Allergen Ara h 1 Occurs in Peanuts as a Large Oligomer Rather Than as a Trimer

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Ara h 1, a major peanut allergen, is known as a stable trimeric protein. Nevertheless, upon purification of native Ara h 1 from peanuts using only size exclusion chromatography, the allergen appeared to exist in an oligomeric structure, rather than as a trimeric structure. The oligomeric structure was independent of the salt concentration applied. Subjecting the allergen to anion exchange chromatography induced the allergen to dissociate into trimers. Ammonium sulfate precipitation did not bring about any structural changes, whereas exposing the allergen to hydrophobic interaction chromatography caused it to partly dissociate into trimers, with increasing amounts of trimers at higher ionic strengths. The (partial) dissociation into trimers led to a change in the tertiary structure of the monomeric subunits of the allergen, with the monomers in Ara h 1 oligomers having a more compact tertiary structure compared with the monomers in Ara h 1 trimers. As structural characteristics are important for a protein's allergenicity, this finding may imply a different allergenicity for Ara h 1 than previously described.

KEYWORDS: Peanut; food allergen; Ara h 1; protein structure

INTRODUCTION

Factors that are considered to be important for the allergenicity of food proteins are the ability of a protein to induce the production of and binding to IgE antibodies and their resistance to digestion in the human gastrointestinal tract. These features largely depend on the structural characteristics of the protein. Accordingly, changes in these characteristics may result in changes in allergenicity, such as changes in epitope structure, epitope accessibility, and protein digestibility (*1*, *2*). It is, therefore, important to investigate allergens in their natural form, as distinct differences in characteristics may result in inaccurate conclusions about their allergenic activity (*3*).

Peanut allergy is the third most prevalent food allergy (*4*), and Ara h 1 is recognized by >90% of individuals sensitized to peanut, rendering it a major peanut allergen (*5*). The protein is described as a 63 kDa glycoprotein that is present in peanuts as a highly structured, stable trimer (*6*−*8*). The trimers are reported to be stabilized mostly by hydrophobic interactions and, to a lesser extent, by ionic interactions (*7*). Throughout the amino acid backbone of the protein 23 linear epitopes have been mapped (*9*). Most IgE epitopes are clustered near the regions of the hydrophobic monomer−monomer contacts and, accordingly, upon formation of trimers ∼40% of the residues within the epitopes are excluded from the surface of the protein. Thus,

the formation of trimers is believed to protect Ara h 1 from protease digestion and denaturation, thereby promoting its allergenic properties (*7*).

Ara h 1 is classified into the 7S globulins, which are often denoted vicilins. Vicilins are seed storage proteins belonging to the cupin superfamily. Members of the cupin superfamily share a conserved domain comprising a six-stranded β-barrel structure (*10*). Together with the 11S globulins (legumins), the vicilins account for the majority of cupin allergens (*11*). Besides Ara h 1, vicilins that are known to have allergic activity are, for example, vicilins from peas (*12*), walnuts (*13*), soybeans (*14*), and cashews (*15*). Because of the structural similarity of vicilins it is not surprising that cross-reactive IgE binding between Ara h 1 and other vicilins exists: Cross-reactivity between Ara h 1 and pea vicilin (Pis s 1) (*12*) and between Ara h 1 and soybean β-conglycinin (*16*) has been described.

Vicilins are reported to be present as trimeric, often glycosylated, proteins ranging from ∼150 to 190 kDa, with subunits from ∼50 to 80 kDa (*11*). Although they share only 35−45% amino acid sequence identity with the 11S globulins, they are similar in their three-dimensional structure: both 11S and 7S globulins have similar N-terminal and C-terminal domains, comprising the cupin β-barrel structure (*17*). Some vicilins undergo post-translational proteolytic processing, yielding various polypeptides next to the intact monomer. However, all subunits are held together by non-covalent interactions, keeping the trimeric structure intact (*18*). During post-translational

processing of Ara h 1 an N-terminal peptide of ∼85 amino acids is cleaved (*19*). Nevertheless, the remaining protein remains intact.

The 7S globulin β-conglycinin shows an association/dissociation behavior, depending on pH and ionic strength: at high ionic strength ($I > 0.5$) the protein occurs as a trimer, but at low ionic strength ($I < 0.2$) the protein reversibly associates into hexamers. At intermediate ionic strengths the two forms coexist. Furthermore, at low ionic strength ($I = 0.01$), together with an associated form, a dissociated form of the protein may exist, caused by the reversible dissociation of one of the subunits from the protein (*20*). This behavior has never been reported for Ara h 1.

For the isolation of Ara h 1 several protocols have been described. Most of these methods include ion exchange chromatography (*5*−*8*), sometimes preceded by ammonium sulfate precipitation (*6*, *7*). Recently, Boldt and co-workers (*21*) investigated a peanut extract for protein complexes, rather than looking at purified proteins. They described that Ara h 1 exists in peanuts as a complex with Ara h 3 isoallergens.

Although Ara h 1 in literature is always described to form stable trimers, after purification of the allergen using commonly used purification techniques, we found indications that at least part of the protein occurred in a higher oligomeric form. To further investigate this finding, the aim of our study was to purify native Ara h 1 and to investigate its possible association/dissociation behavior.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Merck (Darmstadt, Germany), unless stated otherwise. Peanuts of the runner market type were generously provided by Imko Nut Products (Doetinchem, The Netherlands) and were stored at 4 °C until use.

Protein Extraction. Peanuts were ground using a domestic type mechanical high-speed slicer (Kenwood Corp., Tokyo, Japan) and defatted with hexane using Soxhlet extraction. After drying at room temperature, the partially defatted meal was ground using a Waring blender (Waring Products Inc., New Hartford, CT) and was subjected to a second defatting step. Next, the defatted meal was dried at room temperature for 24 h and stored at 4 °C until further use. Defatted meal was extracted by stirring in 50 mM Tris-HCl buffer, pH 8.2, at a meal/solvent ratio of 1:10 (w/v) for 1 h at room temperature. Afterward, the extract was filtered through a cheesecloth and subsequently centrifuged (25 min; 14000*g*; 4 °C). The supernatant obtained was filtered over a 1.2 μm filter and denoted peanut extract.

Size Exclusion Chromatography (SEC). Protein samples were applied onto either a 320 mL Superdex 200 XK 26/60 column (5−13 mL applied per run) or a 25 mL Superose 6 10/30 column (100 μL applied per run; GE Healthcare, Uppsala, Sweden). The columns were equilibrated and eluted with 50 mM Tris-HCl buffer, pH 8.2, with or without 0.3 M NaCl, at a flow rate of 4.3 or 0.5 mL min^{-1}, respectively, using an Äkta purifier system (GE Healthcare). The Superose column was calibrated using a gel filtration calibration kit according to the instructions of the supplier (GE Healthcare). The eluate was monitored at 280 and 325 nm, and appropriate fractions were collected.

Anion Exchange Chromatography (AEC). A 25 mL Source Q XK 26/10 column was equilibrated with 50 mM Tris-HCl buffer, pH 8.2, using an Äkta explorer system (GE Healthcare) and loaded with sample at 10 mL min^{-1}. After washing, a 10 column volume linear gradient from 0 to 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 8.2, was applied at a flow rate of 10 mL min^{-1}. The eluate was monitored at 280 nm, and appropriate fractions were collected.

Hydrophobic Interaction Chromatography (HIC). Ammonium sulfate was added to protein samples to a final concentration of 3.3 M. Samples were stirred for 3 h and left overnight to settle. After centrifugation (15 min; 10000*g*; 4 °C). the supernatant was added to 15 mL of Source 15 phenyl material (GE Healthcare). The suspension was collected on a G3 glass filter and washed with 3.3 M ammonium sulfate in 50 mM Tris-HCl buffer, pH 8.2. The bound material was eluted with 20 mL of water, and the eluate was denoted Ara h 1$_{HIC}$.

Dialysis. Fractions were dialyzed in 10 kDa MWCO slyde-a-lyzer mini dialysis units (Pierce, Rockford, IL) against 50 mM Tris-HCl buffer, pH 8.2. Afterward, samples were stored at 4 °C until use or lyophilized and stored at −20 °C until use.

Protein Quantification. The nitrogen content of samples was determined using the combustion (Dumas) method on a NA 2100 Nitrogen and Protein Analyzer (CE Instruments, Milan, Italy). The instructions of the supplier were followed, and methionine was used as a standard.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein samples were mixed 1:1 with sample buffer [1.4 mL of distilled water, 2.0 mL of 0.5 M Tris-HCl, pH 6.8, 2.0 mL of 10% (w/v) SDS, 2.0 mL of glycerol, and 0.4 mL of 0.05% (w/v) bromophenol blue]. Reducing conditions were obtained by adding β-mercaptoethanol to a final concentration of 10 mM and heating the samples for 5 min at 100 °C. Tris-HCl 10−20% linear gradient Ready Gels (Bio-Rad Laboratories Inc., Hercules, CA) were used. To each lane was applied 10 μL of protein sample, and gels were run at 200 V. Gels were stained according to the Coomassie Brilliant Blue procedure provided by the manufacturer. Low molecular weight protein standards (GE Healthcare) were used according to the instructions of the manufacturer.

Circular Dichroism (CD) Spectroscopy. Far- and near-UV CD measurements were performed in the spectral range from 190 to 260 nm and from 250 to 350 nm, respectively. Protein solutions (0.25 mg mL^{-1} for far-UV CD and 0.5 mg mL^{-1} for near-UV CD) in 5 mM sodium phosphate buffer, pH 7.0, were analyzed. Spectra were recorded on a Jasco-715 spectropolarimeter (Jasco Inc., Easton, MD), thermostated at 20 °C. A step resolution of 0.5 nm, a scan speed of 100 nm min^{-1}, a bandwidth of 1 nm, and a response time of 0.125 s were used. Ten scans were accumulated and averaged. Buffer spectra were subtracted from protein spectra. The secondary structure content from far-UV CD measurements was estimated using a spectral nonlinear least-squares fitting procedure, using reference spectra (*22*).

Fluorescence Spectroscopy. Tryptophan fluorescence spectra of 0.2 mg mL^{-1} protein solutions in 5 mM sodium phosphate buffer, pH 7.0, were recorded on a Perkin-Elmer luminescence spectrophotometer LS 50 B (Perkin-Elmer Corp., Boston, MA) with a pulsed xenon source. Excitation was performed at 295 nm, and emission spectra were recorded as the average of three spectra from 300 to 450 nm, using a scan speed of 100 nm min^{-1} and a resolution of 0.5 nm. Both the excitation and the emission slit were set at 2.5 nm. Spectra were corrected by subtracting the spectrum of a protein free sample obtained under identical conditions.

(In-Gel) Digestion. Protein bands in SDS-PAGE gels were cut manually and destained overnight in 200 μL of 50% (v/v) methanol and 5% (v/v) acetic acid. After a washing with 200 μL of 50% (v/v) acetonitrile in 50 mM Tris-HCl buffer, pH 8, for 15 min at 37 °C, the gel pieces were dehydrated in 200 μL of acetonitrile for 5 min at 37 °C. The acetonitrile was removed, and the gel pieces were dried at ambient temperature in a vacuum centrifuge (Christ, Osterode, Germany). Next, samples were incubated with 30 μL of 10 mM DTT in 100 mM Tris-HCl buffer, pH 8, for 45 min at 65 °C. The solvent was removed, and subsequently 30 μL of 100 mM iodoacetamide in 100 mM Tris-HCl buffer, pH 8, was added. Next, samples were incubated for 30 min in the dark at room temperature. The solvent was removed, and the gel pieces were dehydrated for 5 min in 200 μL of acetonitrile at room temperature. The acetonitrile was removed, and the gel pieces were dried in a vacuum centrifuge. Next, 30 μL of a 2 mg L^{-1} trypsin solution (Roche, Mannheim, Germany, article 11418025001) in 50 mM Tris-HCl buffer, pH 8, was added, and samples were rehydrated on ice for 10 min. Afterward, the excess of trypsin solution was removed, and 50 mM Tris-HCl buffer, pH 8, was added until the gel pieces were completely covered. Digestion was performed overnight at 37 °C. The solution was collected, and 30 μL of 50 mM Tris-HCl buffer, pH 8, was added to the gel pieces. After 10 min at room temperature, this

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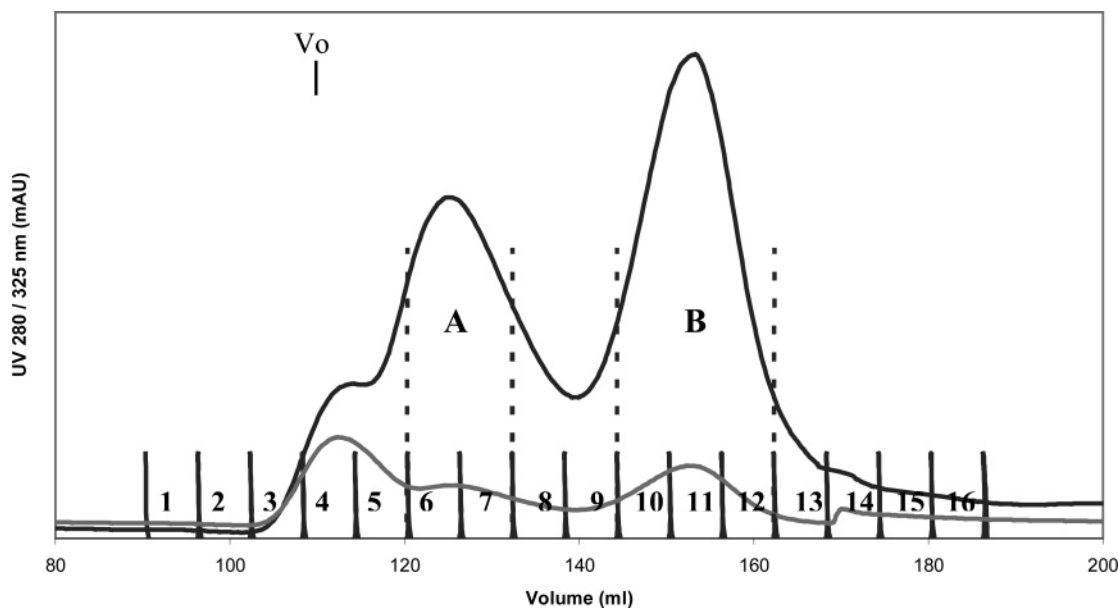
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Figure 1. SEC of peanut extract on a Superdex 200 column in 50 mM Tris-HCl buffer, pH 8.2 ($I = 0.02$ M): (black line) UV absorbance at 280 nm; (gray line) UV absorbance at 325 nm. Indicated are the fractions that were collected for SDS-PAGE (solid lines, fractions 1–16) and SEC peaks A and B (dashed lines, indicated with A and B).

buffer solution was also collected, and 30 μL of 50% (v/v) acetonitrile and 5% (v/v) formic acid was added to the gel pieces. Samples were incubated for 10 min at room temperature before the solution was also collected. This step was repeated once. The collected solutions were combined, and their volume was reduced to <20 μL in a vacuum centrifuge. Acetonitrile and formic acid were added to concentrations of 5 and 0.05% (v/v), respectively, and samples were analyzed with liquid chromatography–mass spectrometry (LC-MS).

Direct digestion of protein samples was performed by adding 2 μL of a 0.5 mg mL^{-1} trypsin solution to 100 μL of protein sample in 50 mM Tris-HCl buffer, pH 8.2 (~1 mg mL^{-1}). Samples were incubated overnight at 37 °C. The pH of the samples was adjusted to ~3 with 10% (v/v) formic acid, and samples were analyzed with LC-MS.

Nanospray LC-MS (Nano-LC-MS). The LC apparatus consisting of an autosampler (Famos), a column switching device (Switchos), and a HPLC pump (Ultimate; all from LC Packings, Sunnyvale, CA), controlled by Xcalibur v1.3 software, was connected to an LCQ Deca XP Max System with a nanospray (NSI) probe (Thermo Electron, Waltham, MA). Trypsin-digested protein samples (1 μL) were applied onto a C18 PepMap μ-precolumn cartridge (5 μm, 100 Å, LC Packings) operating at a flow rate of 0.2 μL min^{-1} at room temperature. After 10 min, the guard column was switched on-line with a C18 PepMap capillary with a pore size of 300 Å (LC Packings). A linear gradient from 95% eluent A [0.05% (v/v) formic acid in water] and 5% eluent B [0.05% (v/v) formic acid in acetonitrile] to 55% A and 45% B in 45 min was applied, followed by a linear gradient from 55 to 5% A and from 45 to 95% B in 25 min. The LCQ was operated in the positive mode using a spray voltage of 3.5 kV. The capillary temperature was 200 °C, and the capillary voltage was 49 V. Mass spectra were collected in a full mass scan, followed by an MS^2 and MS^3 scan of the highest peak in the spectrum.

MS results were analyzed using Bioworks software v3.1 (Thermo Electron). The filter type was single threshold, xcorr versus charge state. For the analysis of the peptides, the Swissprot database (http://www.expasy.org/sprot/) was used containing (1) all annotated sequences, (2) all annotated peanut sequences, and (3) Ara h 1 sequence (P43237) (*23*), respectively.

RESULTS AND DISCUSSION

Protein Purification. The major peanut allergen Ara h 1 is described as a stable trimer (*6–8*) and is classified into the vicilin family, of which some members are known to show association/dissociation behavior depending on the ionic strength
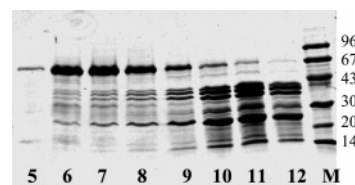

Figure 2. SDS-PAGE of fractions collected during SEC of peanut extract on a Superdex 200 column. The numbers represent the fraction numbers in **Figure 1**. M indicates low molecular weight marker, indicated at right in kilodaltons.

and pH. On the basis of deviant observations during the isolation of Ara h 1, the aim of our research was to investigate the possible association and/or dissociation behavior of Ara h 1. It was chosen to purify the allergen using only SEC at a low ionic strength, to ensure that no structural changes would be induced by the purification method used. Other processing steps such as freezing/thawing and lyophilization were also avoided during the purification.

When the peanut extract was applied to a preparative SEC column, the elution pattern given in **Figure 1** was obtained. The first shoulder peak in the chromatogram eluted in the exclusion volume of the column. This shoulder peak was followed by two larger peaks, which were denoted SEC peak A and SEC peak B, respectively. The shoulder peak showed a relatively high absorption at 325 nm compared to the absorption at 280 nm, indicating the presence of other compounds instead of proteins. This was confirmed by SDS-PAGE: no protein bands were visible in the fractions collected under this peak (data not shown).

SDS-PAGE of fractions under SEC peaks A and B (**Figure 2**) showed that in fractions 5–8, representing SEC peak A, the predominant protein band corresponded to ~65 kDa. This protein band was earlier designated Ara h 1 (*5*). Nano-LC-MS analysis of this protein band after in-gel digestion confirmed that it represented Ara h 1: a comparison of the amino acid sequences of the obtained peptides with a Swissprot-based peanut database showed the highest correlation by mass (~44%) with the deduced amino acid sequence of an Ara h 1 clone
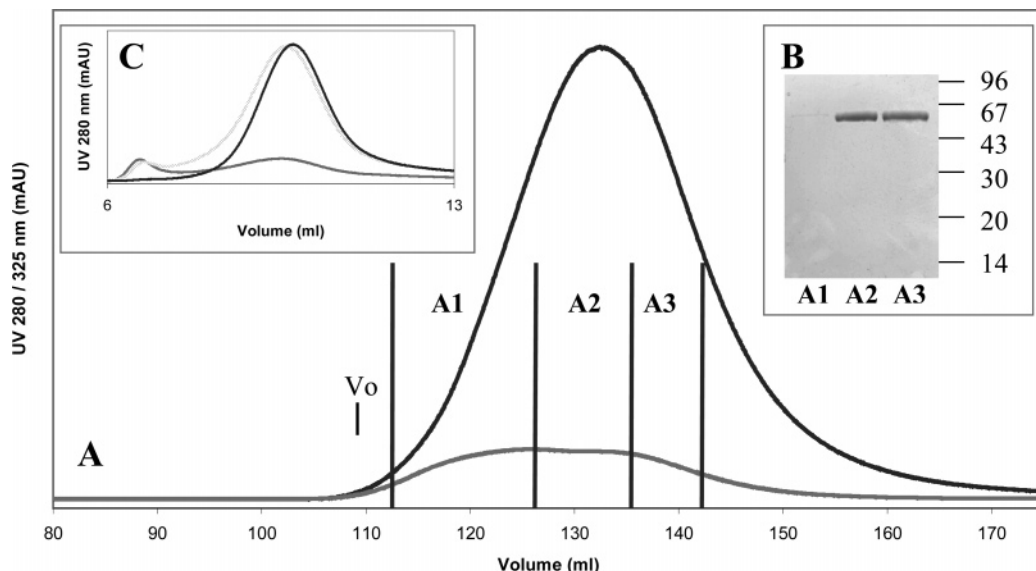
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Figure 3. (**A**) SEC of SEC peak A on a Superdex 200 column in 50 mM Tris-HCl buffer, pH 8.2 (I = 0.02 M): (black line) UV at 280 nm; (gray line) UV at 325 nm. Indicated are the fractions (A1–A3) that were collected. (**B**) SDS-PAGE of fractions A1–A3. Low molecular weight markers are indicated at the right side in kilodaltons. (**C**) SEC of fractions A1–A3 on a Superose 6 column in 50 mM Tris-HCl buffer, pH 8.2 (I = 0.02 M): (gray line) A1; (dashed line) A2; (black line) A3.

P43237 (*23*). The same result was obtained using the complete Swissprot database.

In the subsequent fractions after SEC peak A the Ara h 1 band clearly decreased. In all fractions also several other protein bands were visible, of which the relative intensity increased with the elution volume and which were predominantly present in fractions 10−12, corresponding to SEC peak B. The predominant bands corresponded to 40 and 23 kDa, showing a pattern typical for Ara h 3 (*24*). In-gel digestion was performed, and the amino acid sequences of the peptides were compared with the peanut database. Protein bands that were analyzed were the two most predominant bands around 40 kDa (B1 at ∼40 kDa and B2 at ∼35 kDa) and the protein band of ∼23 kDa (B3). B1 showed the highest correlation by mass (22%) with the deduced amino acid sequence of an arachin-encoding gene (Q5I6T2) (*25*), whereas for B2 the highest correlation by mass (∼18%) was found with the amino acid sequence of a glycinin fragment (Q6IWG5). B3 showed 41% correlation by mass with the amino acid sequence of a cupin protein denoted Gly1 (Q9FZ11), which has been shown to be an Ara h 3 isoallergen (*21*). Results from direct digestion of SEC peak B resulted in 64% correlation by mass with the deduced amino acid sequence of a conarachin gene (Q647H4) and 62% with a gene denoted Ara h 3 (Q8LKN1). These data confirmed that the analyzed protein bands from SEC peak B corresponded to the legumin Ara h 3, which is also called glycinin or arachin (*25*).

Our data thus demonstrated that Ara h 1 on SEC eluted prior to Ara h 3. As Ara h 1 is described as a trimeric protein of ∼180 kDa (*6−8*) and Ara h 3 as a hexamer of ∼400 kDa (*26*), this result was surprising. Because Ara h 1 elutes before Ara h 3 and shortly after the exclusion volume of the column (which has a separation range until ∼600 kDa for proteins), the existence of Ara h 1 in a trimeric structure in peanuts seems to be unlikely: it rather seems that the allergen exists in a higher oligomeric structure. SDS-PAGE analysis of fractions eluting later than SEC peak B did not reveal Ara h 1 in either trimeric or monomeric form. In those fractions only patterns comparable to conglutin proteins were found (Ara h 2 and Ara h 6, data not shown). Peanut extract made from non-defatted peanuts showed

similar results, thus excluding the possibility of Ara h 1 oligomerization induced by defatting (data not shown).

Moreover, next to the observation of Ara h 1 present as higher oligomers, on the basis of our results complex formation of Ara h 1 and Ara h 3, as described by Boldt and co-workers (*21*), seems to be implausible, as an identical SDS-PAGE pattern would then be expected in all fractions. Moreover, as two distinct peaks were recorded from SEC, Ara h 1 and Ara h 3 are likely to exist separately from each other.

To obtain pure Ara h 1 oligomers, SEC peak A was reapplied to the preparative SEC column. The chromatogram is given in **Figure 3A**. At an absorbance of 280 nm a symmetric peak eluted from the column. The peak was fractionated into three parts, denoted A1−A3. The absorbance at 325 nm was relatively low. Nevertheless, the first part of the 280 nm peak showed a comparatively higher absorbance at 325 nm, which was most likely originating from the partially collected shoulder peak as observed in **Figure 1**. SDS-PAGE (**Figure 3B**) showed that Ara h 1 was predominantly present in fractions A2 and A3. Only a faint band at 65 kDa was detected in A1. Furthermore, the purity of Ara h 1 in A2 and A3 was >95%, as estimated using a densitometric scan of the SDS-PAGE gel colored with Coomassie Brilliant Blue.

Analytical SEC of fractions A1−A3 on a Superose 6 column with a theoretical separation range to 2000 kDa for proteins was performed to investigate the purity of the fractions with SEC. It was anticipated that the non-protein shoulder peak, if present in any of the samples, would better be separated from Ara h 1 using this column. Hence, it would be possible to determine which of the samples A1−A3 contained this undesired impurity. The results are given in **Figure 3C**. Both fractions A1 and A2 showed a peak in the exclusion volume of the column, and only A3 showed a symmetric Ara h 1 peak. From these results it was concluded that A3 contained pure Ara h 1 without undesired compounds. The fraction was renamed Ara h 1$_{\text{SEC}}$ and used for further analysis. The apparent molecular weight of the purified Ara h 1 oligomer was estimated to be ∼700 kDa; however, as SEC does not allow a precise deter-
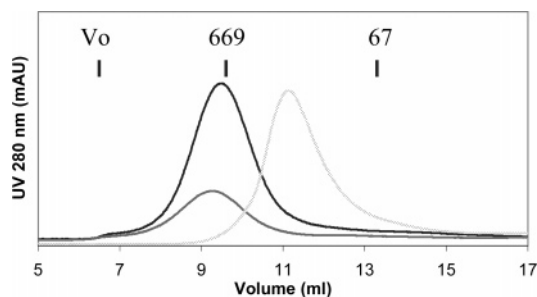
Figure 4. SEC of Ara h 1 on a Superose 6 column in 50 mM Tris-HCl buffer, pH 8.2 ($I = 0.02$ M): (black line) Ara h 1$_{SEC}$; (dashed line) Ara h 1$_{AEC}$; (gray line) Ara h 1$_{ASP}$.

Figure 5. SEC of Ara h 1$_{HIC}$ (black line) and Ara h 1$_{AEC}$ (gray line) on a Superose 6 column in 50 mM Tris-HCl buffer, pH 8.2, containing 0.3 M NaCl ($I = 0.32$ M).

mination of the molecular weight of a protein, no precise estimation of the number of monomers in the protein could be made.

Effects of Purification Methods on Ara h 1 Quaternary Structure. Vicilins are described as trimeric proteins, but reversible association into hexamers is possible, depending on the ionic strength. For β-conglycinin, a lower ionic strength results in a higher amount of hexamers (20). The oligomeric structure of Ara h 1 was found to be independent of the ionic strength applied: the protein eluted as an oligomer when incubated and eluted in buffers with ionic strengths of both 0.02 and 0.32 M. Also, after dialysis from 0.32 to 0.02 M and elution in 0.02 M, still only oligomers eluted from SEC (data not shown).

In the literature Ara h 1 has always been described as a stable trimer of ~180 kDa (7, 8), so our results are contradictory to those described earlier. The structure described for Ara h 1 was in all cases determined after purification of the protein using several other techniques besides SEC. Most of these purification methods included ammonium sulfate precipitation (6, 7), followed by HIC and/or ion exchange chromatography (5–8). As all previously published results point toward trimeric Ara h 1 structures, one would suspect that these generally used purification steps might lead to (ir)reversible dissociation of the naturally occurring oligomeric allergen. To test this hypothesis, various purification techniques were investigated for their influence on the structure of Ara h 1. First, purified Ara h 1 oligomers were applied to an anion exchange column. Ara h 1 appeared to elute as a single peak around 26 mS/cm, corresponding to an ionic strength of ~0.32 M (data not shown). The elution of Ara h 1 at this conductivity corresponds to that reported in the literature (8). The peak was collected and denoted Ara h 1$_{AEC}$ and was analyzed for its quaternary structure with SEC, as can be seen in **Figure 4**. It was shown that Ara h 1$_{AEC}$ eluted as a single peak corresponding to a molecular weight of ~200 kDa. This molecular weight coincides with a trimeric structure of Ara h 1. Thus, anion exchange led to the dissociation of Ara h 1 oligomers into trimers. SEC analysis at various ionic strengths confirmed the irreversible character of the dissociation: Ara h 1$_{AEC}$ eluted as a trimer at ionic strengths of 0.02 M (**Figure 4**) and 0.32 M (**Figure 5**). Also, after dialysis from 0.32 to 0.02 M, the protein only occurred in a trimeric form (data not shown). Accordingly, as it was shown before that adding salt to the protein solution did not induce changes in quaternary conformation, the interactions with the anion exchange material probably led to the irreversible changes in the quaternary structure of Ara h 1.

The presence of a high ammonium sulfate concentration did not induce the association or dissociation of Ara h 1; the protein eluted as an oligomer both at a low (**Figure 4**) and at a high
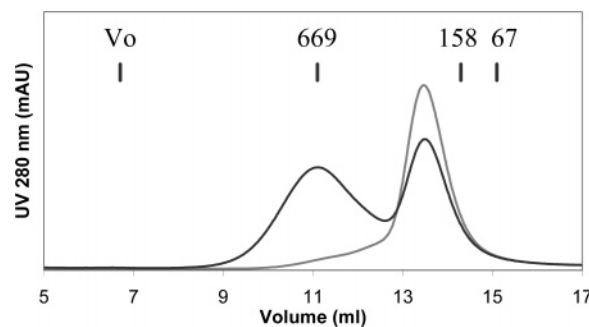
ionic strength (data not shown). Thus, it was concluded that ammonium sulfate did not influence the quaternary structure of Ara h 1.

Furthermore, the effects of HIC on the allergen's structure were investigated. To this end, Ara h 1$_{SEC}$ in 3.3 M ammonium sulfate was bound to and eluted from HIC material and denoted Ara h 1$_{HIC}$. In these samples another phenomenon was observed: at a low ionic strength (0.02 M) only oligomeric Ara h 1 was detected in Ara h 1$_{HIC}$ (data not shown). What was striking, however, were the SEC results at a higher ionic strength (0.32 M): at this ionic strength part of the protein existed in an oligomeric form of ~700 kDa, whereas another part existed in a trimeric form of ~200 kDa (**Figure 5**). After dialysis back to the lower ionic strength, Ara h 1 was again solely present in an oligomeric form (data not shown). Hence, HIC seemed to induce a part of Ara h 1 to dissociate reversibly into trimers at higher ionic strengths.

Structure Analysis. Far- and near-UV CD analysis was performed with Ara h 1$_{SEC}$, Ara h 1$_{HIC}$, and Ara h 1$_{AEC}$ to investigate possible differences in their secondary and tertiary structures. As the presence of Tris buffer disturbed the CD measurement at low wavelengths, Ara h 1 samples were dialyzed and lyophilized and hereafter dissolved in sodium phosphate buffer. SEC analysis before and after lyophilization showed no differences in the elution pattern of the samples (data not shown).

The CD results are given in **Figure 6**. The far-UV CD spectra of all three Ara h 1 samples were similar, having a zero-crossing around 201 nm and a negative extreme around 208 nm, indicative for proteins with a high α-helical content. The spectra were comparable to those described by Koppelman and co-workers (8). With spectral analysis, based on a nonlinear least-squares fitting procedure, it was estimated that the proteins in all three preparations had similar secondary structure contents, consisting of $30 \pm 3\%$ α-helix, $14 \pm 5\%$ β-strand, and $34 \pm 5\%$ β-turn. Thus, the changes in quaternary structure, induced by HIC and AEC, did not result in changes in the secondary structure of Ara h 1.

The near-UV CD spectra of the three Ara h 1 samples were, in contrast to the far-UV CD spectra, not similar, indicating differences in the surroundings of the aromatic amino acid residues. The intensity of the total spectrum of Ara h 1$_{SEC}$ was higher compared to the intensities of the spectra of Ara h 1$_{AEC}$ and Ara h 1$_{HIC}$. Furthermore, Ara h 1$_{HIC}$ and Ara h 1$_{AEC}$ showed similar spectra, except that Ara h 1$_{HIC}$ showed a higher intensity in the 250–260 nm region.

Ara h 1$_{SEC}$ showed a peak around 268 nm, a wavelength at which phenylalanine residues generally absorb (27). A lower intensity at this wavelength, which was observed for Ara h 1$_{HIC}$
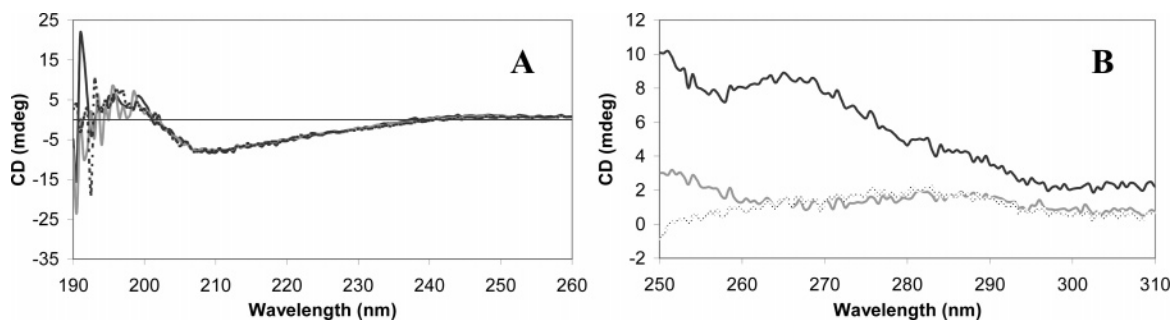
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Figure 6. Far- (**A**) and near-UV (**B**) CD spectra of Ara h 1_{SEC} (black line), Ara h 1_{HIC} (gray line), and Ara h 1_{AEC} (dashed line).
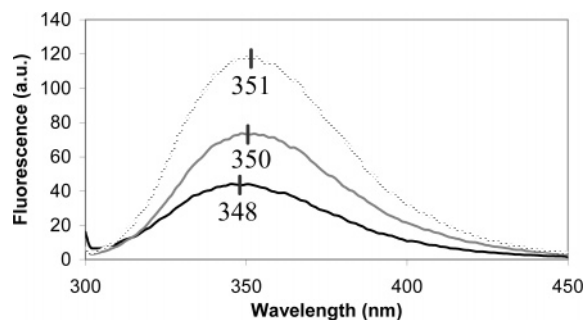

Figure 7. Tryptophan fluorescence spectra of Ara h 1_{SEC} (black line), Ara h 1_{HIC} (gray line), and Ara h 1_{AEC} (dashed line).

Table 1. Quaternary Structure of Ara h 1 after Various Purification Methods at Different Ionic Strengths

ionic strength (M)	purification method[a]			
	SEC	ASP	HIC	AEC
0.02	oligomers	oligomers	oligomers	trimers
0.32	oligomers	oligomers	oligomers + trimers	trimers
$0.32 \rightarrow 0.02$	oligomers	oligomers	oligomers	trimers

[a] SEC, size exclusion chromatography; ASP, ammonium sulfate precipitation; HIC, hydrophobic interaction chromatography; AEC, anion exchange chromatography.

and Ara h 1_{AEC}, implies that the protein core, where the phenylalanine residues are most frequently located, is less compact. Ara h 1_{SEC} also showed a higher intensity in the 270–290 nm region, where tryptophan and tyrosine residues generally absorb (*27*). The lower intensity of Ara h 1_{HIC} and Ara h 1_{AEC} at these wavelengths indicates a lower mobility of the tyrosine and tryptophan residues.

Due to the fact that at the conditions used for CD measurements the proteins in Ara h1$_{AEC}$ were present as trimers, whereas the proteins in Ara h1$_{HIC}$ and Ara h1$_{SEC}$ were present as oligomers, the difference between Ara h1$_{HIC}$ and Ara h1$_{SEC}$ might be explained by the fact that Ara h 1_{HIC} oligomers are partly destabilized, as at a higher ionic strength they partially dissociate into trimers. Taking into account the quite similar near-UV CD patterns of Ara h1$_{HIC}$ and Ara h1$_{AEC}$, the destabilization induced by HIC probably largely corresponds to the destabilization of Ara h 1 induced by AEC.

The intensity of the emission spectra obtained from tryptophan fluorescence spectroscopy measurements (**Figure 7**) was lowest for Ara h 1_{SEC} and highest for Ara h 1_{AEC}. Furthermore, wavelength maxima around 348 nm for Ara h 1_{SEC}, around 350 nm for Ara h 1_{HIC}, and around 351 nm for Ara h 1_{AEC} were measured. These emission wavelengths indicate that the tryptophan residues in all three protein preparations are in a nonpolar environment. An emission maximum at a higher wavelength, as observed for Ara h 1_{HIC} and Ara h 1_{AEC}, indicates that the environment of the tryptophan residues is more polar compared to that of Ara h 1_{SEC}, the latter thus having either a more compact structure or a less exposed tryptophan environment. The increase in fluorescence intensity could be caused by a lower level of quenching, resulting from a lower degree of folding of the tryptophan environment. From these results, which were in agreement with the results from near-UV CD measurements, it could also be concluded that the tertiary structure of stable Ara h 1 oligomers (Ara h 1_{SEC}) is more compact than that of destabilized Ara h 1 oligomers (Ara h 1_{HIC}) and Ara h 1 trimers (Ara h 1_{AEC}): both Ara h 1_{HIC} and Ara h 1_{AEC} have a less compact tertiary structure than Ara h 1_{SEC}, with Ara h

1_{AEC}, consisting solely of trimers, having the least compact protein structure. Thus, subjecting Ara h 1 oligomers to HIC and inducing a reversible and partial dissociation of Ara h 1 oligomers seems to cause a similar but less extensive protein destabilization compared to subjecting the protein to AEC.

On the basis of the results obtained, it can be stated that Ara h 1, a major allergen from peanuts, naturally occurs as an oligomer instead of a trimer. The oligomeric structure of the protein is independent of the ionic strength. Purification techniques have different effects on the allergen's quaternary structure, as summarized in **Table 1**: AEC leads to irreversible dissociation of the allergen, whereas HIC induces a partial and reversible dissociation of oligomers into trimers at higher ionic strengths.

Both AEC and HIC have an influence on the allergen's tertiary structure, and the (reversible) dissociation of Ara h 1 oligomers into trimers induces a less compact protein structure. As all characteristics of Ara h 1 have thus far been determined of the irreversibly dissociated trimeric protein, it could well be that inaccurate conclusions about the protein's characteristics have been drawn, including conclusions about the protein's allergenic capacity. Therefore, further investigation is required of the differences in structural characteristics between oligomeric and trimeric Ara h 1, and these characteristics must be related to the allergen's allergenic activity. Experiments to examine this are currently being conducted at our laboratory.

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