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Govardus A. H. de Jong

Shyamali Jayasena

Phil Johnson

Justin Marsh

Danijela Apostolovic

See next page for additional authors

Authors

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Purification and Characterization of Naturally Occurring Post-Translationally Cleaved Ara h 6, an Allergen That Contributes Substantially to the Allergenic Potency of Peanut

Govardus A. H. de Jong,[1] Shyamali Jayasena,[2] Phil Johnson,[2]

Justin Marsh,[2] Danijela Apostolovic,[3] Marianne van Hage,[3]

Julie Nordlee,[2] Joe Baumert,[2] Steve L. Taylor,[2] Camille Roucairol,[4]

Harmen de Jongh,[5] and Stef J. Koppelman[2]

1. TNO, Utrechtseweg 48, 3704 HE, Zeist, The Netherlands
2. Food Allergy Research and Resource Program, Department of Food Science & Technology, University of Nebraska, 279 Food Innovation Center, Lincoln, Nebraska 68588-6207, United States
3. Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet, 171 77 Stockholm, Sweden
4. DBV Technologies, Development Department, 92120 Montrouge, France
5. Protin consult, 3702 VL, Zeist, The Netherlands

Corresponding author – Stef J. Koppelman, email farrp@unl.edu

ORCID
Phil Johnson: 0000-0002-0462-8933
Steve L. Taylor: 0000-0002-7178-7520
Stef J. Koppelman: 0000-0001-7995-1754

Abstract

The 2S albumin Ara h 6 is one of the most important peanut allergens. A post-translationally cleaved Ara h 6 (pAra h 6) was purified from Virginia type peanuts, and the cleavage site was mapped using high-resolution mass spectrometry. Compared to intact Ara h 6, pAra h 6 lacks a 5-amino acid stretch, resembling amino acids 43−47 (UniProt accession number Q647G9) in the nonstructured loop. Consequently, pAra h 6 consists of two chains: an N-terminal chain of approximately 5 kDa and a C-terminal chain of approximately 9 kDa, held together by disulfide bonds. Intermediate post-translationally cleaved products, in which this stretch is cleaved yet still attached to one of the subunits, are also present. The secondary structure and immunoglobulin E (IgE) binding of pAra h 6 resembles that of intact Ara h 6, indicating that the loss of the nonstructured loop is not critical for maintaining the protein structure. Commercially available monoclonal and polyclonal immunoglobulin G (IgG) antibodies directed to Ara h 6 react with both intact Ara h 6 and pAra h 6, suggesting that the involved epitopes are not located in the area that is post-translationally cleaved. No differences between intact Ara h 6 and pAra h 6 in terms of IgE binding were found, suggesting that the area that is post-translationally cleaved is not involved in IgE epitopes either. For all main cultivars Runner, Virginia, Valencia, and Spanish, intact Ara h 6 and pAra h 6 occur in peanut at similar levels, indicating that pAra h 6 is a consistent and important contributor to the allergenic potency of peanut.

Keywords: peanut, *Arachis hypogaea*, allergen, mass spectrometry, IgE

Introduction

In peanut allergies, a food allergy that affects approximately 0.6% of adults and 1−2% of children/infants in the United States,[1,2] Ara h 2 and Ara h 6 are the dominant allergens.[3] In vitro studies have shown that Ara h 2 and Ara h 6 are the most potent allergens in peanut,[4,5] and this potency has been confirmed in humans in vivo by skin prick tests.[6] Animal models indicate an important role for Ara h 2 and Ara h 6, both in elicitation of reactions in sensitized animals and in therapeutic efficacy of sensitized animals treated with peanut immunotherapy.[7] For humans, immunoglobulin E (IgE) to Ara h 2 and 6 is the best predictor for clinically relevant peanut allergy.[8,9]

Ara h 2 appears on electrophoresis as a doublet at approximately 17 kDa, and Ara h 6 appears as a single band of approximately 15 kDa;[10] but, several isoforms of each allergen have been identified by mass spectrometry.[11,12] Ara h 2 and Ara h 6 belong to the 2S albumin protein family, commonly occurring in plant seeds and nuts, where they serve as storage proteins. 2S albumins are characterized by a high content of disulfide bonds and a highly stable protein core, which is resistant to heat-treatment and digestion.[13] Perhaps due to this stability, many plant 2S albumins are known food allergens, for example those from Brazil nut, walnut, sesame seed, and cashew.[13]

2S albumins are members of the prolamin super family that adopt a common fold; bundles of alpha-helices held together by four or five conserved disulfide bonds.[14] They typically appear in plants as heterodimers consisting of two polypeptide chains derived from a single precursor protein by posttranslational processing.[15] This cleavage occurs in a nonstructured loop which connects alpha-helices.[14] However, in peanut, the 2S albumins are essentially monomeric proteins.[10,16] Apparently, post-translational processing of 2S albumins in peanut is different from that in other plants. Post-translation cleavage has been

observed in other peanut storage proteins, such as Ara h 3,[17] indicating that the peanut plant has the capability for post-translational cleaving of peanut seed storage proteins.

Few reports have described smaller forms of Ara h 2 and Ara h 6[18,11] and revealed some biochemical characteristics as well as evidence of IgE binding. However, the occurrence of smaller forms of Ara h 6 in various peanut types was not investigated. Additionally, it was unclear from these studies whether the presence of such smaller forms of Ara h 6 was a naturally occurring phenomenon or an artifact of purification. In this study we have purified naturally occurring, post-translationally cleaved Ara h 6 (referred to as pAra h 6) and biochemically characterized forms of this protein. The cleavage sites were mapped using mass spectrometry, and the IgE binding properties were assessed using sera from peanut allergic and peanut sensitized patients from USA and Sweden studies. Furthermore, the presence of this cleaved form of Ara h 6 in various peanut market types was investigated.

Materials and Methods

Use of Allergen Names and Allergen Concentration Determination

Peanut (*Arachis hypogaea*) allergen Ara h 6 is listed in the WHO-IUIS Allergen Nomenclature database (www.allergen.org) as conglutin and 2S albumin. The naturally occurring posttranslationally cleaved form of Ara h 6 is here referred to as pAra h 6. Protein concentrations were determined by absorbance spectroscopy at 280 nm, using $A280_{(1mg/mL)}$ of 0.243 for intact Ara h 6, and 0.149 for pAra based on the amino acid composition of the proteins (UniProt accession number Q647G9; signal peptide of first 21 amino acids not included) taking into account that intact Ara h 6 contains amino acids 1−124 and pAra h 6 contains amino acids 1−42 plus 48−124. An intermediate form of pAra h 6 was identified as well and appeared to have heterogeneity at the cleavage site. Due to this heterogeneity the amino acid composition is not known, and therefore, the true protein concentration cannot be determined accurately by measuring A280. As a proxy, the $A280_{(1mg/mL)}$ of pAra h 6 was used, presumably leading to an overestimation of the concentration, because the intermediate form of pAra h 6 may contain a tyrosine residue at position 44.

Peanut Raw Materials and Purification of Intact Ara h 6 and pAra h 6

Raw peanuts (market types Runner, Virginia, Spanish, and Valencia) were from Jimbo's Jumbos, Inc. (Edenton, North Carolina, USA). Peanut butter, the standard reference material (SRM) 2387 was purchased from the National Institute of Standards and Technology (Gaithersburg, Maryland, USA).

For testing by reverse-phase HPLC (rp-HPLC), extracts were made by grinding peanuts and extracting the ground material with 50 mM Tris-HCl buffer (pH 8.2), in 1 to 10 ratio, for 16 h at 4°C. The aqueous layer was collected by centrifugation, diluted in 50 mM Tris-HCl buffer (pH 8.2) filtered (0.22 μm) before injection.

For purification of intact Ara h 6 and pAra h 6, Virginia-type raw peanuts were milled and defatted and subsequently extracted as described earlier.[4] Intact Ara h 6 was purified essentially as described earlier,[4] except that an extra purification step after the anion exchange column was applied by hydrophobic interaction chromatography using a Source Phenyl column (Thermo Fisher Scientific, Uppsala, Sweden). An ammonium sulfate gradient

(from 2.15−0 M in water) was used to fractionate. Peaks containing intact Ara h 6 (identified as a 15 kDa band on reducing SDS-PAGE) were pooled, dialyzed against water, and subsequently lyophilized. Anion exchange fractions identified as containing pAra h 6 (15 kDa band under nonreducing conditions, multiple bands of 5−10 kDa under reducing conditions) were further purified using hydrophobic interaction chromatography, using the same conditions as for intact Ara h 6. Two peaks of approximately equal area were observed. The first peak contained pAra h 6 (identified as a 15 kDa band on nonreducing SDS-PAGE and as a doublet of 5 and 10 kDa on reducing SDS-PAGE), and the second peak contained a protein with similar band pattern plus one extra band. This protein was identified as the intermediate form of pAra h 6 (see Results and Discussion). Peaks were pooled and dialyzed against water and subsequently lyophilized. Lyophilized powders were stored refrigerated in airtight containers. Pure fractions were about 100 mg each.

SDS-PAGE Analysis and Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on Mini-PROTEAN-Tris Tricine gels (Bio-Rad, Hercules, California, USA) operated in the Mini-PROTEAN system according to the instructions of the manufacturer. Protein samples were mixed 1:1 (v/v) with Tris Tricine sample buffer either in the presence of a reducing agent (350 mM dithiothreitol (DTT)) or in absence of a reducing agent and boiled for 10 min. Samples aliquots normalized to a constant protein amount were loaded onto the gels and run at a constant current of 100 V for ∼2 h. Following electrophoresis, gels were either fixed in 40% methanol and 10% acetic acid for 30 min and stained for 1 h with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, California, USA) or used for immunoblotting.

For immunoblotting, proteins were transferred from gels onto 0.2 μm polyvinylidene difluoride (PVDF) membranes at a constant current of 100 V for 1 h. The unbound sites on the membranes were blocked by incubating for 2 h at RT in blocking buffer (0.01 M phosphate buffer containing 0.85% NaCl, 0.2% BSA, and 0.05% Tween 20, pH 7.4) followed by washing the membranes twice (5 min each) with wash buffer (0.01 M phosphate buffer containing 0.85% NaCl and 0.05% Tween 20). Membranes were incubated for 1 h at RT with either the monoclonal antibodies (3B8 and 3E12) from the Indoor Ara h 6 ELISA kit (Indoor Biotechnologies Inc., Manchester, UK) or polyclonal antibodies from the Morinaga peanut protein ELISA kit (Morinaga Institute of Biological Science, Inc. Yokohama-Shi, Japan) at an appropriate dilution. The monoclonal antibodies supplied with the Indoor kit are used at a 1000-fold dilution for ELISA but were used diluted 1:200 (v/v) (in blocking buffer) because more dilution than 1:200 resulted in too faint bands. The polyclonal antibodies supplied with the Morinaga ELISA were diluted 1:10 in blocking buffer (v/v) and used for immunoblotting. Following the 1 h incubation, membranes were washed four times (5 min each) with wash buffer. The membranes incubated with the monoclonal antibodies were incubated with horseradish peroxidase (HRP) labeled goat antimouse immunoglobulin G (IgG; Thermo Scientific, Waltham, Massachusetts, USA) diluted 1:10 000 (v/v) in blocking buffer for 1 h at RT and washed four times (5 min each) with wash buffer. A secondary detector antibody was not used for the membranes that were incubated with the polyclonal antibodies from the Morinaga ELISA as these antibodies are supplied as

HRP conjugated antibodies for the ELISA format. The reactive bands were developed by applying the SuperSignal West Dura Extended Duration substrate (Thermo Scientific, Waltham, Massachusetts, USA) and visualizing the signal using a BioSpectrum 815 imaging system (UVP, Upland, California).

Mass Spectrometry

Purified Ara h 6 samples were diluted to 0.05 mg/mL in 30% (v/v) acetonitrile/0.1% (v/v) formic acid. tris(2-Carboxyethyl)phosphine (TCEP) was added to a final concentration of 50 mM where noted for reducing conditions. The intact mass of proteins was investigated by direct infusion of samples at a flow rate of 10 μL/min into a Thermo Q Exactive Plus Hybrid Quadrupole MS (Thermo Scientific, Waltham, MA, USA) fitted with a Nanospray Flex ion source with capillary temperature set at 320°C, spray voltage at 4.0 kV, and sweep gas of 12. Additional settings used were resolution 240 000, m/z range of 800−3000, automatic gain control (AGC) target of 1×10^6, and 100 ms maximum injection time. Positive ion mode was used for all data acquisition.

Intact protein data was deconvoluted using Xtract software (Thermo Scientific, California, USA) with settings for monoisotopic data. Deconvoluted masses were analyzed using mMass ver 5.5[19] using Ara h 6 isoform sequence data identified from the NCBI Genbank database. Cleavage sites were identified by searching experimental masses against in silico digests of each sequence isoform with no assumed cleavage rules and an infinite number of allowed cleavages.

IgE Binding by ELISA

Sera from 16 Swedish peanut-sensitized patients with IgE to Ara h 6 and/or Ara h 2 (ImmunoCAP ISAC, Thermo Fisher Scientific, Uppsala, Sweden) were selected at the Department of Clinical Immunology, Karolinska University Hospital, Stockholm (Table 1; Swedish study group). The study was approved by the local ethics committee of Karolinska Institutet (ethical approval certificate numbers 2011/2085031/4 and 2012/4:1, Stockholm, Sweden). Sera of seven patients from a US-sponsored study were also used (Table 1; US study group). The patients were from the EU and US (ethical approval certificate number IRB Project ID 6029, Lincoln, Nebraska, USA). To qualify for this study, subjects required a convincing history of a type I allergic reaction when peanuts were consumed as well as a positive skin prick test within the past 6 months or a ImmunoCAP greater than 15 kU_A/L performed within the last 6 months before study participation. The US study was approved by the University of Nebraska−Lincoln Institutional Review Board and by the ethics committee of each of the participating clinical collaborators. When serum pools were used, sera from the US study were pooled (same volume for all sera) and sera from the Swedish patients were pooled (same volume for all sera). Sera from nonpeanut allergic donors served as negative controls.

Table 1. Patient Description

ID	Study country	Total peanut ImmunoCAP (kU/L)	Ara h 2 ImmunoCAP (kU/L)	Ara h 2 ISAC (ISU)	Ara h 6 ISAC (ISU)
S-1	Sweden	5.7	ND	3.9	3.5
S-2	Sweden	2.1	ND	1	0.3
S-3	Sweden	3.5	ND	2.3	2.8
S-4	Sweden	3	ND	1.7	1.8
S-5	Sweden	> 100	ND	90	55
S-6	Sweden	> 100	> 100	ND	ND
S-7	Sweden	> 60	58	ND	ND
S-8	Sweden	> 100	> 100	ND	ND
S-9	Sweden	> 100	> 100	ND	ND
S-10	Sweden	> 100	> 100	ND	ND
S-11	Sweden	> 100	83	ND	ND
S-12	Sweden	75	58	ND	ND
S-13	Sweden	> 100	> 100	ND	ND
S-14	Sweden	75	56	ND	ND
S-15	Sweden	> 100	83	ND	ND
S-16	Sweden	> 100	66	ND	ND
US-1	US	77.3	76.3	51.3	45.8
US-2	US	71.1	40.8	32.5	34.9
US-3	US	53	39.6	64.5	76
US-4	US	139	56.7	6.7	3.45
US-5	US	575	317	31.3	64.2
US-6	US	72.5	27.4	24	50.2
US-7	US	787	259	98.5	124.3

Direct ELISA was done as follows. Half-area microtiter plates (96 wells, Greiner bio-one, Frickenhausen, Germany) were coated with 0.1 μg of corresponding antigen overnight at 4°C. After blocking with 1% BSA in 0.01 M phosphate buffer containing 0.85% NaCl and 0.05% Tween 20, plates were incubated with sera from peanut-sensitized patients and two healthy controls (dilution 1:50) for 2 h at RT. Bound IgE was detected by using mouse antihuman IgE conjugated to horseradish peroxidase (Abcam, UK) for 1 h at RT and 3,3′,5,5′-tetramethylbenzidine (TMB) was added as substrate. The absorbance was measured at 450 nm. Assays were performed in triplicate.

Inhibition ELISA was done with a coating of 0.1 μg of intact Ara h 6, blocking, washing, and IgE-detection steps as were used for the direct ELISA. The sera pools (for the Swedish and US study groups separate) were diluted 1:25 and mixed 1 to 1 with various concentrations of intact Ara h 6 and pAra h 6, resulting in a final serum pool dilution of 1:50, and allergen concentrations ranging from 10 to 0.01 μg/mL. These mixes were incubated on the coated and blocked ELISA plates and incubated for 2 h at RT. After washing plates were stained for IgE as described for the direct ELISA. Concentrations of peanut protein required for 50% inhibition (IC_{50}) of IgE binding were calculated as described previously.[20]

Reversed-Phase HPLC

Reversed-phase HPLC (rpHPLC) was performed based on chromatographic method described earlier,[21] with some modification as follows. Analyses were performed on a Waters UPLC system operated by Empower 3.0 software. Aqueous peanut extracts were analyzed on a BEH C4 column (Waters, 150 × 2.1 mm, 1.7 μm; 300 Å) with a 215 nm UV detection and a gradient elution from 95% phase A/5% phase B to 10% phase A/90% phase B, (mobile phase A H_2O/0.2% TFA; mobile phase B ACN/0.17% TFA).

Results and Discussion

Purification and Structural Analysis of Post-Translationally Cleaved Forms of Ara h 6

From side fractions obtained during a purification of Ara h 6 as described earlier,[4] two previously unidentified proteins were purified. For both proteins, > 96% of the parent ion mass spectrum could be assigned to masses derived from Q647G9 at ± 5 ppm mass accuracy, demonstrating Ara h 6 identity. Given the protein identity and the protein profile (Fig. 1), the two proteins are likely post-translationally cleaved forms of Ara h 6. One adopted the classical model of 2S albumins,[15] i.e., a 15 kDa protein band under nonreducing conditions that dissociates into ∼9 and 5 kDa bands upon reduction (Fig. 1, lanes marked "ph6") and is hereafter referred to as post-translationally cleaved Ara h 6, or pAra h 6. The other protein fraction exhibited the same bands on SDS-PAGE, plus one additional band under both nonreducing and reducing conditions (Fig. 1, lanes marked "ph6-int") and is referred to as intermediately processed Ara h 6, or intermediately pAra h 6. The purity of both proteins is estimated to be > 95%, based on SDS-PAGE and the observation that 96% of the parent ion mass spectrum could be assigned to masses derived from Q647G9 (± 5 ppm mass accuracy).

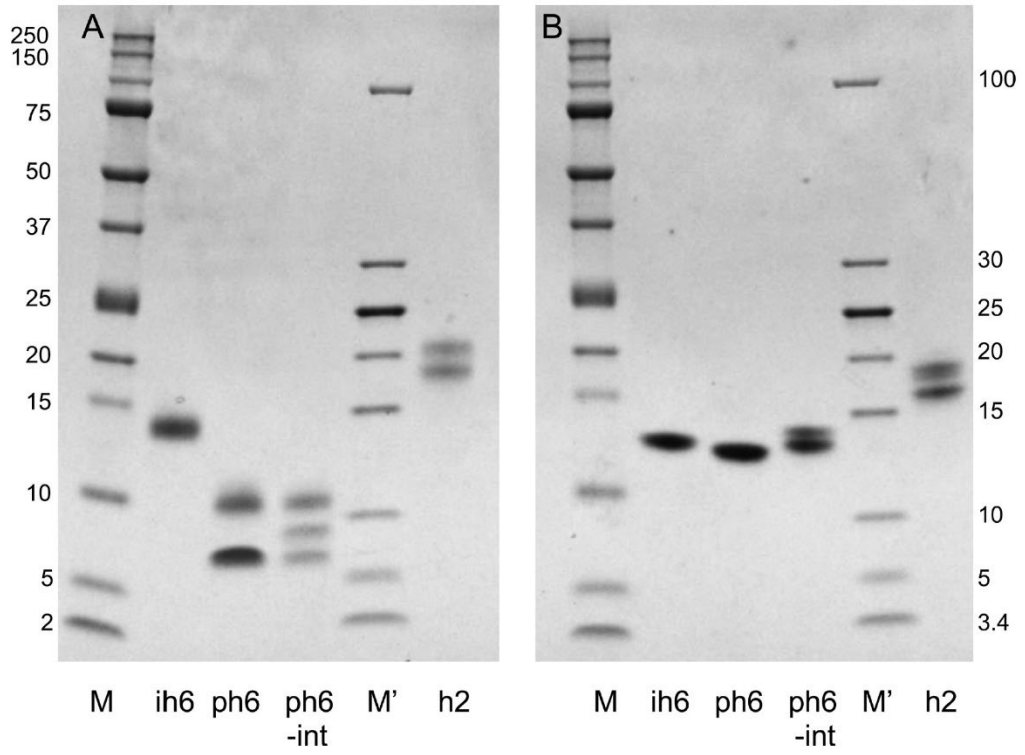
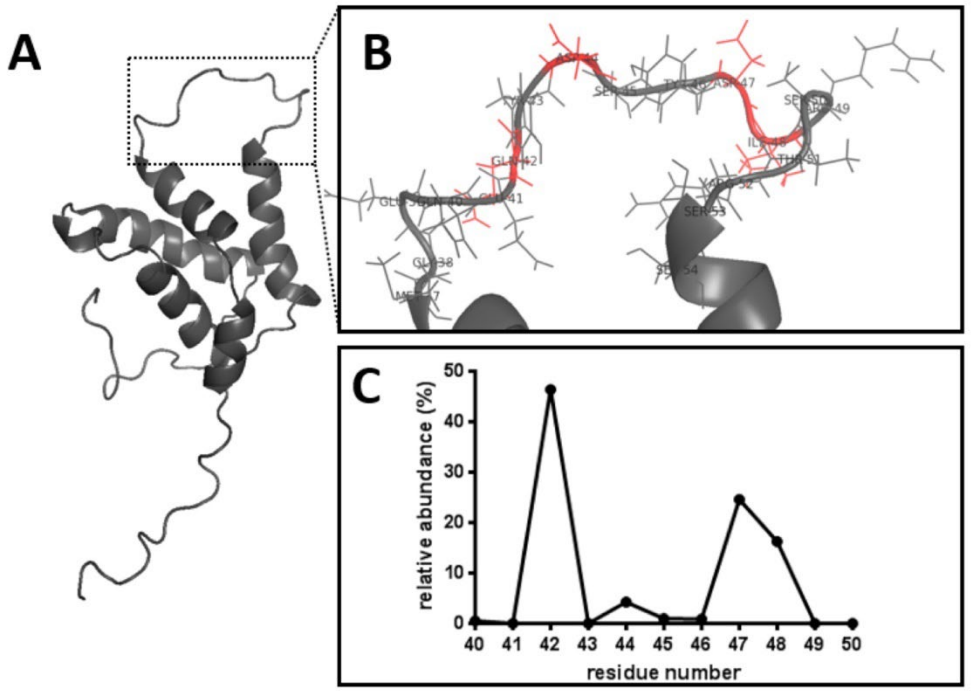


Figure 1. SDS-PAGE of intact and post-translationally cleaved Ara h 6. (A) Reducing conditions. (B) Nonreducing conditions. M marker proteins (indicated in left margin in kilodalton); M′ marker proteins (indicated in right margin in kilodalton); ih6 intact Ara h 6; ph6 post-translationally cleaved Ara h 6; ph6-int intermediate form of post-translationally cleaved Ara h 6; h2 Ara h 2.

The peptides present were mapped by intact mass analysis of pAra h 6 fractions under reducing and nonreducing conditions using a peptide mass fingerprinting approach, with assignment of individual peptides using the Q647G9 sequence. This revealed that part of the pAra h 6 forms results from two cleavage events resulting in the removal of part the nonstructured loop (residues 43−47). This is considered completely post-translationally cleaved Ara h 6 because no species with more extensive cleaving in this area were found. Another form of pAra h 6 appears to result from a single cleavage within the flexible loop, resulting in distinct N- and C-terminal peptides under reducing conditions, and the mass of the intact Ara h 6 protein +18 Da (corresponding to one hydrolysis) when unreduced (Fig. 2). This is considered intermediately post-translationally cleaved Ara h 6, because further cleaving in this area is still possible. Both of these processed forms of pAra h 6 have N- and C-terminal peptides originating from intact Ara h 6, held together by disulfide bridging when unreduced.



D

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        10         20         30         40         50         60
MRRERGRQGD SSSCERQVDR VNLKPCEQHI MQRIMGEQEQ YDSYDIRSTR SSDQQQRCCD

        70         80         90        100        110        120
ELNEMENTQR CMCEALQQIM ENQCDRLQDR QMVQQFKREL MNLPQQCNFR APQRCDLDVS GGRC
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Figure 2. Identification of cleavage sites in post-translationally cleaved Ara h 6. (A) Model of Ara h 6 with the nonstructured loop boxed. (B) Zoom-in on nonstructured loop with cleavage sites indicated in red. (C) Observed cleavage sites in the intermediate form of post-translationally cleaved Ara h 6. (D) Sequence of Ara h 6. The N- and C-terminal peptide of post-translationally cleaved Ara h 6 is underlined in black; the ragged end of the N-terminus observed for both intact and post-translationally cleaved Ara h 6 is underlined by a single gray line; and the amino acids that can either be with the N-terminal peptide or C-terminal peptide in the intermediate form of post-translationally cleaved Ara h 6 are underlined by a double gray line.

The nonstructured loop where cleavage occurs is also the domain where Ara h 6 is susceptible to hydrolysis when exposed to digestion with pepsin or trypsin.[22] This confirms that this nonstructured loop, which is post-translationally cleaved completely for 2S albumins from other sources than peanut, is indeed susceptible for proteolysis. Yet, in peanut an intact form of Ara h 6 and a post-translationally cleaved form apparently coexist. Based on homology with other plant seed storage proteins, the presence of post-translationally cleaved forms of Ara h 6 is expected; however, only the intact form received extensive attention in peanut allergen research. One report described some characteristics of a post-

translationally cleaved form,[18] but this protein was not characterized in detail due to limited amounts obtained by HPLC-based purification. The material we purified allows for a more extensive characterization and furthermore allows to investigate if multiple forms of post-translationally cleaved Ara h 6 exist in the peanut seed.

One form of pAra h 6 adopts the classical band pattern in SDS-PAGE (Fig. 1, lanes marked "ph6"), i.e., Fifteen kDa at nonreducing conditions and 5 and 9 kDa at reducing conditions.[15] The other form shows at reducing conditions an extra band between the 5 and 9 kDa bands (Fig. 1A, lane marked "ph6-int"), which may indeed be an intermediately cleaved form based on molecular weight. Under nonreducing conditions, there is an extra band slightly above the 15 kDa band (Fig. 1B, lane marked "ph6-int"). Given the identification by mass spectrometry (see above), this ought to be Ara h 6, but a higher molecular weight than the intact form is counterintuitive for a cleaved form. One explanation for the higher molecular weight may be a larger hydrodynamic radius due to the cleavage: in the intermediately cleaved form, part of the nonstructured loop is still present and located at the termini of the subunits. These subunits are held together by disulfide bonds, but the detergent SDS has disrupted the protein folding, and an extra amino acid stretch at the termini may increase the radius. This is not the case for pAra h 6 that is completely cleaved. An increase in apparent molecular weight has earlier been shown for a denatured Ara h 6,[20] supporting our hypothesis that cleaved forms of Ara h 6 may have a higher apparent MW than intact Ara h 6. We have not further investigated what determined the higher apparent MW of intermediate form of pAra h 6.

2S albumins have conserved disulfide bonds that support a protein structure that is dominated by α-helices.[14] Lehmann confirmed by far UV-CD spectroscopy that α-helices indeed dominate the protein structure.[23] Figure 3 shows the far UV CD spectra of intact Ara h 6 and the two forms of pAra h 6. Intact Ara h 6 indeed shows the typical spectrum of an α-helical protein with spectral minima at 210 and 222 nm and a steep increase in ellipticity at a lower wavelength from 200 to 190 nm.[23] Both forms of pAra h 6 show the same spectral characteristics (differences in protein concentration explain that the spectra are not overlapping, which was done to improve readability of the figure). Thus, cleaving of Ara h 6 in its nonstructured loop does not affect the secondary protein structure and the disulfide bonds are apparently capable of maintaining this structure when the loop is cleaved.

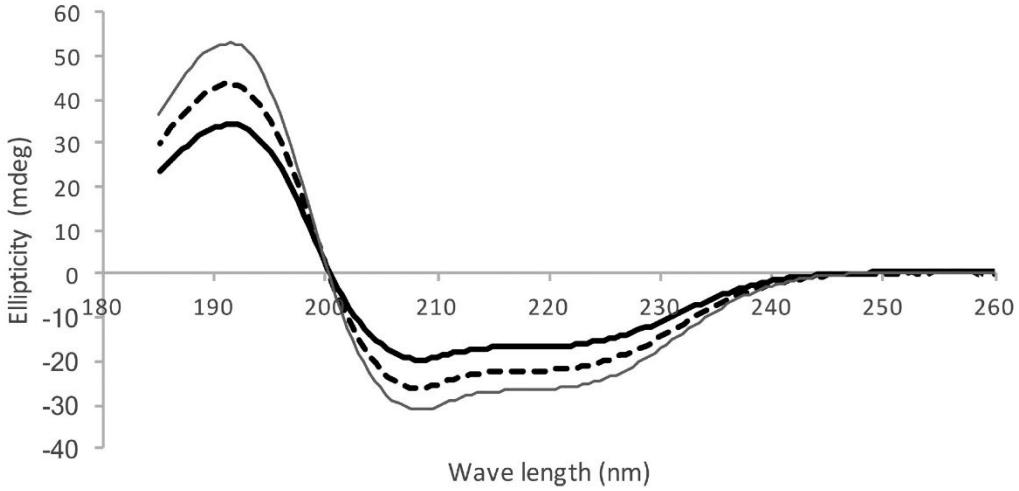

Figure 3. Far UV CD spectrum of intact and post-translationally cleaved Ara h 6. Black line: Post-translationally cleaved Ara h 6. Dotted line: Intact Ara h 6. Gray line: Intermediate form of post-translationally cleaved Ara h 6.

Intermediate forms of pAra h 6 may exist in peanut; however, we used the completely cleaved form for further comparing with intact Ara h 6 because this completely cleaved form will have greater differences with intact Ara h 6 than the intermediately cleaved forms.

Reactivity of Ara h 6 Forms with Monoclonal and Polyclonal Antibodies

Several antibodies against Ara h 6 are commercially available. Figure 4 shows the reactivity of several of these with intact Ara h 6 and pAra h 6. Panel A shows polyclonal antibodies that were developed for use in ELISA. These antibodies appear reactive to both forms of Ara h 6 under reducing conditions; however, when nonreducing conditions are used, only intact Ara h 6 shows reactivity, and this reactivity is lower than for intact Ara h 6 at reducing conditions. No reactivity is observed for the 5 kDa subunit of pAra h 6, indicating that the epitopes are located on the 9 kDa, C-terminal part of the protein. The prerequisite of reducing disulfide bonds for reactivity with this polyclonal antibody is in line with the ELISA protocol for which the polyclonal has been developed; reduction is a sample preparation step for this ELISA (Moringa ELISA peanut product insert, see Materials and Methods). Panels B and C show the reactivity of two monoclonal antibodies directed against Ara h 6, commonly used for ELISA. Both of these monoclonal antibodies show reactivity to intact Ara h 6, at reducing and nonreducing conditions, and reactivity with pAra h 6 at nonreducing conditions (Fig. 4B–C). This is in line with an earlier observation that pAra h 6 is equally reactive to intact Ara h 6 in the ELISA that utilizes these two monoclonal antibodies (sandwich ELISA; nonreducing condition).[24]

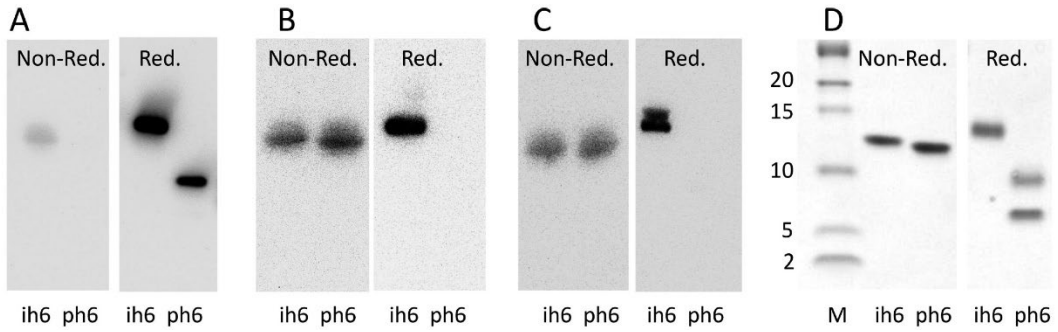

Figure 4. Reactivity of Ara h 6 forms with monoclonal and polyclonal IgG antibodies. (A) Polyclonal antibody (Morinaga). (B) Monoclonal antibody (Indoor 3B8). (C) Monoclonal antibody (Indoor 3E12). (D) Corresponding SDS-PAGE with protein stain. Non-Red.: non-reducing SDS-PAGE conditions. Red.: reducing SDS-PAGE conditions. ih6: intact Ara h 6. ph6: post-translationally cleaved Ara h 6. M: marker proteins (indicated next to marker lane in kilodalton).

Under reducing conditions, only intact Ara h 6 shows reactivity for the monoclonal antibodes, while under nonreducing conditions, both forms of Ara h 6 are being recognized. The monoclonal antibodies were developed for use in ELISA under native, nondenaturing conditions. The reactivity of both monoclonal antibodies for only the intact Ara h 6 under reducing conditions suggests that the epitopes recognized by both monoclonal antibodies on the pAra h 6 form are sensitive to denaturation. While the exact epitopes of these monoclonal antibodies have not been described, the current results allow speculation on the nature of the epitopes. First, the observation that under nonreducing conditions both intact Ara h 6 and pAra h 6 react on Westernblot indicates that the epitopes of both monoclonal antibodies are not located on the nonstructured loop where the cleave occurs (amino acids 43−48, or directedly adjacent). Second, the absence or reactivity of both monoclonal antibodies with pAra h 6 under reducing conditions suggest that the 5 and 9 kDa subunits should be associated for reactivity with the monoclonal antibodies. In the case of pAra h 6 where the peptide bond between the 5 and 9 kDa chains is cleaved, this association depends on disulfide bonds, and reduction of disulfide bonds separates the two chains. For intact Ara h 6 the two domains are associated by the peptide bond, regardless of the presence of disulfide bands, keeping the two domains together at both nonreducing or reducing conditions. Together this suggests that for both monoclonal antibodies a certain structural organization in Ara h 6 is necessary at positions other than the loop at amino acid positions 43−48 that is cleaved in pAra h 6.

IgE Reactivity of Ara h 6 Forms

Binding of allergens to IgE in sensitized patients is a prerequisite of an allergic reaction and is therefore investigated to compare intact and pAra h 6. Several papers have shown that the IgE binding to Ara h 6 is dependent on conformational epitopes,[20,25] and therefore native conditions should be used to compare IgE binding of intact Ara h 6 and pAra h 6. For this reason, an ELISA format is preferred over immunoblotting following SDS-PAGE.

Figure 5 shows binding of IgE from patients from a Swedish and US study group to intact Ara h 6 and pAra h 6 in a direct ELISA. While there is diversity in IgE binding between patients, the IgE binding to the two different forms of Ara h 6 is comparable on a per patient level. This is further illustrated by a correlation plot (Fig. 5B).

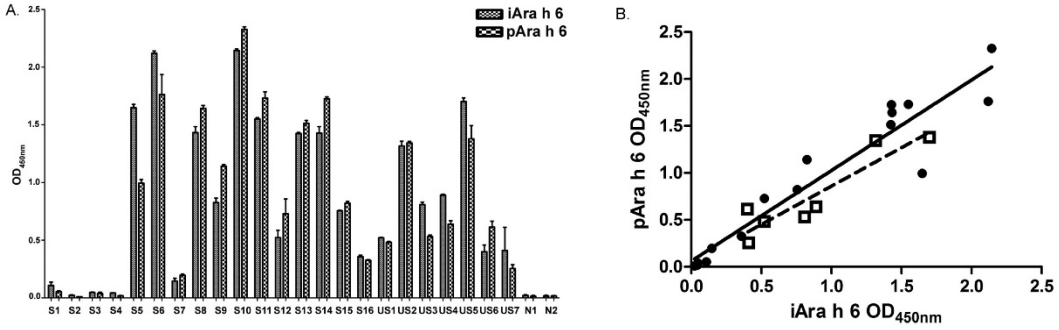

Figure 5. IgE binding to intact and post-translationally cleaved Ara h 6. (A) Numbers on the X-axis refer to patient codes in Table 1. Bars with fine dots: Intact Ara h 6. Bars with large dots: Post-translationally cleaved Ara h 6. OD450nm: optical density at 450 nm. (B) Correlation of IgE binding between intact Ara h 6 and post-translationally cleaved Ara h 6 for sera from the US study (open squares) and Swedish study (filled circles) patients. OD450nm: optical density at 450 nm.

Inhibition ELISA was used to quantify the IgE binding, using pools of patient serum. Figure 6 shows the inhibition lines, which are virtually overlapping for intact and pAra h 6. The concentration required for inhibiting the signal for 50% (IC_{50}) was calculated based on triplicate experiments and was 0.41 μg/mL (± 0.28 μg/mL) for intact Ara h 6 and 0.56 μg/mL (± 0.27 μg/mL) for pAra h 6 for the Swedish study group and 0.26 μg/mL (± 0.13 μg/mL) for intact Ara h 6 and 0.26 μg/mL (± 0.16 μg/mL) for pAra h 6 for the US study group. Relevant differences in IgE binding potency, for example for chemically modified hypoallergic extracts used for immunotherapy, are typically 10−100-fold and preferably more.[20,26–28] Thus, the values we report here indicate that intact and pAra h 6 have highly comparable IgE binding.

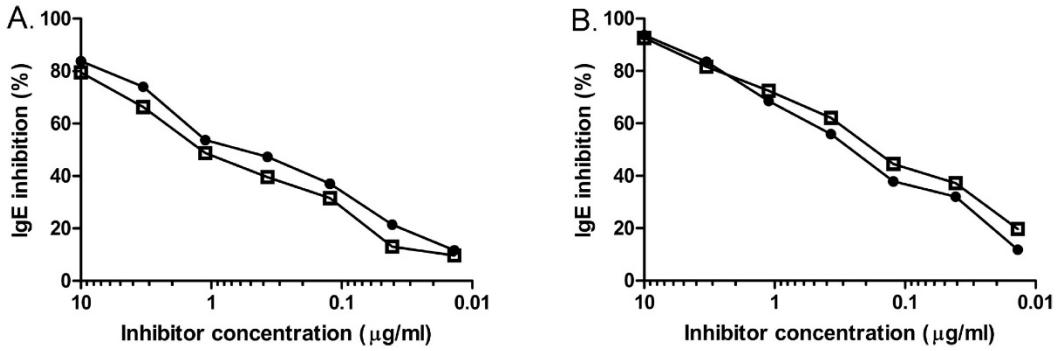

Figure 6. Potency of intact and post-translationally cleaved Ara h 6 to inhibit IgE binding. (A) Sera from Swedish study; (B) Sera from US study. Filled circles: Inhibition with intact Ara h 6. Open squares: Inhibition with post-translationally cleaved Ara h 6.

Presence of Post-Translationally Cleaved Ara h 6 in Various Peanut Market Types

In order to further establish the relevance of pAra h 6, the occurrence and abundance in various peanut types was investigated. The four main market types commonly consumed in the US (i.e., Runner, Virginia, Spanish, and Valencia), as well as SRM 2387 peanut butter, were analyzed for protein profile on SDS-PAGE. By comparing with the purified forms of Ara h 6, it is shown that all samples contain bands corresponding with both intact Ara h 6 and pAra h 6 (Fig. 7A). Reactivity of these bands with the polyclonal antibody reactive for Ara h 6 supports that these bands are indeed of Ara h 6 identify (Fig. 7B, boxed area), although it cannot be excluded that they may contain Ara h 2 as well, based on the specificity of this polyclonal antibody.[29] The reactive band corresponding with pAra h 6 is found in all peanut types as well as in peanut butter and is less intense than the band corresponding with intact Ara h 6 (Fig. 7B). Based on the data presented in Figure 4, it is known that pAra h 6 is less reactive than intact Ara h 6 with this polyclonal antibody, which makes it difficult to estimate the ratio of the amounts of intact Ara h 6 and pAra h 6 in the different samples.

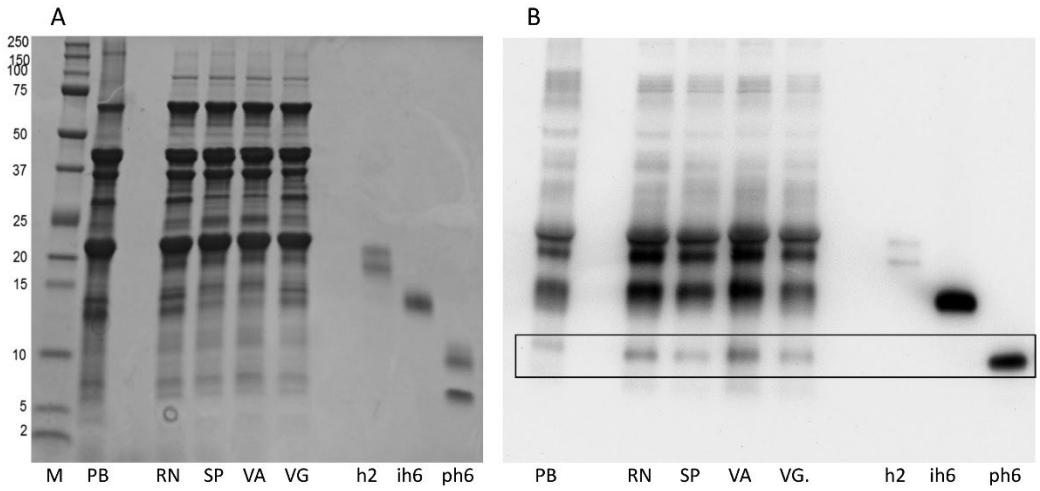

Figure 7. SDS-PAGE profiles of various peanut market types and intact and post-translationally cleaved Ara h 6. (A) SDS-PAGE (reducing conditions). (B) Westernblot using polyclonal antibodies (Morinaga) against Ara h 2 and Ara h 6. M: marker proteins (indicated left in kilodalton). PB: peanut butter. RN: Runner peanut. SP: Spanish peanut. VA: Valencia peanut. VG: Virginia peanut. h2: Ara h 2. ih6: intact Ara h 6. ph6: posttranslationally cleaved Ara h 6. The boxed area in panel B indicates the band of pAra h 6 that was detected by Western blotting.

Reversed-phase-HPLC was used to further investigate the occurrence of pAra h 6 in peanut. Peanut extract shows various peaks in the area where Ara h 6 elutes (Fig. 8A). Using purified intact Ara h 6 and purified pAra h 6 (Fig. 8B), the four main peaks were assigned: Peaks 5 and 6 represent intact Ara h 6, and peaks 2 and 4 represent pAra h 6. The fact that for each form of Ara h 6 two peaks are found may be explained by the presence of different gene products (UniProt IDs Q647G9 and A1DZE9) or by loss of N- or C-terminal amino acids, similar to what has been described for Ara h 2.12 This has not been further investigated. Two smaller peaks can be observed for peanut extract, and these are attributed to the intermediate forms of pAra h 6 that show various peaks matching with peaks 1 and 3 and also several that are overlapping with intact Ara h 6 and pAra h 6 (not shown). Contribution of the individual peaks to the Ara h 6 area as presented in Figure 7A was calculated for the four peanut types. Intact Ara h 6 constitutes approximately half of the Ara h 6 content in peanut, although small differences are observed between peanut types (Table 2). Consequently, the other half of the Ara h 6 content comes from pAra h 6, indicating an important contribution of pAra h 6.

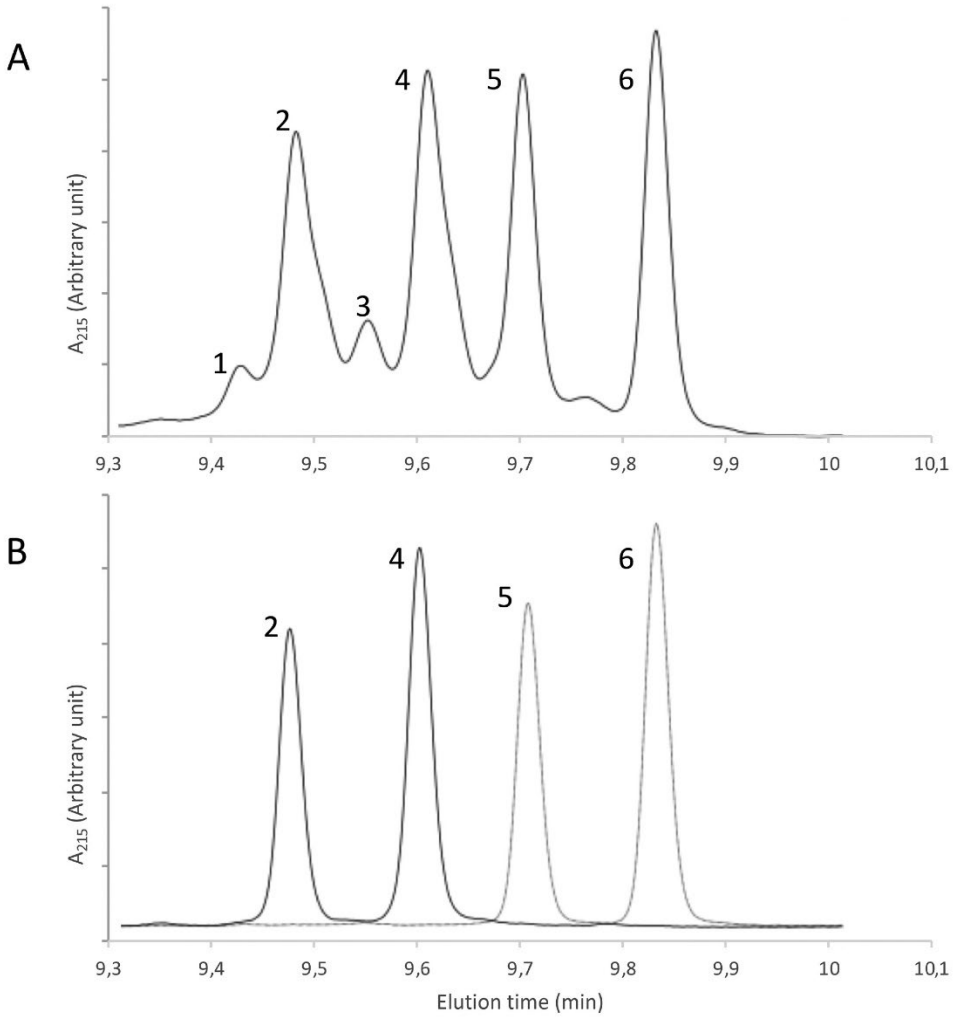

Figure 8. Reversed-phase UPLC profiles of various peanut market types and intact and post-translationally cleaved Ara h 6. (A) Virginia peanut. (B) Intact (light gray chromatogram) and post-translationally cleaved Ara h 6 (dark gray chromatogram).

Table 2. Percentage of Intact and Post-Translationally Cleaved Ara h 6 in Different Peanut Market Types

	rp-UPLC fractions						iAra h6	pAra h 6	
								Fully cleaved	All forms
	P1	P2	P3	P4	P5	P6	(P5 + P6)	(P2 + P4)	(P1 + P2 + P3 + P4)
Runner	2.3	21.4	5.2	24.3	24.9	21.9	46.8	45.7	53.2
Spanish	2.0	16.8	4.6	22.4	26.5	27.7	54.2	39.2	45.8
Valencia	2.8	19.2	5.1	24.1	24.4	24.5	48.8	43.3	51.2
Virginia	2.9	21.9	6.3	25.5	22.6	20.8	43.4	47.4	56.6

Combined with the observation that pAra h6 has comparable IgE binding potency as intact Ara h 6 (Fig. 6), its high abundance in all four main market types of peanut commonly grown and consumed in the US makes pAra h 6 an allergen that must be taken into account when Ara h 6 is investigated in peanut-containing products.

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