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Purification and Characterization of Ara h1 and Ara h3 from Four Peanut Market Types Revealed Higher Order Oligomeric Structures

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ABSTRACT: This study aimed to purify and characterize the peanut allergens Ara h1 and Ara h3 from four cultivars that represent the four major market types to provide better understanding of the molecular organization of oligomers in different market types. The chromatographic profiles of Ara h1 and Ara h3 from the four cultivars obtained from anion exchange chromatography were similar. However, they differed in the distribution of trimeric and hexameric structures of Ara h3 isolated by size exclusion chromatography. The Menzies (Runner market type) and Walter (Spanish market type) cultivars, wherein Ara h3 proteins consist of two acidic subunits, exhibited trimeric and hexameric conformations proportionally. However, the Middleton (Virginia market type) and Kelinci (Valencia market type) cultivars, wherein Ara h3 proteins consist of three acidic subunits, showed predominantly a hexameric structure. The oligomeric structures of the purified Ara h1 demonstrated strong IgE binding properties, whereas the allergenic property of the oligomeric Ara h3 could not be performed due to lack of availability of specific IgE. In addition, the polyclonal antibodies raised against the purified Ara h1 and Ara h3 showed highly specific binding to their respective antigens.

KEYWORDS: *anion exchange chromatography, size exclusion chromatography, peanut allergens, Ara h1, Ara h3, 2D electrophoresis, mass spectrometry, human IgE antibodies, rabbit IgG antibodies*

INTRODUCTION

Peanut protein triggers allergenic reactions in hypersensitive individuals. The prevalence of peanut allergy is about 0.4−0.6% in children and 0.3−0.7% in adults in developed countries.[1,2] Several surveys have indicated a significant increase in peanut allergy around the world, particularly in children.[3] Peanut allergens Ara h1, Ara h2, and Ara h3 are categorized as major allergens because these proteins have proven to be recognized by >50% of the peanut patient sera.[4−7] Ara h1 is recognized by >90% of the peanut-hypersensitive individuals.[4] Ara h3 has been identified as a major or minor allergen, depending on the population sampled. This allergen triggers an IgE-mediated reaction in 44−77% of the peanut allergic population.[7−9]

Ara h1 adopts a homotrimeric form when present at a relatively low concentration, and this form is thought to have an important role in allergenicity.[10,11] The formation and stability of Ara h1 trimer is largely due to hydrophobic interaction between monomers, although the cooperative interaction of monomer also involves ionic interactions.[11] The purification of native Ara h3 as a heteromultimeric protein indicated a molecular weight of ∼400 kDa.[12] The fractionation by anion exchange chromatography of the same allergen shows a number of peptides with molecular weights ranging from 14 to 45 kDa by SDS-PAGE.

Cultivated peanuts (*Arachis hypogaea*) are categorized into four major market types: Virginia, Runner, Spanish, and Valencia.[13] The Virginia and Runner market types are classified under subspecies *hypogaea* var. *hypogaea*, whereas Valencia and Spanish market types belong to the subspecies *fastigiata* var. *fastigiata* and subspecies *fastigiata* var. *vulgaris*. The Virginia type is mainly used for roasted and salted peanuts in shell and shelled and honey-coated snacks. The Spanish type is typically used in peanut butter industries, in shelled roasted peanuts with or without testae, and in confectionery factories. The Valencia type is usually consumed in-shell after roasting and boiling, whereas the Runner type is utilized in peanut butter manufacture, in salted nut snacks, and in confectionary.[13]

Several studies have reported the purification and characterization of specific Ara h proteins. Generally, single- or two-step purification using anion exchange chromatography alone or anion exchange followed by size exclusion chromatography was conducted. Burks et al.[4] purified Ara h1, and a year later, Ara h2 using anion exchange chromatography.[5] De Jong et al.[14] also purified Ara h1 and Ara h2 using a single chromatographic separation. Two sequential steps of anion exchange chromatography, followed by size exclusion chromatography, were conducted to purify Ara h1 and Ara h3.[12,15,16] Each of these previous studies generally conducted their experiments on a single cultivar. No study has been conducted on the purification of Ara h1 and Ara h3 from the four main market types to compare their relative abundance of oligomeric structures as well as isomeric numbers of dissociated forms of Ara h1 and Ara h3. This study, therefore, was conducted to closely examine the molecular organization of the Ara h1 and Ara h3 oligomers, isolated from the four main cultivars representing four market types.

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MATERIALS AND METHODS

Sample Preparation. Raw peanuts of four market types, namely, Virginia (Middleton cultivar), Runner (Menzies cultivar), Spanish (Walter cultivar), and Valencia (Kelinci cultivar), were ground with a mortar and pestle. They were defatted three times using *n*-hexane (1:5 w/v) by shaking on a rotary shaker at 200 rpm for 15 min, and the *n*-hexane layer was discarded. The defatted peanut meal was dried in a fume hood overnight. Extraction of the defatted peanut meal was carried out by shaking in 20 mM Tris buffer (pH 7.2) containing 1 mM EDTA and 20 μg/mL leupeptin hemisulfate salt for 1 h at 4 °C. The extract was centrifuged initially at 3000 rpm and then at 20000 rpm for 20 min, at 4 °C. The supernatant was filtered through 0.45 μm membranes and was concentrated using an Amicon centrifugal filter unit using a membrane with a 1000 kDa cutoff. The concentration of protein was determined by a bicinchoninic acid kit (Sigma) using BSA as a standard. The extract was stored at −80 °C until used.

Purification of Ara h1 and Ara h3 Using Anion Exchange Chromatography. Purification of Ara h1 and Ara h3 was carried out by anion exchange chromatography followed by size exclusion chromatography. For anion exchange chromatography, a single 5 mL High Q column (Bio-Rad) fitted on the Biological LP (Bio-Rad) setup was used. The column was initially equilibrated using 20 mM Tris-HCl (pH 7.2), and the purification was carried out as follows. Sample dissolved in a volume of loading buffer that was 2.5 times the column volume (CV) was applied at a flow rate of 1.5 mL/min. After column washing, bound proteins were then eluted using a NaCl gradient from 0 to 0.4 M at a flow rate of 1.5 mL/min. Strongly bound proteins were eluted by applying 1.5 CVs of 1 M NaCl at the same flow rate. The eluted proteins were monitored at 280 nm, and 1−5 mL fractions were collected. SDS-PAGE was subsequently carried out for each of the fractions.

Purification of Ara h1 and Ara h3 Using Size Exclusion Chromatography. Prior to use, the Superdex 200 column was calibrated using a gel filtration standard (Sigma) containing the following molecular weight markers; bovine carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase from yeast (150 kDa), β-amylase from sweet potato (200 kDa), apoferritin from horse spleen (443 kDa), bovine thyroglobulin (669 kDa), and blue dextran (2000 kDa). Fractions that contain Ara h1 and Ara h3 were loaded onto the Superdex 200 with a flow rate of 0.4 mL/min using an AKTA purifier system (Pharmacia). An elution buffer of 20 mM Tris-HCl−0.15 M NaCl was used during the purification, and fractions were collected in 2 mL volumes.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was conducted in accordance with the technique reported by Laemmli[17] involving 15% hand-cast gels and 10−20% Tris-HCl gradient precast gels (Bio-Rad). Protein samples were reduced and denatured in Laemmli sample buffer (1:1 v/v). Prior to loading, the samples were preheated to 90 °C for 15 min. Prestained molecular weight markers (6.5, 14.2, 20, 24, 29, 36, 45, 55, 66, 97, 116, and 200 kDa) (Sigma) were used as reference. Electrophoresis was performed at a constant voltage of 150 V in Tris glycine buffer.

Acetone−TCA Precipitation with DTT. Defatted peanut meal (1 g) was extracted in 10 mL of a chilled solution of 20 mM Tris-HCl (pH 7.2) containing 1 mM EDTA and 20 μg/mL leupeptin hemisulfate salt, for 1 h at 4 °C with constant stirring. Centrifugation was conducted at 15000 rpm at 4 °C for 20 min, and the supernatant was filtered through a 0.45 μm filter. Then, 300 μL of the supernatant was transferred into a 2 mL tube and mixed with a chilled solution of 10% trichloroacetic acid (TCA) in acetone with 25 mM DTT. After incubating at −20 °C for 15 min, the mixture was centrifuged at 15000 rpm at 4 °C for 5 min. The supernatant was decanted, and the pellet was washed twice with cold acetone containing 25 mM DTT. Residual acetone was removed, and the pellet was stored at −80 °C until used.

Two-Dimensional Electrophoresis. The collected fractions from size exclusion chromatography were precipitated in cold acetone by incubation at −20 °C for 1 h. The solution was centrifuged at 15000 rpm at 4 °C for 15 min. The supernatant was discarded, and the residual acetone was removed by placing the tubes in an ice bath until the pellet was completely dried. The protein pellet was resolubilized in a lysis solution containing 8 M urea, 2% (w/v) CHAPS, 75 mM DTT, and 2% IPG buffer 3−10 prior to sample loading. A 7 cm IPG strip (pH 3−10 linear) was rehydrated in the rehydration solution containing 8 M urea, 2% (w/v) CHAPS, 75 mM DTT, 2% IPG buffer 3−10, and 0.002% bromophenol blue for 12 h. The protein sample was loaded into the rehydration solution (in gel rehydration), and isoelectric focusing was performed.

Prior to performance of the second-dimension electrophoresis, the IPG strip was equilibrated in an equilibration solution containing 6 M urea, 0.5 M Tris-HCl (pH 8.8), 30% glycerol, 2% SDS, and 2% DTT for 15 min. The second equilibration was performed by adding 2.5% iodoacetamide in the absence of 2% DTT for 15 min. SDS-PAGE was conducted on 12.5% gels, and the gels were stained with Coomassie brilliant blue R-250.

In-Gel Digestion and Mass Spectrometry Identification. In-gel digestion of protein spots and mass spectrometric analysis were conducted as follows. Protein spots were excised manually from the SDS-PAGE gel and dehydrated in acetonitrile. After removal of the acetonitrile, the trypsin digestion was performed by placing samples into 50 mM ammonium bicarbonate buffer (pH 8.0) containing trypsin, and the mixture was incubated overnight at 37 °C. The supernatant was collected by centrifugation, and further extraction of peptides was conducted by washing with 20 mM ammonium bicarbonate and washing three times with 5% formic acid in 50% acetonitrile. Pooled supernatant was dried using a vacuum centrifuge. A buffer containing 0.1% heptafluorobutyric acid and 0.5% formic acid was added to the sample prior to mass spectrometric (MS) analysis.

Sequence Analysis. The mass spectral results were sequenced using the BLAST protein database (www.blast.ncbi.nlm.nih.gov). Multiple alignments were performed by Clustal W (1.82) using default parameters (www.ebi.ac.uk/clustalw).

Immunoblotting with Serum IgE. To verify the serum IgE binding of the purified allergens, immunoblotting was performed as follows. A nitrocellulose membrane that was blotted with peanut protein from a SDS-PAGE gel was placed in 10 mL of blocking buffer consisting of 3% BSA in 100 mM Tris-buffered saline (pH 7.5) for 45 min with constant agitation. The blocking buffer was then replaced with the pooled patient sera (the Allergy Unit, Royal Prince Alfred Hospital, NSW) diluted in Tris-buffered saline (1:20). The pooled patient serum was constituted by mixing equal volumes of three sera from selected patients showing strong positive skin pick tests and strong in vitro interaction with higher (e.g., Ara h1) and lower molecular peanut allergens (e.g., Ara h2/6). The membrane was incubated for 90 min at 25 °C with constant agitation. The membrane was washed three times with 100 mL of 0.5% Tween 20, each for 10 min. Then, goat anti-human IgE-HRP in the blocking buffer with 0.1% Tween 20 (1:5000) was incubated for 25 min at 25 °C. Three washings were conducted with the last washing using Milli-Q water. For color development, the membrane was incubated with 5 mL of 3,3′,5,5′-tetramethylbenzidine solution for 7−10 min until the desired color was achieved.

Raising Polyclonal Antibodies. Polyclonal antibodies specific to the purified Ara h1 and Ara h3 were raised in rabbits. Four female white New Zealand rabbits were injected subcutaneously at multiple sites with the purified Ara h1 and Ara h3 emulsified in TiterMax gold (Sigma) adjuvant. Booster injections with Freund's incomplete adjuvant were given at 4 week intervals. Sera were harvested 1 week after each booster injection.

The titer of the specific antibodies was determined by titrating against their respective antigens immobilized on a microtiter plate. Briefly, purified Ara h1 or Ara h3 (1 μg/well) was coated on the mirowell plate. After the plate had been washed three times as previously described, the diluted sera were incubated for 30 min, and the plates were washed three times. Goat anti-rabbit IgG−HRP conjugate (at 1:10000 dilution, 100 μL per well) was applied for 30 min. After the washing step, color was developed by incubating the 3,3′,5,5′-tetramethylbenzidine substrate solution (100 μL per well) for 30 min. The color development was stopped by adding 0.125 M H_2SO_4 (50 μL per well), and absorbance was measured at 450 and 650 nm.

Immunoblotting with Rabbit Antibodies. Proteins separated on SDS-PAGE were blotted onto a nitrocellulose membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) at 10 V. Free binding sites of the membrane were blocked with 3% BSA in 100 mM Tris buffer (pH 7.5) containing 0.9% sodium chloride (TBS) for 45 min at room temperature with constant agitation. The membrane was incubated with rabbit serum (1:1000) for 90 min at 25 °C with constant agitation. The blot was washed three times using 100 mL of 0.5% Tween 20. Goat anti-rabbit IgG−HRP in 1% BSA in TBS with 0.1% Tween 20 (1:5000) was incubated for 25 min. Finally, three washings were performed, with the last washing using Milli-Q water. For visualization, the membrane was incubated in 10 mL of the substrate solution containing 3,3′,5,′5′-tetramethylbenzidine and urea hydrogen peroxide for 20 min.

RESULTS AND DISCUSSION

Purification of Ara h1 and Ara h3 Using Anion Exchange Chromatography. The initial attempt to purify Ara h1 and Ara h3, which employed a Uno Q column (Bio-Rad), showed poor resolution (data not shown). Purification of peanut allergens was subsequently conducted using a High Q column (Bio-Rad). This column separated Ara h1 and Ara h3 from the protein extract of Middleton with a linear salt gradient of 0−0.4 M followed by a rapid increase to 1 M and maintained for 100 min (Figure 1). Ara h3 bound more strongly to the solid phase than Ara h1 and needed a higher salt concentration to elute. This finding was similar to the previous studies using the Source Q column (Pharmacia).[12,18]

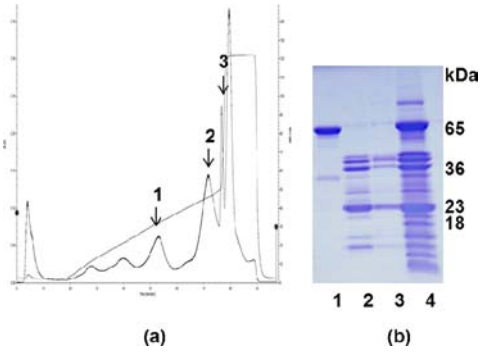

Figure 1. (a) Ion exchange chromatography of crude peanut protein from Middleton using a 5 mL High Q (Bio-Rad) column. The slope is the conductivity of the eluate, representing the salt gradient. The numbers indicate the collected fractions. (b) SDS-PAGE (10−20% gradient gel) of fractions 1−3. Lanes 1−3 are the collected fractions, and lane 4 is the total peanut protein.

Fraction 1, eluted at ∼0.25 M NaCl, contained a high quantity of Ara h1 (Figure 1). The acidic (∼36, ∼38, ∼42 kDa) and basic (∼23 kDa) subunits of Ara h3 were eluted in fractions 2 and 3 at 0.34−0.36 and 0.44−1.0 M NaCl, respectively. Protein extracts of Menzies, Walter, and Kelinci were fractionated using similar conditions. No apparent differences in the ion exchange chromatographic profiles of the protein extracts from the four cultivars were observed.

Purification of Ara h1 and Ara h3 Using Size Exclusion Chromatography. Further purification of partially purified Ara h1 and Ara h3 obtained from the anion exchange chromatography was conducted using a Superdex 200 column as a second chromatographic step. In general, Ara h1 from the four cultivars resulted in similar elution profiles with one major peak after 150 min (Figure 2a). Middleton had a small shoulder peak eluting just before the major peak, whereas one small

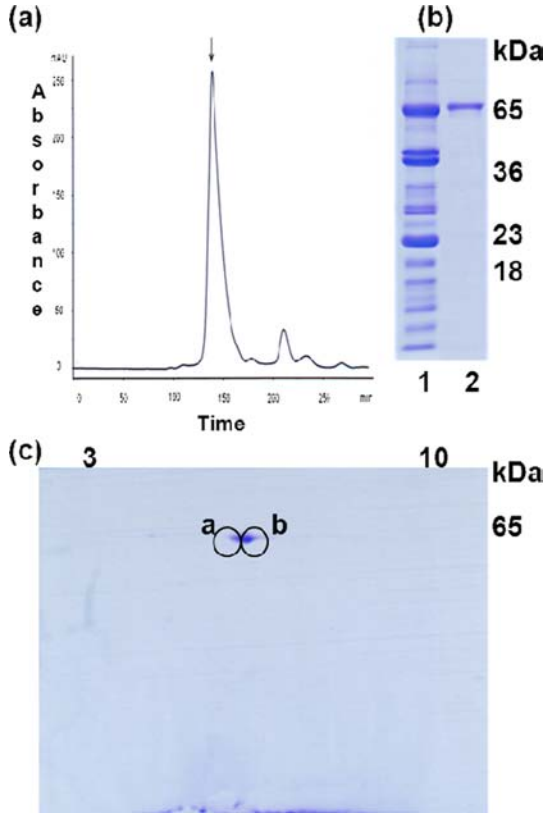

Figure 2. (a) Size exclusion chromatography of Ara h1 from Walter using a Superdex 200 column. The arrow shows the position of the collected fraction. (b) SDS-PAGE of the purified Ara h1. Lane 1 is the crude peanut protein, and lane 2 is the purified Ara h1. (c) 2D electrophoresis of the purified Ara h1 using 3−10 linear IPG strips, 15% SDS-PAGE. Circled areas a and b represent protein spots excised for MS analysis.

shoulder peak was observed just after the major peak for Kelinci. Both the major and shoulder peaks elicited a ∼65 kDa band on SDS-PAGE (Figure 2b), suggesting the peaks are likely to be part of Ara h1. Aggregation or dissociation of Ara h1 during the purification may result in the shoulder peak.

The apparent molecular weight of Ara h1, as calculated by the protein markers on the size exclusion chromatography, varied between ∼430 and ∼550 kDa, suggesting that the native Ara h1 may exhibit an oligomeric structure rather than a trimeric conformation (∼180 kDa) as previously reported.[10,11,19] Elution of oligomeric Ara h1 of an estimated molecular weight of ∼660 kDa via size exclusion chromatography also has been previously reported.[15] However, the same study observed an interaction between Ara h1 with an anion exchange material subsequently affecting the quaternary structure and resulting in trimeric Ara h1. Our results with anion exchange chromatography did not show any changes in the quaternary structure and the presence of trimeric Ara h1. This could be due to different anion exchange materials being used, even though they may have similar properties (i.e., quaternary ammonium bead-type), that give rise to the observed difference in association/dissociation behavior of Ara h1.

Unlike Ara h1 and more interestingly, the four peanut cultivars exhibited different distributions of Ara h3 oligomers from the size exclusion chromatography. The two major peaks of Ara h3 eluting at ∼150 and ∼165 min were found in

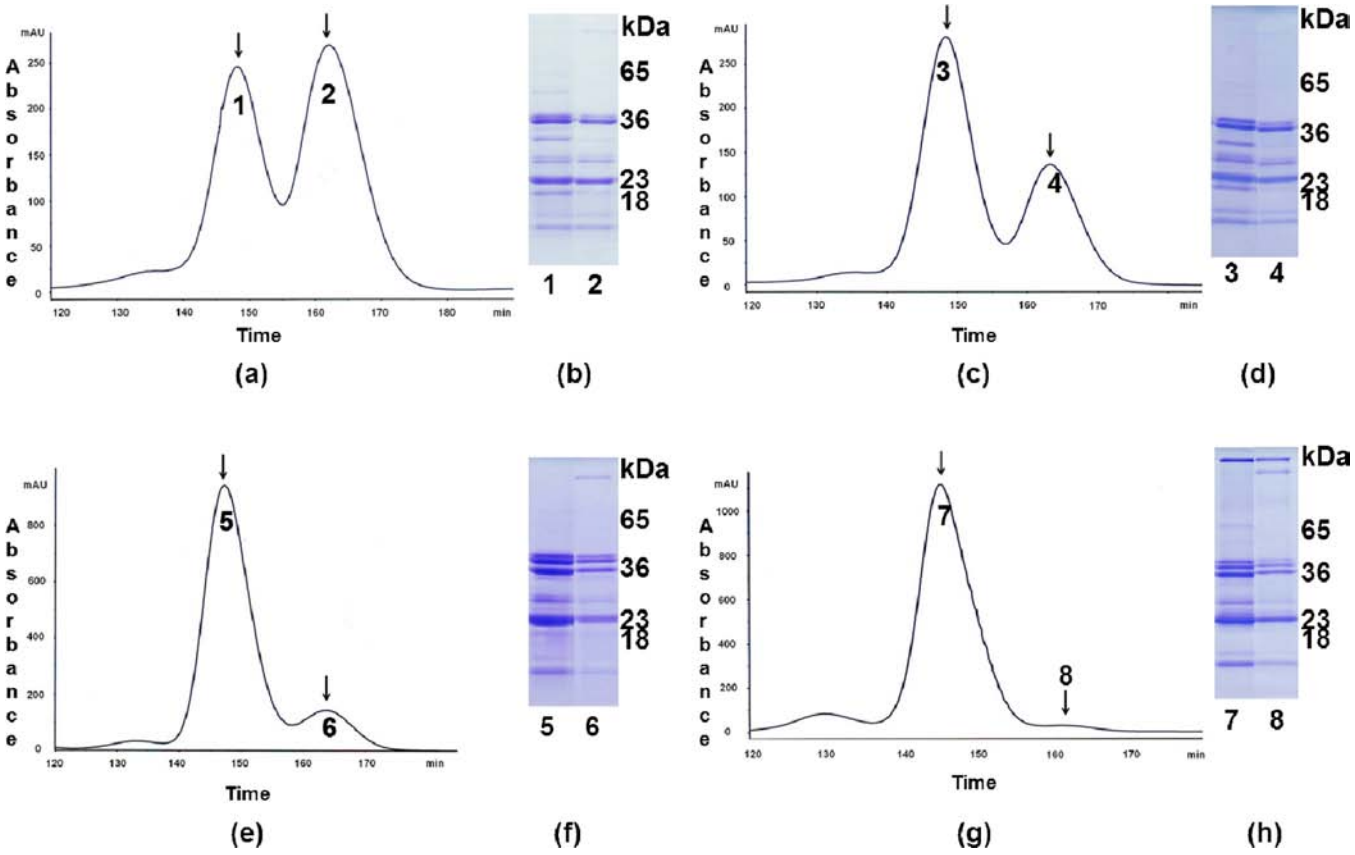

Figure 3. (a) Size exclusion chromatography of Ara h3 from Menzies; (b) SDS-PAGE of the first peak (lane 1) and the second peak (lane 2) of Menzie's Ara h3. (c) Size exclusion chromatography of Ara h3 from Walter; (d) SDS-PAGE of (lane 3) the first peak and (lane 4) the second peak of Walter's Ara h3. (e) Size exclusion chromatography of Ara h3 from Middleton; (f) SDS-PAGE of (lane 5) the first peak and (lane 6) the second peak of Middleton's Ara h3. (g) Size exclusion chromatography of Ara h3 from Kelincil (h) SDS-PAGE of (lane 7) the first (main) peak and (lane 8) the second (minor) peak. Size exclusion chromatography was performed using a Superdex 200 column. The arrows indicate the positions of the collected fractions.

Menzies and Walter, but in different proportions between the first and second peaks (Figure 3a,c). Middleton and Kelinci had one major peak and one minor peak eluted at around similar times (Figure 3e,g).

The differential distribution of Ara h3 trimer and hexamer may be due to the differences between post-translational modification of 11S globulin storage proteins and stability of this conformation in the experimental conditions (i.e., 0.15 M NaCl). These 11S storage proteins are approximately 350 kDa, and they consist of both acidic and basic subunits. These storage proteins are the product of a synthesis of 60 kDa preglobulins as precursors, consisting of covalently linked acidic and basic polypeptides, which are deposited in storage bodies.[20] In these storage bodies, ~180 kDa trimers are generally produced.[20,21] Asparagine-dependent endopeptidase then cleaves the structures, resulting in an NH_2-terminal acidic chain and a COOH-terminal basic chain. These two polypeptides appear as ~35 and ~20 kDa on SDS-PAGE, respectively. A disulfide bond links these two polypeptides to form a subunit that appears as the ~60 kDa on SDS-PAGE.[22] Hexamers of this structure are then created as a matured form of the protein.[23] Consistent with this finding, Rabjohn et al.[8] found that Ara h3 existed as a hexameric structure of ~400 kDa. In addition, Ara h3 also was found to exist partly as trimers and mostly as hexamers at an ionic strength of 0.2 M NaCl.[12]

The molecular weights of the two peaks were estimated in our study to be ~170 and ~310 kDa, on the basis of the protein markers. The ~170 kDa form was likely to be a trimeric structure of Ara h3, whereas ~310 kDa represents higher order oligomers, probably a hexamer. The SDS-PAGE of the two major peaks of Menzies and Walter showed two major protein bands with molecular weights of ~42 and ~38 kDa (Figure 3b,d), whereas Middleton and Kelinci had three major protein bands of acidic subunits at ~42, ~38, and ~36 kDa, respectively (Figure 3f,h). A band at ~23 kDa (Figure 3b,d,f,h) and several minor bands ranging from ~12 to ~35 kDa were also observed. The bands of ~24, ~26, ~36, ~38, and ~42 kDa were probably N-terminal polypeptides of the acidic subunit of Ara h3 that were truncated C-terminally at different positions.[18] Similarly, the ~15 and ~16 kDa bands were C-terminal polypeptides of the acidic subunit that were truncated N-terminally. On the basis of the results obtained, it is suggested that cultivars (or market types) that consisted of three acid subunits of Ara h3 either exhibit mostly as hexameric conformation or may be more stable against pH and ionic strength of the extraction and purification conditions, whereas cultivars (or market types) that consisted of two acidic subunits demonstrate Ara h3 in both trimeric and hexameric structures. Ara h3 oligomers in these cultivars may also be more susceptible to extraction and purification conditions and, thus, are more easily dissociated into trimeric structure.

Two-Dimensional Electrophoresis of Ara h1 and Mass Spectrometric Identification. To verify the purity and identity of the purified Ara h1, 2D electrophoresis was

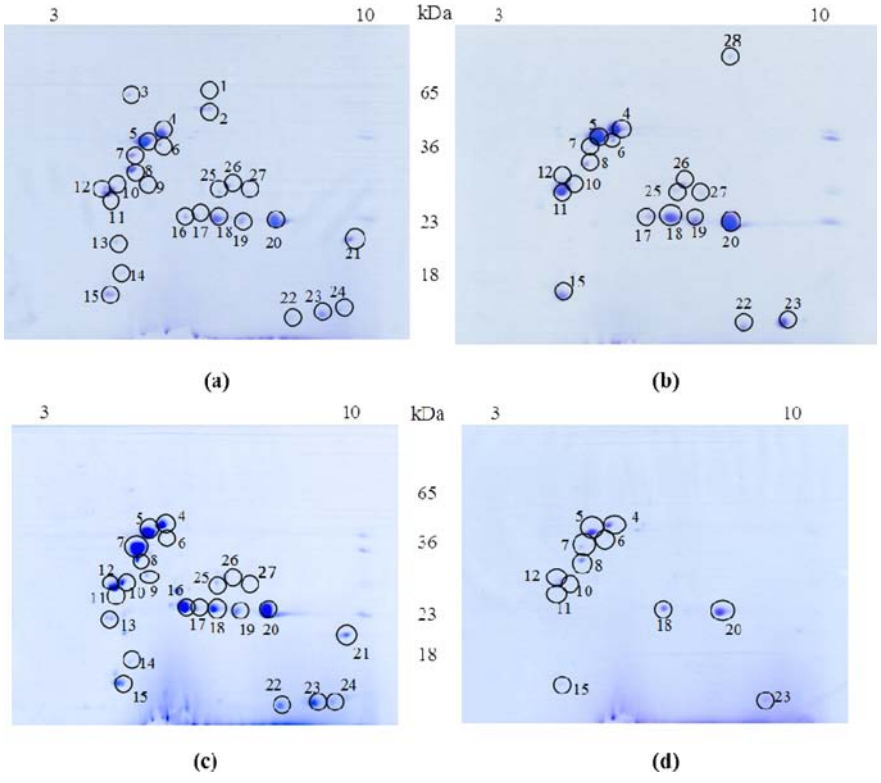

Figure 4. 2D electrophoresis of (a) the first peak and (b) the second peak of Ara h3 isolated from Menzies and (c) the major peak and (d) the minor peak of Ara h3 from Middleton using 3−10 linear IPG strips, 15% SDS-PAGE. The numbers represent protein spots excised for the MS analysis.

Table 1. Analysis of the 14 Accessions (GenBank or Swiss-Prot) Using Alignment Sequences of Protein BLAST with the Full Allergens Ara h1 and Ara h3

protein spots	GenBank (gb) or Swiss-Prot (sp)	protein	identity (%)	positive (%)	gap (%)
1, 2	sp|P43237.1|ALL11_ARAHY	allergen Ara h1	22	48	16
5, 11, 12, 15, 18, 19, 20, 21, 28	gb|ABL14270.1|	arachin 6 [*Arachis hypogaea*]	90	93	0
5, 18, 19, 20, 21, 23	gb|ACH91862.1|	arachin Ara h3 isoform [*Arachis hypogaea*]	95	96	0
4, 6, 7, 14	gb|AAM46958.1|AF510854_1	allergen Ara h3/Ara h4 [*Arachis hypogaea*]	88	92	2
26, 27	gb|AAM93157.1|	trypsin inhibitor [*Arachis hypogaea*]	94	96	0
4, 5, 24	gb|AAC63045.1|	glycinin [*Arachis hypogaea*]	100	100	0
16, 17, 19, 20, 28	gb|AAR02860.1|	storage protein [*Arachis hypogaea*]	88	92	2
9, 16, 20, 22	gb|AAT39430.1|	glycinin [*Arachis hypogaea*]	70	77	8
5, 18, 19, 20, 21, 23	gb|AAU21491.1|	arachin Ahy-2 [*Arachis hypogaea*]	88	91	2
3, 13, 21	gb|AAU21493.1|	conarachin [*Arachis hypogaea*]	41	54	16
22, 24	gb|AAD47382.1|	glycinin [*Arachis hypogaea*]	91	93	0
5, 10, 12, 27, 28	gb|AAW56067.1|	arachin Ahy-4 [*Arachis hypogaea*]	97	97	0
9, 16	sp|Q647H2.1|AHY3_ARAHY	arachin Ahy-3	65	74	9
10, 18, 19, 20, 21, 25, 27	gb|AAG01363.1|	Gly1 [*Arachis hypogaea*]	92	94	0

performed. The purified Ara h1 fraction from Middleton and Menzies migrated to the same p*I* at ∼5.5 (Figure 2c). The two major spots with an estimated molecular weight of ∼65 kDa were verified as the full-length Ara h1 by the MS analysis. Hence, the two-step chromatography was successful in isolating native Ara h1 with an estimated purity of >95%.

The 2D electrophoresis of peanut protein also resulted in the fragmentation of Ara h1 into acidic (∼33 kDa, p*I* values ∼5) and basic fragments (∼18 kDa, p*I* values ∼9.5) with different p*I* value and molecular weights.[24] No protein spots at ∼18 and ∼33 kDa, however, were observed in our study. This may be due to two factors: (1) Ara h1 remained stable under the storage conditions and conditions of isoelectric focusing and (2) Coomassie blue staining may not be sufficiently sensitive to detect these low abundant proteins.

Two-Dimensional Electrophoresis of Ara h3 and Mass Spectrometric Identification. The Ara h3 from Middleton and Menzies, which represent Ara h3 with three and two acidic subunits, respectively, were investigated using 2D electrophoresis. The two acidic subunit bands (∼38 and ∼42 kDa) of Menzies cultivar migrated to p*I* values ∼5 (Figures 3b and 4a,b). The third acidic subunit (∼36 kDa) was slightly more acidic than the other two subunits and migrated to a p*I* lower than ∼5 (Figures 3f and 4c,d). The additional band in Middleton resolved on SDS-PAGE was observed at 23 kDa and was distributed into several spots, with p*I* values between ∼7 and ∼9 (Figure 4). The second chromatographic peak of

Menzies and Middleton resolved into fewer spots in the 2D gel than that of the main peak.

Of the total 28 protein spots derived from the first and second peaks of Ara h3 (Figure 4a), the spots numbered 1 and 2 were identified as Ara h1 by the MS analysis. Ara h1 eluted just before Ara h3 and tailing of Ara h1 peak coeluted with the Ara h3 peak. Reducing sample load on chromatographic purification as a remedy to this tailing issue resulted in purer protein, but also resulted in lower quantity that could be isolated per run.

Only 12 protein spots were recovered from the minor peak of Ara h3 from the Middleton cultivar (Figure 4d), whereas 19 protein spots were recovered from the second major peak of Ara h3 from the Menzies cultivar (Figure 4b). Spots 9, 13, 14, 16, 21, and 24 were not observed in the second major peak of Ara h3 (Menzies). Either these proteins exist in lower quantities in this cultivar, or the original second peak's protein structure was less complex compared to the first peak. Protein spots 3−28 were identified by the MS analysis and are listed in Table 1.

Immunoblotting with Serum IgE. To determine whether the purification performed has significantly altered the structure of the purified allergens, the allergenicity of the purified allergens was evaluated by IgE binding study using the pooled sera from patients showing hypersensitivity toward peanuts. All of the purified Ara h1 (∼65 kDa) from the four cultivars interacted positively with the pooled human sera (data not shown). This indicated that the purified Ara h1 probably retained its native form and remained allergenic. Unfortunately, allergenicity of the purified Ara h3 could not be confirmed as the IgE from patient sera used are specific only to Ara h1 and lower molecular weight and lack the specific binding to Ara h3.

Polyclonal Antibodies against the Purified Ara h1 and Ara h3. Rabbits immunized with the purified Ara h1 and Ara h3 from Middleton and Menzies produced high-titer antibodies within 3 months of immunization. The antibodies against Ara h3 from Menzies and Middleton generated consistently high titer at each booster immunization. Similar results were observed in the antibodies raised against Ara h1 from Menzies and Middleton. Both Ara h1 and Ara h3 from the two cultivars/market types were evidently immunogenic, able to elicit strong adaptive immune response in the animals, and the antibody maturity occurred within 3 months of immunization.

Immunoblotting with Rabbit Antibodies. Unfortunately, the antibodies raised against Ara h1 from Menzies showed binding with the ∼42 kDa basic subunit of Ara h3. This may be due to a trace amount of Ara h3 that remained in the Ara h1 immunogen preparation, even though it was not detectable by the 2D electrophoresis. On the other hand, the antibodies raised against Ara h1 from Middleton were highly specific to Ara h1, showing no detectable nonspecific binding with other peanut proteins (Figure 5a).

Similarly, the antibodies raised against Ara h3 from Menzies detected Ara h1 (∼65 kDa). This could be due to a trace of Ara h1 remaining in the purified Ara h3 as fragmented conarachin (spot 8 at ∼34 kDa, p*I* ∼ 4.5). On the other hand, the antibodies raised against Ara h3 from Middleton demonstrated specific binding to all three subunits of Ara h3.

To investigate the epitopes of the antibodies generated from Middleton's Ara h1 and Ara h3, immunoblotting of proteins fractionated by 2D electrophoresis was conducted. Figure 5a demonstrates the specific binding of the antibodies raised against Ara h1 to ∼65 kDa protein at p*I* values from ∼4 to ∼6.5. The antibodies also detected two small spots at ∼34 kDa, p*I* ∼5.5. The denaturing agent (e.g., urea) and reducing agent (e.g., DL-dithiothreitol) used in the sample preparation may further lead to denaturation and dissociation of Ara h1 into fragmented proteins at ∼34 kDa (p*I* ∼ 5.5), which is approximately half the molecular weight of the intact Ara h1 monomer.

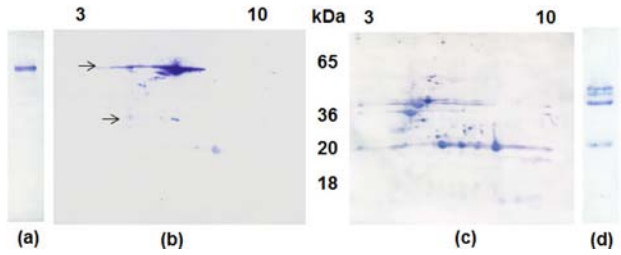

Figure 5. (a, b) Immunoblotting of IgG raised against Ara h1 from Middleton versus total peanut protein in (a) SDS-PAGE and (b) 2D electrophoresis. Arrows show the protein spots recognized by the patient IgE. (c, d) Immunoblotting of IgG raised against Ara h3 from Middleton versus total peanut protein in (d) SDS-PAGE and (c) 2D electrophoresis.

As expected, the antibodies raised against Ara h3 detected spots of Ara h3 acidic subunits and several spots of basic subunits (Figure 5b). The antibodies raised against the purified Ara h3 immunogen detected all of these polypeptides. The basic subunits at ∼15 kDa were detected, however, with low intensity. This could be due to the low immunogenicity of Ara h3 basic subunit polypeptides.

In summary, the purification of Ara h1 from the four peanut cultivars (market types) using anion exchange chromatography followed by size exclusion chromatography presented Ara h1 in multimeric forms in all of the cultivars/market types, rather than a trimer as generally accepted. The purified Ara h3 from the same cultivars/market types, however, exhibited different quaternary structures, showing differential distribution of trimeric and hexameric structures. On the basis of the results obtained, it can be speculated that peanut cultivars consisting of two acidic subunits of Ara h3 display both trimeric and hexameric conformation, whereas peanut cultivars consisting of three acidic subunits display predominantly hexameric structures of Ara h3. However, the current study could not examine the allergenicity of the purified Ara h3 oligomers due to lack of availability of Ara h3 specific serum IgE and whether such differences in the quaternary structures observed in different cultivars/market types play a role in the protein's allergenic potency. The purified Ara h1 still demonstrated strong IgE binding, suggesting much of the native conformational structure and its IgE binding epitopes must have been retained. The antibodies from the immunized rabbits showed high specificity with their respective immunogens prepared from the purified Ara h1 and Ara h3. The successful elicitation of specific antibodies (IgG) in rabbits against the native Ara h1 and Ara h3 suggests the strong antigenic potency of these proteins.

The present study could not deduce whether the observed differential distribution of different oligomeric structures of Ara h1 and Ara h3 are purely genetic variation or whether certain degrees of genetic and environmental interaction also play an important role. Such structural variation of the allergens in different cultivars/market types could influence how food processing might affect the final presentation of these allergens

to our digestive and immune systems. Further studies to understand the implications of such structural variation on allergenicity of different cultivars/market types are warranted.

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ABBREVIATIONS USED

BSA, bovine serum albumin; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G; IgE, immunoglobulin E; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; 2D electrophoresis, two-dimensional electrophoresis.

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