



Reduction and alkylation of peanut allergen isoforms Ara h 2 and Ara h 6; characterization of intermediate- and end products

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ABSTRACT

Conglutins, the major peanut allergens, Ara h 2 and Ara h 6, are highly structured proteins stabilized by multiple disulfide bridges and are stable towards heat-denaturation and digestion. We sought a way to reduce their potent allergenicity in view of the development of immunotherapy for peanut allergy. Isoforms of conglutin were purified, reduced with dithiothreitol and subsequently alkylated with iodoacetamide. The effect of this modification was assessed on protein folding and IgE-binding. We found that all disulfide bridges were reduced and alkylated. As a result, the secondary structure lost α -helix and gained some β -structure content, and the tertiary structure stability was reduced. On a functional level, the modification led to a strongly decreased IgE-binding. Using conditions for limited reduction and alkylation, partially reduced and alkylated proteins were found with rearranged disulfide bridges and, in some cases, intermolecular cross-links were found. Peptide mass finger printing was applied to control progress of the modification reaction and to map novel disulfide bonds. There was no preference for the order in which disulfides were reduced, and disulfide rearrangement occurred in a non-specific way. Only minor differences in kinetics of reduction and alkylation were found between the different conglutin isoforms. We conclude that the peanut conglutins Ara h 2 and Ara h 6 can be chemically modified by reduction and alkylation, such that they substantially unfold and that their allergenic potency decreases.

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1. Introduction

Peanut allergens Ara h 2 and Ara h 6 are 2S albumins and members of the conglutin family of seed storage proteins [1]. Ara h 2 and Ara h 6 are isoforms of each other, and have been identified as major peanut allergens in *in vitro*, by solid-phase immunoassays and effector cell-based assays [2–7], and *in vivo*, by skin prick tests in peanut-allergic patients [3]. Furthermore, in a mouse model it was shown that immunotherapy with Ara h 2 and Ara h 6 resolved peanut allergy [8], demonstrating that Ara h 2 and Ara h 6 together represent the most relevant peanut allergens in terms of immunotherapy. Ara h 2 and Ara h 6 contain multiple disulfide-bridged cysteine residues, resulting in at least four helical structures that are tightly coiled, heat- and protease-stable [9]. It has been suggested that the tightly coiled protein core may be important for allergenicity of peanut proteins [3,10–12]. Other peanut allergens described in the past, Ara h 1 and Ara h 3, have been shown to be less potent *in vitro*, by effector cell-based assays and

in vivo, by skin prick tests in peanut-allergic patients [13,14]. Together, Ara h 2 and Ara h 6 represent 80–90% of the allergenic potential of the peanut and are now considered as the most relevant peanut proteins [6,15].

Ara h 2 has two isoforms (Ara h 2.01 and Ara h 2.02), with molecular masses of around 17 and 19 kDa, respectively, coded by homologous genes. The Ara h 2.02 has insertion of 12 amino acids in the middle of the sequence that contains linear IgE-binding epitope [8,16–18]. The mass difference between these two isoforms is 1413 Da [16]. Determination of site-specific proline hydroxylation, disulfide linkages and C-terminal variation of Ara h 2 has been reported previously [19]. It has been shown that Ara h 2 undergoes C-terminal proteolytic processing by endogenous peanut protease [20], resulting in the occurrence of heavy and light isoform of Ara h 2 lacking the C-terminal dipeptide RY. Ara h 6 has been shown to be a potent allergen, sharing epitopes with Ara h 2, with 59% amino acid overall homology and 75% homology in the α -helical regions, resulting in the high degree of immunological cross-reactivity between these allergens (Fig. 1.) [3,21]. Ara h 6 may undergo posttranslational proteolytic processing that involves removal of a dipeptide (IR) from the middle part of the sequence [22], resulting in two polypeptide chains held together by disulfide bonds. Because Ara h 2.02 isoform contains an extra copy of

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A

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Ara h 2.02  MAKLTILVALALFLLAAHASARQ-Q-WELQGDRRRC33QSQLERANLRPC45EQHLMQKIQRDED
Ara h 2.01  MAKLTILVALALFLLAAHASARQ-Q-WELQGDRRRC33QSQLERANLRPC45EQHLMQKIQRDED
Ara h 6      MAKSTILVALLALVLVAHASAMRRERGRQGDSSSC35ERQVDRVNLKPC47EQHIMQIRIMGEQE
***  *****  :*:*****  :  .  .  *  :  *:*.**:**  ***:***  :  :

Ara h 2.02  SYGRDPYSPSQDPYSPSQDPDRRDPYSPSPYDRRGAGSSQHQRCC103C104NELNEFENNQRCC116MC118
Ara h 2.01  SYGRDPYSPSQDP-----YSPSPYDRRGAGSSQHQRCC91C92NELNEFENNQRCC104MC106
Ara h 6      QYD-----SYDIRSTRSSDQQQRCC79C80DELNEMENTQRCC92MC94
. *                               ** .:  **:***  *  :****:***  **

Ara h 2.02  EALQQIMENQSDRLQGRQQEQFKRELRLNLPQQC152GLRAPQRC160DLEVESGGGRD
Ara h 2.01  EALQQIMENQSDRLQGRQQEQFKRELRLNLPQQC140GLRAPQRC148DLEVESGGRD
Ara h 6      EALQQIMENQC105DRLQDRQMVQQFKRELMNLPQQC128NFRAPQRC136DLDVSGGRC145---
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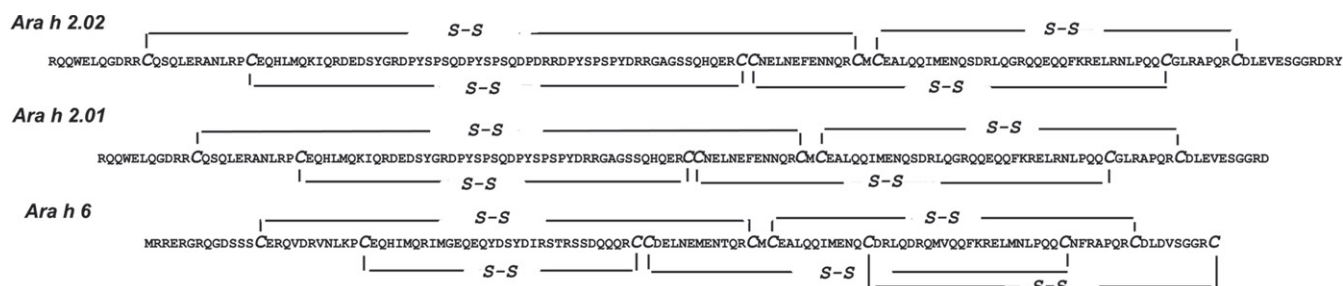
B

Fig. 1. Primary structure of peanut conglutins. Panel A. Sequence alignment of peanut conglutinin isoforms. The following protein identifiers were used in the web-based program ProtParam (www.expasy.org/protparam/), and the protein sequences were taken from www.uniprot.org: Q6PSU2-1, Q6PSU2-4 and Q647G9. Dark letter area: covered sequences by peptide mass finger printing. Panel B. Scheme for disulfides in native conglutinin isoforms.

Figure based on the information taken from Li et al. [19] for Ara h 2 isoforms, and from Lehmann et al. [10] for Ara h 6.

an immunodominant epitope, DPYSPS, it has been speculated that this isoform could be more potent than Ara h 2.01 [23,24]. This is supported by Hales et al. [25], who showed that IgE-binding to Ara h 2.02 is more pronounced than for Ara h 2.01. Also, Ara h 2.02 is more efficient in an IgE competition assay [25]. However, Chen et al. [26] reported that Ara h 2.01 has slightly greater allergenic potency than Ara h 2.02, and that repeating linear sequences does not contribute to the allergenic activity of Ara h 2. These differences may be explained by differences in the serology of peanut allergic patients included in these studies. Taken together, peanut derived allergens Ara h 2 and Ara h 6 represent a diverse group of different isoforms of peanut conglutinin, the most important allergens in peanut, whose allergenicity is not completely understood.

Within the group of isoforms of peanut conglutinin, it has been shown that reduction of the disulfides and subsequent alkylation of the resulting sulfhydryl groups leads to diminished IgE-binding [11,27]. Apparently, the IgE-binding is mainly depending on the protein structure for these allergens. The reduced and alkylated molecules are hypo-allergenic but still immunogenic [12] and potentially suitable for immunotherapy in peanut-allergic patients. The aim of this study is to investigate the critical steps in the reduction and alkylation of peanut allergens to enable the development of consistently modified peanut conglutinins.

2. Materials and methods

2.1. Purification of conglutinin isoforms

The mix of conglutinin isoforms Ara h 2.01, Ara h 2.02, and Ara h 6 was purified as described earlier [3]. To the mix of conglutinin isoforms 4 M ammonium sulfate in 20 mM Tris/HCl pH 8 was added to achieve a

final concentration of 1.6 M ammonium sulfate. This was applied on a SourcePhenyl 15 column (GE Healthcare, Uppsala, Sweden, 15 mg of protein per 1 ml of column material) previously equilibrated with 1.6 M ammonium sulfate in 20 mM Tris, pH 8.0 (loading buffer). After washing with loading buffer, the column was eluted with a gradient (10 column volumes) of 1.28 M to 0.12 M ammonium sulfate in 20 mM Tris/HCl pH 8. The purity of the collected fractions was tested by SDS-PAGE shown in Fig. 2.

2.2. Kinetics of reduction and alkylation

To assess the adequate concentration of reducing and alkylating reagents, as well as the period necessary for complete modification, kinetic studies were performed. First, 0.5 mg/ml of peanut conglutinins in 50 mM sodium phosphate buffer pH 8 were reduced with 0.5 and 5 mM dithiothreitol (DTT). The protein samples were preheated at 60 °C for 0.5 min before reagent addition. Reaction proceeded at 60 °C with continuous shaking, and aliquots (100 µl) were periodically withdrawn (after 0.5, 1, 2, 5, 10, 30, 60 min). The reduction was stopped by immediately adding iodoacetamide (IAA), final concentration of 5 mM and 50 mM, at room temperature in the dark for 90 min. All aliquots were analyzed by SDS-PAGE (non-reducing conditions).

2.3. SDS-PAGE analysis of native and reduced/alkylated conglutinin isoforms

Proteins (Ara h 2.02 – heavy isoform, Ara h 2.01 – light isoform, and Ara h 6) were analyzed using a Hoefer Scientific Instrumentation apparatus (Amersham Biosciences, Uppsala, Sweden), on 14% polyacrylamide gel. Samples were analyzed under both, non-reduced and

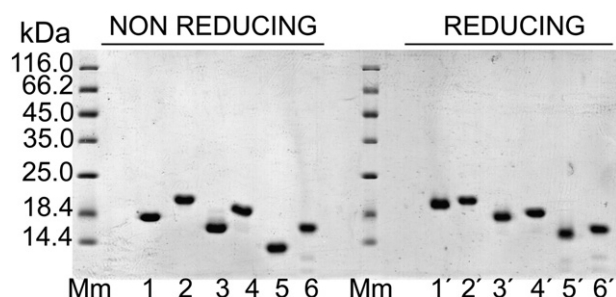


Fig. 2. SDS-PAGE of native and of reduced (5 mM DTT) and alkylated (50 mM IAA) peanut conglutinin isoforms. Panel A: Non-reducing SDS-PAGE conditions. Lane Mm: molecular weight markers (indicated in kDa); lane 1: Ara h 2.02; lane 2: reduced and alkylated Ara h 2.02; lane 3: Ara h 2.01; lane 4: reduced and alkylated Ara h 2.01; lane 5: Ara h 6; lane 6: reduced and alkylated Ara h 6. Panel B: Reducing SDS-PAGE conditions. Lane M: molecular markers (indicated in kDa); lane 1': Ara h 2.02; lane 2': reduced and alkylated Ara h 2.02; lane 3': Ara h 2.01; lane 4': reduced and alkylated Ara h 2.01; lane 5': Ara h 6; lane 6': reduced and alkylated Ara h 6. Panel B: Reducing SDS-PAGE conditions. Lane M: molecular markers (indicated in kDa); lane 1': Ara h 2.02; lane 2': reduced and alkylated Ara h 2.02; lane 3': Ara h 2.01; lane 4': reduced and alkylated Ara h 2.01; lane 5': Ara h 6; lane 6': reduced and alkylated Ara h 6.

reduced conditions. Gel was stained using Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Germany).

2.4. Mass spectrometry analysis of intact conglutins

Masses of native and reduced/alkylated (red/alk) proteins were analyzed on EASY nLC II system coupled with LTQ Orbitrap XL (Thermo scientific, Waltham, Massachusetts, USA), in order to determine exact mass differences of the proteins and if all disulfides were modified. Five microliters (protein concentration of 100 µg/ml) of each sample was injected onto the trapping column (EasyColumn C18, 2 cm length, ID 100 µm, 5 µm particle size) and separation was performed on an EasyColumn C18 (length 10 cm, ID 75 µm, particle size 3 µm). Solvent A contained 0.1% formic acid in water (v/v) and B – 0.1% formic acid in 98% acetonitrile (v/v) (water, acetonitrile and formic acid were MS grade from Sigma-Aldrich). Proteins were washed from trapping column with 95% solvent A and 5% solvent B, and eluted from separating column, into the LTQ system, using a gradient of 5–65% solvent B, with a flow rate of 300 nL/min. The total run time was 100 min. Spray was generated with a stainless steel emitter with tip voltage set at 2.0 kV, capillary voltage 9 V and capillary temperature of 275 °C. A high-resolution full FTMS profile spectrum (scan range = 300–4000 m/z, resolving power = 60 000, 1 microscan) was acquired using Xcalibur (version 2.1) software (Thermo Scientific). The experiments of reduction/alkylation were done at a minimum of duplicate.

2.5. Circular dichroism spectroscopy of native and reduced and alkylated conglutins

In order to detect changes in the secondary structure of proteins during modification, native and reduced and alkylated proteins (50 µg/ml in Mili Q water) were recorded on JASCO J-815 spectropolarimeter (JASCO, Japan). Far-UV spectra were recorded at 20 °C using quartz cuvettes with path length of 0.5 mm. Recording was done in 0.1 nm steps at the rate of 100 nm/min over the wavelength range of 190–260 nm. The results are expressed as mean residue weight (MRW) molar ellipticity $[\theta]_{MRW} = \theta / (c \times d \times N)$, where θ is the observed ellipticity, c is the molar concentration of the protein, d is the optical path length and N is the number of amino acid residues.

2.6. Spectrofluorimetric monitoring of native and reduced/alkylated conglutins

Fluorimetric monitoring was performed using HORIBA Scientific Fluoromax-4 spectrofluorimeter (Jobin Yvon, Japan). Excitation was

done at 280 nm and emission followed in the range of 290–450 nm at 25 °C. Spectra of native and reduced and alkylated proteins (concentration of 25 µg/ml) in 50 mM sodium phosphate buffer pH 8 and 50 mM Tris buffer pH 7.2 were recorded.

2.7. Peptide mass fingerprinting

Tryptic digestion of totally and partially reduced/alkylated proteins was performed in 100 mM ammonium bicarbonate buffer pH 8.5 with trypsin from porcine pancreas (proteomic grade, dimethylated; Sigma-Aldrich). Enzyme to substrate ratio of 1:50 (w/w) was used. Digestion was carried out with 50 µg/ml of proteins at 37 °C for 16 h, after which the reaction was stopped by adding formic acid to a final concentration of 1%. Peptide desalting and separation were achieved on the EASY nLC II system. Linear gradient from 5 to 95% of solvent B with flow rate of 300 nL/min was performed for 62 min of total run time. Injection volume was 2 µL. Spray was generated with a stainless steel emitter, with tip voltage set at 2.0 kV, capillary voltage 6 V and capillary temperature of 250 °C. The peptide masses are measured in a survey scan with a maximum resolution of 30,000 in the Orbitrap over the scan range of m/z 300–2000. To obtain a maximum mass accuracy, a prescan is used to keep the ion population in the Orbitrap for each scan approximately the same. During the high-resolution scan in the Orbitrap, the five most intense monoisotopic peaks in the spectra were fragmented and measured in the linear ion trap in a data dependent acquisition mode. For fragmentation, a collision-induced dissociation (CID) was performed with collision gas normalized energy of 35 and duration of 30 ms. Data acquisition was performed using Xcalibur (version 2.1) software (Thermo Scientific). MS data analysis was performed in Proteome Discoverer 1.3.0339 (Thermo Scientific). Search was performed against Uniprot-sprot FASTA database with SEQUEST algorithm. Two missed trypsin cleavages were allowed per peptide. Peptide tolerance and MS/MS tolerance were set to 10 ppm and 0.5 Da, respectively. Fixed amino acid modification was carbamidomethylation as static modification. For dynamic modifications, oxidations of Met and Pro were used. False Discovery Rate (FDR) was set to 0.01 for strict and 0.05 for relaxed modes of peptide discovery. Results were filtered to find a minimum of two peptides per protein with high confidence. MassMatrix (www.massmatrix.net) software was used (version 2.4.2.) for MS kinetic determination of reduction and alkylation during purification of intermediate product(s) and identification of disulfide bond in peptides obtained from trypsin digestion [28]. The files were searched against custom protein database (UniProtKB/Swiss-Prot v57.10 2009-11-03) using the MassMatrix search engine and decoy protein sequences were randomized. Spectra with double, triple and more charges were searched. Peptide sequence length was set up from 3 to 50 amino acid residues and two missed cleavage sites, precursor ion tolerance and product ion tolerance to 10 ppm and 0.5 Da, respectively. For crosslink search option, disulfide option was used in exploratory mode with the maximum number of cross linked peptides of set at two. The experiment of reduction/alkylation was done in duplicate and obtained duplicate files containing MS/MS spectra were merged and searched through database.

2.8. IgE binding properties of native and reduced and alkylated conglutins

The IgE binding properties of the modified and native allergens were analyzed using an inhibition ELISA, in order to compare their allergenicity. A serum pool with sera from 14 patients with proven peanut allergy was constructed following the EMEA Note for Guidance on Allergen Products (EMA/CHMP/BWP/304831/2007). Peanut-specific IgE in this pool was 39.6 kU/l (ImmunoCap F13). Plates (NUNC MaxiSorp, Nunc, Denmark) were coated with 100 µL of 3 µg/ml native proteins per well o/n at 4 °C in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃ pH 9.6). The remaining binding sites were blocked with 1% BSA in TBS-T (20 mM Tris containing 0.9% NaCl pH 7.4 containing 0.1% of Tween 20 (w/v)), for 1 h at 37 °C in an

incubator shaker. Dilution series of the native and modified samples in 1% BSA in TBS-T were incubated with the serum pool (dilution factor was 3), directly on the plate for 1 h at 37 °C. The concentration range of the dilution series was varied per allergen, to focus on the typical S-shaped curve of each allergen (for native proteins starting concentration was 10 µg/ml, and for reduced/alkylated 500 µg/ml). Detection was performed with 100 µl mouse-anti-human IgE monoclonal antibody (Abcam, UK) (2000 times diluted in TBS-T containing 1% BSA) conjugated to horse radish peroxidase (HRP). Finally, staining was performed by enzymatic conversion of 3,3',5,5'-tetramethylbenzidine (TMB). The reaction was stopped after 15 min with 50 µl of 0.5 M sulfuric acid per well. The plates were read at 450 nm. IgE binding potencies were calculated using the horizontal displacement compared to the inhibition curve of native conglutinin isoform as reference (defined as 100%). Inhibition curves had correlation coefficients ≥ 0.985 .

3. Results

3.1. Electrophoretic mobility of native and reduced/alkylated peanut conglutinins

SDS-PAGE analysis was used to compare the protein profiles of Ara h 2 heavy (Ara h 2.02) and light (Ara h 2.01) isoforms and Ara h 6, before and after modification. Fig. 2 shows profiles of native and modified peanut allergens under non-reducing and reducing conditions. The modified heavy and light Ara h 2 and Ara h 6 show a characteristic shift in apparent molecular weight upon reduction of disulfide bridges using 5 mM DTT. The proteins contain 8 (Ara h 2), or 10 (Ara h 6) cysteine residues that are subsequently alkylated by exposure to an excess of iodoacetamide (50 mM), causing a significant increase in apparent molecular weight under non-reducing conditions, compared to native proteins. The shift in apparent molecular weight is larger than expected based on the mass addition by the alkylation (approximately 116 Da per disulfide bridge). The larger difference in apparent molecular weight is most likely due to the loss of protein structure impacting the mobility during electrophoresis. Under reducing SDS-PAGE conditions, shift in the molecular weight of investigated proteins was only minor, representing the mass addition by the alkylation. Under reducing conditions small amounts of low molecular weight bands are visible, probably conglutinin peptides resulting from posttranslational processing [20] that are held together by disulfide bridges under native conditions.

3.2. Mass of native and reduced and alkylated peanut conglutinins

In order to assess exact masses of native and reduced and alkylated proteins, high-resolution mass spectrometry was done for Ara h 2 heavy and light isoforms and Ara h 6. Fig. 3 shows the ESI-MS spectra for native and modified Ara h 2 heavy isoform as an example. Reduced and alkylated proteins show higher charge states (mean = 21+) compared to native proteins (mean = 10+) because more protonable groups are exposed to the solvent than are exposed in a native protein. Table 1 summarizes the retrieved masses of all three proteins. Masses of reduced and alkylated Ara h 2.02, Ara h 2.01, and Ara h 6, increased by 465.58, 464.21, and 579.56 Da, respectively. Those increases correspond to eight, or ten modified (carbamidomethylated) cysteine residues in the Ara h 2, and Ara h 6 respectively. This suggests complete modification of all residues in three analyzed proteins. No unmodified protein was observed in MS spectra of reduced/alkylated proteins.

3.3. Secondary and tertiary structure aspects of native and reduced and alkylated peanut conglutinins

The secondary structure of native and reduced/alkylated conglutinins was analyzed by far UV CD spectroscopy (Fig. 4 panels A, B and C). Native proteins show minima at 208 and 220 nm, and a steep increase

in ellipticity below 200 nm, indicative for a high content of secondary structure, in particular α -helices. This is similar for all three isoforms. After modification, all proteins showed characteristic change of far UV CD spectra reflecting substantially unfolded structures. CD spectra demonstrate that reduction and alkylation decrease the α -helix content of all three conglutinin isoforms resulting in an increase in β -structures and in random coil. Similar CD spectra were obtained by Starkl et al. [11], after reduction/alkylation of Ara h 2 and Ara h 6.

The intensities and maxima of intrinsic fluorophores of Ara h 2 light and heavy isoforms, as well as Ara h 6 were monitored by spectrofluorimetry (Fig. 5). Both Ara h 2 isoforms contain Trp ($n = 1$) and Tyr ($n = 4, 5$, or 6 depending on the isoform), while Ara h 6 only contains Tyr ($n = 2$). Fluorescence spectra of the two isoforms of native Ara h 2 show typical maxima at 350 nm, indicative of a fully solvent-exposed Trp. Furthermore, a Tyr fluorescence peak around 305 nm is observed, caused by the high number of Tyr residues, and apparent inefficient resonance energy transfer from Tyr to Trp in native Ara h 2. Native Ara h 6 only shows a Tyr fluorescence peak at around 305 nm. Upon unfolding of the two Ara h 2 isoforms, induced by modification, no change in λ_{max} for the Trp is observed, as Trp was already completely water-exposed. However, the shoulder at 305 nm, which corresponds to Tyr emission, disappears and spectra are dominated by Trp emission only (Fig. 5, upper panel), probably due to increased emission quenching, observed for modified Ara h 6 as well. Further for modified Ara h 6, at pH 8.0, a new emission maximum is observed at 345 nm, while this peak is not observed at neutral pH. Probably, this peak at 345 nm at pH 8.0 is due to tyrosinate emission, which is pronounced in basic conditions. The observation that this peak is absent at neutral pH excluded the possibility that the fluorescence at 345 is due to contamination with a Trp-containing protein.

We hypothesize that the differences in charge state distribution (CSD) observed between native and reduced and alkylated conglutinins by mass spectroscopy (Fig. 3) can be explained by the changes in secondary and tertiary structure aspects caused by the reduction and alkylation. CSD of the native protein exhibits a bell-shaped curve with maximum at 10+, whereas modified species showed predominantly a distribution at higher CSD (21+), possibly explained by more exposure of protonable groups for reduced and alkylated conglutinin due to the partially denatured character of the reduced and alkylated forms of conglutinin.

3.4. Kinetics of reduction and alkylation assessed by apparent molecular weight

Time course of allergen reduction with two different concentrations of DTT, and a molar excess of iodoacetamide of at least ten-fold, is shown in Fig. 6. SDS-PAGE analysis shows that modification with 5 mM DTT is fast and complete reduction is achieved after 5 min. On the other hand, ten times lower concentration of reducing reagent requires longer incubation time (30 min) to achieve complete reduction. DTT in a concentration of 0.5 mM, in the first 2 min, induces reduction of some disulfide bonds, but only after 5 min a significant portion of protein is completely reduced, while the major part remains unmodified. Comparing SDS-PAGE profiles obtained after reduction with 0.5 mM and 5 mM DTT, it is clear that the lower concentration gave variable intermediate products up to an incubation time of 30 min. For a subsequent experiment, three different reduction time points with 0.5 mM or 5 mM DTT were selected: 0.5, 5 and 60 min, and the concentration of alkylating reagent varied as well (0.5, 5; 50 mM iodoacetamide already tested in the experiment shown in Fig. 6). 0.5 mM iodoacetamide is not effective to terminate reduction in both cases (0.5 and 5 mM DTT), demonstrated by the continuation of reduction of protein bands (Fig. 7, left hand lanes). On the other hand, 5 mM IAA stops reduction with 0.5 mM DTT, while it could not stop the reaction with 5 mM DTT. Notably, bands at the higher molecular weight (particularly for Ara h 6) could be observed in both

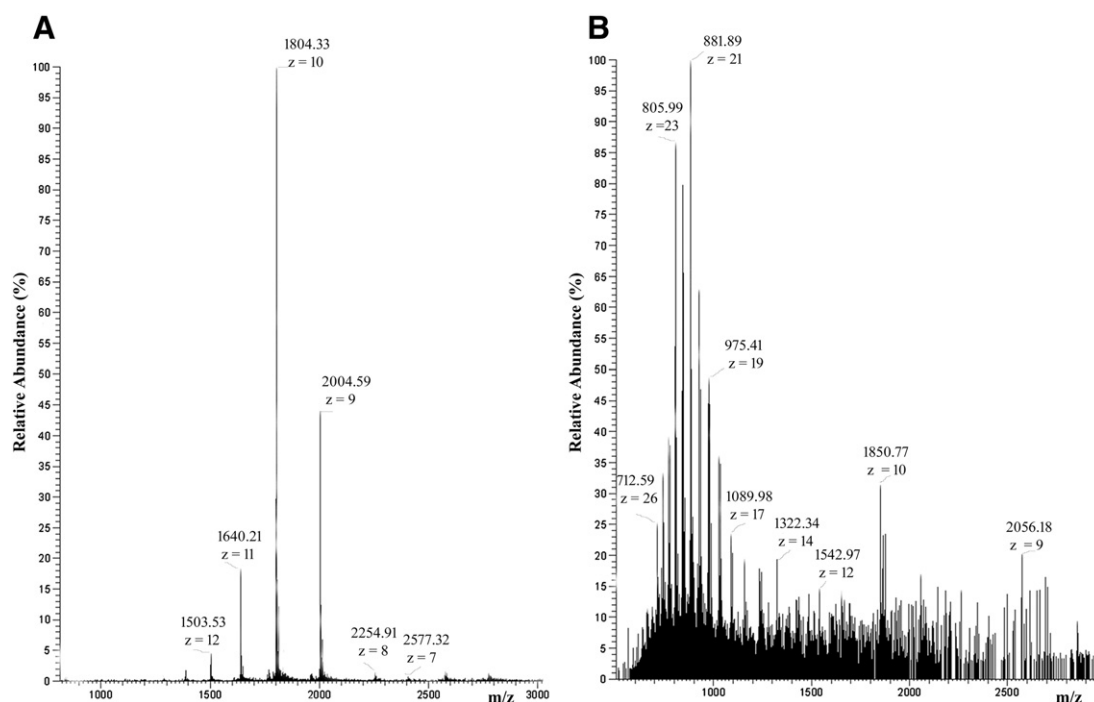


Fig. 3. ESI-MS spectra of native and of reduced and alkylated Ara h 2.02. Panel A: native Ara h 2.02; panel B: reduced and alkylated Ara h 2.02.

cases of lower concentration of IAA (0.5 and 5 mM) indicating intermolecular cross-linking by rearrangement of disulfide bonds. 50 mM iodoacetamide stops reaction with 5 mM DTT and no high molecular weight bands are observed at this condition (Fig. 6). In Fig. 7C some low molecular weight bands are visible, probably originating from breakdown products of Ara h 6 protein originating from peptides that are held together by disulfide bridges under native conditions [20]. These low molecular weight bands are not present in control sample (native, non-modified Ara h 6). See also Fig. 2 in Section 3.1.

3.5. Kinetics of reduction/alkylation assessed by mass increase

In order to observe if there is a critical disulfide that was modified during first steps of reduction, we recorded mass spectra of Ara h 2.02 in 0.5 min of reduction with 0.5 mM and 5 mM DTT (Fig. 8). Using the conditions of 0.5 min of reduction with 0.5 mM DTT, the apparent molecular weight of the conglutin has not changed substantially but a faint band at somewhat higher apparent molecular weight is present (Fig. 6A). Far UV CD spectroscopy data indicate that the conglutin modified at limiting conditions (in 0.5 min of reduction with 0.5 mM DTT) has a secondary structure resembling that of its native counterpart (data not shown). MS data showed that not only the mass of the native protein was present in the sample, but also one more mass peak

appeared (mass difference between native and 0.5 min of reduced and alkylated protein was 116.27 Da and this corresponds exactly to one modified disulfide, data not shown). For 0.5 min of reduction with 5 mM DTT, four bands were observed Fig. 6A, and mass spectrum showed five different masses in the same ion charge state (Fig. 8). More precisely, first peak corresponds to native Ara 2.02 (ion population of 1640.40, 1804.34, 2004.71 m/z), second peak showed that one disulfide was affected by alkylation, (ion population of 1513.45 m/z), third intermediate has the mass that corresponds to two modified disulfides, (ion population of 1406.87, 1828.43 and 2285.74 m/z), fourth had three affected disulfides, (ion population of 1671.21 and 2042.36 m/z), and finally, last peak showed totally modified Ara h 2.02 (ion population of 805.29, 841.80, 925.88, 974.56, 1028.65 and 1850.72 m/z) (Fig. 8). As can be noticed in Fig. 8, in the region of charge states of 23+ to 18+ (maxima at 22+) the most abundant species are Ara h 2.02 with four modified disulfides, while in the region of charge states of 13+ to 8+ (maxima at 10+) the most abundant species are unmodified and Ara h 2.02 with one, two and three disulfides modified. Although in lower charge state region all modified species were found, in the higher charge state region unmodified and one/two disulfide modified proteins were not detected. These results suggest that one and two disulfide modified Ara h 2.02 mostly retained native conformation, in line with the far UV CD

Table 1
Masses of peanut conglutin isoforms.

Proteins	Accession number ^a	Published mass determined by MALDI (Da)	Published mass determined by ESI-Q-TOF (Da) ^d	Experimental mass of native protein (Da)	Experimental mass of red/alk protein (Da)	Difference in experimental mass between native and reduced and alkylated (Da)
Ara h 2.02	Isoform1/Q6PSU2-1	18,050 ^b	18,035	18,032.13	18,497.71	465.58
	Isoform3/Q6PSU2-3	NR	17,716	NR	NR	NR
Ara h 2.01	Isoform2/Q6PSU-2	16,670 ^b	16,662	NR	NR	NR
	Isoform4/Q6PSU-4	NR	16,344	16,341.26	16,805.47	464.21
Ara h 6	Q647G9	14,843 ^c	NR	14,835.12	15,414.68	579.56

NR: Not reported.

^a Accession number from www.uniprot.org.

^b Reported by Chatel et al. [17].

^c Reported by Bernard et al. [22].

^d Reported by Li et al. [19].

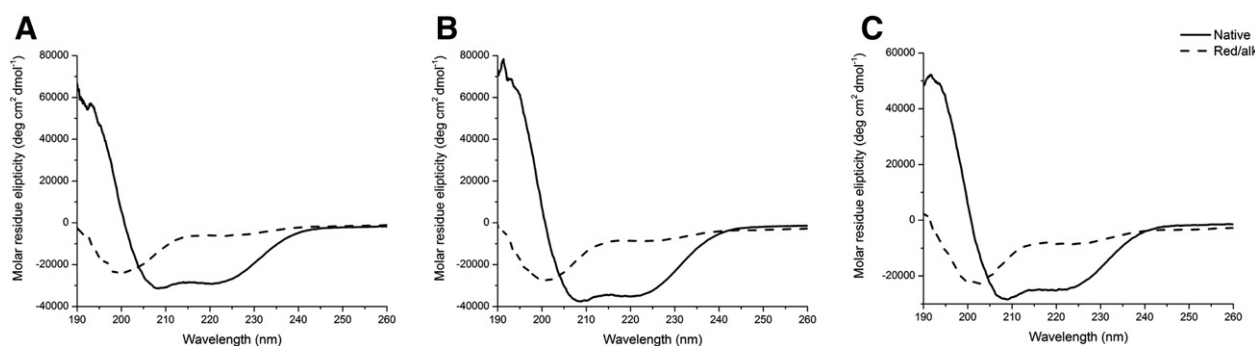


Fig. 4. Far-UV CD spectra of native and of reduced and alkylated peanut congrutins. Panels A, B, and C: Ara h 2.02, Ara h 2.01, and Ara h 6 respectively. Solid line, native allergens. Dashed line, reduced and alkylated allergens.

spectroscopy data of Ara h2.02 modified for 0.5 min of reduction with 0.5 mM DTT (not shown). On the other hand population of Ara h 2.02 with four modified disulfides is distributed between native and partially unfolded conformation. These observations are in accordance with SDS-PAGE results demonstrating that under limiting reducing/alkylating conditions (0.5 min) there are species with a minimum of two distinct conformations (native and partially unfolded) resulting in bands of apparently different masses. After complete modification the modified species are in partially unfolded conformation, in line with the determination of secondary structure content.

3.6. Investigation of modification of individual disulfide bonds

To investigate if there is a specific critical disulfide that undergoes modification as the first step of the modification, the congrutin isoforms were reduced with 0.5 mM DTT for 0.5 min. First, a tryptic digestion of completely reduced/alkylated congrutins was prepared and analyzed by peptide mass fingerprinting using nanoLC-MS/MS. The peptide coverages with high confidence were 57.56%, 31.40% and 67.59% for Ara h 2.02, Ara h 2.01 and Ara h 6, respectively (underlined area sequences shown in Fig. 1). All cysteine-containing peptides are

shown as modified. In a subsequent experiment, congrutins were reduced at limiting conditions (0.5 min reduction with 0.5 mM DTT) followed by standard alkylation and then analyzed by peptide mass fingerprinting. Data were analyzed by MassMatrix program in order to distinguish between modified disulfides and preserved disulfides. MS/MS spectra for this time point of modification provided for all Cys residues evidence of occurrence of both, reduced form and as semi-disulfide, indicating that there was no specific disulfide that was modified first (Table 2). Interestingly, we found that during modification with limiting reduction/alkylation conditions scrambling of disulfides occurred. Comparing to Fig. 1 that provides the disulfide organization in the native congrutin isoforms, we identified a number of new disulfides as shown in Table 2. Also, some disulfides representing the native organization were found, in particular the disulfide that involved Cys33 for Ara h 2.01 and Ara h 2.02. For Ara h 2.02, new disulfides are found between Cys116 and Cys118, and between Cys103 and Cys104, representing disulfide formation between Cys residues that are very closely positioned to each other. Also, new disulfides between Cys residues that are spatially separated from each other are formed (for example between Cys33 and Cys116 and Cys33 and Cys160). Another observation made in this regard is that disulfide

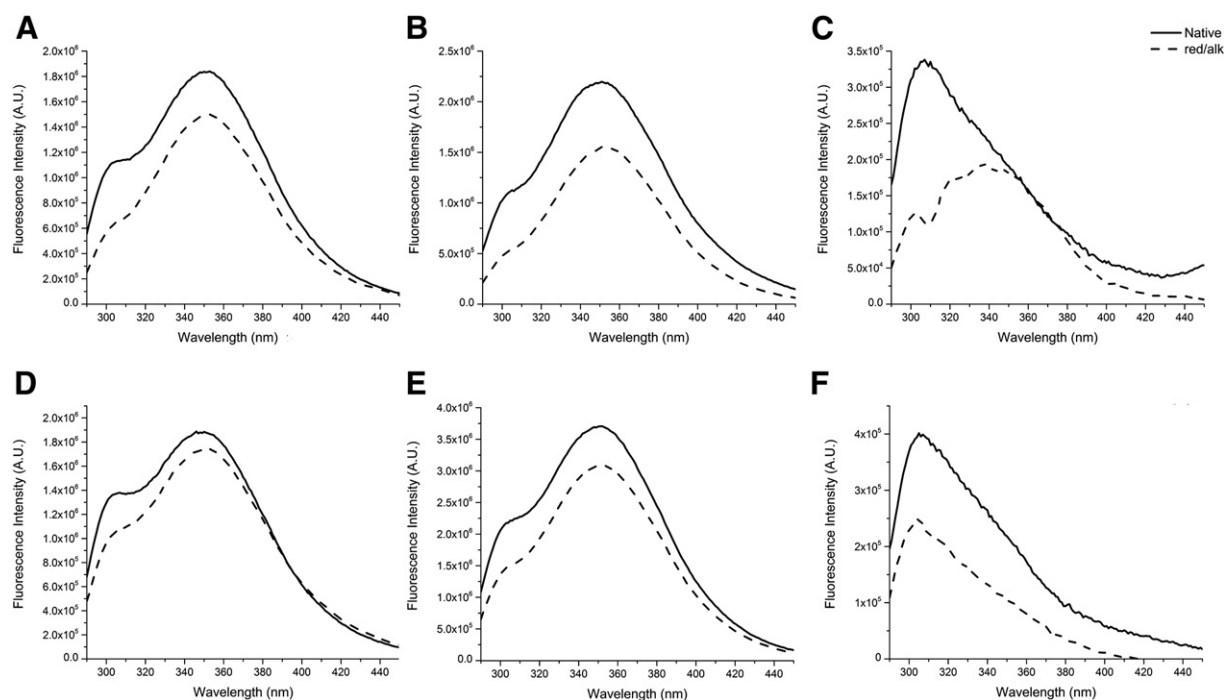


Fig. 5. Intrinsic fluorescence spectra of native and of reduced and alkylated peanut congrutins. Panels A, B, and C: Ara h 2.02, Ara h 2.01, and Ara h 6, respectively, in 50 mM Na-phosphate buffer pH 8. Panels D, E, and F: Ara h 2.02, Ara h 2.01, and Ara h 6, respectively, in 50 mM Tris buffer pH 7.2. Solid line, native allergens. Dashed line, reduced and alkylated allergens.

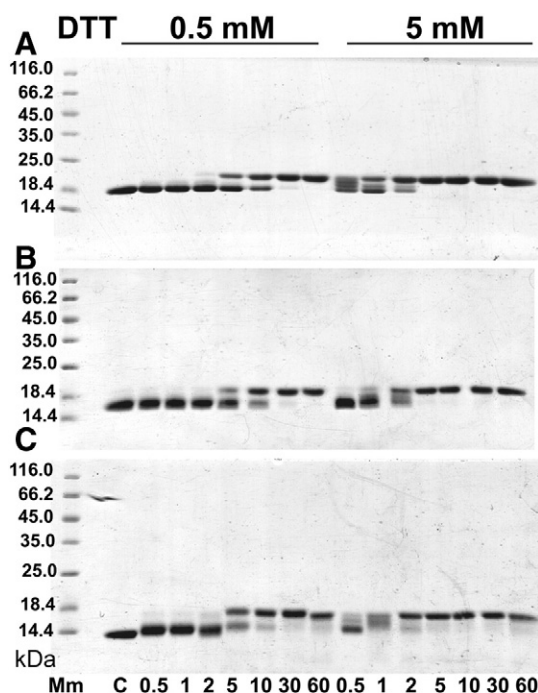


Fig. 6. Time course of reduction of peanut congrutin isoforms assessed by SDS-PAGE under non-reducing conditions. Panel A: Ara h 2.02; panel B: Ara h 2.01; panel C: Ara h 6. Lane Mm: Molecular weight marker (indicated in kDa) in left margin; lane C: Control, i.e. native congrutin. Numbers under lanes correspond to reduction time (minutes).

formation upon reduction of a Cys residue is not restricted to a specific other Cys residue. For example, Cys33 can react with Cys116, and Cys160. Cys116, on its turn, can form disulfides with Cys118 and Cys160, next to Cys33. Similar observations are made for the other congrutin isoforms too. The identified peptides for all congrutin isoforms are shown in Table 2.

3.7. IgE-binding properties

In order to examine the allergenic potency of the modified proteins, IgE-binding properties were tested by IgE-inhibition ELISA. All congrutin isoforms show a decrease in allergenic potency after reduction and alkylation (Fig. 9), demonstrated by the higher concentration of protein needed to inhibit the IgE-binding. While higher concentrations of reduced and alkylated Ara h 2 (both isoforms) lead to an almost complete inhibition, as observed for their native counterparts, the reduced and alkylated form of Ara h 6 is not able to completely inhibit the IgE binding (Fig. 9C). Testing reduced and alkylated Ara h 6 at higher concentrations (>1 mg/ml) was not possible due to limitations in solubility. Analysis of the horizontal displacement of the inhibition curves was used to determine the decrease in IgE-binding potency. This analysis could only be done for Ara h 2.02 and Ara h 2.01, because for Ara h 6, the inhibition curves for native and modified Ara h 6 were not parallel. By comparing displacement of inhibition curves the reduction in potency was calculated: For Ara h 2.02 the decrease in IgE-binding potency was 98.8%; for Ara h 2.01 this was 98.9%. For Ara h 6, the inhibition curve for modified must be horizontally displaced for at least 3 logs before one point touches the inhibition curve of native. Although not truly quantitative, one could say that the decrease in IgE-binding potency is therefore at least 99.9%. For the three congrutin isoforms, it is clear that the effect of reduction and alkylation is the largest for Ara h 6.

4. Discussion

There are currently no therapeutic options to treat peanut allergy [29]. Subcutaneous immunotherapy, as practiced for inhalant

allergens like grass pollen and mites, has been evaluated for peanut, but subcutaneous injection of peanut extract has been shown to be unacceptable due to the high risk of anaphylaxis [30].

The most important allergens in peanut allergy are Ara h 2 and Ara h 6, isoforms of each other and member of the congrutin seed storage proteins [1]. We have selected these for the development of immunotherapy. A common procedure to reduce the allergenicity of allergens, while maintaining the clinical efficacy in immunotherapy, is cross-linking of allergens with glutaraldehyde [31]. This is however not suitable for peanut congrutins due to a low content of Lys residues. Glutaraldehyde-treated peanut congrutin retained 37 to 52% of the IgE-binding potency [27]. Therefore, we considered if disrupting the disulfide bonds would be an option to substantially reduce the allergenic potential.

Initial investigations on a similar protein from Brazil nut showed that this approach maintained immunogenicity while the protein structure was denatured [32]. For peanut congrutins it was shown that this approach strongly reduced allergenicity, as well as in mixtures of 2 [11] or 3 [12] congrutin isoforms. Because these isoforms differ in number of disulfide bonds, IgE epitopes, and structural stability toward digestion [33], we aim to investigate the effect of reduction and alkylation on the individual isoforms of peanut congrutin, and to study the kinetics of this modification.

Peanut allergens Ara h 2 and Ara h 6 were modified by means of reduction and alkylation of their disulfide bonds. We studied the effect of this modification on the structure and allergenicity of these proteins. Upon reduction and alkylation, all three isoforms of peanut congrutin show an increase in apparent molecular weight in SDS-PAGE analysis,

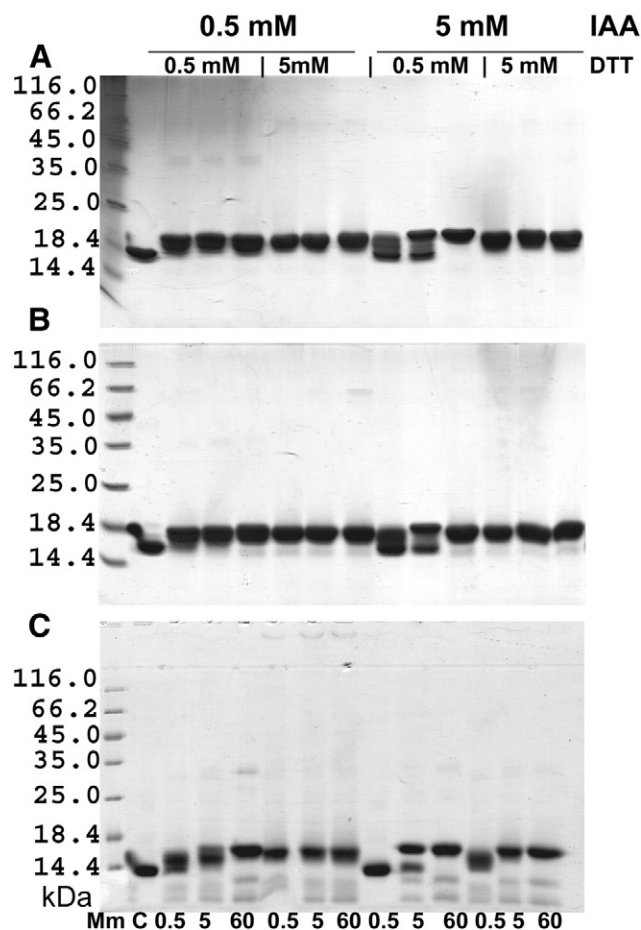


Fig. 7. Limiting concentration of alkylation agent leads to crosslinking of peanut congrutins. SDS-PAGE analysis applying non-reducing conditions. Panel A: Ara h 2.02; panel B: Ara h 2.01; panel C: Ara h 6. Lanes: Mm: Molecular weight marker (indicated in kDa) in left margin; C: Protein only as control; time of reduction (minutes): 0.5, 5, and 60.

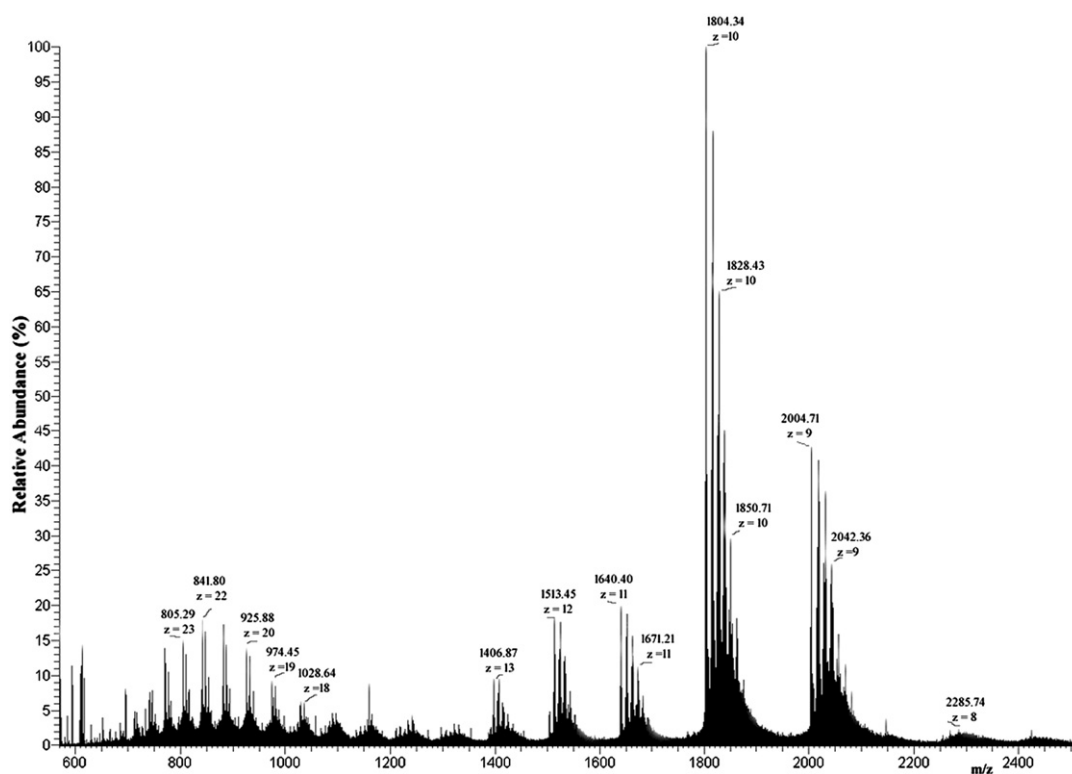


Fig. 8. Mass spectrum for Ara h 2.02 reduced for 0.5 min with 5 mM DTT and subsequently alkylated.

and increase in mass as shown by mass spectroscopy. We demonstrate that the mass increase exactly matched the alkylation of 4 and 5 disulfides for the two Ara h 2 isoforms and Ara h 6, respectively. Furthermore, all three isoforms showed a denaturation on secondary folding level, *i.e.* a loss of α -helices, for all three isoforms to the same extent. Similar results were reported for reduced and alkylated Pru p 3 allergen of the peach, recombinant Ara h 6 and Ara h 2 [12,34,35], and Ber e 1 allergen from Brazil nut [32], all proteins of the 2S albumin protein family with disulfide bond-stabilized structure. This decrease in α -helix content led to an increase not only in random coil, but also in β -sheet and turn, indicating helix-to-sheet transition. Maleki et al. [36] noticed that partially reduced and roasted Ara h 2 decreases in α -helices in favor of β -strands and random-coil or loop formation. It was reported that high pressure microfluidization treatment of Ara h 2 converts the disulfide bonds to sulfhydryl groups and results in partially unfolded protein with some α -helices converted to β -sheets [37]. Taken together, our results on changes in secondary structure as an effect of reduction and alkylation are in line with previous reports. The unfolding on secondary folding level is associated with tertiary structure rearrangement as shown by intrinsic fluorescence spectroscopy. Ara h 6 has no Trp, while both Ara h 2 isoforms have a single Trp, at position 4 spatially separated from the protein core [10]. The emission from Trp in Ara h 2 dominates in the recorded fluorescence spectra of both forms of Ara h 2 with emission maximum around 350 nm, suggesting both polar environment and hydrogen bonding of the single fully solvent exposed Trp of Ara h 2. Therefore, Trp fluorescence is a poor indicator for unfolding of Ara h 2. Some further evidence for weaker tertiary interactions came from charge state distribution observed in mass spectroscopy. The reduced and alkylated conglutins exhibited higher z -values than their native counterparts, suggesting a more open structure upon reduction and alkylation [38].

At limiting conditions for reduction and alkylation we found a variety of intermediate reduced proteins for all three conglutin isoforms, with 1, 2, 3, 4 or 5 disulfides reduced. Lowering the concentration of the reducing agent 10 fold, and investigating the degree of modification after short incubation time, we found that only one disulfide was

reduced. We used peptide mass fingerprinting to map the free Cys and found that all Cys residues could be in either the free form or semi-disulfide form. This indicates that there is no preference for the first disulfide to be targeted by reduction. Using a limiting concentration of iodoacetamide, we observed for Ara h 6 high-molecular weight bands suggesting disulfide re-arrangements leading to intermolecular cross-links. We also observed extensive disulfide rearrangement intramolecularly, similar to what has been described for other proteins such as insulin [39]. Reduced Cys residues could react with various other Cys residues in a non-specific way. This non-specificity of Cys-reactivity after reduction at limiting conditions suggests that the order of reduction is random, and that the structure of partially reduced conglutin is unfolded to a large extent. Disulfide scrambling is a known phenomenon for processed proteins, and hampers characterization of proteins [40]. When hydrolyzed with digestive enzymes, Ara h 2 forms large (approximately 10 kDa) N-terminal- and C-terminal-derived peptides that are held together by disulfide bonds [9,10,33]. Given the sequences of the digestion-resistant peptides, the disulfides Cys33–Cys116 and Cys45–Cys103 (for Ara h 2.02) are responsible for linking these peptides, and interestingly, these disulfides are found partially in their native form when limiting modification conditions are used, suggesting more resistance to reduction than other disulfides. Digested conglutin is equally allergenic as intact conglutin [9,10] because the structure is essentially intact owing to the disulfides. Therefore the disulfide bonds linking the digestion-resistant peptides are important in maintaining the allergenicity. From the safety perspective of immunotherapy it is therefore important to have a complete and consistent modification of the disulfides of peanut conglutin. This can be achieved with the conditions set out in this paper, avoiding disulfide scrambling. Compared to the gold standard of allergen modification by glutaraldehyde, where heterogeneous protein aggregates are formed, our technique of reduction and alkylation results in monomeric allergens that can be characterized biochemically to a greater level of detail.

The effect of the unfolding induced by complete reduction and alkylation on the allergenicity was evaluated in an IgE-binding assay. Our results point out that for each individual conglutin the allergenicity

Table 2
List of all peptides in conglutin isoforms that have exchanged disulfides.

Ara h 2.02												
No	Crosslink	Score	pp value*	pp2 value*	pptag value*	m/z	Charge	Mw observed	Mw	ΔM	Missed cleavage	Sequence
1.	C116-C118	24	11.9	13.2	5.6	632.9288	+3	1896.7718	1806.7707	0.0011	0	C ₁₁₆ M C ₁₁₈ EALQQIMENQSDR
2.	C103-C104	69	20.9	18.2	7.6	806.8222	+2	1610.6271	1610.6322	0.0049	0	C ₁₀₃ C ₁₀₄ NELNEGENNQ
3.	C33-C160	29	6.1	9.0	3.1	642.2902	+3	1924.8561	1924.8488	0.0074	0	C ₃₃ QSQLER C ₁₆₀ DLEVSGGR
4.	C33-C118	62	11.9	13.9	5.8	939.4041	+3	2816.1978	2816.1889	0.0089	0	C ₃₃ QSQLER C ^{IAA} M C ₁₁₈ EALQQIMENQSDR
5.	C116-C160 C33-C118	22	3.6	5.2	2.1	713.1540	+6	4273.1540	4273.8458	0.0418	1	APQRC ₁₆₀ DLEVSGGR C ₁₁₆ M C ₁₁₈ EALQQIMENQSDR C ₃₃ QSQLER
Ara h 2.01												
No	Crosslink	Score	pp value*	pp2 value*	pptag value*	m/z	Charge	Mw observed	Mw	ΔM	Missed cleavage	Sequence
1.	C104-C106	146	29.6	29.1	12.1	632.9304	+3	1896.7765	1896.7707	0.0058	0	C ₁₀₄ M C ₁₀₆ EALQQIMENQSDR
2.	C91-C92	71	27.3	18.9	8.7	805.8219	+2	1610.6365	1610.6322	0.0043	0	C ₉₁ C ₉₂ NELNEGENNQ
3.	C45-C91	28	2.2	6.6	2.7	855.1862	+5	4271.9020	4271.8934	0.0085	1	ANLRP C ₄₅ EQHLMK GAGSSQHQR C ₉₁ C ^{IAA} NELNEGENNQ
4.	C33-C106	20	4.1	6.0	1.4	705.3057	+4	2818.2011	2818.1763	0.0248	0	C ₃₃ QSQLER C ^{IAA} M C ₁₀₆ EALQQIMENQSDRLQGR
5.	C33-C92 C91-C140	25	3.3	6.2	1.8	757.3406	+6	4539.0071	4538.9719	0.0352	1	C ₃₃ QSQLER GAGSSQHQR C ₉₁ C ₉₂ NELNEGENNQ NLPQQ C ₁₄₀ GLR
Ara h 6												
No	Crosslink	Score	pp value*	pp2 value*	pptag value*	m/z	Charge	Mw observed	Mw	ΔM	Missed cleavage	Sequence
1.	C79-C80	72	22.3	23.3	5.3	791.8029	+2	1582.8029	1582.5986	0.0055	0	C ₇₉ C ₈₀ DELNEMENTQR
2.	C92-C94	59	16.5	13.9	7.1	828.0196	+3	2482.0442	2482.0400	0.0042	1	C ₉₂ M C ₉₄ EALQQIMENQC ^{IAA} DRLQDR
3.	C128-C136	12	7.4	4.5	2.5	804.3707	+3	2411.0974	2411.0901	0.0074	0	C ₁₂₈ DLDVSGGR ELMNPQQ C ₁₃₆ NFR
4.	C47-C80	24	2.6	5.2	4.3	809.6121	+4	3235.4267	3235.4057	0.0209	0	VLNKP C ₄₇ EQIMQR C ^{IAA} C ₈₀ DELNEMENTQR

*pp, pp2: Two pp scores based on two statistical models. High maximum value of pp and pp² indicates a good match. The pp scores should be used along with pptag score.
pptag: A statistical score that evaluates the match based on its color tags. A better score indicates better match quality. Significant statistical scores (a pp or pp² value greater than six).
^{IAA}: Modified Cys.

was strongly reduced compared to the native counterparts, as described earlier for mixtures of conglutins [11,12]. Chen et al. [26] investigated the allergenicity rank order of peanut conglutins and found Ara h 2.01 > Ara h 2.02 > Ara h 6. Our data for native conglutin are in line with this observation, and furthermore show that this order is retained

after reduction and alkylation. The largest effect of the modification is found for Ara h 6. A possible explanation can be that Ara h 2 contains linear IgE-binding epitopes in the section not present in Ara h 6, i.e. SQDPYSPSPY (Fig. 1) [23]. Such linear epitopes will remain intact after reduction and alkylation. Nevertheless the data show that the

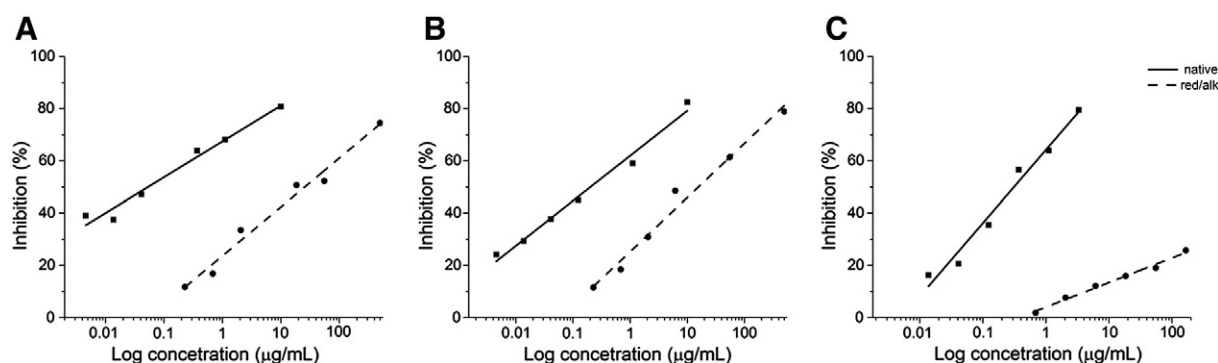


Fig. 9. IgE-binding potency of native and of reduced and alkylated peanut conglutin isoforms. Panel A: Ara h 2.02; panel B: Ara h 2.01; panel C: Ara h 6. Solid lines: Native peanut conglutins; dashed lines: reduced and alkylated peanut proteins.

IgE-binding to conformational epitopes is more important than IgE-binding to linear epitopes, as about 99% of the IgE-binding is lost upon reduction and alkylation, also for both Ara h 2 isoforms.

In conclusion, our data show that all peanut conglutin isoforms can be completely reduced and alkylated resulting in a denatured protein structure on secondary and tertiary folding level, with a concomitant loss of IgE-binding potency. Applying limiting conditions for the alkylating step, disulfide exchange occurs leading to inhomogeneous reaction products, undesirable for pharmaceutical development.

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References

- [1] H. Breiteneder, C. Radauer, A classification of plant food allergens, *J. Allergy Clin. Immunol.* 113 (2004) 821–830(quiz 831).
- [2] H. Bernard, E. Paty, L. Mondoulet, A.W. Burks, G.A. Bannon, J.M. Wal, P. Scheinmann, Serological characteristics of peanut allergy in children, *Allergy* 58 (2003) 1285–1292.
- [3] S.J. Koppelman, G.A. de Jong, M. Laaper-Ertmann, K.A. Peeters, A.C. Knulst, S.L. Hefle, E.F. Knol, Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2, *Clin. Exp. Allergy* 35 (2005) 490–497.
- [4] A.E. Flinterman, E. van Hoffen, C.F. den Hartog Jager, S. Koppelman, S.G. Pasmans, M.O. Hoekstra, C.A. Bruijnzeel-Koomen, A.C. Knulst, E.F. Knol, Children with peanut allergy recognize predominantly Ara h 2 and Ara h 6, which remains stable over time, *Clin. Exp. Allergy* 37 (2007) 1221–1228.
- [5] F. Blanc, K. Adel-Patient, M.F. Drumare, E. Paty, J.M. Wal, H. Bernard, Capacity of purified peanut allergens to induce degranulation in a functional in vitro assay: Ara h 2 and Ara h 6 are the most efficient elicitors, *Clin. Exp. Allergy* 39 (2009) 1277–1285.
- [6] H.S. Porterfield, K.S. Murray, D.G. Schlichting, X. Chen, K.C. Hansen, M.W. Duncan, S.C. Dreskin, Effector activity of peanut allergens: a critical role for Ara h 2, Ara h 6, and their variants, *Clin. Exp. Allergy* 39 (2009) 1099–1108.
- [7] X. Chen, Y. Zhuang, Q. Wang, D. Moutsoglou, G. Ruiz, S.E. Yen, S.C. Dreskin, Analysis of the effector activity of Ara h 2 and Ara h 6 by selective depletion from a crude peanut extract, *J. Immunol. Methods* 372 (2011) 65–70.
- [8] M. Kulis, X. Chen, J. Lew, Q. Wang, O.P. Patel, Y. Zhuang, K.S. Murray, M.W. Duncan, H.S. Porterfield, W.A. Burks, S.C. Dreskin, The 25 albumin allergens of *Arachis hypogaea*, Ara h 2 and Ara h 6, are the major elicitors of anaphylaxis and can effectively desensitize peanut-allergic mice, *Clin. Exp. Allergy* 42 (2012) 326–336.
- [9] M. Sen, R. Kopper, L. Pons, E.C. Abraham, A.W. Burks, G.A. Bannon, Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes, *J. Immunol.* 169 (2002) 882–887.
- [10] K. Lehmann, K. Schweimer, G. Reese, S. Randow, M. Suhr, W.M. Becker, S. Vieths, P. Rosch, Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions, *Biochem. J.* 395 (2006) 463–472.
- [11] P. Starkl, F. Felix, D. Krishnamurthy, C. Stremnitzer, F. Roth-Walter, S.R. Prickett, A.L. Voskamp, A. Willensdorfer, K. Szalai, M. Weichselbaumer, R.E. O'Hehir, E. Jensen-Jarolim, An unfolded variant of the major peanut allergen Ara h 2 with decreased anaphylactic potential, *Clin. Exp. Allergy* 42 (2012) 1801–1812.
- [12] H.P.M. van der Kleij, J. Smit, H. Sleijster-Selis, R. van den Hout, L. Gilmartin, R. Pieters, E. Kerkvliet, S. Koppelman, A peanut allergoid with increased safety and maintained immunogenicity, *J. Allergy Clin. Immunol.* 127 (2011) Ab32.
- [13] S.J. Koppelman, M. Wensing, M. Ertmann, A.C. Knulst, E.F. Knol, Relevance of Ara h 1, Ara h 2 and Ara h 3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h 2 is the most important peanut allergen, *Clin. Exp. Allergy* 34 (2004) 583–590.
- [14] K.A. Peeters, S.J. Koppelman, E. van Hoffen, C.W. van der Tas, C.F. den Hartog Jager, A.H. Penninks, S.L. Hefle, C.A. Bruijnzeel-Koomen, E.F. Knol, A.C. Knulst, Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? *Clin. Exp. Allergy* 37 (2007) 108–115.
- [15] Y. Zhuang, S.C. Dreskin, Redefining the major peanut allergens, *Immunol. Res.* 55 (2013) 125–134.
- [16] A.W. Burks, L.W. Williams, C. Connaughton, G. Cockrell, T.J. O'Brien, R.M. Helm, Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge, *J. Allergy Clin. Immunol.* 90 (1992) 962–969.
- [17] J.M. Chatel, H. Bernard, F.M. Orson, Isolation and characterization of two complete Ara h 2 isoforms cDNA, *Int. Arch. Allergy Immunol.* 131 (2003) 14–18.
- [18] W.G. Shreffler, D.A. Lencer, L. Bardina, H.A. Sampson, IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2, *J. Allergy Clin. Immunol.* 116 (2005) 893–899.
- [19] J. Li, K. Shefcheck, J. Callahan, C. Fenselau, Primary sequence and site-selective hydroxylation of prolines in isoforms of a major peanut allergen protein Ara h 2, *Protein Sci.* 19 (2010) 174–182.
- [20] J. Radosavljevic, D. Dobrijevic, M. Jadranin, M. Blanus, J. Vukmirica, T. Cirkovic Velickovic, Insights into proteolytic processing of the major peanut allergen Ara h 2 by endogenous peanut proteases, *J. Sci. Food Agric.* 90 (2010) 1702–1708.
- [21] M.L. Ramos, G. Fleming, Y. Chu, Y. Akiyama, M. Gallo, P. Ozias-Akins, Chromosomal and phylogenetic context for conglutin genes in *Arachis* based on genomic sequence, *Mol. Genet. Genomics* 275 (2006) 578–592.
- [22] H. Bernard, L. Mondoulet, M.F. Drumare, E. Paty, P. Scheinmann, R. Thai, J.M. Wal, Identification of a new natural Ara h 6 isoform and of its proteolytic product as major allergens in peanut, *J. Agric. Food Chem.* 55 (2007) 9663–9669.
- [23] J.S. Stanley, N. King, A.W. Burks, S.K. Huang, H. Sampson, G. Cockrell, R.M. Helm, C.M. West, G.A. Bannon, Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2, *Arch. Biochem. Biophys.* 342 (1997) 244–253.
- [24] I.H. Kang, P. Srivastava, P. Ozias-Akins, M. Gallo, Temporal and spatial expression of the major allergens in developing and germinating peanut seed, *Plant Physiol.* 144 (2007) 836–845.
- [25] B.J. Hales, A. Bosco, K.L. Mills, L.A. Hazell, R. Loh, P.G. Holt, W.R. Thomas, Isoforms of the major peanut allergen Ara h 2: IgE binding in children with peanut allergy, *Int. Arch. Allergy Immunol.* 135 (2004) 101–107.
- [26] X. Chen, Q. Wang, R. El-Mezayen, Y. Zhuang, S.C. Dreskin, Ara h 2 and Ara h 6 have similar allergenic activity and are substantially redundant, *Int. Arch. Allergy Immunol.* 160 (2013) 251–258.
- [27] S.J. Koppelman, R.H.J.A. van den Hout, H.E. Sleijster-Selis, D.M.A.M. Luijkx, US patent application US2011229523, 2008.
- [28] H. Xu, L. Zhang, M.A. Freitas, Identification and characterization of disulfide bonds in proteins and peptides from tandem MS data by use of the MassMatrix MS/MS search engine, *J. Proteome Res.* 7 (2008) 138–144.
- [29] A. Nowak-Węgrzyn, H.A. Sampson, Future therapies for food allergies, *J. Allergy Clin. Immunol.* 127 (2011) 558–573(quiz 574–555).
- [30] J.J. Oppenheimer, H.S. Nelson, S.A. Bock, F. Christensen, D.Y. Leung, Treatment of peanut allergy with rush immunotherapy, *J. Allergy Clin. Immunol.* 90 (1992) 256–262.
- [31] J.L. Ceuppens, D. Bullens, H. Kleinjans, J. van der Werf, Immunotherapy with a modified birch pollen extract in allergic rhinoconjunctivitis: clinical and immunological effects, *Clin. Exp. Allergy* 39 (2009) 1903–1909.
- [32] S.J. Koppelman, W.F. Nieuwenhuizen, M. Gaspari, L.M. Knippels, A.H. Penninks, E.F. Knol, S.L. Hefle, H.H. de Jongh, Reversible denaturation of Brazil nut 2S albumin (Ber e1) and implication of structural destabilization on digestion by pepsin, *J. Agric. Food Chem.* 53 (2005) 123–131.
- [33] S.J. Koppelman, S.L. Hefle, S.L. Taylor, G.A. de Jong, Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides, *Mol. Nutr. Food Res.* 54 (2010) 1711–1721.

- [34] S. Hazebrouck, B. Guillon, M.F. Drumare, E. Paty, J.M. Wal, H. Bernard, Trypsin resistance of the major peanut allergen Ara h 6 and allergenicity of the digestion products are abolished after selective disruption of disulfide bonds, *Mol. Nutr. Food Res.* 56 (2012) 548–557.
- [35] M. Toda, G. Reese, G. Gadermaier, V. Schulten, I. Lauer, M. Egger, P. Briza, S. Randow, S. Wolfheimer, V. Kigongo, M. Del Mar San Miguel Moncin, K. Fotisch, B. Bohle, S. Vieths, S. Scheurer, Protein unfolding strongly modulates the allergenicity and immunogenicity of Pru p 3, the major peach allergen, *J. Allergy Clin. Immunol.* 128 (2011) e1021–e1027.
- [36] S.J. Maleki, O. Viquez, T. Jacks, H. Dodo, E.T. Champagne, S.Y. Chung, S.J. Landry, The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function, *J. Allergy Clin. Immunol.* 112 (2003) 190–195.
- [37] C.Q. Hu, H.B. Chen, J.Y. Gao, C.P. Luo, X.J. Ma, P. Tong, High-pressure microfluidisation-induced changes in the antigenicity and conformation of allergen Ara h 2 purified from Chinese peanut, *J. Sci. Food Agric.* 91 (2011) 1304–1309.
- [38] A. Konijnenberg, A. Butterer, F. Sobott, Native ion mobility-mass spectrometry and related methods in structural biology, *Biochim. Biophys. Acta* 1834 (2013) 1239–1256.
- [39] H.R. Morris, P. Pucci, M. Panico, G. Marino, Protein folding/refolding analysis by mass spectrometry. Scrambling of disulphide bridges in insulin, *Biochem. J.* 268 (1990) 803–806.
- [40] J. Echterbille, L. Quinton, N. Gilles, E. De Pauw, Ion mobility mass spectrometry as a potential tool to assign disulfide bonds arrangements in peptides with multiple disulfide bridges, *Anal. Chem.* 85 (2013) 4405–4413.