JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Peanut Allergen Ara h 1 Interacts with Proanthocyanidins into Higher Molecular Weight Complexes

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Mildly extracted peanut allergen Ara h 1 was previously reported to occur as an oligomeric complex. In this paper we describe how the protein in this oligomeric complex interacts noncovalently with phenolic compounds of the proanthocyanidin type. These interactions are being disrupted during anion exchange chromatography, resulting in the dissociation of the oligomeric Ara h 1 complex into protein trimers. By use of the known three-dimensional structure of β-conglycinin, a soy protein homologous to Ara h 1, proline-rich regions were observed in silico on both faces of its trimeric structure, which are conserved in Ara h 1. These proline-rich regions could explain the binding of proanthocyanidins to Ara h 1 and the formation of multiple Ara h 1 trimer complexes. This was supported by the observation that the addition of peanut proanthocyanidins to trimeric Ara h 1 and to β-conglycinin resulted in the formation of soluble oligomeric protein complexes. The structurally related legumin proteins do not contain such proline-rich regions on both sides of the protein, and proanthocyanidins were shown to have a lower affinity for legumin proteins from peanuts and soybeans (peanut allergen Ara h 3 and soy glycinin, respectively). Ara h 1 present as the oligomeric complex is assumed to be the representative form of the allergen in which it is consumed by humans.

KEYWORDS: Peanut allergy; Ara h 1; protein-polyphenol interaction; proanthocyanidins

INTRODUCTION

Resistance to digestion in the human gastrointestinal tract as well as stability upon cooking and industrial processing are properties considered to be prerequisites for food allergens. These properties depend to a large extent on the structural characteristics of the allergenic protein. In addition, these characteristics can be influenced by other components present in food. For example, phenolic components are known to be able to associate with proteins, resulting in conjugates affecting both the solubility (*1*) and digestibility (*2*) of the proteins.

Peanut allergy is the most prevalent food allergy in older children, adolescents and adults. In addition, allergic reactions to peanuts are usually persistent for life and can cause life-threatening situations. In recent years much research has been devoted to identify and characterize the allergens present in peanuts. One of the major allergens in peanuts, Ara h 1, is described as a 63 kDa glycosylated seed storage protein (*3*). The gene encoding for Ara h 1 has high homology (60–65%) with those of the vicilin seed storage protein family (*4*). Members of this family are all described to be trimeric proteins (*5*), either or not reversibly associating into hexamers at low ionic strength (*6*). Ara h 1 has been assumed to occur as a trimer (*7*). However, it was recently reported (*8*) that upon extraction the allergen occurs as a larger oligomer. It was observed that irreversible dissociation of the oligomers into trimers is induced by applying the allergen to anion exchange chromatography (AEC) (*8*), a commonly used purification technique.

The occurrence of Ara h 1 in a stable oligomeric structure is not consistent with other data described in the literature for Ara h 1 and for other vicilin proteins. Therefore, it was hypothesized that other compounds are present in the oligomeric protein complex, causing the complexation of the trimeric protein. This complexation occurs via noncovalent interactions, the latter ones being disrupted during anion exchange chromatography. Phenolic compounds are able to interact noncovalently with proteins, which may result in complexation (*9*, *10*). Phenolic compounds are described to be present in peanuts. The predominant monomeric phenolic compound in peanuts is *p*-coumaric acid (*11*). Besides, oligomeric flavan-3-ols, also known as proanthocyanidins or condensed tannins, are reported to be present in (roasted) peanuts (*12*) and in peanut skins (*13*), with degrees of polymerization varying between 1 and 5 (*12*).

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10.1021/jf071585k CCC: $37.00
Published on Web 09/21/2007

Noncovalent interactions between proteins and monomeric phenolic compounds require relatively high molar ratios of phenolic compounds to proteins (>100) in order to affect the functional properties of the proteins (*14*). The average content of phenolic compounds in raw peanuts is 47 mg kg^{-1} (*11*), next to 24–29% (w/w) protein, with Ara h 1 representing 12–16% thereof (*15*). Thus, per mol of Ara h 1 there would be a maximum of only 0.5 mol *p*-coumaric acid available per Ara h 1 molecule, and this ratio would be too low to affect the functional properties of Ara h 1 (*14*). Proanthocyanidins, on the other hand, can affect the functional properties of proteins at much lower phenolic compound to protein ratios. Besides, proanthocyanidins, because of their larger sizes, are able to interact with more than one protein site, which may result in the formation of cross-links between proteins, likely resulting in aggregation (*16*). Interactions between proanthocyanidins and proteins have been reported to be noncovalent, and larger proanthocyanidins normally have a higher ability to bind to proteins than smaller ones (*17*).

In this study we aimed to further characterize the previously identified oligomeric structure of Ara h 1 (*8*) and investigated if proanthocyanidins are responsible for the oligomerization of the allergen.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO), unless stated otherwise. Peanuts of the runner market-type and peanut skins were provided by Imko Nut Products (Doetinchem, The Netherlands) and were stored at 4 °C until use. Soy β-conglycinin and glycinin were purified from Hyland soybeans as described by Kuipers and co-workers (*18*). Millipore water was used for all experiments (Millipore Corp, Bedford, MA).

Ara h 1 Purification. Ground peanuts were defatted with hexane using Soxhlet extraction. Defatted and nondefatted ground peanuts were extracted (10 g/100 mL or 10 g/20 mL, respectively) for 1 h under continuous stirring, in 15 mM sodium phosphate buffer, pH 6.2, containing 0.3 % (w/v) sodium metabisulfite. Afterward, the extracts were sieved through cheese-cloth and centrifuged (25 min; 14000*g*; 4 °C). The supernatants obtained were filtered over a 1.2 μm filter and applied onto a Superdex 200 column (10 cm × 52 cm; 150 mL of extract applied per run; GE Healthcare, Uppsala, Sweden). The column was equilibrated and eluted with 15 mM sodium phosphate buffer, pH 6.2, at a flow rate of 40 mL min^{-1} using an Äkta Explorer system (GE Healthcare) operated at room temperature. Eluates were monitored at 280 and 325 nm, and appropriate fractions were collected and reapplied onto the same column. Again, fractions containing pure Ara h 1, as analyzed by SDS-PAGE and analytical size exclusion chromatography, were pooled and stored at −20 °C until use. The Ara h 1 preparation purified from defatted peanuts with skins was denoted Ara h $1_{oligomers}$, and the one purified from nondefatted peanuts without skins was denoted Ara h $1_{oligomers,NS}$.

Ara h 1 trimers were induced from Ara h $1_{oligomers}$ using anion exchange chromatography, as described before (*8*), and denoted Ara h $1_{trimers}$.

Ara h 3 Purification. For the purification of Ara h 3, defatted peanuts were extracted (10 g/100 mL) for 1 h at room temperature under continuous stirring, in 50 mM Tris/HCl buffer, pH 8.2. Afterward the extract was sieved through cheese-cloth, and centrifuged (25 min; 14000*g*; 4 °C). The supernatant obtained was filtered over a 1.2 μm filter and subsequently applied onto a 320 mL Superdex 200 XK 26/60 column (13 mL applied per run; GE Healthcare). The column was equilibrated and eluted with 50 mM Tris/HCl buffer, pH 8.2, at a flow rate of 4.3 mL min^{-1}, using an Äkta Purifier system (GE Healthcare) operated at room temperature. Eluates were monitored at 280 nm, and fractions containing Ara h 3, as analyzed by SDS-PAGE, were collected, pooled, and applied onto a 1.2 L Source Q Fineline column, using an Äkta explorer system. The column was equilibrated with 50 mM Tris/HCl buffer, pH 8.2, and the flow rate used was 40 mL min^{-1}. After sample application and washing, a 10 column volumes linear gradient from 0.1 to 1 M NaCl in 50 mM Tris/HCl buffer, pH 8.2, was applied. The eluate was monitored at 280 nm and samples containing pure Ara h 3, as analyzed by SDS-PAGE, were collected and pooled. The pooled fractions were dialyzed in 10000 MWCO dialysis tubings (Medicell Int. Ltd., London, U.K.) against water. Afterward samples were lyophilized and stored at −20 °C until use.

Analytical Size Exclusion Chromatography. Analytical size exclusion chromatography was performed on a Superdex 200 10/300 column (0.1 mL of sample applied per run, GE Healthcare). The column was equilibrated and eluted with 15 mM sodium phosphate buffer, pH 6.2, at a flow rate of 0.5 mL min^{-1}, using an Äkta purifier system operated at room temperature. The eluate was monitored at 280 and 325 nm.

SDS-PAGE. Protein samples were analyzed using on a Mini-PROTEAN II system (Bio-Rad Laboratories Inc., Hercules, CA) according to the instructions of the supplier. 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) were used. 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.

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. A protein conversion factor of 6.03 for Ara h 1 was calculated from the part of the amino acid sequence remaining after post-translational processing (*19*) (accession number P43238) and its content and composition of linked *N*-glycans (*20*).

Peanut Proanthocyanidin Purification. Peanut skins were defatted with hexane using Soxhlet extraction. Defatted peanut skins (60 g) were extracted three times with 1 L of 20% (v/v) aqueous methanol. Afterward, the methanol was evaporated in a rotating vacuum evaporator and samples were freeze-dried. Next, samples were dissolved in water and proanthocyanidins were extracted three times with ethyl acetate at a ratio of water to ethyl acetate of 1:1 (v/v). The ethyl acetate fractions were collected and combined. The ethyl acetate was evaporated, and the remaining material was dissolved in a mixture of acetone, hexane, and ethanol (volume ratio 7:3:2). Samples of 10 mL (700 mg) were applied onto a preparative Inertsil column (30 × 250 mm, GL Sciences Inc., Tokyo, Japan), operated at a flow rate of 27.22 mL min^{-1}. The eluents used were (A) hexane and (B) acetone. A linear gradient from 40% to 60% B was executed in 30 min, followed by 20 min of eluting with 60% B, and a linear gradient from 60% to 75% B in 20 min. UV spectra were recorded from 210 to 300 nm. Fractions containing proanthocyanidin pentamers and higher molecular weight oligomers, as determined by analytical normal phase chromatography with mass detection (masses equal to or higher than 1434 Da), were collected, pooled, evaporated, and freeze-dried. Analytical normal phase chromatography was performed on a Thermo Spectra system (Thermo Separations Products Inc., San Jose, CA). Samples (dissolved in methanol) were injected onto a Luna Silica column (4.6 × 250 mm; Phenomenex Inc., Torrance, CA) operated at room temperature. The eluents used were (A) methanol, (B) dichloromethane, and (C) 50% (v/v) acetic acid. The flow rate was 1.0 mL min^{-1} and detection was performed at 280 nm. A gradient from 82% A, 14% B, and 4% C to 61% A, 35% B, and 4% C was followed in 50 min. Mass spectra were recorded with an electrospray ionization mass spectrometer (LCQ Classic; Thermo Finnigan, San Jose, CA), with detection in the negative mode. The capillary spray voltage was 5 kV, and the capillary temperature was 270 °C.

Batchwise Anion Exchange Chromatography. Source Q material (0.75 mL; GE Healthcare) was washed with water, followed by 50 mM Tris/HCl buffer, pH 8.2 (washing buffer). Next, 5 mL of Ara h 1 solution (2 mg mL^{-1}) in 15 mM sodium phosphate buffer, pH 6.2, of which the pH was adjusted to 8.2 with 1 M NaOH, was added to the column material. Next, the column material was washed twice with 0.75 mL of washing buffer. Subsequently, the column material was eluted twice with 0.75 mL of 50 mM Tris/HCl buffer, pH 8.2,

containing 0.5 M NaCl, and twice with 0.75 mL of 50 mM Tris/HCl buffer, pH 8.2, containing 1 M NaCl. Finally, the column material was washed twice with washing buffer and the remaining column material was air-dried. All buffers were removed from the anion exchange material after centrifugation (22000*g*; 5 min; 20 °C).

Proanthocyanidin Quantification. Samples were analyzed using the HCl–butanol assay (*21*). Samples were mixed with 0.1–0.5 mL of butanol, containing 5% (v/v) HCl and incubated in a water bath at 95 °C for 2 h. After incubation, the color of the samples was visually analyzed or samples were centrifuged (22000*g*; 5 min; 4 °C) and the absorbance of the supernatant was measured at 550 nm (BioTek Instruments Inc., Winooski, VT). A calibration curve was made using pentameric proanthocyanidins that were purified as described above.

Sequence Analysis. The amino acid sequences of Ara h 1 (accession number P43238, amino acids 85–626) and Ara h 3 (accession number O82580, amino acids 1–507) were aligned with the amino acid sequence of the β subunit of β-conglycinin (accession number P25974, amino acids 26–439) and glycinin G1 (accession number P04776, subunit A1a, B2, amino acids 1–495) from soybean, respectively, using the program MegAlign (DNAStar, Inc., Madison, WI). A Clustal W alignment was performed. The weight table PAM 250 was used, and the parameters gap length and gap penalty were set at 0.2 and 10, respectively. Next, the proline residues of Ara h 1 and Ara h 3 were projected onto the X-ray three-dimensional structure of soybean β-conglycinin (PDB code 1IPJ) and soybean glycinin (PDB code 1FXZ), respectively, using the program Deepview (www.expasy.org/spdbv/).

Reconstitution of Protein–Proanthocyanidin Mixtures. Solutions of pentameric peanut proanthocyanidins (purified as described above, using a molar mass of 1434 for calculation) in 100 mM sodium phosphate buffer, pH 8.0, were added to separately weighted amounts (5 mg) of Ara h $1_{trimers}$, Ara h 3, soybean β-conglycinin, soybean glycinin, and mixtures of two of these proteins, resulting in samples with molar ratios of proanthocyanidins to proteins varying from 3:1(:1) to 10:1(:1). Samples were mixed head-over-tail overnight at room temperature. Afterward, samples were centrifuged (22000*g*; 5 min; 4 °C) and the supernatants were analyzed for their nitrogen content (Dumas) and their molecular size distribution. For the latter, aliquots (100 μL) were analyzed on a Superdex 200 10/300 column, equilibrated and eluted with 100 mM sodium phosphate buffer, pH 8.0, at a flow rate of 0.7 mL min^{-1}, using an Äkta purifier system operated at room temperature. The column was calibrated using a high molecular weight calibration kit (GE Healthcare), and eluates were monitored at 280 nm.

RESULTS AND DISCUSSION

Ara h 1–Proanthocyanidin Interactions. The recently reported occurrence of Ara h 1 in an oligomeric structure after purification using mild extraction techniques (*8*) is contrary to previous observations, stating that Ara h 1 occurs as trimers (*7*). As proanthocyanidins are able to interact with proteins at relatively low molar ratios, possibly resulting in the formation of complexes (*16*), we investigated if proanthocyanidins were present in the oligomeric Ara h 1 protein complex. The results showed that per milligram of Ara h $1_{oligomers}$ 0.060 ± 0.008 mg of proanthocyanidins was present, while in Ara h $1_{trimers}$ no proanthocyanidins ($<5.000 \times 10^{-5}$ mg of proanthocyanidins per mg protein) were detected. Considering a molecular mass of flavan-3-ols of ~300 Da, and monomeric Ara h 1 being ~67 kDa, this would eventuate in a molar ratio of ~14 mol of flavan-3-ols to 1 mol of monomeric protein, or ~3 mol of pentameric proanthocyanidins to 1 mol of monomeric protein, the pentamers being the proanthocyanidins with the highest degree of polymerization detected in peanuts (*12*).

As Ara h $1_{oligomers}$ eluted as a single peak from a SEC column upon detection at 280 nm (*8*), it can be stated that the proanthocyanidins and proteins are associated. The absence of proanthocyanidins in Ara h $1_{trimers}$ indicates that AEC, used in the purification procedure of Ara h $1_{trimers}$, causes a disruption of the protein–proanthocyanidin interactions. This disruption would subsequently result in the separation of the proanthocyanidins from Ara h 1 and the dissociation of the oligomeric Ara h 1 complex into a trimeric one.

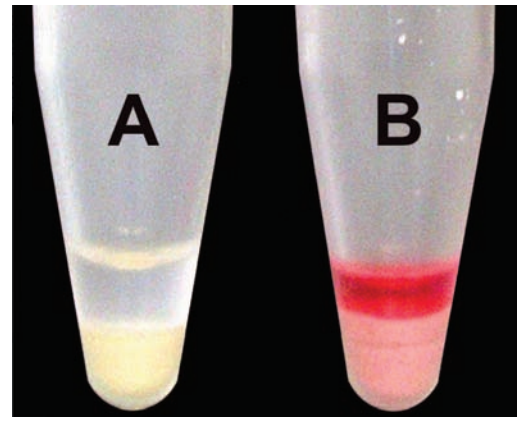

Figure 1. Source Q anion exchange material applied to the HCl–butanol assay, before (A) and after (B) binding of Ara h 1 oligomers and elution of the protein with 1 M NaCl.

Effect of Anion-Exchange Chromatography on Ara h 1–Proanthocyanidin Interactions. The binding of Ara h $1_{oligomers}$ to an AEC column did not result in an increase in the UV absorbance at 280 nm in the flow-through. Also, only a single peak eluted from the column during the salt gradient applied, corresponding to trimeric Ara h 1 (*8*). It thus seemed that the proanthocyanidins present in the Ara h $1_{oligomers}$ preparation bound to the AEC column and were not released during the salt gradient applied. A batchwise AEC experiment was performed to investigate whether the proanthocyanidins linked to Ara h 1 indeed bound to the column material. The results showed that none of the fractions collected (nonbound, wash, eluates) except the starting material, appeared to contain proanthocyanidins when analyzed with the HCl–butanol assay (data not shown). However, when the washed anion exchange material after application of Ara h 1 oligomers was analyzed, the column material turned pink (**Figure 1**). This indicates the presence of proanthocyanidins. Thus, the proanthocyanidins that were previously linked to Ara h 1 bound to the AEC column material, resulting in the dissociation of the oligomeric Ara h 1 complex into a trimeric one (*8*).

Origin of Ara h 1–Proanthocyanidin Interactions. The Ara h $1_{oligomers}$ were purified from peanuts with skins. As peanut skins are reported to contain more proanthocyanidins than peanuts themselves (*13*), the proanthocyanidins from peanut skins could have caused the oligomerization of Ara h 1 during extraction. Nevertheless, extracts of peanuts without skins on SEC showed the same results as extracts of peanuts with skins when analyzed on SEC, with Ara h 1 eluting at the same volume, corresponding to an oligomeric structure (data not shown). Besides, we have previously reported that in peanut extract from nondefatted peanuts Ara h 1 also occurs solely as oligomers (*8*).

Although oligomerization was not induced by the proanthocyanidins in peanut skins during extraction, we could not determine whether the Ara h 1–proanthocyanidin interactions are induced during protein extraction, by enabling contact between the protein and the proanthocyanidins, or if these complexes already exist in the peanut itself. Flavan-3-ols are described to be synthesized in the cytosol, but they are accumulated in cell vacuoles, where they undergo condensation (*22*). Proteins are also accumulated in vacuoles. However, they are reported to be stored in protein storage vacuoles, while all

other compounds are described to be stored in so-called vegetative vacuoles (*23*). This indicates that Ara h 1 and proanthocyanidins in peanuts occur independently from each other. However, since peanuts are either processed or masticated before consumption, thereby enabling contact between proanthocyanidins and Ara h 1, the oligomeric structure of Ara h 1 seems representative for the structure of Ara h 1, which is normally consumed by humans. The pH used for the protein extraction (6.2) resembles the pH of human saliva, which ranges between 6 and 7 (*24*). Although the time of mastication generally is short, we assume that complexation (if it had not already occurred) may take place, as interactions between proteins and salivary proline-rich proteins are also assumed to occur in the time span that food resides in the oral cavity (*25*). Consequently, we consider the oligomeric structure of Ara h 1 as the structure of the allergen in which it is generally consumed by humans.

Tentative Mechanism of Ara h 1–Proanthocyanidin Interactions. The molecular mass of Ara h $1_{oligomers}$ was earlier estimated to be ~700 kDa (*8*) using SEC. This mass suggests a complex bearing multiple trimeric Ara h 1 proteins, most probably four. Although most proanthocyanidin–protein interactions result in precipitated complexes (*1*), the Ara h 1–proanthocyanidin interactions thus result in soluble complexes. In peanuts, the allergen Ara h 3 is also present. This protein belongs to the hexameric legumin family, which is structurally related to the vicilin family (*26*). Upon extraction at pH 8.2, and $I = 0.02$ M, this protein occurs solely in a hexameric structure (*8*), indicating that this protein is not able to form larger complexes because of interactions with proanthocyanidins.

The ability of proteins to form complexes with proanthocyanidins varies considerably, and proanthocyanidins predominantly form complexes with proline-rich proteins (*25*). Hydrophobic interactions have been described to be the major driving force for the interaction between various small polyphenols, for example, epigallocatechin gallate (EGCG), and proteins (*1, 27, 28*). However, hydrogen bonding has been reported to be the main driving force for proanthocyanidin–protein interactions (*1, 29*). On the basis of these results, the interaction of peanut proanthocyanidins with Ara h 1 is also likely to be mainly driven by hydrogen bonding.

After post-translational processing, monomeric Ara h 1 contains 31 proline residues (*19*). To explain the apparent affinity of proanthocyanidins for Ara h 1, we looked at the three-dimensional structure of β-conglycinin, a homologous protein from soybeans (containing 21–38 proline residues, depending on the monomer subunit type), as the three-dimensional structure of Ara h 1 has not been determined. However, molecular modeling studies with Ara h 1 have shown that its three-dimensional structure is highly similar to that of β-conglycinin (*30*).

The three-dimensional structure of β-conglycinin has been determined for the trimer built up of three β subunits, each containing 21 proline residues. Alignment of the amino acid sequence of Ara h 1 with that of this β-conglycinin subunit (**Table 1**) reveals that the first 80 N-terminal amino acids of Ara h 1, containing 11 proline residues, are not covered. The other two subunits of β-conglycinin (α and α') also have an N-terminal extension, containing 14 and 17 proline residues, respectively. However, the three-dimensional structures of the N-terminal extensions of these subunits of β-conglycinin have not been determined.

In the three-dimensional structure of β-conglycinin, which is depicted in **Figure 2**, most of the proline residues are located on the surface of the molecule. Many of the surface-located prolines are centered around the hole in the middle of the trimeric complex. These proline-rich regions in the middle of the trimeric complex are present on both faces of the protein (indicated as A and B in **Figure 2**), and are highly conserved in Ara h 1 (**Table 1**). On face A, four conserved residues are present per monomer, thus 12 residues in total, and on face B two conserved residues per monomer are present, thus six residues in total. The presence of these proline-rich regions on Ara h 1 might explain the binding of proanthocyanidins to the allergen. Moreover, the occurrence of proline-rich regions on both faces of the trimer likely explains the oligomerization of Ara h 1 into soluble complexes bearing several Ara h 1 molecules, as proanthocyanidins may act as a linker between the Ara h 1 trimers. Soy β-conglycinin in that case is also expected to be able to interact with proanthocyanidins and to form soluble higher molecular weight complexes. Yet, the structure of β-conglycinin, a protein that has been purified and characterized several times without anion exchange chromatography (*18, 31, 32*), has never been reported to occur as larger complexes or to interact with proanthocyanidins. The most obvious reason for this would be that soybeans do not to contain proanthocyanidins (*12*). However, if proanthocyanidins would be present in samples containing β-conglycinin, the protein would be expected to interact with these compounds in a similar manner as Ara h 1.

Table 1. Alignment Data with Respect to Proline of (the β-subunit of) Soy β-Conglycinin and Ara h 1 after Post-translational Processing[a]

	β-conglycinin	Ara h 1
prolines in amino acid sequence	21	31
conserved prolines in amino acid sequence		16
prolines in 3D structure	17	16
conserved prolines in 3D structure		14
prolines on surface 3D structure	12	10
conserved prolines on surface 3D structure		9
prolines in proline-rich region on face A	4	4
prolines in proline-rich region on face B	3	2

[a] Faces A and B are indicated in Figure 2.

Tentative Mechanism for Ara h 3–Proanthocyanidin Interactions. Large oligomeric complexes were previously not detected for peanut allergen Ara h 3 (containing 28 prolines) present in the same extract as oligomeric Ara h 1 (*8*). To this end, we investigated this protein and looked at the three-dimensional structure of the homologous soy glycinin (containing 37 proline residues), also because the three-dimensional structure of Ara h 3 is not known. The alignment data are given in **Table 2**, and the three-dimensional structure of glycinin is shown in **Figure 2**. The three-dimensional structure of glycinin does not cover the complete amino acid sequence of the protein: four disordered regions (varying in lengths from 5 to 48 residues) are not covered in the three-dimensional protein structure. In **Table 2** their proline contents are given, but no further investigation was performed into these sequences.

Most of the prolines on the surface of glycinin are conserved in Ara h 3. Furthermore, these conserved prolines are situated mostly on one face of the protein (indicated as face A in **Figure 2**). This face of the legumin protein is the hydrophobic part of the protein and interacts in the plant with the same side of another trimeric molecule, to form a hexameric structure (*33*). Thus, the prolines present on that face of Ara h 3 will not be available for interaction with proanthocyanidins as they will be shielded in the hexameric protein structure. The other face of the trimeric molecule (face B) contains four proline residues, of which three are conserved in Ara h 3. These proline residues are not situated in close proximity of each other and cons-

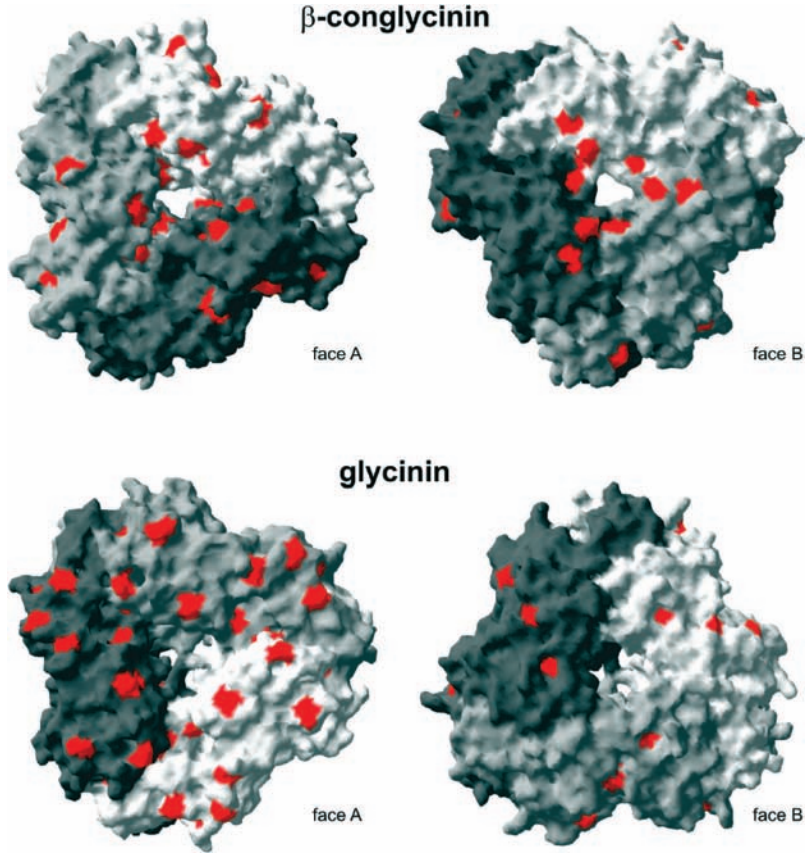

Figure 2. The three-dimensional structures of soybean β-conglycinin and glycinin. Prolines are indicated in red, and monomeric subunits are indicated in white, gray, and dark gray.

Table 2. Alignment Data with Respect to Proline of Soy Glycinin and Ara h 3[a]

	glycinin	Ara h 3
prolines in amino acid sequence	37	28
conserved prolines in amino acid sequence		20
prolines in 3D structure	20	18
conserved prolines in 3D structure		17
prolines on surface 3D structure	15	13
conserved prolines on surface 3D structure		12
prolines on face A	12	10
prolines on face B	4	3
prolines in disordered region 90–109	4	0
prolines in disordered region 179–197	3	3
prolines in disordered region 249–296	0	5

[a] Faces A and B are indicated in Figure 2.

equently do not comprise a proline-rich region. These data indicate that proanthocyanidins will not have a high affinity for Ara h 3 and glycinin. As a result, higher molecular weight complexes, due to cross-linking Ara h 3 by proanthocyanidins, are not likely to occur. This hypothesis is in agreement with our earlier reported results, describing that Ara h 3 upon extraction from peanuts at pH 8.2, $I = 0.02$, has a lower molecular weight compared to Ara h 1 oligomers and solely occurs in a hexameric structure (*8*).

Induction of Interactions between Proanthocyanidins and Vicilin and Legumin Proteins. In order to verify our hypotheses concerning the affinity and interaction of proanthocyanidins with vicilin and legumin type proteins, we investigated whether the addition of proanthocyanidins to these proteins would result in an interaction, either or not eventuating in soluble oligomeric complexes. To this end, proanthocyanidins were added to Ara h $1_{trimers}$, β-conglycinin, Ara h 3, glycinin, and combinations of these proteins. Subsequently, the effects of these additions on the size and solubility of the proteins were analyzed. The results for Ara h $1_{trimers}$ and Ara h 3 are given in **Figure 3**, and the proportions of soluble protein at the conditions applied are given in **Table 3**. The Ara h $1_{trimers}$ preparation clearly increased in size with increasing concentrations of proanthocyanidins added, as the protein peak eluted after smaller elution volumes with increasing proanthocyanidin concentrations, thus corresponding to higher molecular weight complexes. The proportion of soluble protein in all Ara h 1 samples did not change much, indicating that almost exclusively soluble complexes were formed. The fact that an absolute increase in peak height was observed with increasing proanthocyanidin concentrations also points to the proanthocyanidins being bound to the protein, as proanthocyanidins contribute to the UV absorbance at 280 nm.

At pentameric proanthocyanidin to protein ratios higher than those calculated for Ara h $1_{oligomers}$ (3:1) soluble complexes with masses higher than 700 kDa were detected, the latter being the mass of Ara h $1_{oligomers}$ (*8*). Furthermore, at every ratio tested, it appeared that Ara h 1–proanthocyanidin complexes with only a relatively small size distribution were obtained. Our data thus indicate that in the peanut itself the concentration of proanthocyanidins available for interaction with Ara h 1 is limiting, thereby constraining the size of the soluble complex to ~700 kDa.

The addition of proanthocyanidins to β-conglycinin showed results similar to those for Ara h 1: With increasing concentrations of proanthocyanidins soluble larger complexes were formed (data not shown). In the sample with the highest concentration of proanthocyanidins, the proportion (%) of soluble protein was somewhat lower compared to that of the other samples. However, it could very well be that the small

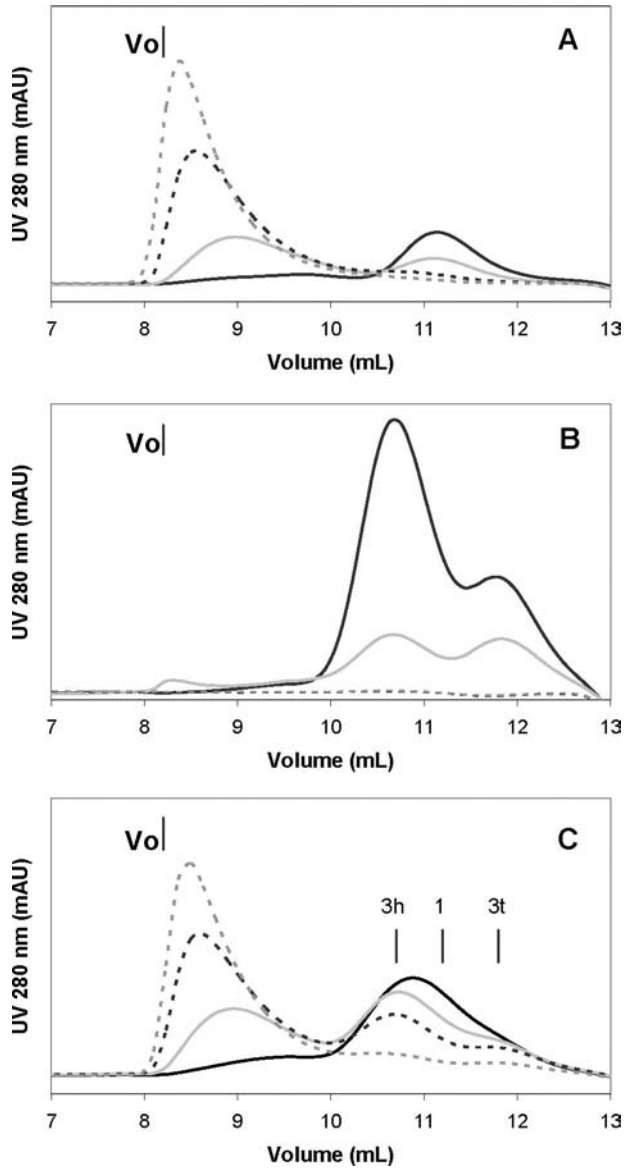

Figure 3. Size exclusion chromatogram of (A) Ara h $1_{trimers}$, (B) Ara h 3, and (C) Ara h $1_{trimers}$ and Ara h 3 with added peanut proanthocyanidins, on a Superdex 200 column. Molar ratios of proanthocyanidins to proteins (to proteins) in samples: 0:1(:1) (black lines); 3:1(:1) (gray lines); 7:1(:1) (black lines, dashed) 10:1(:1) (gray lines, dashed). Indicated is the void volume of the column (V_o), and the elution volume of Ara h 3 hexamers (3h), Ara h 3 trimers (3t), and Ara h 1 trimers (1).

Table 3. Proportion (%) of Soluble Protein (at pH 8.0, $I = 0.3$ M), with Standard Deviations, of Protein Samples with Increasing Molar Ratios of Proanthocyanidins to Proteins (PA:P)

ratio P:PA	Ara h $1_{trimers}$	Ara h 3	β-conglycinin	glycinin
0:1	100 ± 3	100 ± 2	100 ± 4	100 ± 2
3:1	100 ± 2	38 ± 1	103 ± 3	92 ± 4
7:1	101 ± 1	32 ± 2	123 ± 3	32 ± 2
10:1	97 ± 3	15 ± 3	62 ± 0	2 ± 3

amount (12% w/w) of glycinin present in the sample (*18*) was responsible for this insolubilization. Altogether, it was concluded that interactions of proanthocyanidins with β-conglycinin and Ara h $1_{trimers}$ result in the formation of larger (mostly) soluble complexes, which can likely be explained by the distribution of prolines on the surface of these proteins. Batchwise AEC of

Table 4. Proportion (%) of Soluble Protein (at pH 8.0, $I = 0.3$ M), with Standard Deviations, of Protein Samples with Increasing Molar Ratios of Proanthocyanidins to Proteins to Proteins (PA:P:P)

ratio PA:P:P	Ara h $1_{trimers}$ /Ara h 3	β-conglycinin /glycinin
0:1:1	100 ± 9	100 ± 7
3:1:1	117 ± 0	114 ± 3
7:1:1	94 ± 2	88 ± 3
10:1:1	75 ± 2	66 ± 2

(the soluble part of) Ara h 1 and β-conglycinin with proanthocyanidin to protein ratios 10:1 showed that the interactions between the proteins and proanthocyanidins were disrupted, resulting in the binding of proanthocyanidins to the column material (data not shown).

Samples of Ara h 3 with increasing proanthocyanidin concentrations showed that the total area corresponding to Ara h 3 (mainly hexamers, but also few trimers) decreased with increasing proanthocyanidin concentrations (Figure 4). In line with these results a decrease in protein solubility with increasing proanthocyanidin concentrations was observed (**Table 3**). Similar results were obtained for soy glycinin. The interactions of proanthocyanidins with glycinin and Ara h 3 thus resulted in the formation of insoluble protein–proanthocyanidin complexes, implying that an interaction between Ara h 3 and proanthocyanidins did occur. Next to prolines, other amino acids are able to interact with proanthocyanidins, although with a lower affinity (*28*). These interactions could have caused the precipitation of the legumin proteins.

In **Figure 3** and **Table 4** the results from combinations of proteins with added proanthocyanidins are given. When proanthocyanidins were added to a combination of Ara h 1 and Ara h 3, with increasing proanthocyanidin concentrations the peak corresponding to Ara h 1 decreased, and larger soluble complexes were formed, comparable to the results for only Ara h 1 with proanthocyanidins. The content of soluble protein did not decrease much. Also, at the highest proanthocyanidin concentration added, still Ara h 3 peaks (hexamers and trimers) were visible. Proanthocyanidins thus have a higher affinity toward Ara h 1 and form soluble complexes, rather than toward Ara h 3 and precipitate this protein. The same was found for combinations of β-conglycinin and glycinin with added proanthocyanidins (data not shown). Apparently, the affinity of proanthocyanidins for vicilin type proteins is higher than that for legumin type proteins, which is in agreement with the abovementioned hypotheses.

In conclusion, in this study we have shown that Ara h 1 upon extraction from peanuts occurs as an oligomeric complex bearing noncovalent interactions with proanthocyanidins. The distribution of proline residues over the surface of vicilin proteins, to which Ara h 1 belongs, likely explains the propensity of proanthocyanidins to interact with vicilin proteins and form soluble high-molecular weight complexes. The oligomeric structure of such mildly extracted (pH 6.2, $I = 0.02$) Ara h 1 is considered to be representative for the structure of Ara h 1 in which it is consumed by humans, as consumption of peanuts always occurs after processing and/or mastication. As the interaction of proanthocyanidins with proteins may alter protein characteristics, like digestibility (*2*), the latter one being an important characteristic for allergenic proteins, it would be interesting to investigate those characteristics of Ara h 1 which are important for its allergenic activity (like digestion and heat stability) and compare them to trimeric Ara h 1. These experiments are currently being carried out at our laboratory.

ACKNOWLEDGMENT

The authors thank Maaike Appeldoorn and Mark Sanders for the purification of peanut proanthocyanidins and Bas Kuipers for the purification of soy glycinin and β-conglycinin.

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Received for review May 30, 2007. Revised manuscript received July 23, 2007. Accepted August 2, 2007.

JF071585K