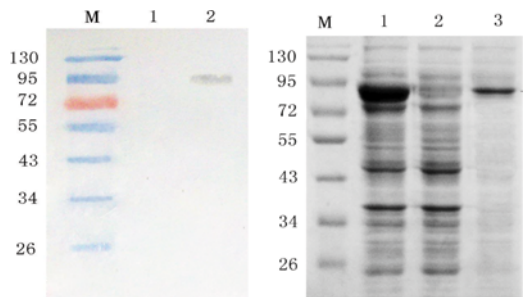


FIGURE 4 Western blotting analysis and affinity chromatography purification of rAra h 1. Left, Western blotting analysis, M: prestained protein marker, 1: protein expression without IPTG induction, 2: protein expression with IPTG induction;. Right, Purification of rAra h 1, M: protein marker, 1: total protein expression with IPTG induction, 2: protein expression with IPTG induction, 3: affinity chromatography purified rAra h 1 from protein expression.

the right image showed the purification of rAra h 1. With the His tag, the rAra h 1 was easily separated from the total expression.

Besides immunological comparison, both nAra h 1 and rAra h 1 were analyzed by MS. In Figure 5, the peptide fragments of two proteins were in accordance with each other, indicating that the two protein have similar primary structure.

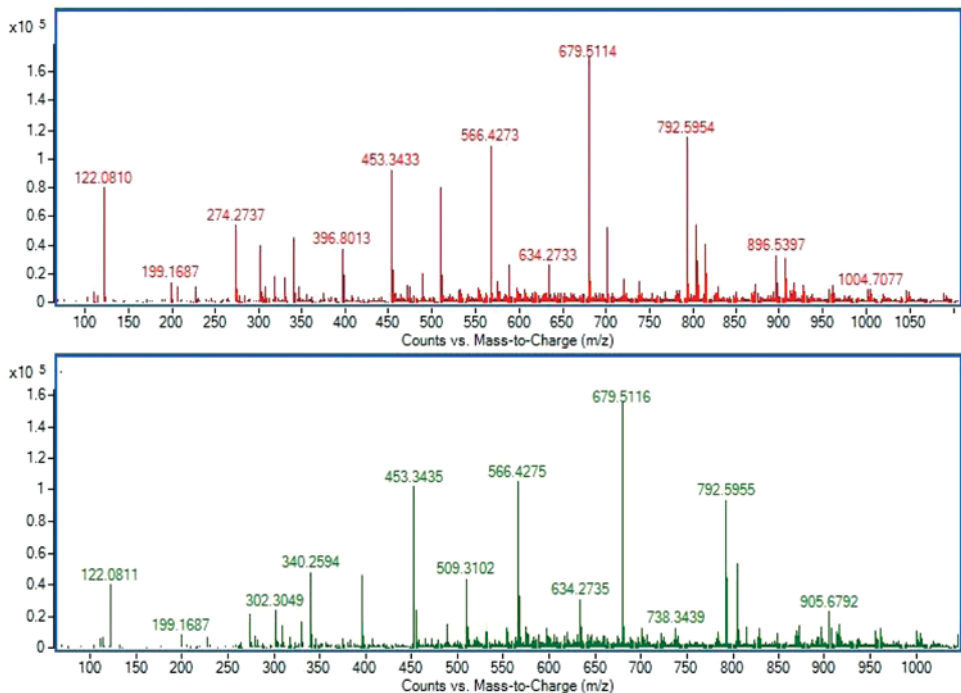


FIGURE 5 Mass analysis of natural peanut allergen Ara h 1 and recombinant Ara h 1: (A) nAra h 1; (B) rAra h 1.

The immunological and structural property of rAra h 1 is similar to nAra h 1, which made it possible to use rAra h 1 in allergen diagnosis or treatment. Moreover, the immunological property of rAra h 1 can be adjusted by mutation,^[13] so that hypoallergenic protein could be prepared for treatment.

As a major allergen in peanut, Ara h 1 was purified or prepared with recombinant expression.^[6,9,10,22–25] In previous reports, Ara h 1 was used for biochemical, structural, and immunologic research, or process, and diagnosis. Few works focused on sample preparation, which made the price of pure Ara h 1 too expensive for research and other applications. Here, the presented work aimed at preparing inexpensive Ara h 1. Both purification and expression construction could be performed in a regular laboratory to get enough Ara h 1 sample.

CONCLUSIONS

The purification, cloning, expression, and immunological analysis of peanut allergen Ara h 1 were performed in this work. By either purification or expression, Ara h 1 could be prepared at high purity and low cost. SDS-PAGE, MALDI-TOF MS, and immunological analysis confirmed the two proteins were Ara h 1. Both the expressed Ara h 1 and the purified Ara h 1 reacted positively with antisera from rabbit, and they were proposed as a source of the protein for immunological or clinical studies for peanut allergy.

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