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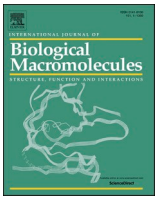

Novel post-translationally cleaved Ara h 2 proteoforms: Purification, characterization and IgE-binding properties

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ABSTRACT

The 2S albumins Ara h 2 and Ara h 6 have been shown to be the most important source of allergenicity in peanut. Several isoforms of these allergens have been described. Using extraction and liquid chromatography we isolated proteins with homology to Ara h 2 and characterized hitherto unknown Ara h 2 proteoforms with additional post-translational cleavage. High-resolution mass spectrometry located the cleavage site on the non-structured loop of Ara h 2 while far UV CD spectroscopy showed a comparable structure to Ara h 2. The cleaved forms of Ara h 2 were present in genotypes of peanut commonly consumed. Importantly, we revealed that newly identified Ara h 2 cleaved proteoforms showed comparable IgE-binding using sera from 28 peanut-sensitized individuals, possessed almost the same IgE binding potency and are likely similarly allergenic as intact Ara h 2. This makes these newly identified forms relevant proteoforms of peanut allergen Ara h 2.

1. Introduction

Amongst food allergies, peanut allergy is one of the most common in Western countries and its prevalence is 1 to 2 % [1]. Peanut allergy is notorious for its persistence, and because traces of peanut in food products can induce severe, life-threatening reactions [2,3]. Dominant allergens are members of the 2S albumin protein family [4], which often play an important role in allergenicity of other plant foods such as tree nuts [5]. In the case of peanut, major 2S albumins are Ara h 2 and Ara h 6 [6], which have been shown to be the most potent allergens *in vitro* [7,8] and *in vivo* [9] studies. For diagnostics purposes, and as an OIT prognostic tool, IgE to Ara h 2 was shown to be the best predictor for clinical relevance in peanut allergy [10–13]. Ara h 6 is highly homologous to Ara h 2 and is recognized by many peanut-allergic individuals' IgE [10]. Other peanut allergens such as Ara h 1 and Ara h 3 are more abundant than Ara h 2 and Ara h 6 in peanut seeds [14,15], but show lower allergenic potency.

Ara h 2 in peanut is represented by two main isoforms, Ara h 2.01 and Ara h 2.02, with the former lacking a number of amino acids residing in the loop region, resulting in a smaller molecular weight. Typically, Ara h 2 isolated from peanut is as mix of these isoforms, presenting on SDS-PAGE as a doublet of approximately 20 kDa [16]. *C*-terminal proteolysis can occur in both isoforms, resulting in truncated forms that lack 2 terminal amino acids. This difference is not sufficient to distinguish the intact and *C*-terminally cleaved forms, but high-resolution analytical chromatography can separate these four isoforms [17].

2S albumins typically appear in plants as heterodimers consisting of two polypeptide chains derived from a single precursor protein by post-translational processing [18]. This cleavage occurs in a non-structured loop, resulting in a large and small subunit. However, in peanut, the 2S albumins are essentially monomeric proteins [19,20]. Apparently,

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post-translational processing of 2S albumins in peanut is different to that in other plants. Post-translational cleavage has been observed in other peanut storage proteins, such as Ara h 3 [21], illustrating that the peanut plant is capable of post-translational modifications and that Ara h 2 is not *per se* inert to such modifications. Also, post-translational modifications such as *C*-terminal truncation and hydroxylation of prolines have been described for Ara h 2 [15,17].

Cleaved 2S albumins on non-reducing SDS-PAGE are seen as single protein band, because the disulphide bonds hold the two subunits together. Upon reduction, two bands appear on SDS-PAGE, for example, at 8 and 4 kDa in the case of Brazil nut representing the large and small subunit. Up to now, no cleaved forms of Ara h 2 have been described [7]. Fragments of Ara h 2 have been identified on 2-dimensional electrophoresis [22], however without further characterization. For Ara h 6 we recently identified and characterized a cleaved form of Ara h 6 [23,24], and we therefore speculated that cleaved forms of Ara h 2 exist in peanut as well.

In this work, we set out to search for, purify and characterize a cleaved form of Ara h 2, and to determine its relevance in for the peanut's allergenicity.

2. Materials and methods

2.1. Use of allergen names and allergen concentration determination

Peanut (*Arachis hypogaea*) allergen Ara h 2 is listed in the WHO-IUIS allergen Nomenclature database (www.allergen.org) as conglutin and 2S albumin, with two main isoforms; Ara h 2.01 (GenBank: AAK96887.1, small isoform, mature protein is 139 amino acids) and Ara h 2.02 (GenBank: AAN77576 large isoform, mature protein is 151 amino acids). These sequences correspond to NP_001363146.1 and NP_001392340.1 derived from the peanut genome. Both these isoforms can be C-terminally truncated, losing 2 amino acids [17]. The two preparations of post-translationally cleaved Ara h 2 described in this study are denoted pAra h 2 S1 and pAra h S2, for sample 1 and sample 2, respectively. This notation does not coincide with published names for Ara h 2 isoforms. These isoforms are denoted using the WHO-IUIS allergen nomenclature (*e.g.* Ara 2.01 and Ara h 2.02). When 'Ara h 2' is used in this paper, the mix of Ara 2.01 and Ara h 2.02 is meant. Protein concentrations were determined by absorbance spectroscopy at 280 nm, using A280(1 mg/mL) of 0.83, based on NP_001392340.1.

2.2. Peanut raw materials and purification of intact Ara h 2 and pAra h 2 proteoforms

Peanut proteins were obtained from an extract of raw Virginia type peanuts using anion exchange and hydrophobic interaction chromatography, as described earlier [25]. Fractions from the hydrophobic chromatography were analyzed by SDS-PAGE, under reducing and non-reducing conditions. Fulfilling criteria for common plant 2S albumins [18], candidates of post-translationally cleaved Ara h 2 were fractions that show two protein bands at approximately 12 and 8 kDa (at reducing conditions) while no intact band at approximately 20 kDa is visible. Such fractions were found in the elution area between 130 and 110 mS/cm. This crude sample, denoted as 'C' in Fig. 1A, was analyzed by mass spectrometry to confirm Ara h 2 identity and by far-UV CD spectroscopy to investigate if the typical secondary structure content of plant 2S albumins was still present. A side fraction with similar protein profile but with an extra band was kept as well (denoted as 'S' in Fig. 1A).

These fractions C and S were combined with replicate fractions from other hydrophobic chromatography runs, resulting in Pool C and Pool S. These pools were further independently purified using anion exchange chromatography using a Source Q column (8 mL volume) (Thermo Fisher Scientific, Uppsala, Sweden). Fractions were eluted using a salt gradient from 0 to 1 M NaCl in 20 mM Tris buffer, pH 8.0. Fractions showing two protein bands at approximately 12 and 8 kDa (under reducing conditions), in the absence of a 20 kDa band, were collected. This resulted in two preparations of pAra h 2, referred to as Sample 1 (from Pool C) and Sample 2 (from Pool S) and are referred to as pAra h 2 S1 and pAra h 2 S2, respectively. Materials were lyophilized and stored refrigerated in airtight containers.

Samples of different market types and cultivars were taken from a previous study [14].

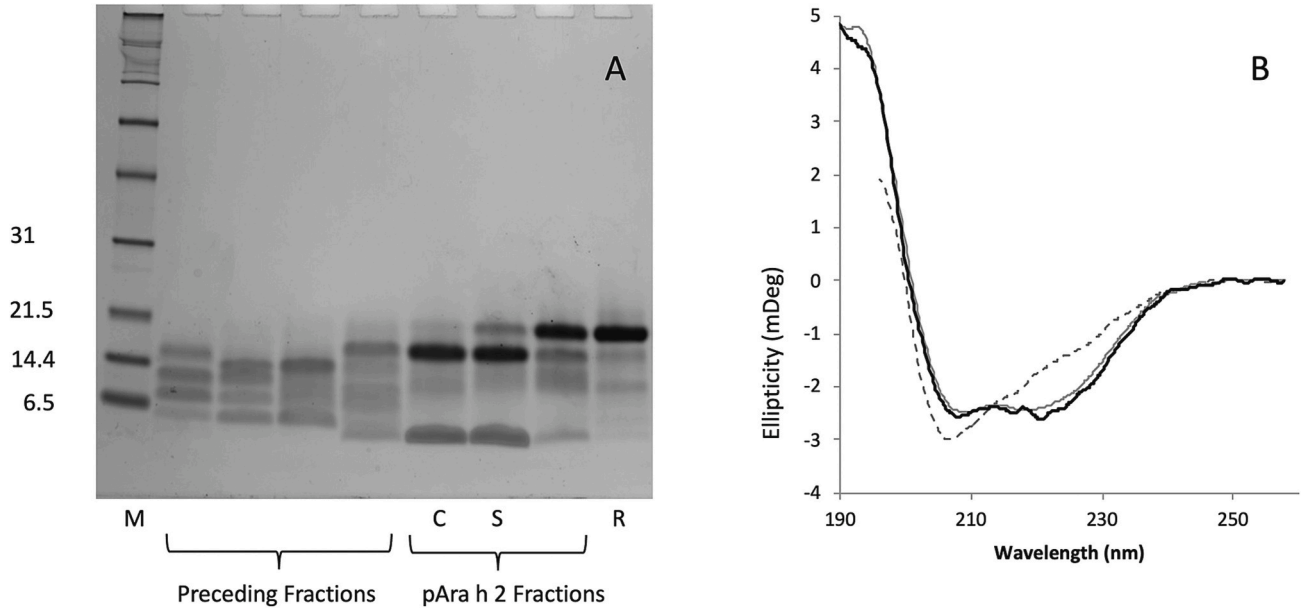

Fig. 1. *Characteristics of crude pAra h 2.* Panel A: Protein profile by reducing SDS-PAGE. Fractions from hydrophobic interaction chromatography column. M: Marker proteins (indicated in left margin in kDa), Preceding fractions: fractions eluting before the pAra h 2 candidate is eluting, pAra h 2 Fractions: fractions containing a pAra h 2 candidate, C: the crude pAra h 2 candidate, S: Side fraction that contains crude pAra h 2 as well as other protein bands, R: residual fractions (one of series) that do not contain the pAra h 2 candidate. Panel B: Far UV CD spectra of (crude) pAra h 2 candidate (lane C from Panel A) and other peanut allergens. Black line: (crude) pAra h 2 candidate. Gray line: Ara h 2. Striped line Ara h 1.

2.3. SDS-PAGE analysis

SDS-PAGE analysis was performed on Mini-PROTEAN-Tris Tricine gels (Bio-Rad, Hercules, CA, 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 (350 mM 1,4-Dithio-D-threitol (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, CA, USA) or used for immunoblotting.

2.4. Native and reduced intact mass spectrometry

Purified pAra h 2 samples were diluted to 0.25 mg/mL in 5 % (*v*/v) acetonitrile/0.1 % (v/v) formic acid. For reduced intact mass analysis, DTT, was added to a final concentration of 50 mM, heated to 95 °C for 5 min and cooled prior to analysis. The samples were separated by one-dimensional microscale liquid chromatography (LC) using an UltiMate 3000 RSL® liquid chromatography (UPLC) system (Thermo Scientific™), equipped with a Acquity UPLC Protein BEH C4, 300 Å, 1.7 μm, 2.1 mm × 150 mm and an equivalent VanGuard™ pre-column, 2.1 mm × 150 mm (Waters Corporation, Milford). Mobile phase A consisted of water containing 0.1 % (v/v) formic acid, while mobile phase B was 100 % (v/v) acetonitrile containing 0.1 % (v/v) formic acid. The sample was injected (8 μL on-column) and proteins were eluted from the column and separated using a gradient of 5–35 % mobile phase B over 24.5 min at a flow rate of 250 $\mu L.min^{-1}$. The column temperature was maintained at 35 °C.

The eluted proteins were directed into a Thermo Q Exactive Plus Hybrid Quadrupole MS (Thermo Scientific, Waltham, MA, USA) set in Full MS mode, fitted with a microspray flex ion source. The capillary temperature was set at 320 °C, spray voltage at 3.5 kV, sheath gas of 50, auxiliary gas of 10 and the probe heater set at 200 °C. Additional settings used were resolution 140,000, *m/z* range of 600–2500, automatic gain control (AGC) target of 1×10^6, and 200 ms maximum injection time. Positive ion mode was used for all data acquisition.

Data was deconvoluted using Xtract software (Thermo Scientific, CA, USA) using a sliding window algorithm. Monoisotopic data output was selected. Resultant deconvoluted masses, with >3 charge states, were analyzed using mMass ver 5.5 [26] using Ara h 2 isoform sequence data (GenBank: NP_001363146.1 and NP_001392340.1). Native intact mass data was assumed to have intact cystine, while reduced samples were assumed to have cysteine residues. Cleavage sites were identified by matching experimental monoisotopic masses against predicted monoisotopic masses of the *in silico* mass prediction of each sequence. This was conducted by *in silico* digests of the sequences with the following rules: infinite number of allowed cleavages and variable hydroxyproline. Matching monoisotopic masses with an error tolerance of less than ±10 ppm established proteoform identification. Relative amounts of cleaved proteoforms were estimated by comparing fractional abundances of deconvoluted mass intensities.

2.5. Analysis of 20 cultivars of common peanut market-types by bottom-up proteomics

A previously published bottom-up dataset [15] was re-analyzed to determine if there was supporting evidence for the Ara h 2 cleavage sites identified herein by intact mass spectrometry. The data analysis parameters of PEAKS were modified to allow cleavage between any two residues ('no enzyme' setting). This was followed by a PEAKS PTM search. This semi-quantitative MS1 data of the unique peptides between Cys-45 and Cys-91 of Ara h 2.01 and between Cys-45 and Cys-103 of Ara h 2.02 were collated. This region, which holds the putative cleavage site, is also the variably modified hydroxyproline (HyP) region in Ara h 2, complicating the analysis [15]. Matching peptides showing an intensity of $>1e^5$, were further assessed by inspection of their resultant MS2 spectra. Peptides without ions detected on both sides of a hydroxyproline modification in at least 1 spectrum were not accepted and removed from the assessment. Furthermore, an A score > 10 was deemed sufficient. Eight peptides met these criteria, including 2 tryptic peptides from Ara h 2.01 and Ara h 2.02. Three "non-tryptic" peptides were derived from Ara h 2.01 and 3 "non-tryptic" peptides were derived from Ara h 2.02.

Estimation of Ara h 2 cleaved *vs* non-cleaved in the raw peanut extracts was carried out by normalizing the MS1 peak area intensity of the selected peptides (see above) to the MS1 peak area intensity of the top3 rabbit glycogen phosphorylase peptides, spiked into the sample, allowing estimation of fmol (on column) relative amount of each selected peptide across all genotypes studied. Peptides with a similar HyP motif were grouped, and the tryptic peptide (assumed to originate from a non-cleaved Ara h 2 proteoform) was compared *via* ratio to the non-tryptic peptides (assumed to originate from cleaved Ara h 2 proteoforms) allowing an estimation of the frequency of cleavage for a particular Ara h 2 HyP proteoform. This method of determining the relative amount of cleavage relies on all detected peptides giving similar ion abundances at equivalent amounts of peptide and can therefore only be considered an estimate.

2.6. Patient serum and IgE-binding tests

Sera from 28 peanut-sensitized patients to Ara h 2, (tested by ImmunoCAP Thermo Fisher Scientific, Uppsala, Sweden), were selected at the Department of Clinical Immunology, at Karolinska University Hospital, Stockholm. Table S1 shows the serum IgE values for Ara h 2 (f423) and total peanut protein (f13). Sera were used individually for direct IgE-binding and as pool for IgE-inhibition studies. The serum pool contains all sera where the same volume was used for construction. The pool had an Ara h 2-IgE (f423) titer of 88 kU_A/L, and peanut (f13) titer of >100 kU_A/L. Sera from non-peanut allergic donors, sensitized to house dust mites (C1) and α-Gal (C2), served as negative controls.

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 allergen 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 2 healthy controls in three different dilutions (1:25, 1:50 and 1:100) for 2 h at room temperature (RT). Bound IgE was detected by using mouse anti-human 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 the same coating, blocking, washing, and IgE-detection steps as were used for the direct ELISA. The serum pool was diluted 1:25 and mixed 1 to 1 with various concentrations of intact Ara h 2, pAra h 2 S1 and pAra h 2 S2, resulting in a final serum pool dilution of 1:50, and allergen concentrations ranging from 10 μg/mL to 0.01 μg/mL. These mixes were incubated on the plates for 2 h at RT. After washing plates bound IgE was detected as above for the direct ELISA. Concentrations of peanut protein required for 50 % inhibition (IC_{50}) of IgE-binding were calculated as described previously [27].

2.7. Mediator release assay

The allergenic activity was measured using humanized rat basophil leukaemia cells (hRBL clone RS-ATL8), kindly provided by Prof. Ryosuke Nakamura [28]. hRBL cells were cultivated in cell culture medium MEM supplemented with 10 % heat-inactivated FBS, 100 U/mL penicillin-streptomycin, 0.2 mg/mL geneticin, 0.2 mg/mL hygromycin B, 0.2 mM L-Glutamine (Gibco). For the assay, hRBL cells were loaded

with a 1:50 diluted serum pool (heat-inactivated at 56 °C for 5 min) in a cell culture medium overnight at 37 °C. Six allergen dilutions (10000–0.1 pg/mL) of Ara h 2, pAra h 2 S1 and pAra h 2 S2 were used for stimulation. In addition, total ß-hexosaminidase release induced by adding 10 % Triton X-100 and serum without allergens were used as controls. The release of ß-hexosaminidase was detected by adding substrate 4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma, Merck), incubating 1 h at RT. The plate was read on a microplate reader (Varioskan™ LUX, Thermofisher Scientific) for fluorescent intensity at 360 nm excitation/465 nm emission. Results are shown as the percentage of total ß-hexosaminidase release achieved (complete cell lysis using Triton-X 100). The experiment was done in duplicate.

3. Results

3.1. Purification of post-translationally cleaved forms of Ara h 2

Following a protocol for the purification of peanut 2S albumins [25], crude preparations of pAra h 2 were isolated after hydrophobic chromatography. Fig. 1A shows the electrophoretic pattern at reducing conditions. Several fractions with one band at approximately 16 kDa and one band at approximately 5 kDa are observed. In Fig. 1A, these fractions are denoted as 'C' for the crude pAra h 2 candidate and 'S' for a side fraction that contains pAra h 2 candidate as well as other protein bands. (fractions C and S in Fig. 1A). One other fraction shows a single band at approximately 20 kDa (potentially intact Ara h 2, (lane R in Fig. 1A), and other fractions show a mixed pattern, potentially a mix of intact Ara h 2 and pAra h 2. Fraction C was further studied with label-free MS analysis confirming Ara h 2 identity and showing a minor contamination of Ara h 6 and Ara h 7 (Supplementary material and Table S2). Ara h 1 identity was not detected. Far UV CD spectroscopy showed that the structure of the crude pAra h 2 protein is dominated by α-helices, as is known for plant 2S albumins [29]. The spectrum is essentially overlapping with that of intact Ara h 2 (Fig. 1B), suggesting that the cleavage does not result in a major change in secondary structure of the protein, as was earlier also demonstrated for a post-translationally cleaved form of Ara h 6 [23] and *in vitro* digested forms of Ara h 2 [30].

Fractions corresponding to C and S, from multiple runs, were further purified using anion exchange chromatography to remove traces of Ara h 6 and Ara h 7, and traces of potentially present intact Ara h 2. This purification was done with two independent pools because the different crude pAra h 2 fractions contain different levels of contaminants (Fig. 1A compare lane C with lane S), and because they may have different Ara h 2 isoform distribution. From each anion exchange run one main peak eluted and pools were made by conservatively cutting off the shoulders (chromatographs shown as Supplemental Figs. 1 and 2). The protein profiles of the resulting preparations are shown in Fig. 2. Panel A shows for reference Ara h 2, isoform Ara h 2.01 and isoform Ara h 2.02 (all at reducing conditions). Panel B shows the two preparations of pAra h 2, both at reducing and non-reducing conditions. Both preparations of pAra h 2 fulfill the criteria for cleaved 2S albumins, *i.e.* a large and small subunit upon reduction, while associated if not reduced [18].

3.2. Characterization of Ara h 2 proteoforms in S1 and S2 purifications by intact mass spectrometry

The samples were subjected to native intact mass analysis and the data was deconvoluted to give experimental monoisotopic masses. *In silico* mass prediction used the sequences of both Ara h 2.01 and 2.02 (GenBank: NP_001363146.1 and NP_001392340.1), minus the signal peptide, which were assumed to have 4 disulphide bonds. We also considered the known post-translational modifications of Ara h 2. These include variable HyP modification (between 1 and 3) in the loop region and variable *C*-termini trimming of the RY residues. Furthermore, as the samples were known to separate on SDS-PAGE into a small and large subunit (see Fig. 2), the hydrolysis reaction would be expected to

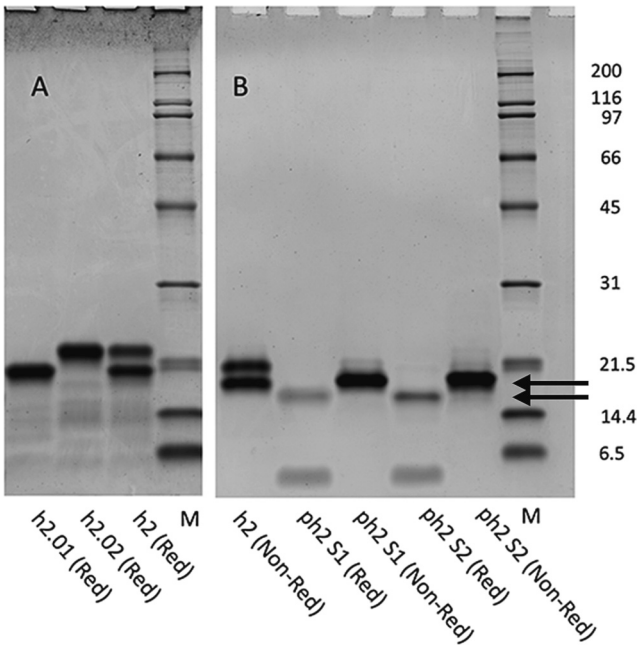

Fig. 2. *Protein profiles of purified pAra h 2.* Panel A: SDS-PAGE of reference proteins Ara h 2.01 ('h2.01'), Ara h 2.02 ('h2.02') and Ara h 2 ('h2'), M are marker proteins (indicated in right Margin in kDa). Panel B: SDS-PAGE of pAra h 2 sample 1 ('h2 S1) and sample 2 ('h2 S2) and Ara h 2 ('h2'). Either reducing (indicated 'Red') or non-reducing (indicated 'Non-Red') conditions were used for SDS-PAGE. Arrows points two protein bands in non-reducing condition of pAra h 2S1 and S2 samples, where the dominant band represent cleaved forms from Ara h 2.01 proteoform.

increase the monoisotopic mass in native intact mass analysis by 17.99 Da (H_2O addition). The experimental and predicted monoisotopic masses were compared. An error tolerance of ±10 ppm was used to establish identification of Ara h 2 proteoforms.

We were able to identify 84 % of the signal (fractional abundance) in the S1 sample and 88 % of the signal in S2 sample. Both samples were predominantly composed of an Ara h 2.01 proteoform with 2 HyP modifications and a proteolytic cleavage (see Table 2 and Supplementary Table S3). The predominant proteoforms in the 2 samples differed in their C-termini trimming. The RY residues in Ara h 2.01-S1 are absent, while they are present in Ara h 2.01-S2. These proteoforms accounted for 71 % of the signal in S1 and 73 % in S2.

Other Ara h 2.01 proteoforms (six in total) were also identified in both samples. The less abundant proteoforms ranged from 0.3 to 8.1 % of the signal. These proteoforms differed in HyP modification (1 or 3) or C-termini trimming from the predominant proteoform. We also identified 2 proteoforms of Ara h 2.02 in sample S2, both showing 3 HyP modifications. Together they accounted for 1 % of the signal (see Supplementary Table S3). Proteoforms showing a deletion of sequence were not detected, suggesting that proteolytically cleaved Ara h 2 undergoes a single cleavage, unlike proteolytically cleaved Ara h 6 [23].

The cleavage site is located in the non-structured loop (between the second and third cysteine of Ara h 2) that connects the α- helices. This is similar to what is seen in other plant 2S albumins [18] and in peanut allergen Ara h 6 [23]. Also from *in vitro* digestion studies it is know that this non-structured loop is susceptible for proteolysis [30]. In contrast to 2S albumins from other plants, Ara h 2 coexist as an intact form and a post-translationally cleaved form.

The samples were subjected to reduced intact mass analysis. *In silico* mass prediction of reduced Ara h 2 used the same considerations as the native intact mass analysis, except that Ara h 2 was assumed to have 8 reduced cysteines and that the large and small subunits were separated. We were able to identify 92 % of the signal in the reduced S1 sample and

90 % of the signal in reduced S2 sample. The signal could be attributed to unique proteoforms. This included 5 Ara h 2.01 small subunits, 2 Ara h 2.02 small subunits and 11 Ara h 2.01 large subunits (see Supplementary Table S3). Both samples showed 2 predominant signals, presumably from the predominant proteoform. They matched a small (68 % in reduced S1; 59 % in reduced S2) and a large subunit (9 % in reduced S1; 11 % in reduced S2) (see Table 1). The experimental masses show that the predominant proteoform small subunit terminates at Asp-58 and the large subunit, with 2 HyP modifications, begins at Ser-59. A difference of 319.17 Da between the large subunits of the 2 samples reflects the RY removal, as seen before in the native intact mass analysis (see Table 1).

The less abundant Ara h 2.01 proteoforms detected in the reduced samples ranged from 0.01 to 8.7 % of the signal. These proteoforms differed from the predominant proteoform in their HyP modification (1 or 3), *C*-termini trimming and position of cleavage site. The two Ara h 2.02 proteoforms in the samples ranged from 0.1 to 0.5 % of the signal. Their experimental masses indicate that Ara h 2.02 small subunit terminate at either Asp-63 or Asp 70.

Peanut contains similar amounts of Ara h 2.01 and Ara h 2.02 [17]. We found our pAra h 2 to be predominantly Ara h 2.01. Ara h 2.01 is smaller than Ara h 2.02, due to the absence of 12 amino acids ([17], and Fig. 2A). Thus, pAra h 2 derived from Ara h 2.01 is expected to migrate at a somewhat lower molecular weight than pAra h 2 derived from Ara 2.02. Indeed, Fig. 2B shows that both pAra h 2 samples, under non-reducing conditions, have a main band at approximately 20 kDa, and a minor band at 19 kDa, representing a high content of pAra h 2 derived from Ara h 2.01 and low content of pAra h 2 derived from Ara h 2.02, in line with the mass spectrometry data (Table 1). It is possible that Ara h 2.02 is not processed in the same way as Ara h 2.01, but also that the purification employed removed processed Ara h 2.02.

In analogy with Ara h 6 in peanut, and other plant 2S albumins, it is not surprising that a cleaved form of Ara h 2 exists in peanut. However, such a proteoform has not been reported. Schmidt et al. [22] showed five Ara h 2 fragments on 2D electrophoresis, derived from variable cleavage at from the *C*-terminal part of the protein. However, these protein fragments were not purified or further characterized.

3.3. *Post-translationally cleaved forms of Ara h 2 are present in common peanut cultivars*

The intact mass analysis of the purified samples provides good evidence of the novel cleaved forms of Ara h 2 in peanuts and identify their site of cleavage. However, intact mass analysis does not address how much Ara h 2 is cleaved as a proportion to the total in the peanut seed, especially as proteoforms may be selectively purified. Techniques to investigate this are limited due to the complication of Ara h 2 having variable HyP modification and variable cleavage site. We chose to use semi-quantitative label-free quantification as this MS technique can discriminate both cleavage site and HyP modification. However, resultant quantitation can only be considered an estimate.

Using the criteria described in the methodology, 8 peptides were identified (Table 2) and quantitation of each peptide was conducted for each of the genotypes. Supplementary Table S4 includes further detail of the selected peptides. Supplementary Fig. 3 shows an example spectrum of each of the chosen peptides. The 2 most abundant peptides detected in the peanut cultivars at the cleavage region were Tryp 2.01 $HyP^{67/74}$ (126.3 ± 56.8 fmol on column) and Tryp 2.02 $HyP^{67/74}$ (121.1 ± 58.8 fmol on column). These peptides would be released from the intact form of Ara h 2.01 and Ara h 2.02, respectively. Three non-tryptic peptides align to each of these tryptic peptides and are assumed to be representative of cleaved proteoforms of Ara h 2. Label-free quantitation of each peptide allows an estimation of the 8 detected proteoforms of Ara h 2. Fig. 3 shows an alignment of these peptides to their respective unique Ara h 2 isoform.

The most abundant non-tryptic peptide for Ara h 2.01, NonTryp 2.01

Table 1
Predominant Ara h 2.01 proteoforms detected in samples by intact mass spectrometry.

Sample	HyP addition (Da)	Cleavage addition (Da)	C-termini removal (RY) (Da)	Predicted monoisotopic mass (Da)	Experimental monoisotopic mass (Da)	Error tolerance (ppm)	Fractional abundance (%)	Ara h 2.01 proteoform
S1 Native	+ 2 × 15.99	+17.99	−319.17	16,349.49	16,349.468	+1.33	71.74	h2.1–1
S2 Native	+ 2 × 15.99	+17.99	None	16,668.65	16,668.580	+4.21	73.49	h2.1–2

Sample	HyP addition (Da)	**Derived Cut site**	C-termini removal (RY) (Da)	Predicted monoisotopic mass (Da)	Experimental monoisotopic mass (Da)	Error tolerance (ppm)	Fractional abundance (%)	Ara h 2.01 proteoform
S1 Reduced	None	**DED/SYE**	Small subunit	4620.24	4620.224	+3.46	67.53	rh2.1-A ss
	+ 2 × 15.99	**DED/SYE**	−319.17	11,737.27	11,737.262	+0.68	8.88	rh2.1-A' ls
S2 Reduced	None	**DED/SYE**	Small subunit	4620.24	4620.249	−2.00	58.71	rh2.1-A ss
	+ 2 × 15.99	**DED/SYE**	None	12,056.44	12,056.430	+0.85	10.73	rh2.1-B' ls

Table 2
Peptides used in the assessment of Ara h 2 proteolytic cleavage sites in 20 peanut genotypes of which 11 Virginia, by bottom-up proteomics.

Isoform	Unique peptide sequence (HyP:p) (Mis-cleavage: _)	Acronym[a]	Cleavage Site	Virginia Mean Abundance (fmol)[a,b] (St.Dev)	Virginia Mean Percent Ratio Detected[c] (St.Dev) (CV)[d]	All Peanut Mean Abundance (fmol)[a] (St.Dev)	All Peanut Mean Percent Ratio Detected[c] (St.Dev) (CV)[d]
Ara h 2.1	DEDSYERDPYS**p**SQDPYS**p**SPYDR	Tryp 2.01 HyP$^{67/74}$	None	126.3 (56.8)	**88.3 (1.8) (2.0)**	121.7 (54.7)	**89.6 (2.9) (3.2)**
	SYERDPYS**p**SQDPYS**p**SPYDR	NonTryp 2.01 HyP$^{67/74}$ (D^{58}/S^{59})	DED-SYE	10.7 (5.5)	**7.4 (1.1) (14.7)**	8.6 (5.1)	**6.4 (2.0) (30.9)**
	PYS**p**SQDPYS**p**SPYDR	NonTryp 2.01 HyP$^{67/74}$ (D^{63}/P^{64})	ERD-PYS	4.0 (2.0)	**2.9 (1.1) (31.0)**	3.7 (1.8)	**2.7 (0.8) (29.1)**
	DEDSYERDPYS**p**SQD	NonTryp 2.01 HyP^{67} (D^{70}/P^{71})	SQD-PYS	2.0 (0.8)	**1.4 (0.3) (25.1)**	1.8 (0.7)	**1.4 (0.5) (33.4)**
Ara h 2.2	DEDSYGRDPYS**p**SQDPYS**p**SQDPDRR	Tryp 2.02 HyP$^{67/74}$	None	121.1 (58.8)	**84.7 (2.0) (2.4)**	105.9 (52.0)	**86.0 (3.5) (4.1)**
	SQDPYS**p**SQDPDRR	Non-tryp 2.02 HyP^{74} (P^{68}/S^{69})	YSP-SQD	12.0 (4.6)	**8.3 (0.8) (9.3)**	10.3 5.0)	**7.7 (1.6) (20.6)**
	PYS**p**SQDPYS**p**SQDPDRR	Non-tryp 2.02 HyP$^{67/74}$ (D^{63}/P^{64})	GRD-PYS	7.3 (2.8)	**5.1 (1.2) (23.2)**	6.6 (2.7)	**5.1 (1.1) (21.7)**
	PYS**p**SQDPDRR	Non-tryp 2.02 HyP^{74} (D^{70}/P^{71})	SQD-PYS	2.6 (0.8)	**1.9 (0.5) (26.2)**	2.6 (0.7)	**2.1 (0.5) (24.8)**

[a] Acronym describes whether peptide is tryptic (Tryp) or non-tryptic (Nontryp), unique isoform origin (2.01 or 2.02), site of HyP modification by amino acid number and site of putative cleavage site assigned by P1 and P1' amino acid number.
[b] Mean abundance of peptide was calculated by normalizing the intensity of the MS1 peak area to the average intensity of the top3 peptides from the Glycogen phosphorylase spike (100 fmol on column) in each sample. The selected peptide abundance was then averaged across the peanut samples.
[c] Mean percentage ratio of each peptide was calculated by assuming that the abundance of the 4 peptides for each isoform is equivalent to 100 %. The selected peptide ratio was then averaged across the peanut samples.
[d] Coefficient of variation of the percentage ratio of each peptide was calculated across the peanut samples.

Fig. 3. *Alignment of Ara h 2 tryptic (Tryp) and non-tryptic (Non-tryp) peptides indicating potential cleavage sites.* Panel A: selected unique peptides of Ara h 2.01. Panel B shows selected unique peptides of Ara h 2.02. Cleavage sites, as mapped by the non-tryptic peptides and intact mass analysis, are black font and arrow. Cleavage sites, as mapped by intact mass analysis *only* are green font and arrow. Cleavage sites, as mapped by non-tryptic peptides *only* are blue font and arrow. Hydroxyproline (HyP) modification is indicated by 'p'. Underlined R indicates mis-cleavage. Red font indicates non-tryptic peptides. Numbered HyP indicates modified amino-acid number and bracketed amino acids indicate cleavage site. Panel C: Location of cleavage site in Ara h 2, modelled from 3OB4 (Q6PSU2-1) using SWISS-MODEL and visualized with PyMol. Hyp residues were added using the PyTMs plugin. The cleavage site (DED/SYE) is on a flexible loop adjacent to an a-helix. The minimal distance between the cleavage site (D residue) and most proximate Hyp residue is 9.3 Å.

HyP$^{67/74}$ (D^{58}/S^{59}), is presumably representative of the proteoform cleaved between Asp-58 and Ser-59. This correlates to the predominant Ara h 2.01 proteoform found in samples S1 and S2 by reduced intact mass analysis (see Supplementary Table S3: 2.1-A' ls; B' ls and A ss). The other 2 non-tryptic peptides, although of lesser abundance, also correlate to proteoforms found in samples S1 and S2, by intact mass analysis. Proteoforms cleaved between Asp-63 and Pro-64 are indicated by 2.1-D' ls; I' ls and D ss; while proteoforms cleaved between Asp-70 and Pro-71 are indicated by 2.1-C' ls, H' ls and E ss in Supplementary Table S3.

The Ara h 2.02 non-tryptic peptide: Non-tryp 2.02 HyP^{74} (P^{68}/S^{69}), although abundant is not correlated to any detected mass event in either of the intact mass analyzed purified samples. It would correlate to an Ara h 2.02 proteoform cleaved between Pro-68 and Ser-69. The less abundant non-tryptic peptides though, can be correlated to Ara h 2.02 proteoforms found in the S1 and S2 intact mass analysis. The Ara h 2.02 proteoform cleaved between Asp-63 and Pro-64 are indicated by 2.2-B ss, while proteoforms cleaved between Asp-70 and Pro-71 are indicated by 2.2-A ss in Supplementary Table S3.

Peptide abundances may be used to estimate the ratio of non-cleaved to cleaved Ara h 2 proteoforms (see Table 2). Although semi-

quantitative, the ratios would suggest approximately 10 % and 14 % of Ara h 2.01 and Ara h 2.02, respectively, are cleaved. The predominant Ara h 2.01 cleaved form appears to be cleaved between Asp-58 and Ser-59, which would indicate that the S1 and S2 purifications are representative of the most abundant Ara h 2.01 cleaved proteoform. Schematic overview of Ara h 2.01 proteoforms as informed by intact mass analysis detection and previous bottom-up MS analyses by Marsh J. et al. [15], could be found in Fig. 4.

The relative normalized abundances of peptides selected to represent cleaved proteoforms were similar across genotypes (Supplementary Figs. S4 and S5), indicating that Ara h 2 cleavage is similar across peanut genotypes. Further experimentation such as absolute quantitation by use of stable-isotope labelled peptide spikes, using the unique Ara h 2 isoform HyP modified peptides described herein, would confirm these estimations.

In a previous study on the allergen content of peanut cultivars [14], protein profiles of all samples show low molecular weight bands on electrophoresis when reducing conditions are applied. Such bands were not present under non-reducing conditions. This suggests the presence of cleaved 2S albumins [18]. These bands are at least in part derived from Ara h 6, for which a post-translationally cleaved form was also described in common peanut types [23]. With the current observation that pAra h 2 is present in these same samples, another part of these low molecular weight bands can be attributed to pAra h 2. [22] investigated Virginia type peanuts and found several Ara h 2 fragments by 2D electrophoresis. These could in part be explained by the presence of pAra h 2, however, the identified fragments were all from the C-terminal part, probably representing the large subunit of pAra h 2. No *N*-terminal subunits were identified [22], and this may be due the cut-off of the electrophoresis gel used (molecular weight range from 10 to 150 kDa). Our current data

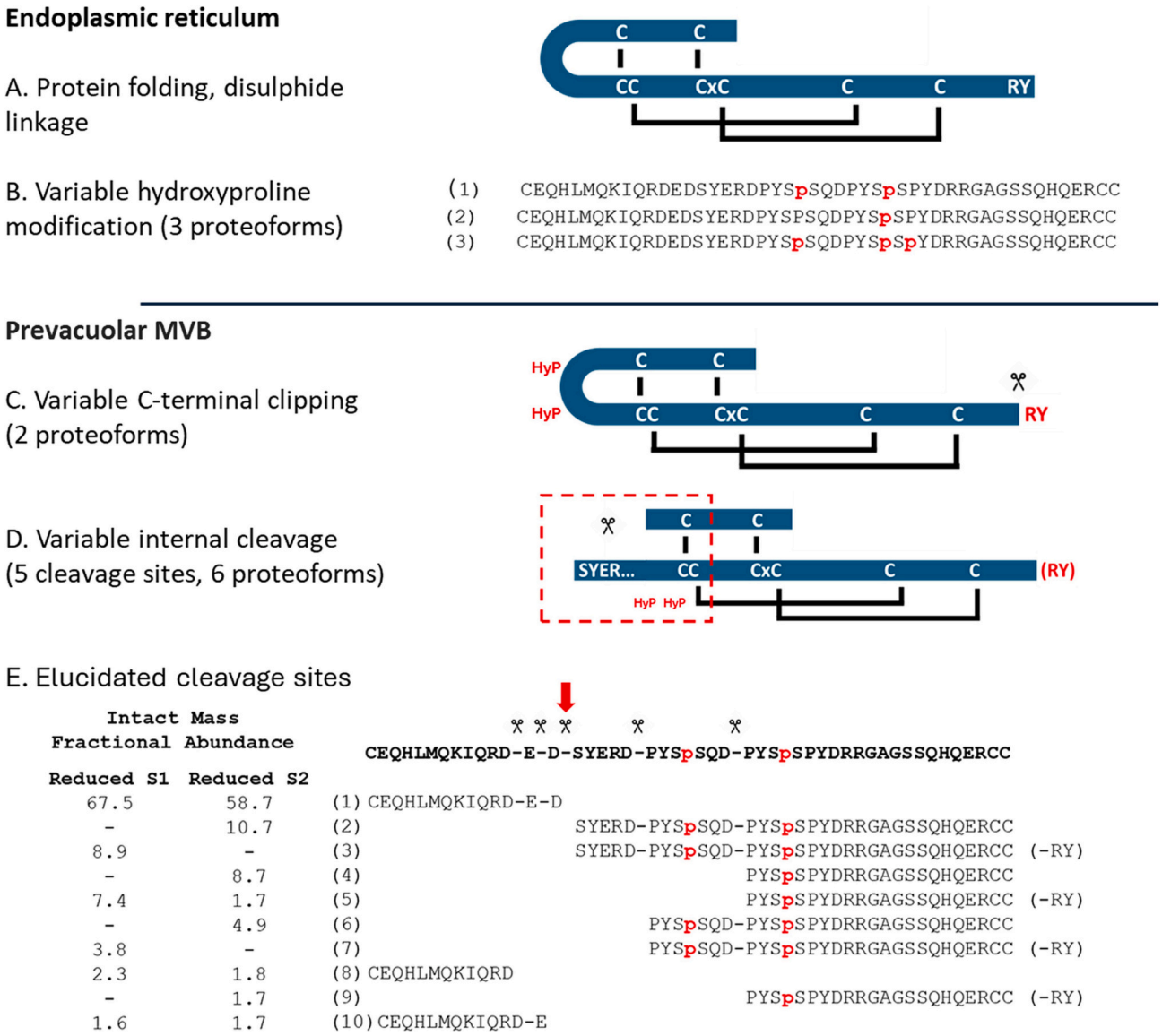

Fig. 4. Schematic overview of post-translational cleavage process of Ara h 2.01 proteoforms and their identifications by bottom-up mass spectrometry approach. Ara h 2.01, a 2S albumin, is translated and imported into the endoplasmic reticulum (E.R.) lumen. (A-B) In the E.R., folding, disulphide linkage and variable hydroxyprolination occurs. The sites and predominant HyP proteoforms are listed in order of abundance (1–3). The 2S albumins are sorted and trafficked through the Golgi to secretory vesicles. (C—D) Secretory vesicles of the 2S albumins are fused with prevacuolar multivesicular bodies (MVB) where proteolytic processing is thought to occur [43]. The processed 2S albumins are then deposited into the protein storage vacuole. (E) The cleaved proteoforms elucidated from the intact mass analysis of reduced pAra h 2 samples (S1 and S2) are shown in the bottom panel. Table 1 and Supplementary Table S3 lists the identified masses (predicted *vs* experimental) from pAra h 2 S1 and S2. The red arrow indicates the predominant cleavage site. (-RY) indicates the large subunit lacked the C-terminal RY amino-acids.

provides evidence that *N*-terminal subunits of Ara h 2 also exist in peanut.

3.4. IgE reactivity and allergenicity of pAra h 2 proteoforms

Intact Ara h 2 is known as one of the main peanut allergens, with the majority of peanut allergic patients having IgE to Ara h 2. Serum from 28 patients sensitized to peanut were included in this study (Table S1). Using a direct ELISA, IgE binding to intact Ara h 2 and the two forms of pAra h 2 was tested (Fig. 5A). IgE-binding to Ara h 2 correlates well with the IgE values from the ImmunoCAP analysis (Table S1), with an R^2 of 0.904 (Supplementary Fig. S6). In most cases, sera with high IgE-reactivity to Ara h 2 have also high reactivity to both pAra h 2 forms, and *vice versa*, except in serum #2 where Ara h 2 was substantially more reactive than pAra h 2, and in sera #3, 16,18, 19, 21, 23, and 27 where pAra h 2 was more reactive than Ara h 2. For all sera the magnitude of IgE-binding was the same for both forms of pAra h 2 (Fig. 5B). The same pattern of IgE binding was observed when sera were diluted 1:25 or 1:100 (Supplementary Fig. S7A and B respectively).

To investigate cross-reactivity on the IgE-level between intact Ara h 2 and pAra h 2 forms, we used a serum pool which had a similar IgE reactivity towards the intact Ara h 2 and the two pAra h 2 forms as tested in direct ELISA (Supplementary Fig. S7C). For IgE-inhibition experiments, a dilution of 50-fold was selected to allow observing changes in IgE-binding in both directions. Fig. 4 shows a representation of IgE inhibition with cleaved forms of Ara h 2 towards the intact allergen. Both forms of cleaved Ara h 2 were able to inhibit the IgE binding somewhat better than Ara h 2. There was a slight difference between the two pAra h 2 forms: pAra h 2 S2 showed higher potency to inhibit than pAra h 2 S1. Based on our mass spectrometry data, pAra h 2 isoforms do not lack any known epitopes and comparable IgE-binding potencies between intact Ara h 2 and pAra h 2 was expected. IgE inhibition was also done with the two different forms of cleaved pAra h 2 coated to plates, using intact or the two cleaved forms of Ara h 2 as an inhibitor to further investigate IgE cross-reactivity. IC50 values were calculated (Table 3) and show detectable differences in the potency, in line with inhibition curves where the most potent from highest to lowest are: pAra h 2 S2 > pAra h 2 S1 > Ara h 2. This pattern of potency was seen irrespective of the set-up of the IgE inhibition experiment. The higher potency of pAra h 2 compared to intact Ara h 2 could be due to better accessibility of IgE antibodies towards specific epitopes. Earlier work showed that Ara h 2 exposed to digestive proteases is cleaved in the same area where the post-translational cleaving takes place, and that these cleaved forms have increased mobility compared to uncleaved Ara h 2 [30]. An important IgE-binding epitope in Ara h 2, R^{62}DPYSPSQDPYSPS^{75} [31,32] is located near the cleavage site of the most abundant Ara h 2 proteoforms (Fig. 3A) and may be better accessible in pAra h 2 compared to intact Ara h 2. For the less abundant proteoforms of pAra h 2, the cleavage site was found within this epitope (Figs. 3B and 4). Current *in vitro* diagnostics for peanut allergy uses IgE-binding to either peanut extract or individual recombinant peanut allergen such as Ara h 1, Ara h 2 and Ara h 6. Using Ara h 2 alone leads to a higher specificity but somewhat lower sensitivity and interestingly, adding four Ara h 2-peptides increased this sensitivity [13]. Two of these peptides represent parts of Ara h 2 in close proximity of the cleavage site we describe here, suggesting that availability and accessibility of epitopes close to the cleavage site is important for *in vitro* diagnosis. However, since we have not mapped epitopes and and have used a serum pool which could have multiepitope recognition, the impact of post-translational cleavage remains unknown.

Nevertheless, the observed differences in the IgE binding potency are minor; up to 5-fold. Relevant differences in IgE-binding potency, for example, for chemically modified hypoallergic extracts used for immunotherapy, are typically 10 to 100-fold and preferably more [27,33–35]. In addition, the occurrence of proteoforms is lower than intact Ara h 2 forms in the peanut extract and does not vary a lot between different peanut variants, which might not affect the allergenicity, as investigated before [14]. However, this has been evaluated with a serum pool as well. Thus, the values we report here indicate that intact and pAra h 2 have highly comparable IgE binding and demonstrate that the post-translational cleavages have no detrimental effect on IgE-binding. We also addressed this on the allergenicity level, where we performed a mediator-realized assay in humanized RBL cells by measuring levels of β-hexosaminidase in percentages (Fig. 5C). The serum pool used in inhibition ELISA was used to sensitized cells prior to cells were activated with intact and cleaved forms of Ara h 2. A higher mediator release towards intact and cleaved forms of pAra h 2 was noticed at higher concentrations (10000–10 pg/mL), while there was no mediator release with lowest concentrations (1 and 0.1 pg/mL). All three 2S albumin preparations showed almost the same allergenicity, indicating that the novel cleaved forms of pAra h 2 are as potent as intact Ara h 2. In addition, in inhibition ELISA, we found minimal difference in IgE-binding between intact Ara h 2 and cleaved pAra h 2. These minor differences could be due to greater conformational epitope exposure in the cleaved forms of Ara h 2, which are not easily accessible for FcεRI crosslinking in the mediator assay. Both linear and conformational IgE epitopes have been described for Ara h 2 and another peanut 2S albumin Ara h 6 [36].

Today there are more studies showing the importance of conformational epitope in allergenicity but also in the development of tolerance in oral immunotherapy [36,37]. For immunotherapy for peanut allergy, several products have proven successful in double-blind, placebo-controlled phase 3 clinical trials [38,39]. The active ingredient used in these therapies is peanut flour [38] or peanut extract [39], both by definition including all proteoforms of individual allergens. Immunotherapy with Ara h 2, or peptides thereof, as single allergen is also in development and show promising results [40–42]. No double-blind, placebo-controlled efficacy trials using Ara h 2 to treat peanut allergy have been published so far, so whether immunotherapy with single isoforms of Ara h 2 is comparably safe and effective as immunotherapy with peanut protein comprising different proteoforms of Ara h 2 remains unknown.

4. Conclusion

We have purified and characterized post-translationally cleaved forms of Ara h 2, that exist in peanut in tandem with the intact form. The cleavage site is in the non-structured loop connecting the α-helical domains. The secondary structure known to be important for IgE-binding by conformational epitopes, is maintained in the cleaved forms of Ara h 2, and IgE binding is not substantially different for the cleaved and intact form. The cleaved form resembles the common structure of plant 2S albumins, *e.g.* a smaller *N*-terminal subunit linked *via* disulphide bridges to a larger *C*-terminal subunit. However, up to now, Ara h 2 was only known as a single-chain protein, and this is the first time the cleaved form has been purified and characterized. Being present in the main peanut market types, having similar IgE-binding characteristics, and capable to producing mediator realize as the intact protein, post-translationally cleaved Ara h 2 represents a relevant Ara h 2 isoform. We showed that cleaved forms of Ara h 2 are important proteoforms in terms of allergenicity, contributing to a further understanding as to why Ara h 2 is the most important peanut allergen. Better molecular description of allergenic proteins assists the design of clinical materials for diagnosis or treatment of peanut allergy by allowing the use of the most relevant allergen proteforms.

CRediT authorship contribution statement

Stef J. Koppelman: Writing – original draft, Supervision, Resources, Methodology, Conceptualization. **Govardus A.H. de Jong:** Writing – review & editing, Investigation. **Justin Marsh:** Writing – original draft, Validation, Software, Methodology, Investigation. **Phil Johnson:**

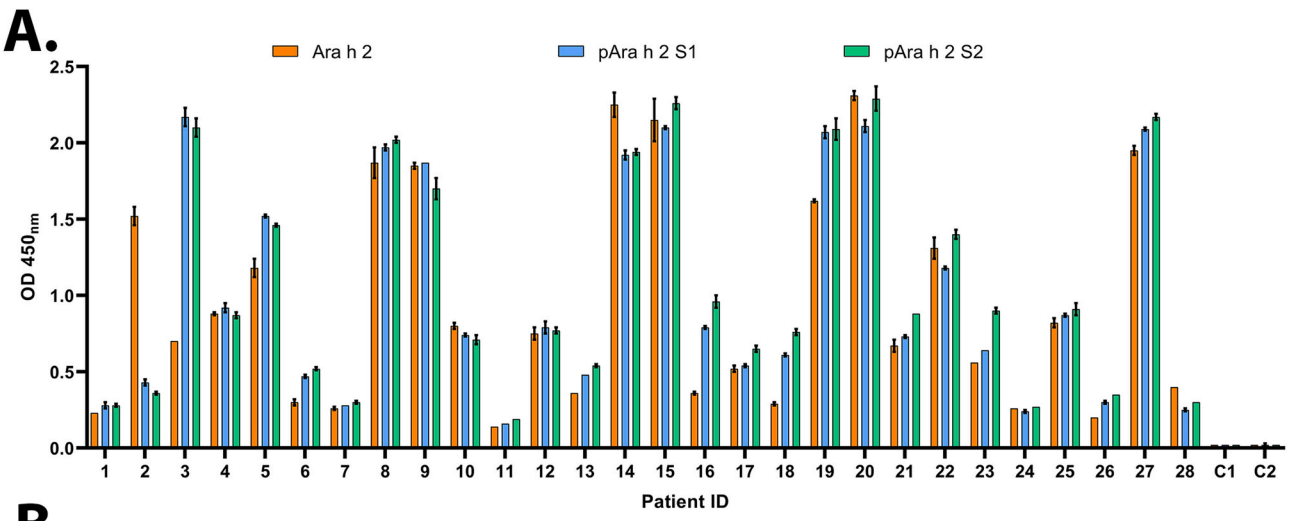

Fig. 5. *Allergenic characteristics of pAra h 2 proteforms.* IgE-binding to Ara h 2 and pAra h 2 proteoforms was assessed in A: direct ELISA using sera from peanut sensitized patients diluted 50-fold. B: Inhibition ELISA. IgE binding to Ara h 2 was inhibited by Ara h 2 (orange), pAra h 2 S1 (blue) or pAra h 2 S2 (green) using the serum pool in dilution of 1:50. C. Allergenic activity of Ara h 2 (orange), pAra h 2 S1 (blue) or pAra h 2 S2 (green) measured by mediator release assay.

Table 3
IC50 values from IgE inhibition ELISA.

IC50 ± SD (μg/mL)	Ara h 2	pAra h 2 S1	pAra h 2 S2
Ara h 2 coated	1.29 ± 0.27	0.67 ± 0.37	0.45 ± 0.36
pAra h 2 S1 coated	0.9 6 ± 0.10	0.72 ± 0.01	0.52 ± 0.04
pAra h 2 S2 coated	2.61 ± 0.79	0.88 ± 0.11	0.55 ± 0.12

Writing – review & editing, Validation, Software, Methodology, Funding acquisition. **Emily Dowell:** Investigation. **Marija Perusko:** Writing – review & editing, Methodology. **Adrie Westphal:** Investigation. **Marianne van Hage:** Writing – review & editing, Methodology. **Joseph Baumert:** Writing – review & editing, Funding acquisition, Conceptualization. **Danijela Apostolovic:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Koppelman Stef reports a relationship with DBV Technologies that includes: consulting or advisory. Marianne van Hage reports a relationship with Thermo Fisher Scientific ImmunoDiagnostics Division that includes: speaking and lecture fees. Marianne van Hage reports a relationship with AstraZeneca AB that includes: speaking and lecture fees. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ethic statement

In this study we used serum from 28 peanut-sensitized patients and two controls (non-peanut allergic). The Swedish Ethics Review authority approved the study (Etikprövningsmyndigheten) with permit number No. 20112085-31/4. The study was performed following the declaration of Helsinki.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.130613.

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