

High-pressure microfluidisation-induced changes in the antigenicity and conformation of allergen Ara h 2 purified from Chinese peanut

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Abstract

BACKGROUND: Peanut allergy is one of the most serious food allergies, and Ara h 2 is one of the most important peanut allergens as it is recognised by serum immunoglobulin E from more than 90% of peanut-allergic individuals. Dynamic high-pressure microfluidisation has been widely used in food processing as a new technology. The aim of this study was to investigate the effect of high-pressure microfluidisation on the antigenicity and structure of Ara h 2. Extracted peanut allergen Ara h 2 was treated under a continuous pressure array of 60, 90, 120, 150 and 180 MPa. Immunoreactivity was measured by indirect enzyme-linked immunosorbent assay with rabbit polyclonal antibodies. Secondary structure was analysed by circular dichroism. Surface hydrophobicity and sulfhydryl groups were assessed via fluorescence and UV absorption spectra respectively.

RESULTS: High-pressure microfluidisation treatment decreased the antigenicity of peanut allergen Ara h 2, changed its secondary structure and increased its UV absorption intensity and surface hydrophobicity.

CONCLUSION: The change in conformation contributed to the decrease in antigenicity of Ara h 2, and the spatial conformation of peanut allergen Ara h 2 plays a critical role in its antigenicity.

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Keywords: Ara h 2; high-pressure microfluidisation; antigenicity; conformation; peanut allergy

INTRODUCTION

Peanut allergy is one of the most serious food allergies because it is severe, common and typically permanent.¹ Surveys have shown that peanut allergy appears to be increasing both in some western countries and in China.^{2–4} Thus peanut allergy has become a public health issue and is receiving increased attention.

So far, 11 peanut allergens have been identified and named by the Allergen Nomenclature Sub-committee.⁵ Among these allergens, Ara h 1 and Ara h 2 are important as they are recognised by serum immunoglobulin E (IgE) from more than 90% of peanut-allergic individuals.^{6,7} Moreover, Ara h 2 is a more potent allergen than Ara h 1, which has been confirmed by basophil histamine release and percutaneous skin tests with patients.⁸ Ara h 2 is a glycoprotein of about 17.5 kDa⁷ that belongs to the 2S albumin family with eight conserved cysteine residues.⁹

Since foods/food ingredients are often subjected to a variety of processing conditions, the allergenic properties of proteins could be altered by hiding, destroying or disclosing allergenic epitopes through conformational changes or by changing the digestibility of proteins during the process.¹⁰ In the case of Ara h 2 the IgE-binding capacity was altered by heat treatment and in particular was increased by roasting, which is likely due to intramolecular crosslinking or enhanced trypsin-inhibitory activity.¹¹

Dynamic high-pressure microfluidisation is a new technology that is combined with transportation, mixing, ultrarefining, homogenisation, emulsification and many other unit operations and was developed on the basis of the microjet homogeniser and ultrahigh-pressure homogeniser. Forces of strong shear, high-speed bump, instantaneous pressure release and cavitation are all integrated into the sample during the process, and the pressure can be as high as 200 MPa.¹² Thus it differs from high hydrostatic pressure.¹³ Mechanical forces produced in high-pressure homogenisation treatments may affect the tertiary or

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quaternary structure of a protein, which can be used to modify its properties. Studies have shown that the functionality of whey products is improved after high-pressure microfluidisation treatment¹⁴ and that the relative activity of polyphenol oxidase can also be enhanced by this treatment.¹⁵ The effects of processing on the antigenicity of proteins are commonly reported, but no study has yet investigated the effect of high-pressure microfluidisation on protein antigenicity.

The aims of the present study were (i) to investigate the effect of high-pressure microfluidisation on the antigenicity of Ara h 2 and (ii) to analyse the mechanism of the changes in its immunoreactivity by examining the changes in its structure. The results of this study may be helpful for producing hypoallergenic or non-allergenic peanut products.

MATERIALS AND METHODS

Materials and reagents

DEAE-Sepharose Fast Flow Resin (16 mm × 250 mm) was purchased from Amersham Biosciences (Freiburg, Germany). Complete Freund's adjuvant, incomplete Freund's adjuvant, peroxidase-conjugated anti-IgG, Tween 20, *o*-phenylene diamine, 8-aniline-1-naphthalenesulfonate (ANS), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and the sources of electrophoresis chemicals were all obtained from Sigma (St Louis, MO, USA). Prestained Protein Ladder was purchased from MBI Fermentas (Burlington, Canada). Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Billerica, MA, USA). Microplates were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents were of analytical grade and were obtained from China National Medicine Co. Ltd (Beijing, China).

Purification of peanut allergen Ara h 2

Xianghua is a main variety of Chinese peanut cultivated for its high yield and plump fruit. Unshelled Xianghua peanuts were powdered manually using a mortar and pestle with liquid nitrogen and then defatted by stirring in acetone at a powder/acetone ratio of 1:5 (w/v) for 2 h at 4 °C. The supernatant was removed by centrifuging three times at 9000 × *g* for 15 min at 4 °C to obtain the defatted powder. Peanut protein extract was obtained by mixing the defatted powder with 50 mmol L⁻¹ Tris-HCl (pH 7.2) at a powder/solvent ratio of 1:5 (w/v). After 10 h of stirring at 4 °C the supernatant was collected by centrifugation at 9000 × *g* for 15 min at 4 °C and ionic salts were removed by subsequent dialysis with the same buffer for 48 h at 4 °C. The resulting crude extract was stored at -20 °C until use.

Purification of Ara h 2 was performed using anion exchange chromatography. The crude extract was loaded (flow rate 1.5 mL min⁻¹) onto DEAE-Sepharose Fast Flow Resin equilibrated with 50 mmol L⁻¹ Tris-HCl (pH 7.2). Then the column was washed with the same buffer until the peak appeared, proteins were eluted successively with 500 mL of 0–0.2 mmol L⁻¹ NaCl gradient in 50 mmol L⁻¹ Tris-HCl and the fractions were collected for further analysis.

The samples were resuspended in reducing sample buffer (20 mmol L⁻¹ Tris, pH 6.7, 2 mmol L⁻¹ ethylene diamine tetraacetic acid, 20 g L⁻¹ sodium dodecyl sulfate (SDS), 250 mL L⁻¹ glycerol, 30 mL L⁻¹ β-mercaptoethanol, 0.2 g L⁻¹ bromophenol blue) and heated for 5 min in a boiling water bath. Then 50 g L⁻¹ stacking gels and homogeneous 150 g L⁻¹ separating gels were used for SDS polyacrylamide gel electrophoresis (SDS-PAGE)

analysis.¹⁶ Proteins were visualised by staining with Coomassie Brilliant Blue R250. Further confirmation of the proteins was obtained by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) fragmentation analysis of selected tryptic peptides with protein-specific sequences (Applied Biosystems, Framingham, MA, USA). Protein concentrations were measured using the method of Lowry *et al.*¹⁷ with bovine serum albumin as standard.

Preparation of antiserum against Ara h 2

Polyclonal antibodies were obtained in adult New Zealand rabbits by first injecting them with 200 µg of purified Ara h 2 emulsified in 1 mL of complete Freund's adjuvant. After a 2 week interval a booster injection was given with the same amount of purified Ara h 2 in incomplete Freund's adjuvant. Serum samples were collected 1 week after the last injection, tested by indirect enzyme-linked immunosorbent assay (ELISA) and stored at -80 °C¹⁸ until use.

Western blotting

For immunoblot analysis, separated proteins were subsequently electrotransferred onto a PVDF membrane. After blocking with 50 mL L⁻¹ skimmed milk in Tris buffered saline (TBS) (50 mmol L⁻¹ Tris-HCl, 150 mmol L⁻¹ NaCl, pH 7.5) for 1 h at 37 °C, the membrane was incubated for 1 h at 37 °C with rabbit anti-Ara h 2 serum (diluted 1:10 000) as primary antibody, washed in TBS containing 0.5 mL L⁻¹ Tween 20 (TBST) and then incubated with peroxidase-conjugated anti-mouse IgG (diluted 1:5000) as secondary antibody for 1 h at 37 °C. Peroxidase-positive bands were detected by immersing the membrane in 20 mL of TBS containing 12 mg of 4-chloro-1-naphthol, 4 mL of ethanol and 12 µL of 300 mL L⁻¹ H₂O₂ for 5 min at 37 °C.¹⁹

High-pressure microfluidisation treatment

A 0.4 mg mL⁻¹ solution of protein in 50 mmol L⁻¹ Tris-HCl buffer (pH 7.2) was added to the inlet reservoir of an M-110EH-30 microfluidiser (Microfluidics, Newton, MA, USA) and treated under a continuous pressure array of 60, 90, 120, 150 and 180 MPa. A 10 mL sample was removed after passing each pressure; this was repeated three times. The treated solutions passing through the pipe were cooled by icy water and collected for analysis.

Indirect ELISA

Antigenicity was estimated by means of indirect ELISA. The individual wells of a 96-well microtitre plate were coated with 100 µL of either treated or native Ara h 2 sample (1 µg mL⁻¹) in coating buffer (0.015 mol L⁻¹ Na₂CO₃, 0.035 mol L⁻¹ NaHCO₃, pH 9.6), left overnight at 4 °C and subsequently blocked with 50 mL L⁻¹ skimmed milk in phosphate-buffered saline (PBS) for 1 h at 37 °C. Then rabbit antiserum (diluted to 1:10 000 in PBS) was added to each well. After incubation for 1 h at 37 °C, 100 µL of goat anti-rabbit IgG conjugated with horseradish peroxidase (diluted to 1:5 000 in PBS) was added to each well followed by incubation for 1 h at 37 °C to detect the bound immunogen. Between each step the wells were washed three times with PBS containing 0.5 mL L⁻¹ Tween 20. Colour was developed by adding 100 µL of substrate solution (0.5 mg mL⁻¹ *o*-phenylene diamine, 0.3 mL L⁻¹ H₂O₂ in 0.1 mol L⁻¹ citrate buffer, pH 5.5) to each well and incubating for 15 min at 37 °C. The reaction was terminated by adding 50 µL of 2 mol L⁻¹ H₂SO₄. The absorbance at 490 nm was read using a Bio-Rad 1860 microplate reader (Bio-Rad, CA, USA).

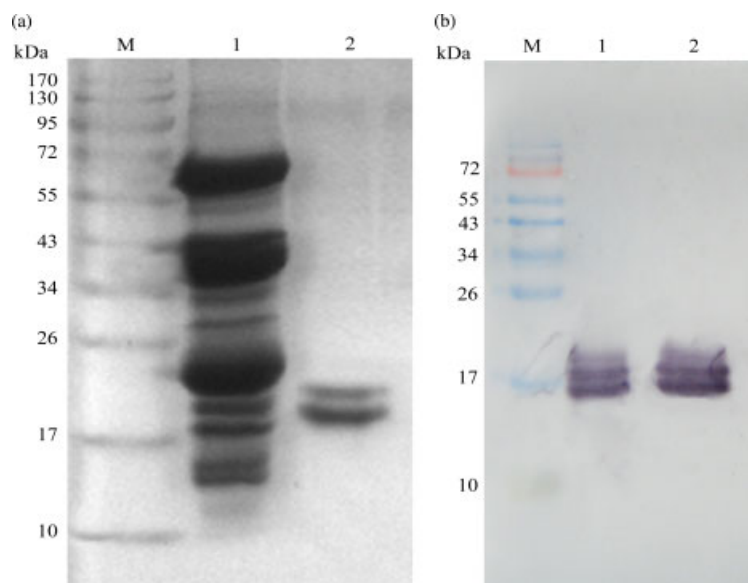


Figure 1. (a) SDS-PAGE analysis of Ara h 2 purified from peanut by anion exchange chromatography and (b) Western blotting performed on crude peanut extract and purified Ara h 2 with serum from rabbit. Lanes: M, molecular weight markers; 1, crude peanut extract; 2, Ara h 2.

Circular dichroism analysis

To define the secondary structure of native and high-pressure microfluidisation-treated Ara h 2, circular dichroism (CD) spectroscopy was performed with a JASCO J810 spectropolarimeter (JASCO, Groß-Umstadt, Germany) on 0.2 mg mL^{-1} protein solutions. CD spectra were scanned in the far-UV range from 250 to 190 nm at a rate of 100 nm min^{-1} with a bandwidth of 1 nm at room temperature. The obtained spectra represented an average of three consecutive scans. The results for each sample were averaged and the contents of different secondary structures were calculated by JASCO secondary structure software.

Determination of protein surface hydrophobicity

Surface hydrophobicity was determined using the hydrophobic fluorescence probe ANS. Measurements were performed according to the method of Takagi *et al.*²⁰ A $10 \mu\text{L}$ aliquot of 8 mmol L^{-1} ANS solution was added to 4 mL of each protein sample solution. The relative fluorescence was measured with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The excitation wavelength was 390 nm and the emission wavelength was from 400 to 650 nm at a scanning speed of 10 nm min^{-1} .

UV absorption spectral analysis

UV absorption spectral analysis was performed using a UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The UV absorption spectra were scanned from 200 to 420 nm.

Determination of sulfhydryl groups

Sulfhydryl group contents on the surface of native and treated Ara h 2 were determined by the method of Ellman.²¹ A 1 mL aliquot of Ara h 2 solution was added to 4 mL of 0.1 mol L^{-1} phosphate buffer (pH 8) containing 0.05 mL of 0.01 mol L^{-1} DTNB solution and incubated for 20 min at room temperature. The absorbance of the mixture at 412 nm was measured. The values of sulfhydryl group content were obtained by dividing the values of absorbance by the molar extinction coefficient of 13,600.

Statistical analysis

Data were analysed for statistical significance using ANOVA procedures (SPSS for Windows 2003 Version 13.0, Microsoft Corp., Chicago, IL, USA) and Fisher's least significant difference (LSD, $P < 0.05$) test.

RESULTS

Purification of natural Ara h 2

The crude peanut extract was fractionated into seven major peaks using anion exchange chromatography (data not shown). The eluted fractions were analysed by SDS-PAGE (Fig. 1(a)), and fraction 3 contained double bands with molecular weights ranging from 17 to 20 kDa, which corresponds to the previously reported Ara h 2.⁸ To further identify the proteins, the proteins of two bands were digested separately with trypsin and the resulting peptides were analysed by MALDI-TOF-MS. The obtained data were matched in the NCBI database with MASOT Version 2.1 (Matrix Science, London, UK). The two proteins show 100% identity with NCBI accession numbers GI:31 322 017 and GI:158 344 597 respectively.

Specificity of anti-Ara h 2 antibodies

An immunoblotting experiment was conducted to identify the specificity of polyclonal rabbit antibodies against Ara h 2 using peanut protein extract and purified Ara h 2 as the antigen respectively. Two dominant bands corresponding to Ara h 2 proteins were detected in both peanut extract and Ara h 2 purified from peanut (Fig. 1(b)). The result suggests that the antiserum against Ara h 2 derived from rabbits had high specificity.

Comparison of antigenicity

Figure 2 shows that the level of IgG binding to Ara h 2 was decreased after any pressure treatment compared with the untreated sample. A significant decrease in Ara h 2 immunoreactivity was induced at a pressure of 60 MPa, while only a slight drop occurred from 60 to 90 MPa. Another significant decrease in immunoreactivity was observed when the pressure increased from 90 to 120 MPa.

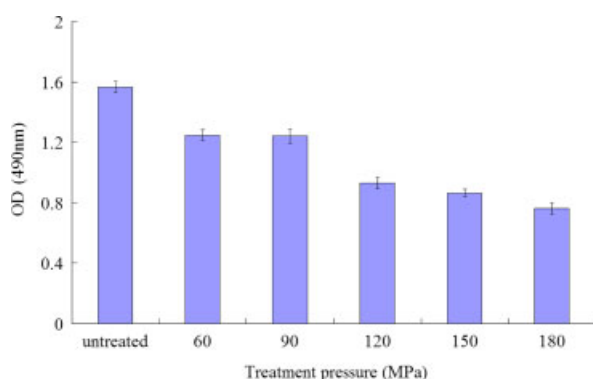


Figure 2. Effect of high-pressure microfluidisation on antigenicity of Ara h 2 defined by ELISA measurement.

Effect of high-pressure microfluidisation on CD spectra

The effect of high-pressure microfluidisation on the secondary structure of Ara h 2 was assessed by far-UV CD spectroscopy (Fig. 3). The two negative peaks at 208 and 222 nm are characteristic of the α -helical configuration, while the positive peak at 214 nm is characteristic of the β -sheet configuration.²² The estimated secondary structure of native Ara h 2 using spectral analysis revealed 28.8% α -helices, 33.9% β -turns and 37.4% random coils, which is in agreement with a previous analysis showing that Ara h 2 consists mainly of α -helices (30–60%) and coils/loops (30–60%) with few or no β -sheets (0–16%).²³ After high-pressure microfluidisation treatment, there are obvious differences between the spectra of native and treated Ara h 2. The increase in ellipticity in the region 190–200 nm reflects a decrease in α -helices in favour of β -sheet formation. Figure 4 shows the changes in percentage content of α -helices, β -sheets and β -turns of Ara h 2 after high-pressure microfluidisation treatment, which indicate a loss of α -helix content with a concomitant increase in β -sheet content. For example, in the case of Ara h 2 treated at 90 MPa, the content of α -helices dropped from 28.8 to 22.8%, accompanied by an increase in the content of β -sheets from 0 to 18.6%.

Effect of high-pressure microfluidisation on hydrophobicity

Hydrophobic interactions play an important role in the stabilisation of the tertiary and quaternary structure of proteins, and ANS has been widely used as a fluorescent probe to measure protein surface hydrophobicity. Since ANS shows a low quantum yield of

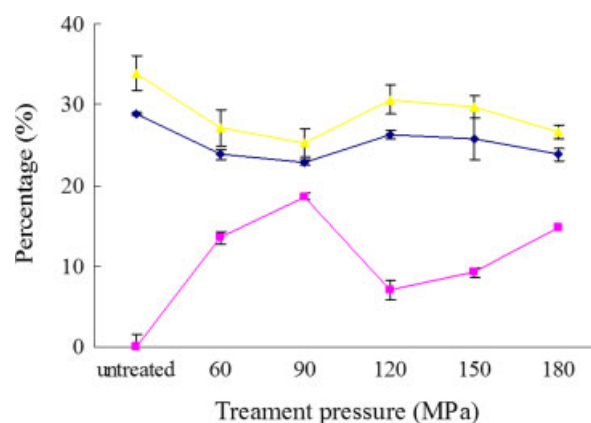


Figure 4. Effect of high-pressure microfluidisation on α -helix ($\blacklozenge$), β -sheet ($\blacksquare$) and β -turn ($\blacktriangle$) percentages.

fluorescence in aqueous solution, a large increase in fluorescence is observed when it binds to the accessible hydrophobic regions of proteins.²³ Fig. 5 shows the ANS-induced fluorescence spectra of Ara h 2 samples treated under different pressures. It can be seen that native Ara h 2 exhibited a low fluorescence intensity, while 60 MPa treatment increased the fluorescence intensity greatly. The intensity of fluorescence continued to increase with increasing pressure up to a maximum value at 150 MPa, then decreased slightly at 180 MPa.

Effect of high-pressure microfluidisation on UV absorption spectra

UV absorption spectra depend mainly on the UV absorption of tryptophan and tyrosine residues located in side-chain groups, and to some extent on that of phenylalanine, histidine and cysteine. The characteristic absorption peak of tryptophan is located at about 280 nm. The effects of high-pressure microfluidisation treatment on the UV absorption spectra of Ara h 2 are presented in Fig. 6. It can be seen that significant changes in UV absorption occurred at 250–280 nm. After 60 MPa treatment the absorption intensity increased. The intensity of absorption continued to increase with increasing pressure up to a maximum value at 180 MPa. The changes in UV absorption indicate changes in the structure of Ara h 2.

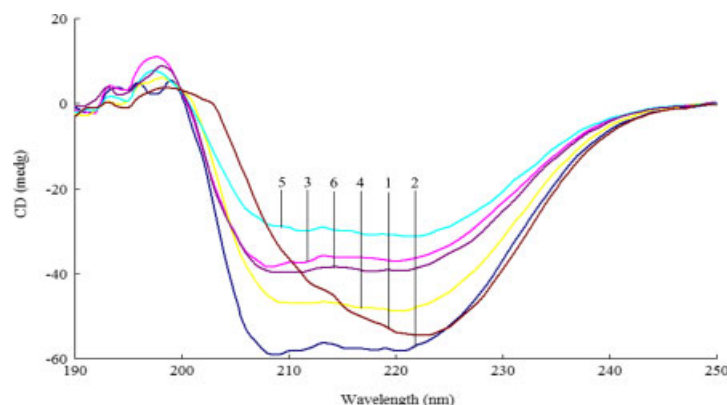


Figure 3. Far-UV CD spectra of untreated and high-pressure microfluidisation-treated Ara h 2. Curves 1–6 represent samples treated under a continuous pressure array of 0, 60, 90, 120, 150 and 180 MPa respectively.

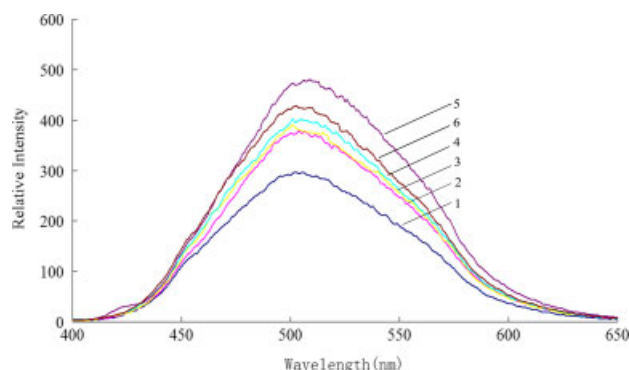


Figure 5. Fluorescence emission spectra of untreated and high-pressure microfluidisation-treated Ara h 2. Curves 1–6 represent samples treated under a continuous pressure array of 0, 60, 90, 120, 150 and 180 MPa respectively.

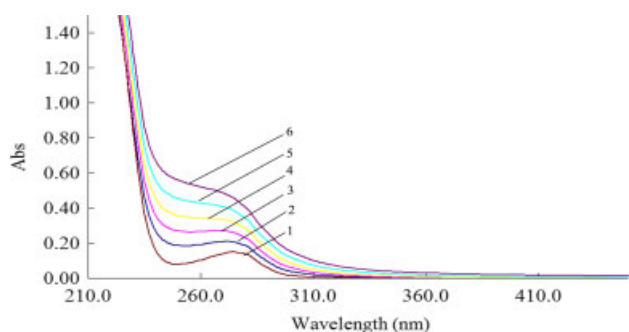


Figure 6. UV absorption spectra of untreated and high-pressure microfluidisation-treated Ara h 2. Curves 1–6 represent samples treated under a continuous pressure array of 0, 60, 90, 120, 150 and 180 MPa respectively.

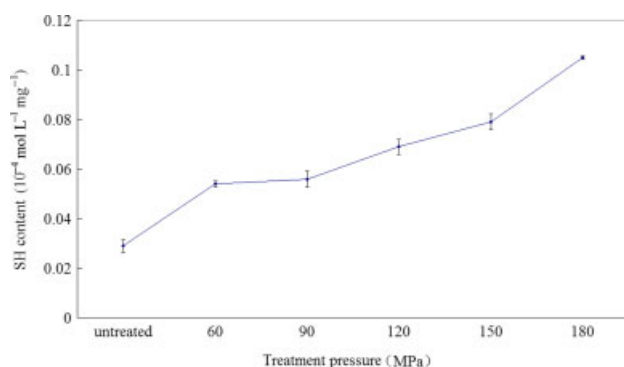


Figure 7. Sulfhydryl group (SH) content on surface of Ara h 2 under high-pressure microfluidisation treatment.

Effect of high-pressure microfluidisation on sulfhydryl groups

Figure 7 shows the changes in sulfhydryl group (SH) content of Ara h 2 resulting from high-pressure microfluidisation treatment. With increasing pressure the SH content continued to increase, showing a maximum value at 180 MPa. Native Ara h 2 contains eight cysteine residues that could form up to four disulfide bonds.²⁴ The rupture of S–S bonds might contribute to the formation of new free SH. The results suggest that high-pressure microfluidisation could modify the conformation of Ara h 2.

DISCUSSION

The effects of high-pressure microfluidisation treatment on the conformation and immunoreactivity of Ara h 2 are presented in this study. The UV absorption intensity did not change at 225 nm, which is the characteristic absorption peak of peptide bonds, indicating that peptide bonds were not affected by high pressure. Thus high-pressure microfluidisation treatment did not affect the primary structure of Ara h 2. However, high pressure changed the tertiary and quaternary structure of Ara h 2, contributing to the increase in hydrophobicity and UV absorption intensity. The increased intensity of fluorescence indicated that the protein was unfolded and more hydrophobic regions were exposed.²⁵ Further increase in pressure (180 MPa) led to a slight decrease in fluorescence intensity, suggesting that the exposed hydrophobic groups may reassociate or aggregate to form a more stable structure. This corroborates a previous report that exposure of hydrophobic surfaces is favoured at relatively low pressures,²⁶ and it was found that Ara h 2 was partially unfolded by high-pressure microfluidisation. This is consistent with the results of analysis of the UV absorption spectra. The increase in UV absorption intensity can likely be explained by the amino acid residues being partially exposed to a less hydrophobic environment by high-pressure processing.²⁷

Typically, the increase in surface hydrophobicity and UV absorption intensity induced by high pressure is not unique to this protein. Similar observations have been made for other proteins such as glycinin²⁸ and ovalbumin.²⁹ It was reported that these were all globular proteins with more compact structures and containing intramolecular disulfide bonds.^{7,30,31} Our observations further confirmed that the changes in structure due to high pressure are related to the amino acid composition and structural configuration of the native protein.

After high-pressure microfluidisation treatment, some α -helices were destroyed and converted to β -sheets, indicating that the secondary structure of Ara h 2 changed. Interestingly, we found that the reduction rates of antigenicity matched well with the changes in secondary structure (Fig. 4). For example, after 60 MPa treatment the secondary structure of Ara h 2 changed significantly, with the percentages of α -helices and β -turns decreasing greatly. At the same time the percentage of β -sheets increased considerably, which corresponded with a significant decrease in antigenicity. The same was true of the results for the sample treated at 120 MPa. The content of α -helices decreased with the reduction in antigenicity, indicating that IgG-binding epitopes are probably localised within the α -helical structure of Ara h 2.

The disulfide bonds of Ara h 2 play an important role in defining its secondary and tertiary structure.³² High-pressure microfluidisation treatment may convert the disulfide bonds into sulfhydryl groups, thus increasing the sulfhydryl group content and decreasing the antigenicity. Therefore we assumed that the disulfide bonds play an important role in maintaining allergen antigenicity. Our study carried out with reduced Ara h 2 indicated that the denatured Ara h 2 shows reduced allergenicity when the disulfide bonds are reduced, because the fragments containing IgE-binding epitopes are resistant to enzymatic digestion, but they become susceptible to rapid digestion when the disulfide bonds are destroyed, resulting in a reduction in the overall allergenicity.³² Thus for Ara h 2 the disulfide bonds were found to be important for its antigenicity. Moreover, there is some evidence that disulfide bonds are important for retaining the conformation of IgG- or IgE-binding epitopes.^{33,34} Therefore we

can hypothesise that high-pressure microfluidisation treatment may reduce the allergenicity of peanut allergen Ara h 2 by changing its conformation. Actually, the same result was found for β -lactoglobulin.^{35,36}

In conclusion, high-pressure microfluidisation treatment led to the denaturation of Ara h 2, as shown by the results of this study. The intermolecular and intramolecular disulfide bonds that originally stabilised the native protein structure were disrupted during protein denaturation. These structural changes in peanut allergen Ara h 2 decreased its antigenicity by either destroying or burying the conformational IgG-binding epitopes. Furthermore, the antigenicity of Ara h 2 is related to its conformation. Further tests on the effect of high-pressure microfluidisation treatment on the allergenicity of Ara h 2 in the future seem worthwhile.

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