



Effect of heat treatment on the conformational stability of intact and cleaved forms of the peanut allergen Ara h 6 in relation to its IgE-binding potency

Harmen H.J. de Jongh^a, Govardus A.H. de Jong^b, Danijela Apostolovic^c, Steve L. Taylor^d, Joseph L. Baumert^d, Stef J. Koppelman^{d,*}

^a ProtIn Consultancy, Rozenstraat 19E, 3702 VL Zeist, The Netherlands

^b TNO, Utrechtseweg 48, 3704 HE Zeist, The Netherlands

^c Clinical Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet, Solnavägen 1, 171 77 Solna, Sweden

^d Food Allergy Research and Resource Program, Department of Food Science & Technology, University of Nebraska, 279 Food Innovation Center, Lincoln, NE 68588-6207, USA

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ABSTRACT

This work reports on the effect of heat treatment on the protein conformational stability of intact and post-translationally cleaved peanut allergen Ara h 6 in relation to IgE-binding. Intact and post-translationally cleaved Ara h 6 are structurally similar and their strong resistance to denaturant-induced unfolding is comparable. Only upon exposure to autoclave conditions the two forms of Ara h 6 demonstrated susceptibility to irreversible denaturation resulting in a significant decrease in IgE-binding potency. This reduction is for the intact protein more pronounced than for the cleaved form. This is attributed to less conformational constraints of the cleaved form compared to intact, as suggested by the 2-fold lower activation energy for unfolding found for the cleaved form. Overall, harsh conditions are required to denature Ara h 6 and to significantly reduce its IgE-binding potency. The cleaved form possesses more resistance to such denaturation than the intact form.

1. Introduction

Approximately 0.6% of adults and 1–2% of children/infants in the US are affected by peanut allergy upon food intake (Dyer et al., 2015; Sicherer, Muñoz-Furlong, Godbold, & Sampson, 2010). *In vitro* studies have shown that Ara h 2 and Ara h 6 are the most potent allergens in peanut (Koppelman et al., 2005; McDermott et al., 2007), as confirmed in humans by *in vivo* skin prick tests (Peeters et al., 2007). It is the IgE-binding to Ara h 2 and Ara h 6 that has been suggested as the best predictor for clinically relevant peanut allergy (Klemans, Knol, Bruijnzeel-Koomen, & Knulst, 2014).

Ara h 2 and Ara h 6 belong to the 2S albumin protein family commonly present in plant seeds and nuts where they serve as storage proteins. Ara h 2 is an approximately 17 kDa protein and Ara h 6 is slightly smaller with a molecular weight of 15 kDa (Suhr, Wicklein, Lepp, & Becker, 2004). Several isoforms of both these allergens have been identified by mass spectrometry (Schmidt et al., 2009; Li, Shefcheck, Callahan, & Fenselau, 2010). 2S albumins are member of the prolamin super-family that adopt a common fold; bundles of α -helices held together by 4 or 5 conserved disulfide bonds (Shewry, Beaudoin, Jenkins, Griffiths-Jones, & Mills, 2002). The presence of these

intramolecular disulfides may explain its resistance to heat-treatment and digestion (Breiteneder & Mills, 2005). In general, 2S albumins appear as heterodimers, where each protein consists of two polypeptide chains derived from a single precursor protein by post-translational cleavage (Shewry, Napier, & Tatham, 1995). This cleavage is in the midst of a non-structured loop connecting two of the α -helices (Shewry et al., 2002). However, in peanut 2S albumins are reported to be essentially monomeric (Burks et al., 1992; Suhr et al., 2004). Post-translational cleavage has been reported for another peanut protein, Ara h 3, indicating that peanut does not lack the machinery for post-translational cleaving (Piersma, Gaspari, Hefle, & Koppelman, 2005). It may be that cleaved forms of peanut 2S albumin do exist, but that their presence was not recognized.

Recently, intact (iAra h 6) and post-translationally cleaved Ara h 6 (pAra h 6) isoforms have been described to be present both in peanuts and have been biochemically characterized in detail (de Jong et al., 2018). In pAra h 6, a 5-amino acid stretch resembling amino acids 43–47 (UniProt accession number Q647G9) in the non-structured loop has been clipped from iAra h 6. Consequently, pAra h 6 consists of a N-terminal chain of approximately 5 kDa, and a C-terminal chain of approximately 9 kDa, held together by disulfide bonds. Intermediate post-

* Corresponding author.

E-mail address: farrp@unl.edu (S.J. Koppelman).

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translationally cleaved products, in which the protein was only cleaved without removing amino acids were also described (de Jong et al., 2018). Under ambient conditions the secondary structure and IgE-binding of pAra h 6 resembles that of intact Ara h 6, indicating that the loss of the non-structured loop is not critical for the (conformational) IgE-epitopes. However, under denaturing and reducing conditions, the reactivity of pAra h 6 with commercially available IgG antibodies was shown to be decreased compared to that of iAra h 6 (de Jongh et al., 2018). The origin of the difference in behavior under denaturing and reducing condition is unclear, and may be associated with different denaturing pathways of the two isoforms. Because both isoforms are readily abundant in peanut in an about equal amount (de Jongh et al., 2018), and because Ara h 6 is recognized as one of the most potent peanut allergens, it is important to understand how heat-treatment (at conditions relevant for the food industry) of both isoforms affects structural characteristics which may lead to differentiation in IgE-binding. This insight is essential to validate the assessment of the allergen-potency in peanut-containing food products where mostly heat-treatment is applied during production and preservation strategies.

The aim of this work is thus to understand the structurally different responses of intact and post-translationally cleaved Ara h 6 under denaturing conditions. For that purpose, both isoforms were isolated and analyzed using spectroscopic (circular dichroism and fluorescence) techniques in time-resolved studies to monitor their responses to applied heat treatments. Moreover, their aggregation state of heated samples was analysed using size exclusion chromatography and SDS-PAGE. Their allergenic potency was tested by IgE-ELISA using serum from peanut allergic patients.

2. Materials and methods

2.1. Purification of iAra h 6 and pAra h 6

Raw peanuts (Virginia type) were used for purification of intact and post-translationally cleaved Ara h 6 (referred to as iAra h 6 and pAra h 6 respectively). The purification has been described in detail elsewhere (de Jong et al., 2018). In short, an extract was made of milled, defatted peanuts (at pH 8), and extracted proteins were separated by ion-exchange chromatography and hydrophobic interaction chromatography. Fractions were evaluated by SDS-PAGE and purified proteins were desalted and stored as lyophilized powders, refrigerated in airtight containers. Identification was done with mass spectrometry (de Jong et al., 2018).

2.2. Protein concentration

Protein concentrations were determined by absorbance spectroscopy at 280 nm, using $A_{280}(1 \text{ mg/ml})$ of 0.243 for intact Ara h 6, and 0.149 for pAra h 6 based on the amino acid composition of the proteins (UniProt accession number Q647G9; signal peptide of first 21 amino acids not included) taking into account that intact Ara h 6 contains amino acids 1–124, and pAra h 6 contains amino acids 1–42 plus 48–124. An intermediate form of pAra h 6 that was identified has heterogeneity at the cleavage site (de Jong et al., 2018). For this the $A_{280}(1 \text{ mg/ml})$ of pAra h 6 was used.

2.3. Heating steps

For heating at 80 °C or 90 °C, iAra h 6 and pAra h 6 at 0.5 mg/ml in 10 mM boric acid buffer (pH = 8.2) were heated in a thermoblock to the target temperature in approximately 15 min and the target temperature was maintained for an additional 20 min. For autoclaving, iAra h 6 and pAra h 6 at 0.5 mg/ml in 10 mM sodium borate (pH = 8.2) were heated in a laboratory bench-top autoclave (Tuttnauer 2840) to 121 °C. Target temperature (121 °C) was reached in approximately 30 min and this temperature was maintained for an additional 20 min,

before cooling down to ambient temperature (in approximately 10 min). The maximum pressure reached approximately 2 bar.

2.4. SDS-PAGE analysis

SDS-PAGE analysis was performed on Mini-PROTEAN-Tris Tricine gels (Bio-Rad, CA) 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 or in absence of a reducing agent (DTT) 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 fixed in 40% methanol and 10% acetic acid for 30 min and stained for 1 h with Coomassie Brilliant Blue G-250 (Bio-Rad, CA).

2.5. Circular dichroism

Protein samples were prepared in 10 mM boric acid buffer (pH 8.2) at concentrations of 0.1 mg/ml for recording regular spectra and 0.2 mg/ml for time-scans at fixed wavelength. Samples were measured in quartz cuvettes with a 1 mm path length, filled for > 95% of its volume and sealed to prevent evaporation of the solvent for prolonged time measurements at elevated temperatures. Samples were analyzed for the secondary structure using a Jasco-815 spectropolarimeter (Jasco Corporation, Japan) by recording the ellipticity in the spectral range from 185 to 260 nm with a spectral resolution of 0.2 nm, a scan speed of 100 nm/min, a time-constant of 0.125 sec and a band width on 1 nm. Spectra were recorded as averages of 16 scans. The spectra were corrected for that of a corresponding protein-free sample. Subsequently noise reduction of the spectra was applied using the inverse Fourier transformation routine according to the supplier's software. The far-UV CD spectra were analyzed for the secondary structural element content using non-linear least-squares fitting procedure as previously described (de Jongh, Goormaghtigh, & Killian, 1994).

The samples analysed for their secondary structure content in the presence of a denaturant were prepared by dilution of a 1 mg/ml protein solution ten-fold at the indicated chaotrope-concentration from a concentrated stock solution (i.e. 10 M for urea and 8 M for guanidinium) in 10 mM boric acid (pH 8.2) solution. Prior to measurement the samples were equilibrated for 2 hrs at room temperature.

2.6. Fluorescence

Intrinsic fluorescence spectra of 50 µg/mL Ara h 6 samples in 10 mM Boric acid (pH 8.2) were obtained using a Cary eclipse fluorescence spectrophotometer (Agilent technologies, the Netherlands) according to a previously described procedure (Munialo, de Jongh, Broersen, van der Linden, & Martin, 2013). Quartz cuvettes with an optical path of 1 cm were used. The spectra of the samples were recorded at the excitation wavelength of 280 nm, and the emission wavelength range from 300 to 400 nm at 20 °C with a resolution of 1 nm. The excitation and emission slit widths were 5 nm and a scan speed of 120 nm/min was used. Typically, three spectra were recorded and subsequently averaged.

2.7. Size exclusion chromatography

Size exclusion chromatography (SEC) was performed on a Superdex G75 column (30 ml) operated on an Äkta FPLC p-920 (Amersham Pharmacia Biotech, Uppsala, Sweden) in 50 mM TRIS buffer (pH 8.0) containing 150 mM NaCl at a flow rate of 0.4 ml/min. Detection of protein in the elution profile was achieved by monitoring the absorbance at 280 nm.

2.8. IgE-binding by ELISA

A serum pool was made from sera of seven peanut allergic patients (range and mean of total peanut-specific IgE: 53–787 kU/L and 254 kU/L respectively) as described by Apostolovic and coworkers (2016) and de Jong et al. (2018). The patients were from the EU and US (ethical approval certificate number: IRB Project ID 6029, Lincoln, NE, USA). To qualify for this study, subjects required a convincing history of a type I allergic reaction when peanuts were consumed as well as a positive skin prick test within the past 6 months or a ImmunoCAP > 15 kU/L performed within the last 6 months before study participation. Sera from non-peanut allergic donors served as negative controls.

Inhibition ELISA was performed as described in detail elsewhere (Apostolovic et al., 2016). The serum pool was diluted 1:50 and mixed 1 to 1 with various concentrations of iAra h 6 and pAra h 6, resulting in a final serum pool dilution of 1:100, and allergen concentrations ranging from 10 µg/ml to 1.5 ng/ml. These mixes were incubated on the coated and blocked ELISA plates and incubated for 2 h at room temperature. After washing, plates were stained for IgE as described (Apostolovic et al., 2016). Concentrations of peanut protein required for 50% inhibition (IC_{50}) of IgE-binding were calculated as described previously (Apostolovic et al., 2013) and two-tailed *t*-test was performed to compare results.

3. Results and discussion

3.1. Heat-induced denaturation of Ara h 6 isoforms

In a previous study (de Jong et al., 2018) it was shown that in raw peanuts Ara h 6 is present in various isoforms, where next to the non-cleaved (intact) protein also a post-translationally cleaved form is present (denoted here as iAra h 6 and pAra h 6 respectively). As IgE-binding to Ara h 6 is used as clinical measure for allergy potency, it is essential to evaluate the possible differences in response of these two isoforms to heat-treatment. Heat treatment is a common procedure in the preparation of food products, and the conformational stability needs thus to be studied accordingly. To determine the effect of post-translational cleavage on the secondary structure of the protein far-UV circular dichroism (CD) spectra were recorded. Fig. 1 shows the spectra for iAra h 6 (panel A) pAra h 6 (panel B) at ambient temperature before and after a heat-treatment at 90 °C for 20 min and upon exposure to autoclave conditions at 121 °C (for details see methods). The CD-spectra at ambient temperature of both isoforms are characterised by three extremes, a positive one at 194 nm, and two negative extremes at 208 and 222 nm. Such spectra are typical for proteins with a high α -helical content, as confirmed by spectral shape analysis (de Jongh et al., 1994); both isoforms contain about 59% α -helical and 29% unordered structure elements. This high helical character was also reported earlier for this protein (Lehmann et al., 2006). Interestingly, the prolonged heating at 90 °C does not have a significant irreversible effect on the secondary structure of both isoforms. Such resistance to heat-denaturation has been reported before for Ara h 6 (Lehmann et al., 2006). Recent work from Zhang et al. (2018) showed that Ara h 2 obtained from peanuts that were boiled for 20 min. showed some degree of denaturation. While this seems conflicting with the work from Lehmann et al. (2006), it should be kept in mind that Zhang et al. (2018) used somewhat higher temperature, and that Ara h 2, although a peanut 2S albumin, is not identical to Ara h 6.

Exposure of both proteins to autoclave conditions results in completely different CD-spectra with a single negative extreme around 200 nm. Such spectra are typical for a highly disordered structure of the protein, as also indicated by spectral shape analysis where only approximately 15% α -helical structures are found and about 73% non-ordered (random coil) structures are present. That the intensities of the spectra prior and after heating to 90 °C are comparable indicates that no material has been lost from the sample for example by precipitation,

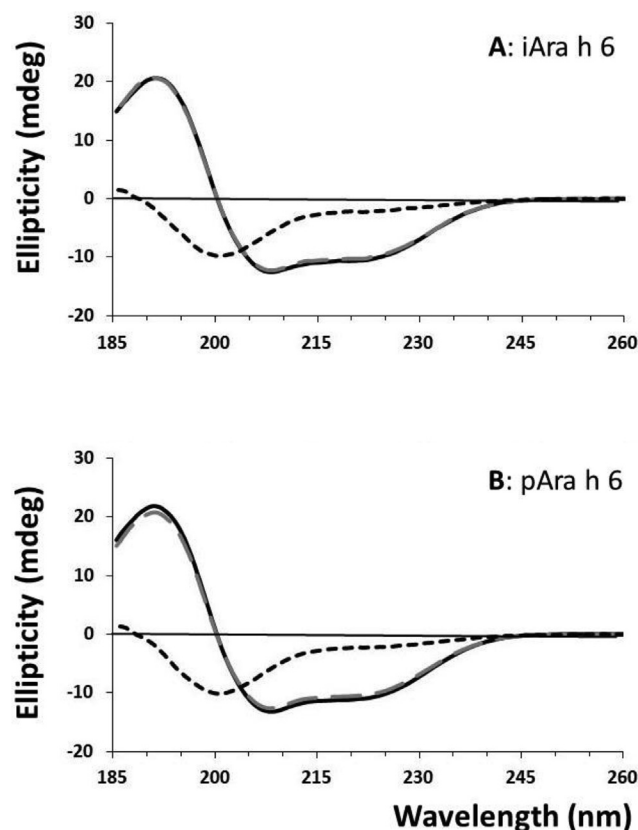


Fig. 1. Far-UV circular dichroism spectra of 0.1 mg/ml Ara h 6 forms (panel A: intact; panel B: post-translationally cleaved) in 10 mM boric acid buffer (pH 8.2). The spectra are recorded at 20 °C. Black solid lines: samples at 20 °C; grey long dashed lines: samples heated for 20 min at 90 °C; black dotted lines: autoclaved samples.

nor that the intensities are reduced due to absorption flattening caused by extensive aggregation of the material. For the autoclaved samples it can be observed that, taking into account the molar ellipticity of the different secondary structure elements (de Jongh et al., 1994), the CD intensity and thereby the protein concentration of the autoclaved sample is comparable that of the non-heated sample. This illustrates again that there is no loss of protein material due to precipitation.

At a tertiary folding level as evaluated by fluorescence spectroscopy (results not shown) a qualitative similar conclusion can be drawn. Due to the absence of a tryptophan in the sequence one needs to rely on the tyrosine fluorescence which is less sensitive to polarity changes, making observed spectral shifts not quantitative. Nevertheless, it can be stated that heat treatment up to 90 °C has for both isoforms no impact on the globular fold of the protein, while autoclaving results for both proteins to a fully unfolded structure where the present aromatics (tyrosine residues) have become more solvent exposed. The impact of heating on the quaternary folding level was first assessed by size exclusion chromatography (Fig. 2). Intact Ara h 6 (Fig. 2, Panel A) elutes as a single peak at 13.5 ml for heat treatment up to and including 90 °C. After heat treatment at autoclave conditions, intact Ara h 6 elutes as two partially overlapping peaks at 12.5 and 13.5 ml, with the peak at 12.5 ml representing higher molecular weight forms and suggesting the formation of aggregates. For post-translationally cleaved Ara h 6, heat treatment up to and including 90 °C does not alter the exclusion behaviour (single peak at 13.5 ml; Fig. 2, Panel B), but autoclaving leads to a second peak at higher elution volume (14.5 ml), indicating lower molecular weight and suggesting dissociation of the two polypeptide chains that post-translationally cleaved Ara h 6 consists of (de Jong et al., 2018). Gel electrophoretic analysis (Fig. 3) confirmed that moderate heating up to

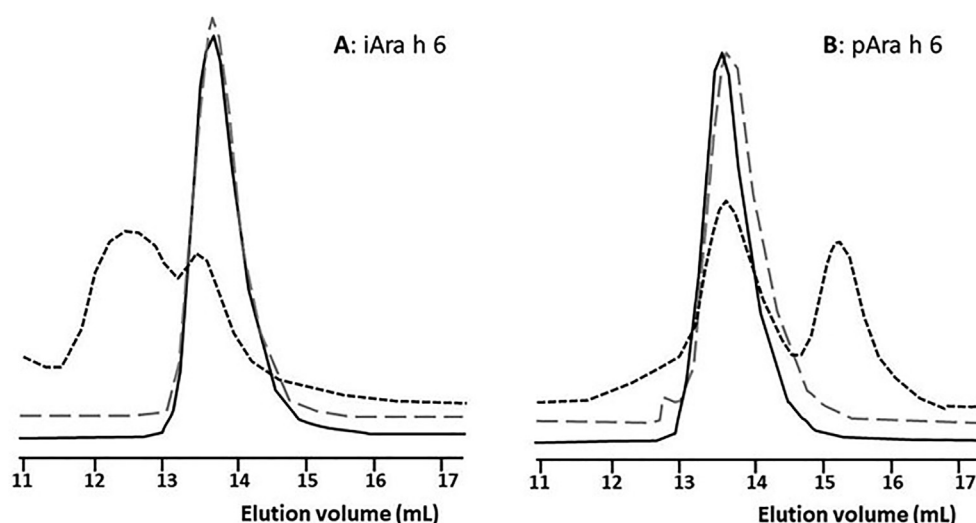


Fig. 2. Size exclusion chromatography elution profiles (redrawn and displaced vertically to improve clarity) of unheated (solid black line), heated (20 min 90 °C; grey long dashed line) and autoclaved (dotted black line) intact and post-translationally cleaved Ara h 6.

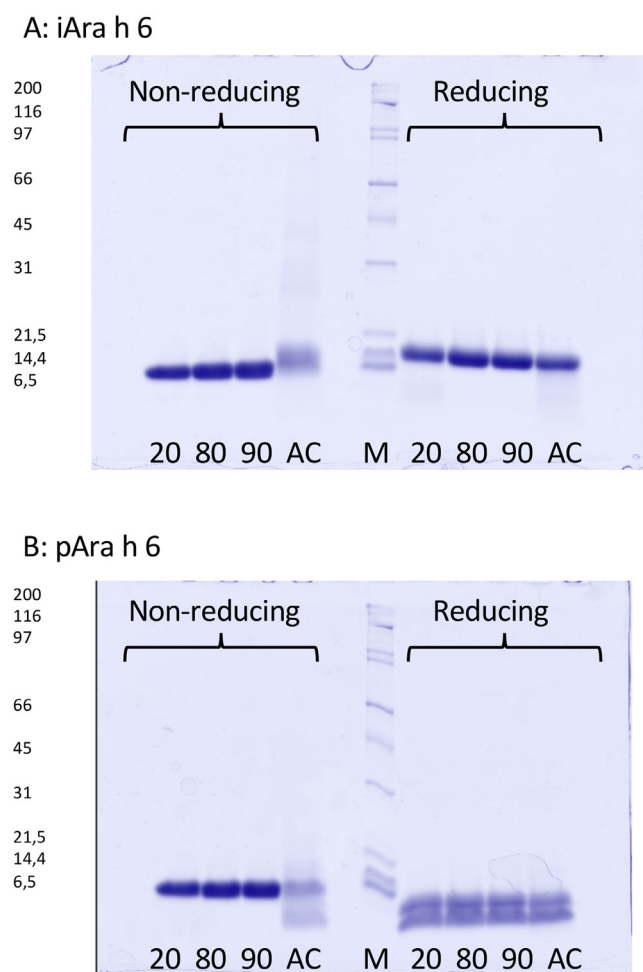


Fig. 3. SDS-gel electrophoretic analysis of unheated (20), heated at 80 °C (80), heated at 90 °C (90) and autoclaved (AC) Ara h 6 forms. Panel A: intact Ara h 6 (iAra h 6); Panel B: post-translationally cleaved (pAra h 6). Left lanes of gels show non-reducing conditions and right lanes of gels show reducing conditions. On the right the molecular weight markers are indicated (in kDa); marker lanes encoded 'M'.

and including 90 °C has no effect on the quaternary structure of both proteins. Clearly SDS-gel electrophoresis analysis under reducing conditions shows that the post-translationally cleaved form of Ara h 6 consists of two separate polypeptide chains as expected based on earlier work (de Jong et al., 2018). Upon autoclave treatment, intact Ara h 6 analyses at non-reducing conditions runs as a slightly higher, more diffuse band, on the gel. This may reflect the structurally unfolded character of the protein subjected to autoclave conditions as observed in Fig. 1. Also, a minor amount of autoclaved iAra h 6 appears as multimers (faint bands at approximately 31 and 45 kDa), however this amount is very small and essentially all iAra h 6 migrates at approximately 15 kDa. Apparently, the aggregates observed with size exclusion chromatography dissociate again to the approximately 15 kDa protein as observed with SDS-gel electrophoresis, essentially independent of the presence of a reducing agent (Fig. 3, panel A). Upon exposure of pAra h 6 to autoclave conditions, however, next to monomeric protein about half of the material elutes at smaller molecular sizes (Fig. 2, right panel). This is confirmed by gel electrophoretic analysis (Fig. 3, panel B) under non-reducing conditions. Obviously under reducing conditions the two chains are separated in the SDS-gel electrophoretic analysis as the two chains are clipped by processing, but no further degradation of the protein is observed to smaller fragments due to the heat treatment. The observation from size exclusion (Fig. 2) that a main part of iAra h 6 after autoclave treatment elutes as a multimeric form may be explained by the native conditions in contrast to the presence of SDS at electrophoresis (Fig. 3); the dimer and multimers dissociate essentially completely by the detergent present in the sample buffer. On reducing conditions no bands reflecting a dimer or multimeric forms are observed at all for iAra h 6 after autoclave treatment suggesting that the formation of dimers and multimeric forms are (partially) disulphide dependent. pAra h 6 after autoclave treatment shows at non-reducing conditions smearable bands of lower MW, confirming that part of the disulphide bonds are lost upon autoclave treatment, in line with the observations by size-exclusion chromatography. It can be concluded that autoclave conditions have a different effect on the quaternary structure, suggesting that there is a different sensitivity to applied heating which may originate from a difference in stability of the protein structure.

3.2. Conformational stability of Ara h 6 assessed by denaturant-titration

To evaluate whether the conformational stability of iAra h 6 and pAra h 6 differ, the proteins were exposed to denaturing conditions by

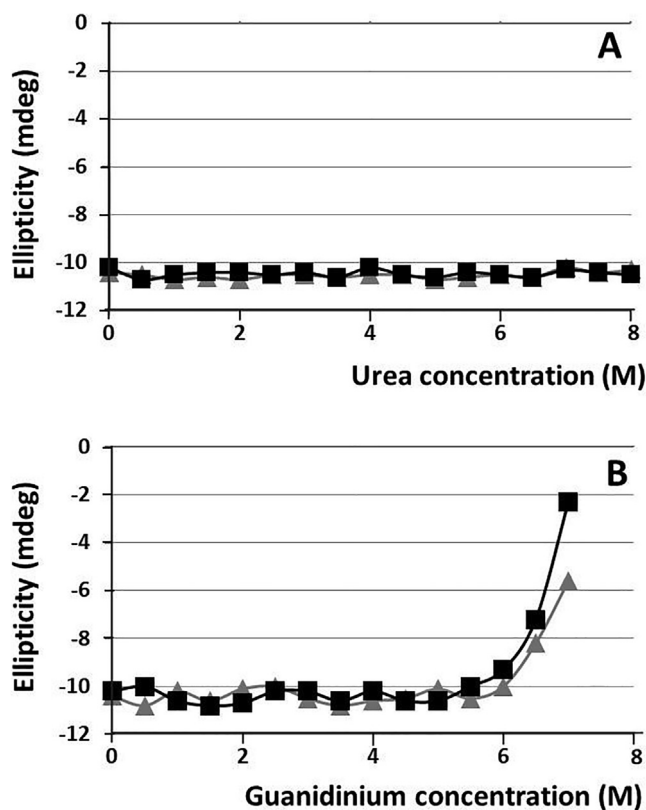


Fig. 4. Ellipticity at 222 nm of 0.1 mg/ml Ara h 6 forms as a function of denaturant concentration (panel A: urea; panel B: guanidinium) in 10 mM boric acid buffer (pH 8.2). Spectra are recorded at 20 °C, where the ellipticity at 222 nm is monitored during 60 sec with a time constant of 4 sec and subsequently averaged. Grey lines and triangles: iAra h 6; Black lines and squared symbols: pAra h 6.

titration with chaotropic agents. Fig. 4 presents the recorded ellipticities in the far-UV regime at 222 nm as a measure for the presence of defined secondary structure elements. In Fig. 4A it can be observed that for both the intact and post-translationally cleaved form of Ara h 6 the presence of up to 8 M urea does not affect the secondary structure of the protein at all. This reflects a remarkable structural stability compared to other globular proteins under such conditions (e.g., the midpoint for unfolding of cow's milk β -lactoglobulin is at 4.2 M (Van Teeffelen, Broersen, & de Jongh, 2005) and of pea vicilins at 5.8 M urea (Munialo, van der Linden, Ako, & de Jongh, 2015)). Titration of the two isoforms with guanidinium (Fig. 4B), known to be a stronger chaotropic agent than urea, illustrates that above 6 M the protein does become structurally destabilized, but complete unfolding is not achieved, not even at the highest concentration guanidinium possible. pAra h 6 seems slightly more unfolded than iAra h 6 above 6 M guanidinium, but a clear indication of a difference in equilibrium stability between the two Ara h 6 forms can not be concluded from these data.

3.3. Conformational stability of Ara h 6 during heating

In an experiment where a sample of Ara h 6 was heated for prolonged incubation times (> hours) exceeding those used in Fig. 1, it was observed by CD that above 50 °C slight and gradual unfolding of the protein could be apparent but with a very slow kinetics (results not shown). This time-dependence was studied in more detail. Fig. 5A shows time-resolved evolution of the ellipticity at 222 nm of a 0.2 mg/ml sample of iAra h 6 heated at different temperatures. It can be observed that the negative ellipticity decreases slowly in time. It follows (in approximation) a single exponential behaviour, reflecting an

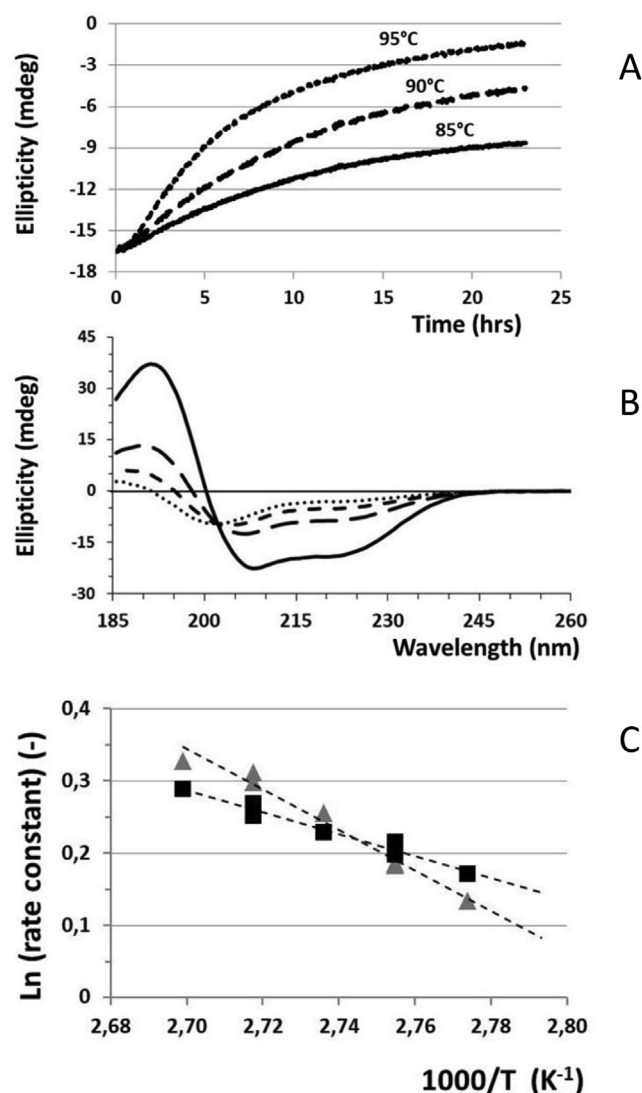


Fig. 5. Panel A shows the time-resolved evaluation of the ellipticity at 222 nm of 0.2 mg/ml iAra h 6 as a function of incubation time at three indicated temperatures 85, 90 and 95 °C. Panel B shows time points (0 (solid), 5 (long dash), 10 (short dash) and 20 (dotted line) hrs) of the trace shown in panel A for the sample incubated at 95 °C. Panel C: Arrhenius plot showing the natural logarithm of the rate constants of the time-resolved profiles of the ellipticity at 222 nm (as exemplified in panel 5A) for intact (grey triangles) and post-translationally cleaved (black squares) Ara h 6 heated for prolonged times at different temperatures, plotted as 1/Temperature. The slope of this plot reflects the E_{act} related to the process according to the Arrhenius equation $k = Ae^{-E_{act}/RT}$. With k as the rate constant, R the gas constant ($= 8.314$ J/mol K), T the temperature (in K) and E_{act} the activation energy (in J) of the process.

unfolding-kinetics on the hour timescale. That this decrease in intensity is related to an ensemble-averaged unfolding of the protein is demonstrated by the three spectra shown in Fig. 5B, where the spectra gradually shift from a more pronounced α -helical spectral shape to that of a more unfolded protein. The fact that an isodichroic point (i.e. a wavelength where the CD-intensity remains unchanged despite an overall spectral change) can be observed at 203.8 nm in Fig. 5B demonstrates that the conformational transition reflects the unfolding of α -helices into unordered structure, as the molar ellipticities for these two secondary structure elements are similar at this wavelength (de Jongh et al., 1994). That this transition is not a result of progressive protein degradation was already demonstrated in Fig. 3 from SDS-PAGE analysis. It can be seen in Fig. 5A that the higher the incubation

temperature, the faster the unfolding takes place. The hours-timescale of these unfolding kinetics explains why the relatively short heating times applied in Fig. 1 (20 min) did not point out to of any detectable unfolding at temperatures up to 90 °C. Moreover, at elevated temperatures the proteins might unfold, but upon cooling they might reversibly refold to their native state.

Analysis of the kinetics profile shown in Fig. 5A using a single exponential decay reveals a rate constant for protein unfolding at a given incubation temperature. These rate constants are presented in Fig. 5C for both the intact and post-translationally cleaved Ara h 6 as a function of $1/T$ in a so-called Arrhenius plot. For both isoforms of Ara h 6 the dependence shows a rather linear behaviour and the slope of the natural logarithm of these rate constants over $1/T$. These slopes provide a measure for the activation energy of the process involved, as described in the figure caption. Interestingly, the slope of iAra h 6 appears to be about twice as steep as that of pAra h 6, providing activation energies of 353 and 171 kJ/mol respectively. This would imply that the process of unfolding requires less energy to overcome to go from a folded to unfolded state of the post-translationally cleaved form of Ara h 6 compared to the intact protein. This activation energy is primarily required during protein unfolding as a desolvation penalty to break ion-pairs on the protein surface to allow spatial swelling of the protein (Rodríguez-Larrea, Ibarra-Molero, & Sanchez-Ruiz, 2006). It has to be noted that the activation energies for folding-unfolding found for pAra h 6 are higher than those reported for lentil lectin (i.e. ~180 kJ/mol; Shnyrov, Marcos, & Villar, 1996), a protein of comparable size, as assembly of heavy and light chains held together by disulphides. A well optimized ion-pairing of charged groups on the protein surface might be the origin of such high activation energy; but this remains to be resolved. In view of the comparable sensitivity of both Ara h 6 forms to chaotropic agents (Fig. 4), based on the onset of unfolding upon increasing chaotropic agent, it can be suggested that the origin of the kinetic difference is related to differences in the polar charged groups at the protein surface rather than the more internal van der Waals interactions (Makhatadze & Privalov, 1993).

It is thus difficult to determine a clear denaturation temperature for these proteins as their unfolding kinetics is that slow that detection of the unfolding is technically not easy to do. It could be suggested that the lower threshold temperature for denaturation may be about 80 °C. Fig. 5C shows that at temperatures above 80–85 °C unfolding will occur and the observation that the dependencies in the Arrhenius-plot follow a linear relation suggest that the mechanism of unfolding remains the same between 85 and 97.5 °C. It needs to be noted that analysis of kinetic data using an Arrhenius equation assumes reversible thermodynamics of the process. However, this will not hold when irreversible aggregation of denatured proteins occurs. Fig. 2 demonstrated, however, that heating at elevated temperatures below the boiling point for 20 min did not result in any detectable formation of protein aggregates.

It can be concluded from these results that although the denaturation process is very slow at heating conditions up to boiling temperatures, there is an inherent difference in the process of unfolding between intact and post-translationally cleaved Ara h 6 reflected in a two times lower activation energy for pAra h 6.

3.4. Impact of conformational dynamics of IgE-binding

At the static conformational level applied heat processing up to 95 °C of Ara h 6 has no significant impact. The noted difference in activation energy between the two isoforms suggests that cleavage of the protein in two separate chains, held together by disulfide bridges, enable the post-translationally cleaved form to fold or unfold more easily compared to the intact form. Whether this kinetic difference, as it may reflect different dynamics of structural epitopes, affects the potency to bind to IgE is evaluated by an IgE-binding ELISA-study using competitive ELISA. Other techniques such as IgE-immunoblotting or direct ELISA were not considered because these techniques lead to

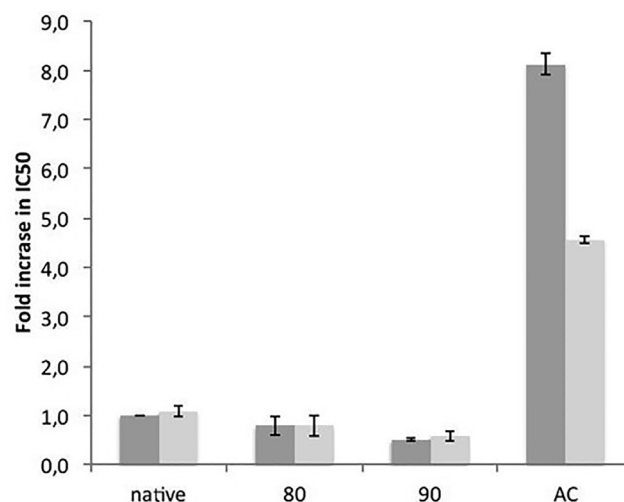


Fig. 6. IgE-binding potency to intact (dark grey) and post-translationally cleaved (light grey) Ara h 6 for non-treated protein, samples heated for 20 min at 80 and 90 °C and those exposed to autoclave conditions (AC).

protein denaturation (by SDS, or due to binding to ELISA plates, respectively), which may prohibit observing denaturing effects of heat treatment. The results are presented in Fig. 6 where the relative fold of increase of protein concentration to achieve 50% binding-inhibition (50% inhibition concentration, or IC₅₀) is presented for untreated protein, and those samples exposed to heating at 80 and 90 °C or to autoclave conditions for intact and post-translationally cleaved Ara h 6. A higher IC₅₀ implies a lower IgE-binding affinity and thereby relates to a reduced allergenic potency. The sample heated at 80 °C is included as it reflects the lower limits for denaturation. For the non-heated and heated (80 or 90 °C) samples no difference between the two isoforms is observed in IgE-binding-potency ($p > 0.05$). In conjunction with the similarity of the protein structures at a secondary (Fig. 1), and quaternary (Fig. 2) folding level, heat-treatment of Ara h 6 does not interfere with binding of IgE. The high structural stability (Fig. 4) and slow dynamics of unfolding at elevated temperature (Fig. 5) does also not differentiate between intact and post-translationally cleaved Ara h 6 for IgE-binding. It could be suggested that moderate heating does marginally increase the ability for IgE to bind, but no explanation at a structural level can be given.

Autoclave conditions, however, do result in a significant reduction of both isoforms in IgE-binding affinity (increase in IC₅₀). For intact Ara h 6 an 8-fold increase in IC₅₀ is observed upon autoclave treatment ($p < 0.05$) and for the post-translationally cleaved form this increase in IC₅₀ was about 4-fold ($p < 0.05$). The difference IC₅₀ between autoclaved intact Ara h 6 and autoclaved post-translationally cleaved Ara h 6 is about 2-fold and statistically significant ($p < 0.05$). This difference is remarkable and could either be the consequence of the higher aggregation state of the intact protein (Fig. 2), or related to the two-fold higher activation energy for the folding-unfolding process. It could be suggested that the post-translationally cleaved form of Ara h 6 does have an easier refolding pathway as reflected by a lower activation energy of the process, thereby regaining more IgE-binding potency upon cooling compared to intact Ara h 6. This difference might be more explicit when the slopes of both forms in the Arrhenius-plot of Fig. 5C are extrapolated to the autoclave temperature of 120 °C ($1000/T \sim 2.54$). However, the secondary structure analysis demonstrated that autoclave conditions lead to an irreversible unfolded state. Apparently, the IgE-binding potency is determined in part by conformational epitopes, explaining why a decreased IgE-binding potency is observed when secondary structure is unfolded, and in part by sequential epitopes, explaining why residual IgE-binding potency remains in this condition. Cleavage and clipping of the small unstructured loop

reduces the association tendency of the protein upon denaturation (Fig. 2) and IgE-binding may thereby be less hindered.

4. Conclusions

Ara h 6 is a renowned peanut allergen, recognized by the vast majority of peanut allergic individuals and more potent than other peanut allergens. Moreover, it is readily abundant in the peanut kernel of all peanut market types commonly consumed in Western countries (Koppelman et al., 2016). Various reports describe the effects of heat treatment on the protein structure in relation to IgE-binding of peanut allergens. In contrast to other food allergens like from milk or egg (Bloom et al., 2014), apple (Okolie, Aryee & Udenigwe, 2018), or hazelnut (Skamstrup Hansen et al., 2003) moderate heat treatment, of peanut allergens under conditions typically applied in industrial food processing does not decrease IgE-binding, and the heat-induced conformational changes are limited (Koppelman, Bruijnzeel-Koomen, Hessing, & de Jongh, 1999). Up to now, the stability of the post-translationally cleaved form of Ara h 6 (pAra h 6) was unknown. In this work, which was in part presented at the annual meeting of the European Allergy and Clinical Immunology meeting in 2018 (de Jong et al., 2018), we showed that pAra h 6 has an IgE-binding comparable to iAra h 6 under non-heated and upon heating up to 90 °C (Fig. 6). pAra h 6 and iAra h 6 are structurally very similar before and after heat-treatment (Figs. 1–3), and their structural stability towards a denaturant is comparable (Fig. 4). The remarkable structural stability is demonstrated by the resilience to unfold in the presence of chaotropic agents as urea (> 8M) or guanidinium (> 6M). Prolonged heating at the hour timescale of the two forms of the protein at elevated temperatures shows a gradual unfolding of the protein, but not a clear denaturation temperature can be identified. This suggests that the structural stabilisation is a kinetically determined step that proceeds on long time scales. From time-resolved unfolding studies at different incubation temperatures and using the Arrhenius equation, it can be concluded that the activation energy for unfolding-refolding is about 2 times lower for pAra h 6 compared to the intact form, suggesting that cleaving and clipping the non-structured loop provides less conformational constraints to rearrange its structure upon denaturation. This work clearly demonstrates that under conditions (time and temperature) that are typically applied in food processing, like pasteurization, will not affect the structural integrity of intact or post-translationally cleaved Ara h 6. Even when the temperature would be above the denaturation temperature (estimated to be here around 80–85 °C; Fig. 5C), the kinetics of unfolding is that slow that no significant population of unfolded proteins will develop. Moreover, the unfolding might still be reversible upon cooling.

Only autoclave conditions differentiate the two forms of Ara h 6. Under these conditions their susceptibility to irreversible denaturation at the structural level was shown (Figs. 1–3), resulting in a decrease in IgE-binding potency, for iAra h 6 more pronounced than for pAra h 6. This is likely related to the more pronounced aggregation state of the intact protein upon exposure to autoclave conditions, where cleavage and elimination of the short unstructured loop may reduce this association behaviour. It could be speculated that typical roasting conditions of peanuts (15 min at 180 °C in a dry state) may therefore also lead to a significant unfolding of the protein. Projection from the Arrhenius plot to such high temperature, when allowed, would already yield a significant population of denatured proteins even after 15 min. The validity of such projection may be argued, but the differentiation in susceptibility to processing under such conditions between intact and post-translationally cleaved Ara h 6 needs to be considered when allergic responses to peanut protein is considered in processed foods.

In short, as illustrated with the example of an important peanut allergen, we conclude that a processing temperature (above the protein's denaturation temperature) is not that relevant in cases where the unfolding kinetics is the conformational determinant that will set the

extent of follow-up actions that may impair with IgE-binding.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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