Heat-induced Conformational Changes of Ara h 1, a Major Peanut Allergen, Do Not Affect Its Allergenic Properties

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Stef J. Koppelman‡, Carla A. F. M. Bruijnzeel-Koomen§, Martin Hessing‡, and Harmen H. J. de Jongh**

From the ‡TNO Nutrition and Food Research Institute, 3700 AJ Zeist, §Department of Dermatology/Allergology, University Medical Center Utrecht, 3508 GA Utrecht, and the ¶Centre for Protein Technology TNO-WAU, 6700 EV Wageningen, The Netherlands

Ara h 1, a major peanut allergen was isolated, and its structure on secondary, tertiary, and quaternary level at ambient temperature was investigated using spectroscopic and biochemical techniques. Ara h 1 appeared to be a highly structured protein on a secondary level, possesses a clear tertiary fold, and is present as a trimeric complex. Heat treatment of purified Ara h 1 results in an endothermic, irreversible transition between 80 and 90 °C, leading to an increase in β-structures and a concomitant aggregation of the protein. Ara h 1 from peanuts that were heat-treated prior to the purification procedure exhibited a similar denatured state with an increased secondary folding and a decreased solubility. The effect of heat treatment on the in vitro allergenic properties of Ara h 1 was investigated by means of a fluid-phase IgE binding assay using serum from patients with a clinically proven peanut allergy. Ara h 1 purified from peanuts heated at different temperatures exhibited IgE binding properties similar to those found for native Ara h 1, indicating that the allergenicity of Ara h 1 is heat-stable. We conclude that the allergenicity of Ara h 1 is unaffected by heating, although native Ara h 1 undergoes a significant heat-induced denaturation on a molecular level, indicating that the recognition of conformational epitopes of Ara h 1 by IgE either is not a dominant mechanism or is restricted to parts of the protein that are not sensitive to heat denaturation.

Peanut allergy is one of the most severe food allergies due to its persistency and the life-threatening character (1). The prevalence of peanut allergy in the Western world has been estimated at 1 in 10,000 up to 1 in 200 (2) and seems to be increasing during the last decades. An explanation for this increased prevalence is controversial. The fact that sensitization routes are not always obvious (3) confuses this phenomenon even more. Doses as low as 100 μg may provoke symptoms (4), indicating that accidental ingestion of minute traces of peanut endanger the life of subjects with peanut allergy. The nature of the allergenic compounds in peanuts has been studied extensively in recent years (5, 6), and two major peanut allergens, Ara h 1 (7, 8) and Ara h 2 (8, 9), have been identified. Purified Ara h 1 has been subjected to some biochemical and immunological studies, and it appeared to be a 63-kDa glycoprotein (7) with distinct IgE binding sites both on the protein part (10–12) and on the carbohydrate moiety of the molecule (13). The gene encoding for Ara h 1 was cloned (14), and the gene product resembled similar biochemical and immunochemical properties (15), although the molecular weight appeared to be somewhat higher (16) possibly due to incorrect processing of a pro-peptide sequence (8). Sequence analysis of Ara h 1 showed a significant homology with the vicilin seed storage protein family (12), and, remarkably, only one cysteine residue was found in the entire protein (14). Several isoforms of Ara h 1 with different iso-electric points and slightly different molecular weights have been described (17), and it has been postulated that Ara h 1 is assembled in di- and trimeric complexes (12), even in the presence of surfactants (17).

Peanuts are widely used in the food industry owing to their nutritive value and to their taste. Consumer product diversification led to an increase in recipes containing peanuts, resulting in an increased risk for inadvertent ingestion of peanuts by allergic individuals. Additionally, contamination of intended peanut-free products with traces of peanuts led to several fatal and near-fatal allergic reactions. Test kits to establish the presence of peanut protein in finished foods are currently commercially available (18, 19), but the lack of legislation concerning labeling of food allergens hampers a systematic control of suspected products (20). The origin of peanut proteins in foods is not always obvious. In most cases, roasted or fried peanuts are used because of their improved flavor and taste compared with their raw counterparts. Foods containing vegetable oil, however, may contain peanut proteins (10, 21) that are not heated during processing. Therefore, peanut allergic individuals are exposed to both native and heat-treated peanut proteins and both can provoke allergic reactions. It is generally accepted that peanuts preserve their allergenic character upon heating, as binding properties of IgE and IgG to a crude peanut extract are neither diminished nor enhanced by heating (22, 23).

However, it is not known whether in these complex systems heat treatment leads to denaturation of the peanut allergens on a molecular level; consequently, the effect of heat denaturation on the allergenic properties of the peanut allergens is not known. The aim of this study was to investigate the heat-induced conformational changes of Ara h 1, and to study the coinciding effects on its allergenic properties. We found that native Ara h 1 is a highly structured protein, on secondary, tertiary, and quaternary folding levels. Both heat-treated purified Ara h 1 and Ara h 1 isolated from heated peanuts show conformational changes, whereas the in vitro allergenic poten-
tial is hardly affected. This study clearly shows that the allergenic character of Ara h 1 is heat-stable, although the structural organization of this major peanut allergen is changed significantly upon heating.

MATERIALS AND METHODS

Peanut Pretreatments

Peanuts (Arachis hypogea) from the Runner cultivar (Cargill, Dawson, GA) were generously provided by Imko Gelria (Doetinchem, The Netherlands) and were stored at 10 °C until use. Peanuts were ground and heat-treated at 50, 80, 90, 110, 140, 155, 170, or 200 °C for 15 min in a thermostated prewarmed hot air oven. Heat treatment at 140 °C and 150 °C resulted in a light brown coloration of the ground peanuts and the release of a typical roasted peanut flavor. At higher temperatures, ground peanuts appeared brown (170 °C) or dark brown (200 °C) under the release of a burning smell. Heat treatment up to 110 °C did not give rise to coloration or scent. After heat treatment, the ground peanuts were stored at 4 °C until use. Peanut protein extracts were made by mixing 20 g of ground peanut with 100 ml of 20 mM bis-Tris-propanic buffer (pH 7.2). After 2 h of stirring at room temperature, the aqueous fraction was collected by centrifugation (3,000 × g at room temperature for 30 min). The aqueous phase was subsequently centrifuged (10,000 × g at room temperature for 30 min) to remove residual traces of fat and insoluble particles. The clear extracts were extensively dialyzed against 20 mM bis-Tris-propanic buffer (pH 7.2) at 4 °C. Protein concentrations were determined using Bradford analysis with BSA as a standard. Reducing SDS-PAGE from extracts from ground peanuts heated up to 140 °C showed similar patterns where Ara h 1 migrated as a single band with an apparent molecular mass of 63 kDa, making up approximately 10% of the total extracted protein based on densitometer analysis of the gel. In extracts from ground peanuts heated at 155 °C and higher temperatures some high molecular mass protein bands were absent. Extracts were stored at −20 °C.

Purification of Ara h 1

Ara h 1 was purified generally as described previously (7, 8) with minor modifications. In short, dialyzed extracts from heat-treated and non-heat-treated ground peanuts were applied on an 8-ml Source Q column (FPLC protein purification system, Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM bis-Tris-propane of pH 7.2 (loading buffer) at room temperature. The column was washed with loading buffer until the A280 of the effluent was less than 0.02. Proteins were eluted using a linear sodium chloride gradient in loading buffer (up to 1 M in 200 ml at a flow of 4 ml/min). Fractions were collected and analyzed on SDS-PAGE. Ara h 1 eluted from 290 to 310 mM sodium chloride and appeared to be essentially pure (>95%) as judged from a densitometer scan of an SDS-PAGE gel stained with Coomassie Brilliant Blue. Comparison of non-reduced and reduced SDS-PAGE gels showed that approximately 10% of Ara h 1 was present as di- and trimers. Further purification steps were omitted in order to maintain the native character of Ara h 1. The N-terminal sequence was determined according to the Edman degradation procedure on an Applied Biosystems Protein sequencing system (SeCU, Utrecht, The Netherlands) and appeared to be identical to the earlier published sequence of Ara h 1 (8). Purified Ara h 1 was stored at −80 °C until use. If not mentioned otherwise, samples were desalted on a PD-10 column (Pharmacia, Uppsala, Sweden), previously equilibrated with a 10 mM phosphate buffer (pH 6.7) containing 50 mM sodium chloride. Concentrations of Ara h 1 were determined by absorbance measurements at 280 nm using a molar extinction coefficient of 36130 M−1 cm−1 (A280 0.1% (1 mg/ml) = 0.59) calculated based on the amino acid composition of Ara h 1 (16).

Patient Sera

Serum from 8 adult patients with a documented peanut allergy was used for studying the interaction of Ara h 1 with IgE. Each of these individuals had a positive skin prick test to peanuts and a convincing history of peanut anaphylaxis. The presence of IgE in the serum specific for Ara h 1 was demonstrated by SDS-PAGE and subsequent Western blotting. Both a non-allergic and an allergic, but not peanut-allergic,

1 The abbreviations used are: bis-Tris, 2-[bis(2-hydroxyethyl)aminol-2-(hydroxymethyl)propane-1,3-diol; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; ATR, attenuated total reflection.

2 S. J. Koppelman and H. H. J. de Jongh, unpublished results.
Heat Denaturation of Ara h 1

Near-UV CD—Near-UV CD spectra of 1.0 mg/ml Ara h 1 in 10 mM sodium phosphate buffer (pH 7.4) were recorded as averages of 25 spectra at temperatures ranging from 20 °C to 90 ± 0.5 °C at intervals of 10 °C. Quartz cells with an optical path length of 0.1 cm were used. The temperature in these cells was measured using a thermocouple wire. The scan range was 350–250 nm, the scan speed 50 nm/min, the data interval 0.5 nm, bandwidth 1.0 nm, and the response time 0.25 s.

Temperature resolved experiments were performed monitoring the ellipticity at defined wavelengths both in the far-and near-UV region by heating samples with a rate of 0.5 °C/min and averaging the CD signal over 16 s.

Fluorescence Spectroscopy—Fluorescence spectra of 0.1 mg/ml Ara h 1 in 10 mM sodium phosphate buffer (pH 7.4) were recorded as averages of three spectra on a Perkin Elmer Luminescence Spectrometer LS 50 B with pulsed xenon source. Spectra were recorded at temperatures ranging from 20 to 70 ± 0.5 °C at intervals of 10 °C. Excitation was at 295 nm, and the resulting emission was measured from 305 to 405 nm with a scan speed of 120 nm/min. Both the excitation and emission slits were set at 3.5 nm. Spectra were corrected for a protein-free spectrum obtained under identical conditions, and the spectra were subsequently smoothed using the software supplied by Perkin Elmer.

FTIR—FTIR measurements were performed on an ATR ZnSe crystal after evaporation of the solvent of a 70-μl sample of 1 mg/ml Ara h 1 to such an extent that the peptide backbone remained at least fully hydrated, based on the shape of the H2O/amide A band at 3400–3100 cm⁻¹. Spectra were recorded as averages of 16 scans on a Bio-Rad FTS 6000 Spectrometer equipped with a KBr beamsplitter, a deuterated triglycerin sulfate detector with an Eurotherm automatic temperature controller. Spectra were recorded from 400 to 6000 cm⁻¹ and stored from 1200 to 2000 cm⁻¹, with a nominal resolution of 2 cm⁻¹. The spectral resolution was enhanced to 1 cm⁻¹ by zero filling prior to Fourier transformation. The interferograms were symmetrized, and the contribution of atmospheric water was eliminated by subtraction of the appropriate spectrum.

Turbidity Experiments

A stock solution of 5 mg/ml Ara h 1 in 10 mM phosphate buffer (pH 6.7) in the presence of 50 mM sodium chloride was heated to 60 °C. Aliquots of this prewarmed stock solution were added to the same buffer in a cuvette with a 1-cm path length, equilibrated at 85 °C making final protein-concentrations from 0.05 to 1.0 mg/ml. Next, the absorbance at 400 nm of the sample was monitored on a Hitachi U-3000 spectrophotometer at 85 °C for 1 h under continuous stirring of the sample.

Ultracentrifugation Experiments

To determine sedimentation coefficients of the Ara h 1 samples 5–20% sucrose step gradients (12 ml total volume) were prepared in 10 mM phosphate buffers (pH 6.7). Prior to the experiments, the gradients were allowed to diffuse to linearity during 24 h at 4 °C, and 0.3-ml aliquots of 4 mg/ml Ara h 1 were loaded on top of the gradient. Next, the tubes were centrifuged in a Beckman L60 centrifuge at 186,000 × g for 16 h at 20 °C. After centrifugation, the gradient was fractionated in 0.5-ml aliquots, of which the absorbance at 280 nm was determined. Sedimentation coefficients were estimated after calibration of the gradient in a separate experiment using proteins with known S values (γ-globulin (11.2 S), catalase (7 S), BSA (4.4 S), trypsin (2.5 S), and ribonuclease (1.78 S)).

Size Exclusion Chromatography

Chromatographic analysis of samples containing 0.1 mg/ml Ara h 1 was performed using a Pharmacia Smart System on a Superdex 200 column (3.2 × 300 mm; Pharmacia Biotech, Uppsala, Sweden), equilibrated and run at 20 °C at 80 μl/min in a 10 mM sodium phosphate buffer (pH 7.0) filtered through 0.2-μm filters (Schleicher & Schuell, Dassel, Germany). Prior to analysis, the Superdex 200 column was calibrated using blue dextran (2.000 kDa), thyroglobulin (667 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), and sodium ascorbate (176 Da). Samples were loaded onto the column using a 50-μl loop.

Differential Scanning Calorimetry (DSC)

DSC was performed on a Micro-DSC III (Setaram, Caluire, France) using 0.9-ml vessels and a detection limit for transitions of minimal 84 μJ g⁻¹ °C⁻¹. A 4 mg/ml Ara h 1 solution in 10 mM sodium phosphate buffer in medium was heated for 15 min at 20 °C isolated from peanuts heated for 15 min at 20 °C (solid line), 50 °C (short dash), 80 °C (medium dash), 90 °C (long dash), 110 °C (dotted line) and 140 °C (dotted/dashed line).

Near-UV CD spectra of 1.0 mg/ml Ara h 1 in 10 mM sodium phosphate buffer (pH 7.4) were recorded as averages of 25 spectra at temperatures ranging from 20 °C to 90 °C at intervals of 10 °C. Quartz cells with an optical path length of 0.1 cm were used. The temperature in these cells was measured using a thermocouple wire. The scan range was 350–250 nm, the scan speed 50 nm/min, the data interval 0.5 nm, bandwidth 1.0 nm, and the response time 0.25 s.

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inhibition of IgE binding as a function of the amount of the different forms of Ara h 1. The concentration needed for half-maximal inhibition were calculated using a semi-logarithmic equation and were used to compare the affinities of the IgE. The plot.

**IgE Binding Experiments**

Affinities of Ara h 1 for IgE were measured using IgE binding experiments generally according to Burks et al. (23). Dilutions of Ara h 1 purified from peanuts treated at different temperatures (final concentrations: 0.003–100 μg/ml, calculated based on the A280 and the molar extinction coefficient) were incubated with a 1:30 dilution of patient serum in phosphate-buffered saline (PBS) containing 1% BSA and 0.1% Tween 20. In this fluid phase, Ara h 1 contained 22 phenylalanines, 6 tyrosines, and 5 tryptophans, which generally absorb in the 260–290 nm, 280–300 nm, and 300–320 nm region, respectively, when these residues are involved in a tertiary interaction network (27). From this spectrum, a distinct tertiary fold can be ascribed to the protein, based on the CD intensities observed, comparable to those found for other plant storage proteins like patatin (29) or glycine. An alternative for studying tertiary interactions is by monitoring the tryptophan fluorescence of Ara h 1 (spectrum not shown). The observed fluorescence maximum of 348 nm, however, is close to that of free tryptophan in an aqueous environment (353 nm), whereas a solvent-buried local environment of the tryptophans would have maxima around 330–335 nm (31). This indicates that the tryptophans of Ara h 1 are relatively on the outside of the protein.

To get insight in the tertiary folding level of Ara h 1, near-UV CD spectra have been recorded (Fig. 3A), which can serve as a measure for the existence of tertiary interactions. Two major spectral bands with a positive ellipticity can be observed, one centered around 280 nm, and one around 312 nm. Ara h 1 contains 22 phenylalanines, 6 tyrosines, and 5 tryptophans, which generally absorb in the 260–290 nm, 280–300 nm, and 300–320 nm region, respectively, when these residues are involved in a tertiary interaction network (27). From this spectrum, a distinct tertiary fold can be ascribed to the protein, based on the CD intensities observed, comparable to those found for other plant storage proteins like patatin (29) or glycine. An alternative for studying tertiary interactions is by monitoring the tryptophan fluorescence of Ara h 1 (spectrum not shown). The observed fluorescence maximum of 348 nm, however, is close to that of free tryptophan in an aqueous environment (353 nm), whereas a solvent-buried local environment of the tryptophans would have maxima around 330–335 nm (31). This indicates that the tryptophans of Ara h 1 are relatively on the outside of the protein.

To investigate the conformational state of Ara h 1 at a quaternary level, ultracentrifugation experiments have been performed to determine the S value. Fig. 3A shows the sucrose-gradient profile, where it can be seen that the protein distribution displays a symmetric band of approximately 8 S, based on calibration of the gradient with various proteins with known S values. S 8 would correspond to a protein-complex of 180–200 kDa, indicative for a trimer form of Ara h 1. Another indication for a trimer organization of this protein is provided by size exclusion chromatography on a calibrated Superose 6 column, which elution profile is displayed in Fig. 4B. The peak observed at 1.25 ml corresponds to a mass of 180 kDa (for globular proteins).

**Structural Changes of Isolated Ara h 1 during Heat Treatment**—To study the heat denaturation of Ara h 1, DSC experiments have been performed as presented in Fig. 5. A clear endothermic transition can be observed with an onset temperature of 83 °C and a maximum at 87 °C. The energy content of this transition is 30 kcal/mol. Lowering the heating rate did not affect the position of the transition, indicating that the sample was always in thermodynamic equilibrium under the conditions used (results not shown). Upon cooling of the sample, no transition was observed, demonstrating that the denaturation was not reversible, and complete, as indicated by the second heating scan. These latter two traces are also displayed in Fig. 5, but are shifted vertically to improve the clarity of the figure.

To get insight in what the effects are of heat treatment on the conformation of the protein at the distinct folding levels, we monitored on-line the changes in CD ellipticity in the far-UV range at 200.8 and at 207 nm as a function of the temperature with a heating rate similar to that used for the DSC experi-

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**Fig. 4.** A, elution profile of Ara h 1 in 10 mM phosphate buffer (pH 7.4) on a 5–20% sucrose density gradient after ultracentrifugation at 186,000 × g for 16 h at 20 °C. The top and bottom of the gradient and the positions of reference proteins with known S values are indicated in the plot. B, elution profile of Ara h 1 in 10 mM phosphate buffer (pH 7.4) on a Superdex 200 column at 20 °C. The excluded and included volume of the column are indicated.

buffer (pH 7.4) was heated from 20 °C to 100 °C with a scan rate of 0.5 °C/min, cooled to 20 °C with 3 °C/min, and subsequently reheated to 100 °C with 0.5 °C/min.

**RESULTS**

**Structural Properties of Ara h 1 at Ambient Temperatures**—Fig. 1A displays the far-UV CD spectrum of Ara h 1 at 20 °C. The spectrum has a negative extreme around 209 nm, with a small shoulder around 222 nm, and crosses zero ellipticity at 199 nm. Such a spectrum is characteristic for a protein with a high degree of structures at a secondary level (25). Spectral analysis to obtain an estimation of the secondary structure content using non-linear least square regression procedures reveals 31% α-helices, 36% β-structures, and 33% random coil. This secondary structure content is qualitatively confirmed by the shape of the amide I band in the IR spectrum of Ara h 1 (Fig. 2A, solid line) where a maximum is observed at 1638 cm⁻¹, indicative for a high degree of β-structures (26, 27). Also, a clear shoulder around 1660 cm⁻¹ is apparent, indicating a comparable amount of helical structures (28).

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3 Lakemond, C. M. M., de Jongh, H. H.-J., Hessing, M., Gruppen, H., and Voragen, A. G. J., submitted for publication.
resulting in a more pronounced shoulder at 1630 cm⁻¹. The shape of the amide I band is affected by the heating step, indicating a change in the secondary structure. The temperature at which the CD-spectra does not change up to 80 °C (not shown), but only the overall intensity increases. This could be caused by a reduced absorption flattening of the spectra at higher temperatures, due to dissociation of the quaternary complex of the protein. Above 80 °C a decrease in negative ellipticity is apparent, but this is accompanied by an increased level of optical density of the sample caused by extensive aggregation of the material. Apparently, denaturation of Ara h 1 results in an immediate aggregation behavior. That this denaturation of Ara h 1 does affect the secondary structure content of the protein, as shown by ATR-FTIR measurements of the heated material after cooling to 20 °C, as shown in Fig. 2A (dashed line). Clearly the shape of the amide I band is affected by the heating step, resulting in a more pronounced shoulder at 1630 cm⁻¹, indicative for the formation of extended β-structure most probably related to the aggregation of the material (32), and an increased intensity around 1658 cm⁻¹. Apparently, denaturation of Ara h 1 leads to a secondary more structured conformation of the protein.

When monitoring the changes in ellipticity in the near-UV CD spectra of Ara h 1 as a function of temperature, it can be seen that whereas the intensity at 288 nm is at 80 °C only reduced by approximately 25%, the ellipticity at 313 nm is reduced by 65% of its original intensity (Fig. 3B). This indicates that the tryptophan residues present gain upon heating more rotationally mobility due to reduced local packing, than the phenylalanine residues. When the tryptophan fluorescence intensity was monitored as a function of temperature an almost linear decrease of intensity was observed (results not shown), which is an intrinsic property of tryptophan fluorescence. No shift of the fluorescence maximum could be observed, indicating that the tryptophan residues, which are readily solvent-exposed at ambient temperatures, preserve this exposed character upon heating.

To investigate the nature of the aggregation phenomenon upon denaturation of the protein, we studied the kinetics of aggregation by monitoring the turbidity of the sample at 85 °C as a function of time (Fig. 6A). It can clearly be seen that the kinetics of aggregation increases with increasing protein concentration. In a sample with a protein concentration of 1 mg/ml 700–800 s are required to obtain a maximal turbidity. Pelleting of the material by centrifugation revealed that all protein was complexed into water-insoluble aggregates, as demonstrated by determining the protein concentration in the supernatant in the cases studied (results not shown).
shown). When the initial slope of the change in turbidity as a function of time is plotted versus the protein concentration (Fig. 6B), a relation is found that can be described by a simple squared function of the protein concentration (dashed line).

These results indicate that the aggregation phenomenon of Ara h 1 is not a cooperative process, but follows a particle collision type of mechanism. Analysis of the heated Ara h 1 by SDS-PAGE demonstrates that, whereas native Ara h 1 has an apparent molecular mass of approximately 63 kDa, the heated material forms stable dimers, trimers, and larger complexes (results not shown).

Structural Properties of Ara h 1 Isolated from Heated Peanuts—To test whether a similar behavior as described above for temperature-induced denaturation of isolated Ara h 1 is also apparent when this protein is heated in peanuts prior to isolation, we heated peanuts for 15 min at various temperatures, cooled them to 20 °C, and isolated Ara h 1 from the peanut as described under “Materials and Methods.” Extraction from peanuts heated at 20, 50, 80, and 90 °C led to similar yields, whereas the extraction yields of peanuts heat-treated at 110 and 140 °C were lower (75% and 32%, respectively). Incubation at these temperatures resulted in no extraction of protein at all from the peanut. Whether this is due to aggregation and destruction of the protein, chemical reactions, a reduced accessibility for extraction or another mechanism is unknown at the present time. Up to 140 °C, Ara h 1 was purified successfully and the protein pattern as analyzed by SDS-PAGE was similar for all samples (not shown). Investigation of the secondary structure of these proteins on ATR-FTIR, as demonstrated in Fig. 2B by analysis of the shape of the amide I band reveals that these are comparable for Ara h 1 isolated from peanuts heated at 20, 50, 80, and 90 °C. The spectrum of the protein from peanuts heated at 110 °C clearly shows an increased intensity around 1630 cm⁻¹, indicative for increased content of extended β-structures. This is even more pronounced for the protein heated at 140 °C in the peanut. From these results and from comparison with the IR spectrum of the isolated protein heated in aqueous solution (Fig. 2A, dashed line), we suggest that in peanuts denaturation of Ara h 1 also can take place, but requires slightly higher temperatures (90–110 °C) compared with that of the isolated protein. The obtained denatured state shows a great resemblance to that of denatured isolated Ara h 1, in that an incremented secondary folding is adopted, with similar IR spectral features.

Recognition of Ara h 1 by IgE from Human Sera—Binding of IgE present in sera from patients with a peanut allergy to Ara h 1 was studied in a binding assay with a fluid phase character, similar to previously described assays (22, 23) in order to maintain the native structure of Ara h 1 optimally. The specificity of this approach was tested using either another major peanut allergen, Ara h 2, or a soy protein extract instead of Ara h 1. Both preparations did not show a dose-dependent effect as was observed for Ara h 1, whereas a peanut protein extract bound IgE in the fluid phase completely (not shown). As a quantitative determination for IgE binding affinity in the fluid phase, the concentration Ara h 1 at the half-maximal signal was used (see “Materials and Methods”). The value for native, not heat-treated Ara h 1 1.41 μg/ml. Affinities of Ara h 1 isolated from peanuts heated at different temperatures were determined in the same way and are shown in Table I. Although it might appear that, from 50 to 140 °C, a small decrease in affinity can be observed, no correlation between the native state of the protein and its IgE-binding affinity is present.

**DISCUSSION**

**Structural Properties of Ara h 1 at Ambient Temperatures**—From the results presented in this work on the structural features of Ara h 1 at the secondary (Figs. 1 and 2), tertiary (Fig. 3), and quaternary (Fig. 4) folding level, we can conclude that this protein is highly structured. Screening of the primary sequences of the Swiss Protein Data base reveals a 46% sequence identity and a 52% sequence weighted similarity with phaseolin, a seed storage protein from the French bean also belonging to the legume family. This latter protein has been crystallized, and the structure is resolved to a 2.2 Å resolution (33, 34) and comprises a β-barrel with a so-called “jelly-roll” folding motif with a solvent-buried character, followed by a helical domain. Interestingly, comparison of the predicted secondary structure of Ara h 1 (35) with the known structure of phaseolin shows a full match of the predicted with the observed β-strands, respectively. Recently, a successful structural alignment between Ara h 1 and phaseolin was presented (12). Whereas in phaseolin (397 amino acids) 172 residues adopt a β-structure, a comparable number is found for Ara h 1 (541 amino acids) based on the 36% found to be β-structured according to the far-UV spectral analysis (Fig. 1). It is also interesting to note that four of the five tryptophans of Ara h 1 are located at residues 30, 44, 51, and 73, an N-terminal region that shows no overlap with the primary sequence of phaseolin (alignment is from residue 77 onward). It can be suggested that this N-terminal domain does not participate in the buried core of the protein, explaining the relatively solvent-exposed character of these tryptophans as observed in their fluorescence characteristics described above. On a quaternary level, Ara h 1 adopts a trimeric complex (Fig. 4), in agreement with recent observations (12, 17).

**Thermal Denaturation of Ara h 1**—From the description of the conformational changes of Ara h 1 studied at the different folding levels upon heat-denaturation, the following picture emerges. The suggested core of the protein comprising β-stranded folding motifs provides the protein with a large stability against heat denaturation, as observed for various other plant storage proteins like soy glycinin. At a secondary level, up to the denaturation temperature no changes in the secondary structure content are observed (Fig. 1B). The larger relative reduction of near-UV CD intensity in the 313 nm region (± 65%) compared with the 288 nm region (± 25%, Fig. 3B) indicates that especially the domains where the tryptophans reside (N-terminal) have a low stability and can gain more flexibility at higher temperatures. The near-UV CD of the phenylalanines, however, is hardly affected by temperatures up to 80 °C, in agreement with the model that they reside in the core of the protein as suggested by the comparison with the aligned structure of phaseolin. The increase of far-UV ellipticity at temperatures higher than 50 °C may reflect a dissociation from the trimer to the monomeric or dimeric form, which gives rise to a lower absorption flattening.

The enthalpy content of 30 kcal/mol for the irreversible denaturation occurring around 85 °C (Fig. 5) is relatively small compared with those reported for complete unfolding of globular proteins, like β-lactoglobulin (105 kcal/mol at 85 °C) (36), lysozyme (129 kcal/mol at 78 °C), or metmyoglobin (135 kcal/mol at 83 °C) (37). This suggest that only a limited part of the protein adopts a different conformation upon heat denaturation and that the major part of the structure elements are preserved. Indeed, it is found that the heat-denatured protein possesses an even higher degree of secondary structure, with an increased content of extended β-structures. The appearance of extended β-structures often reflects the formation of large protein complexes, as indeed observed for Ara h 1 (Figs. 1B, 3B, and 6). For this protein we have not been able to detect

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4 C. M. M. Lakemond, personal communication.
whether, prior to the formation of these large aggregates, a limited unfolding of the protein has to take place, as could be observed for example for a potato storage protein (29), but as the aggregation kinetics points to a particle collision model (Fig. 6), the possible exposure of hydrophobic sites on the partial unfolded protein is expected to be relatively small and only sufficient to result in stable protein-protein interactions if the proteins are already at close range.

Ara h 1 isolated from peanuts heated at 110 °C and 140 °C exhibits a denatured state on secondary level similar to that for partial unfolded protein is expected to be relatively small and only (Fig. 6), the possible exposure of hydrophobic sites on the par-

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Heat Denaturation of Ara h 1

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