



Effect of High Hydrostatic Pressure (HHP) Processing on Immunoreactivity and Spatial Structure of Peanut Major Allergen Ara h 1

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Abstract

Ara h 1 is recognized as a major peanut allergen. The effects of high hydrostatic pressure (HHP) on the immunoreactivity and structure of Ara h 1 were investigated in this study. The immunoreactivity of Ara h 1 was considerably reduced ($P < 0.01$) after HHP treatment (≥ 400 MPa) with the increase of pressure holding time. The maximum reduction of 74.32% was achieved at 600 MPa for 1200 s. The particle size and molecular weight of Ara h 1 increased, and the secondary/tertiary structure changed markedly. The decrease of the immunoreactivity of Ara h 1 was mainly due to the changes in conformation (especially the tertiary structure) and the formation of new multimers, resulting in the inactivation of immunoreactive sites. Results indicate that HHP technology has the potential to be applied to the preparation of hypoallergenic peanut products.

Keywords Ara h 1 · High hydrostatic pressure (HHP) · Structure · Immunoreactivity · Multimer

Introduction

Peanuts are an excellent source of plant protein that is beneficial for the human diet. They are also recognized as one of “big eight” allergens. In several European countries and in North America, peanuts are the most common cause of fatal food-related anaphylaxis (Sicherer and Sampson 2018; Sicherer and Sampson 2007). According to the Allergen Nomenclature Sub-committee, 17 peanut allergens have been identified and named so far. Ara h 1 is a major peanut allergen that makes up 12–16% of the total peanut protein and induces more than 90% peanut-sensitive individual reactions (Koppelman et al. 2001; Burks and Fuchs 1995). Natural Ara h 1 is a stable trimer (180 kDa or above) (Shin et al. 1998) that results from the large hydrophobic

interaction among monomers (63.5 kDa) (Maleki et al. 2000); it features good thermal stability (Koppelman et al. 1999), and it is not easily digested by enzymes (Maleki et al. 2000). At present, doctors can treat the symptoms of allergies, but no cure is available, and even the smallest amount of allergens can cause an allergic response; for food allergies, the only effective treatment is to avoid allergens (Wu et al. 2017). Hence, various processing methods, such as heat, radiation, ultrasound, pulsed ultraviolet light, enzymatic hydrolysis or crosslinking, and so on (Yang et al. 2011; Rahaman et al. 2016), were expected to eliminate immunoreactivity of proteins, and they would either destroy other nutrients in the product or have little effect on reducing the immunoreactivity of some proteins. For Ara h 1, the processing methods for reducing its immunoreactivity have been rarely reported (Blanc et al. 2011).

High hydrostatic pressure (HHP) is a burgeoning nonthermal food processing technology, which has been widely concerned for its good performance in modification of food macromolecules in recent years (Sun-Waterhouse et al. 2014). The HHP refers to the material sealed in a flexible container that is placed in a pressure vessel (which is usually filled with water or other fluids as the pressure-transmitting medium) to be treated for some time under static high pressure (> 100 MPa) to change its properties (Huang et al. 2014a, b). HHP is safer

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and healthier than other food processing methods as it does not damage the small molecule nutrients in food (San Martín et al. 2002). It is also an energy-saving technology because it does not require additional energy once the target pressure is reached (Estrada-Girón et al. 2005). The immunoreactivity of proteins is inevitably related to their structure, and experts have theorized that HHP can change immunoreactivity by altering the structure of food allergens, especially their secondary and tertiary structures (Jiménez-Saiz et al. 2015). Dhakal et al. (2014) discovered that the conformational and linear immunoreactivity epitopes of almond milk decrease at 450–600 MPa for up to 600 s. Meng et al. (2017) explained that the immunoreactivity of bovine β -lactoglobulin drops after HHP treatment (200 MPa) and that its tertiary structure is highly correlated with sensitization. HHP treatment results in varying degrees of changes in the allergenic proteins of apple (Johnson et al. 2010), egg (Hildebrandt et al. 2008), almond (Li et al. 2013), and others; different proteins show different levels of changes in their structures. HHP may reduce the immunoreactivity of food allergens by protein denaturation, induction of protein conformational change, extraction of allergens, and facilitation of enzymatic hydrolysis (San Martín et al. 2002; Estrada-Girón et al. 2005). Therefore, HHP shows immense potential in the manufacture of low allergenic and even desensitized products. However, the relationship between the structure and the immunoreactivity of proteins after HHP treatment has not been clearly described. Are immunoreactive sites destroyed or buried inside the proteins after HHP treatment? The illustration of reduced immunoreactivity in this work can guide the production of desensitized products.

To obtain hypoallergenic or desensitized Ara h 1, this work aims to alter its structure by HHP technology and further clarify the correlation between its structure and immunoreactivity. Sandwich enzyme-linked immunosorbent assay (ELISA) was used to assess the immunoreactivity of Ara h 1. Sodium dodecyl sulfate (SDS)-PAGE, Native PAGE, fast protein liquid chromatography (FPLC), and particle size distribution (PSD) were employed to detect the molecular weight and particle size of Ara h 1. The tertiary structure was characterized by UV absorption spectra, exposed free sulfhydryl (–SH) content, and fluorescence emission spectra. The secondary structure was analyzed through far-UV circular dichroism (CD) and spectroscopy.

Materials and Methods

Materials

Raw peanut seeds (Luhua 9) were purchased from the market (Xiamen, Fujian, China). Defective seeds were removed from the batch, and the good ones were stored at -20°C until use. 8-Anilino-1-naphthalenesulfonic acid (ANS) and 5,5-dithiobis[2-nitrobenzoic acid] (DTNB) were purchased from

Sigma-Aldrich Pty Ltd. (St Louis, MO, USA). NaCl, Tris(hydroxymethyl)aminomethane, SDS, and other chemicals were from Sinopharm (Shanghai, China). All chemical reagents used in our work were of analytical grade.

Extraction and Purification of Ara h 1

Peeled peanut seeds (without red tegument) were ground into powder with liquid nitrogen and then defatted by stirring in acetone at a powder/acetone ratio of 1:10 (w/v) for 2 h at 4°C . The supernatant (fat and acetone) was removed by centrifugation ($10,000\times g$ at 4°C for 30 min). Defatting was repeated twice, and the precipitate was placed in a fume hood to be dried overnight to obtain defatted peanut powder. Acetone in this method is only used in the laboratory to purify proteins for research and not for food industry production. The crude sample of peanut protein was extracted as follows: 5 g of defatted peanut powder was mixed with 50 ml of 50 mM Tris-HCl (pH 8.0) by stirring for 12 h at 4°C , and then the supernatant was collected by centrifugation ($16,000\times g$ at 4°C for 30 min) and stored at -20°C until use.

The crude extract was first subjected to ammonium sulfate precipitation with 70% saturation. The supernatant was collected by centrifugation ($16,000\times g$ at 4°C for 10 min) and then added with ammonium sulfate up to 100% saturation. After centrifugation ($16,000\times g$ at 4°C for 10 min), the protein precipitation was solubilized in the buffer solution (50 mM Tris-HCl, pH 8.0) and then dialyzed against the buffer solution (50 mM Tris-HCl, pH 8.0) overnight for further purification. The solubilized proteins were applied to DEAE Sepharose Fast Flow resin equilibrated with gradient elution at a flow rate of 1.0 ml/min, and the concentration of NaCl was increased from 0 to 0.4 M in Tris-HCl buffer (50 mM, pH 8.0) (Wu et al. 2015). The collected eluate peak was analyzed by SDS-PAGE. The protein was identified by LC-MS (Eksigent HPLC TripleTOF™ 5600+; AB Sciex, Canada). The obtained data were 100% matched with the peptides of Ara h 1 in the protein database (www.uniprot.org). Protein concentration was determined using a modified Bradford Protein Assay Kit (Sangon Biotech Co., Ltd., Shanghai, China). The purified protein was dialyzed against the buffer solution (50 mM Tris-HCl, pH 8.0) overnight and stored at -20°C until use.

HHP Treatments

High-pressure equipment (model HPP 600 MPa/5 l; maximum pressure, 600 MPa; KeFa Hi-Tech Food Machine Co., Ltd., Baotou, China) was used for the HHP treatments, and water served as the pressure-transmitting medium. Protein samples (0.2 mg/ml) were vacuum-packed in polyethylene bags, which were treated with different pressure levels (200 MPa, 400 MPa, and 600 MPa) for 150 s, 300 s, 600 s, and 1200 s. The pressurization rate was 7 MPa/s, the

decompression rate was instantaneous (within 3 s), and the temperature was kept at $20\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ during the pressure processing. After HHP treatment, the samples were moved to a marked centrifuge tube and stored at $-20\text{ }^{\circ}\text{C}$ for further analysis. The untreated sample was used as the experimental control.

Immunoreactivity Analysis

Immunoreactivity was determined using sandwich ELISA kits (Shanghai Du Ma Biological Technology Co., Ltd., China). The operation was carried out by following the kit instructions with slight modifications described by Montserrat et al. (2013). Briefly, the anti-Ara h 1 antibody-immobilized plate was warmed to room temperature in the original aluminum pouch, and testing sample (10 μl) then sample diluent (0.5% bovine serum albumin) (40 μl) were added to testing sample wells. Afterward, 100 μl of HRP-labeled anti-Ara h 1 antibodies was added to each well, covered with an adhesive strip, and incubated for 60 min at $37\text{ }^{\circ}\text{C}$; then, the liquid was discarded and pat dry on the absorbent paper. After the plates were washed five times with PBST; 50 μl chromogen solution A (1.175% urea hydrogen peroxide) and 50 μl chromogen solution B (3,3',5,5'-tetramethylbenzidine (TMB)) were added to each well, gently mixed and incubated for 15 min at $37\text{ }^{\circ}\text{C}$, and protected from light. Finally, 50 μl Stop Solution (0.5% H_2SO_4) was added to each well and the optical density (OD) was read at 450 nm using a microtiter plate reader within 15 min. Each sample was analyzed in triplicate. The results were calculated by the following equation:

$$\text{Immunoreactivity (\%)} = \frac{\text{OD}_{450} \text{ of sample}}{\text{OD}_{450} \text{ of control}} \times 100\%$$

where OD_{450} is the absorbance value of the sample at 450 nm.

Analysis of Protein Molecular Weight and Particle Size

SDS-PAGE and Native PAGE

SDS-PAGE was performed using 10% separating gel and 5% stacking gel on a discontinuous buffer system in reducing conditions according to the method reported by Laemmli (1970). The sample was mixed with the loading buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 1.5% DTT) at a ratio of 1:1 and heated at $100\text{ }^{\circ}\text{C}$ for 5 min in a water bath. After centrifugation (16,000 $\times g$) for 1 min, 10 μl sample and marker were loaded at channels. The gels were run at 80 V for 15 min and then at a constant voltage of 120 V for 1.5 h. They were stained with Coomassie Brilliant Blue G-250 and destained until the protein band was clearly visible. Then, the gels were photographed with a Gel Doc XR imaging system (Bio-Rad, Hercules, CA, USA). Unlike that done in SDS-PAGE, 6%

separating gel and 5% stacking gel were used for Native PAGE. The samples and buffers without SDS which did not require heating were run at $4\text{ }^{\circ}\text{C}$ in the experiments. The other operations were the same as those in SDS-PAGE.

FPLC

The FPLC experiment was performed using an AKTA FPLC system (GE Healthcare Life Sciences) fitted with a Superdex 200 10/300 GL column, which was eluted with a buffer solution (50 mM Tris-HCl, pH 8.0) at $4\text{ }^{\circ}\text{C}$. Exactly 500 μl HHP-treated Ara h 1 (5 mg/ml) was loaded onto the column and eluted at a rate of 0.75 ml/min. The UV absorbance was set to 280 nm to monitor the protein elution.

PSD

The PSD of the HHP-treated protein was measured using static laser light scattering (Malvern Mastersizer 2000 with Hydro 2000S(A); Malvern Instruments Ltd., Worcestershire, UK) at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. The refractive indexes of water and protein were 1.33 and 1.47, respectively (Perreault et al. 2017). Each sample was diluted to 0.25 mg/ml and determined in triplicate.

Analysis of Protein Tertiary Structure

Analysis of UV Absorption Spectra

The UV absorption spectra were measured by a UV-2600 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The UV absorption spectra of each sample (0.2 mg/ml) were scanned from 200 to 400 nm at 100 nm/min.

Exposed Free Sulfhydryl (–SH) Contents

Exposed free –SH contents were determined by the method of Ellman (1959). Each sample (1 ml) was mixed with 2 ml Tris-glycine buffer (0.086 M Tris, 0.09 M glycine, and 0.004 M EDTA; pH 8.0) and 20 μl Ellman's reagent (0.04 g DTNB in 10 ml Tris-glycine buffer). After reaction for 15 min at room temperature, the absorbance of the mixture was measured at 412 nm in a UV-2600 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and the Tris-glycine buffer (3 ml) with Ellman's reagent (20 μl) was used as the blank. Each sample was determined in triplicate.

The exposed free –SH content was calculated by the following equation:

$$\mu\text{mol (–SH)/g (protein)} = \frac{(73.53 \times \text{OD}_{412})}{C}$$

where OD_{412} is the absorbance value of the sample at 412 nm and C is the sample concentration (mg/ml).

Analysis of Fluorescence Emission Spectra

The surface hydrophobicity was determined according to the method of Wang et al. (2008). Exactly 15 µl of ANS solution (8.0 mM in 0.01 M PBS buffer, pH 8.0) was added to 3 ml of each sample (0.1 mg/ml) as the fluorescence probe. After 2 h of light avoidance at room temperature, an F-7000 fluorescence spectrometer (Hitachi, Tokyo, Japan) was used to measure the fluorescence intensity of the samples. The bandwidth was 5 nm, the excitation wavelength was 390 nm, and the emission wavelength ranged from 400 to 650 nm at 1200 nm/min.

Analysis of Far-UV CD Spectroscopy

Far-UV CD spectroscopy was performed with a J810 spectropolarimeter (Jasco Corp., Tokyo, Japan) in accordance with the method of Meng et al. (2016). A quartz cell with an optical path length of 0.1 cm was used for the measurements at room temperature, and the protein concentration in each sample was 0.20 mg/ml. The scan range was 250–190 nm, the scan speed was 100 nm/min, and the bandwidth was 1.0 nm. The CD spectra were measured thrice, and the contents of different secondary structures were calculated by JASCO secondary structure software.

Statistical Analysis

Statistical analysis was performed by SPSS[®] computer program (SPSS Inc., Chicago, IL, USA). The data were tested with two-way factorial ANOVA (factors: pressure and time). Mean comparisons were performed using Duncan's test, and the significance level was established at $P < 0.05$ (Huang et al. 2014a, b).

Results and Discussion

Effects of HHP on the Immunoreactivity of Ara h 1

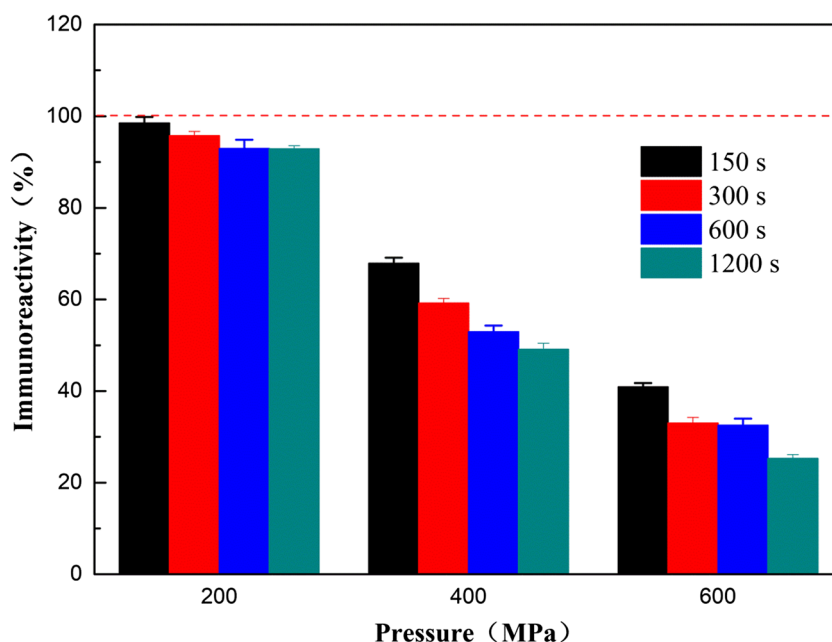
Compared with the result of the control (the immunoreactivity of the control was considered as 100%), the immunoreactivity of the HHP-treated Ara h 1 obviously decreased ($P < 0.01$) at 400 MPa and 600 MPa, and no changes were observed at 200 MPa ($P > 0.05$), as shown in Fig. 1. The immunoreactivity was also reduced significantly as the holding time was extended at 400 MPa ($P < 0.05$). However, for 600 MPa, no significant differences were noted between 300 and 600 s ($P > 0.05$). The pressure of 600 MPa caused the most significant reduction in immunoreactivity, with the maximum reduction being 74.7% when the holding time reached 1200 s. Other studies have uncovered similar results. Huang et al. (2014a, b) discovered that the immunoreactivity of peanut

allergens (Ara h 1 and Ara h 2) is obviously reduced after HHP treatment. Unlike the control group, the treatment group showed a 64.3%, 69.2%, and 73.3% reduction in immunoreactivity at pressure levels of 400 MPa, 600 MPa, and 800 MPa, respectively, for a holding time of 600 s, meanwhile the immunoreactivity was lower for holding 600 s than 300 s. The study of Li et al. (2018) showed that the soy protein isolate (SPI) achieved its lowest allergenicity at 15 min with the holding time increasing from 0 to 20 min under 300 MPa. However, the 20-min dwell time did not have any significant discrepancy with the allergenicity of SPI treated for 15 min. Long et al. (2016) also reported that the immunoreactivity of Ara h 2 treated with different pressure levels for various periods correspondingly decreases, while the immunoreactivity is no longer decreased when the pressure holding time for 10 min or more. In the present study, the immunoreactivity of Ara h 1 was considerably decreased with the increase of pressure holding time by the HHP treatment (≥ 400 MPa); moreover, due to the high bactericidal ability of HHP, the shelf life of food can be prolonged, and it has certain potential in producing safety and health peanut products with low sensitization. However, the mechanism of the effect of HHP treatment on the reduced immunoreactivity of peanut proteins remains unknown. Reports have indicated that HHP treatment could not destroy linear epitopes, although it could change the conformational epitopes of allergens in proteins (Ditto et al. 2010); hence, the decrease of immunoreactivity of Ara h 1 has been associated with its structure. Further studies are necessary to reveal the relationship between protein structure and immunoreactivity.

Effects of HHP on the Molecular Weight and Particle Size of Ara h 1

The pretreatment of peanuts in this study is based only on the laboratory level to obtain higher purified Ara h 1 protein effectively (Wu et al. 2015), and the specific extraction methods applied to industry are up to the characters of products. The SDS-PAGE and Native PAGE profiles of HHP-treated Ara h 1 and untreated Ara h 1 (control) are shown in Figs. 2 and 3, respectively. On the SDS-PAGE profiles, the bands of the HHP-treated proteins exhibited no changes relative to the control, thus proving that the structure of the Ara h 1 monomer was not altered by the HHP treatment; the same has been reported about most proteins (Rastogi et al. 2007). As the bands of 400 MPa and 600 MPa on the Native PAGE profiles moved up and the high molecular weight protein aggregates were blocked in the well, the molecular weight of Ara h 1 increased (> 245 kDa). This condition explained the change in the original trimer structure of Ara h 1, which could form new multimers under HHP. However, the distribution of bands in the Native PAGE profiles was related not only to the molecular weight of the protein but also to the amount of charge

Fig. 1 Effects of HHP on the immunoreactivity of Ara h 1 defined by ELISA measurement. The immunoreactivity of the control was considered as 100%



and particle size (Arndt et al. 2012); therefore, the increase in molecular weight needs further evidence.

FPLC and PSD were employed to evaluate the molecular weight and size of Ara h 1, respectively. In the FPLC profile (Fig. 4), a short retention time equated to a large molecular weight. The curve showed a shift at 200 MPa (Fig. 4a) compared with the untreated Ara h 1 (~12.9 min), while the results of Native PAGE (Fig. 3) and PSD (Fig. 5a) indicated that the molecular weight and particle size of Ara h 1 did not obviously increase, so a shift of the curve at 200 MPa vs control in Fig. 4 was probably because of the changes in the protein structure or particle size rather than the increase of the molecular weight. Moreover, changes in intensity were observed

and attributed to the changes in the protein's tertiary structure. For FPLC, the UV absorbance at 280 nm was set to monitor the protein elution. According to the result of UV absorption spectra (Fig. 6), more buried amino acid residues were exposed after HHP treatment, which caused an increase of UV absorption, so the intensity was increased in Fig. 4 of FPLC. Interestingly, it can be observed that the protein was getting bigger from Fig. 4 b and revealed two peaks (~10.0 min and 11.1 min) at 400 MPa for a holding time of 150 s. The later peak began to diminish for a holding time of 300 s (~10.6 min), and only one peak appeared at 600 s and 1200 s (~10.6 min and 11.1 min, respectively). This outcome indicated that it needs adequate time (≥ 300 s) and pressure ($\geq$

Fig. 2 SDS-PAGE of untreated Ara h 1 and HHP-treated Ara h 1. M, marker; C, control; lanes 1–4, treated at 200 MPa for 150 s, 300 s, 600 s, and 1200 s, respectively; lanes 5–8, treated at 400 MPa for 150 s, 300 s, 600 s, and 1200 s, respectively; lanes 9–12, treated at 600 MPa for 150 s, 300 s, 600 s, and 1200 s, respectively

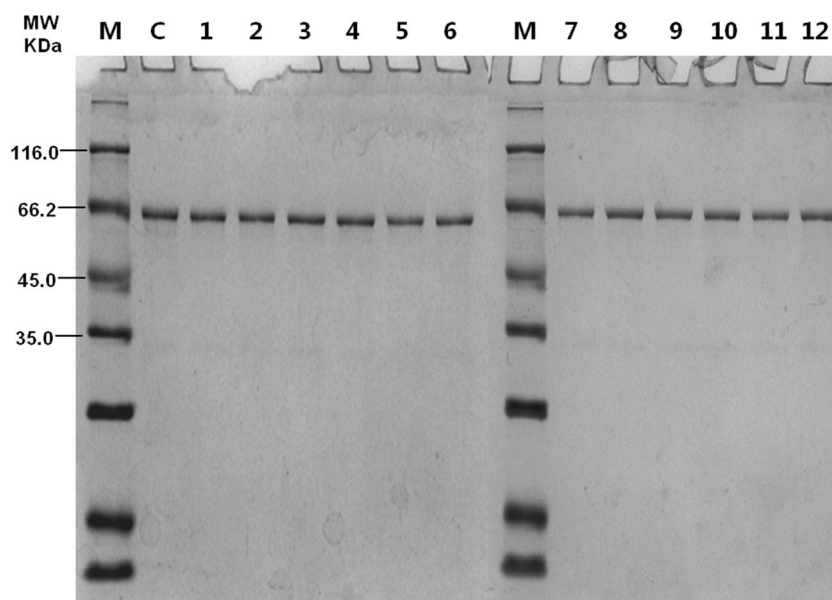
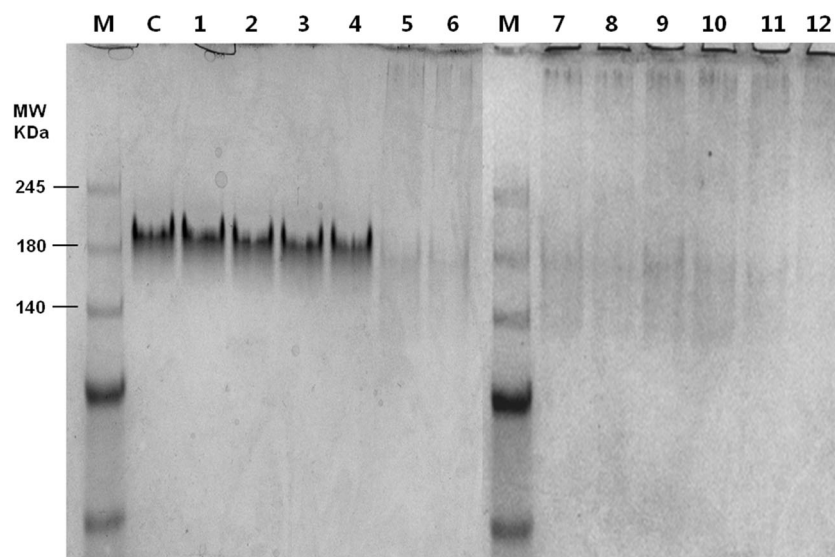


Fig. 3 Native PAGE of control Ara h 1 and HHP-treated Ara h 1. M, marker; C, control; lanes 1–4, treated at 200 MPa for 150 s, 300 s, 600 s, and 1200 s, respectively; lanes 5–8, treated at 400 MPa for 150 s, 300 s, 600 s, and 1200 s, respectively; lanes 9–12, treated at 600 MPa for 150 s, 300 s, 600 s, and 1200 s, respectively



400 MPa) to make Ara h 1 enlarged. Therefore, the molecular weight of Ara h 1 was gradually increased with the increase of holding time at 400 MPa. In Fig. 5 b, the particle size of Ara h 1 was increased obviously at 400 MPa. It can be suspected that the structure of Ara h 1 has undergone remarkable changes at 400 MPa. No significant differences were noted in Fig. 4

c with the increase of holding time at 600 MPa. Thus, the excessive pressure was enough to cause protein enlargement in a short period.

All the retention times of Ara h 1 under HHP treatment for 1200 s were shorter than the control values (Fig. 4d), and the higher the pressure, the greater the molecular weight.

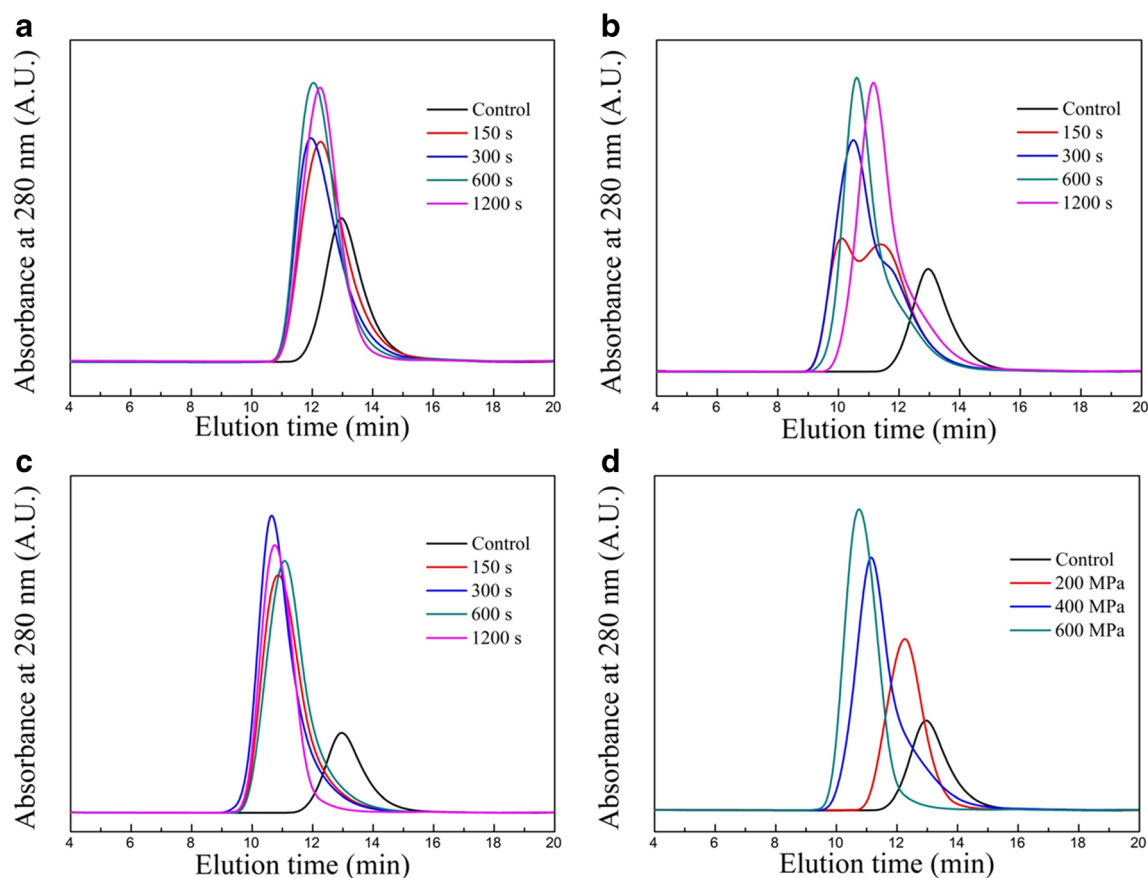


Fig. 4 FPLC profile of untreated and HHP-treated Ara h 1. Untreated Ara h 1 as control. **a** Various holding times with 200 MPa. **b** Various holding times with 400 MPa. **c** Various holding times with 600 MPa. **d** Various pressure holding times for 1200 s

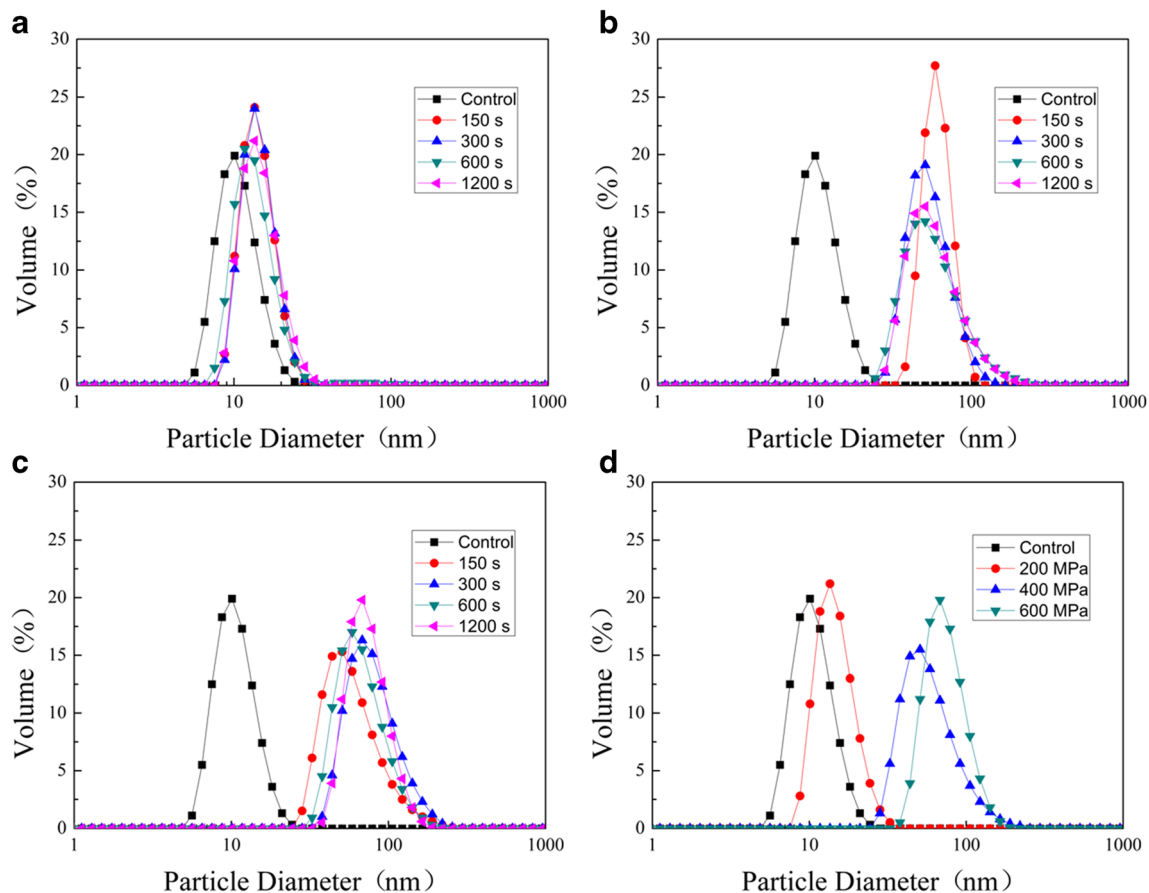


Fig. 5 PSD of untreated and HHP-treated Ara h 1. Untreated Ara h 1 as control. **a** Various holding times with 200 MPa. **b** Various holding times with 400 MPa. **c** Various holding times with 600 MPa. **d** Various pressure holding times for 1200 s

Obviously, the same trend of enlargement was observed in particle size (Fig. 5d). This result demonstrated that the molecular weight and particle size of Ara h 1 increased after HHP treatment (≥ 400 MPa); the same was noted in the results of electrophoresis (Fig. 3). Similar observations have also been made in β -lactoglobulin and β -conglycinin, both of which were aggregated and found to have formed multimers after HHP treatment (Yang et al. 2001; Wang et al. 2011). Blanc et al. (2011) reported that Ara h 1 forms aggregates by boiling and that its immunoreactivity in such a case decreases. These results demonstrate that HHP treatment (≥ 400 MPa) could alter the natural conformation of Ara h 1 and cause it to form large multimers. Remarkably, Ara h 1 protein was gradually enlarged with the increase of holding time at 400 MPa, but it only needed a short time at 600 MPa. Furthermore, the aggregation of proteins would affect the allergic immune response. We can thus infer that the decrease of the immunoreactivity of Ara h 1 under HHP is related to its aggregation. Moreover, some parts of immunoreactive sites may be hidden inside the protein, thereby causing the formation of hypoallergenic Ara h 1. Unlike that at 400 MPa, the molecular weight and particle size of Ara h 1 at 600 MPa did not obviously increase while the immunoreactivity continued to decrease. Hence, the

decrease of immunoreactivity was due not only to the formation of multimers but also to other factors, such as the conformational degeneration or destruction of the protein structure.

Effects of HHP on the Tertiary Structure of Ara h 1

UV absorption spectra mainly depend on the UV absorption of Tyr (280 nm) and Trp (275 nm) residues located in side-chain groups and on the absorption of Phe, His, and Cys to some extent (Sen et al. 2002). Figure 6 shows that the UV absorption changed only slightly at 200 MPa for a holding time of 0–1200 s. At 400 MPa, the UV absorption presented a considerable increase, which was positively correlated with the holding time (Fig. 6b). Although the UV absorption also increased at 600 MPa (Fig. 6c), it had little change with the increase of treatment time and showed a lower degree of change relative to that at 400 MPa. The analysis showed that a large amount of amino acid residues was buried inside the protein and then exposed after HHP treatment (≥ 400 MPa), especially at 400 MPa, exposed amino acid residues increased as the pressure holding time increased. This condition resulted in the increase of UV absorption. The HHP treatment could thus destroy the protein's tertiary structure. At the same time, the

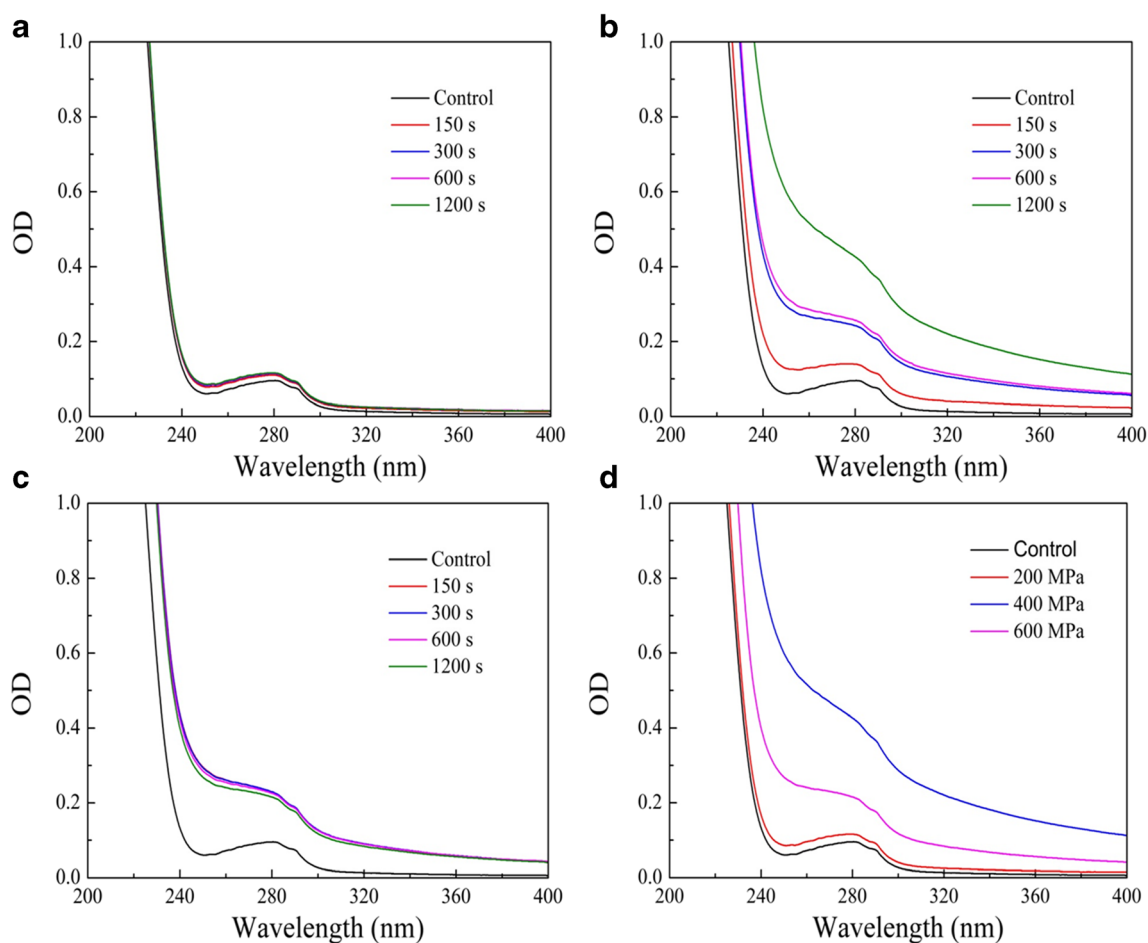
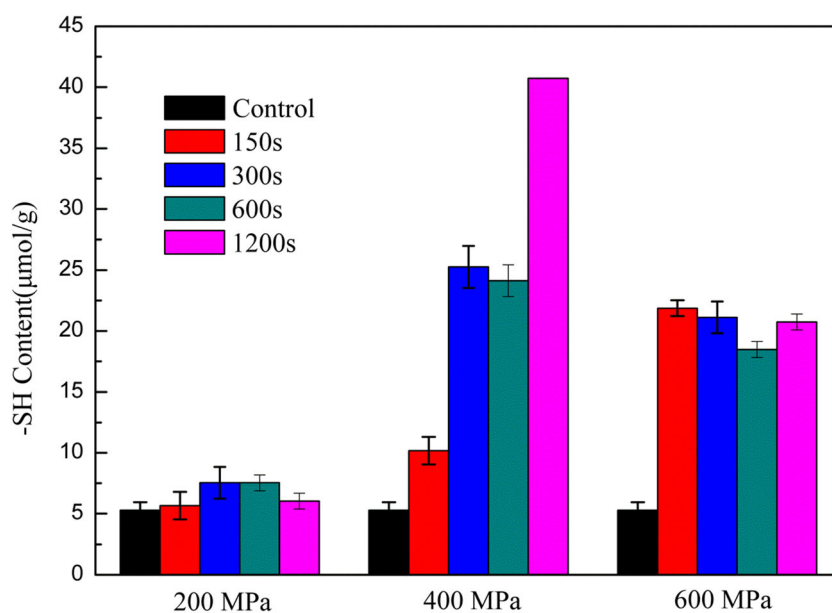


Fig. 6 UV absorption spectra of untreated and HHP-treated Ara h 1. Untreated Ara h 1 as control. **a** Various holding times with 200 MPa. **b** Various holding times with 400 MPa. **c** Various holding times with 600 MPa. **d** Various pressure holding times for 1200 s

excessive pressure treatment (≥ 600 MPa) might have caused the amino acid residues to be reaggregated and buried again,

leading to the decrease of UV absorption at 600 MPa relative to the condition at 400 MPa. The rearrangement of amino

Fig. 7 Effects of pressure and holding time on free sulfhydryl (–SH) content of Ara h 1 after HHP treatment. Untreated Ara h 1 as control



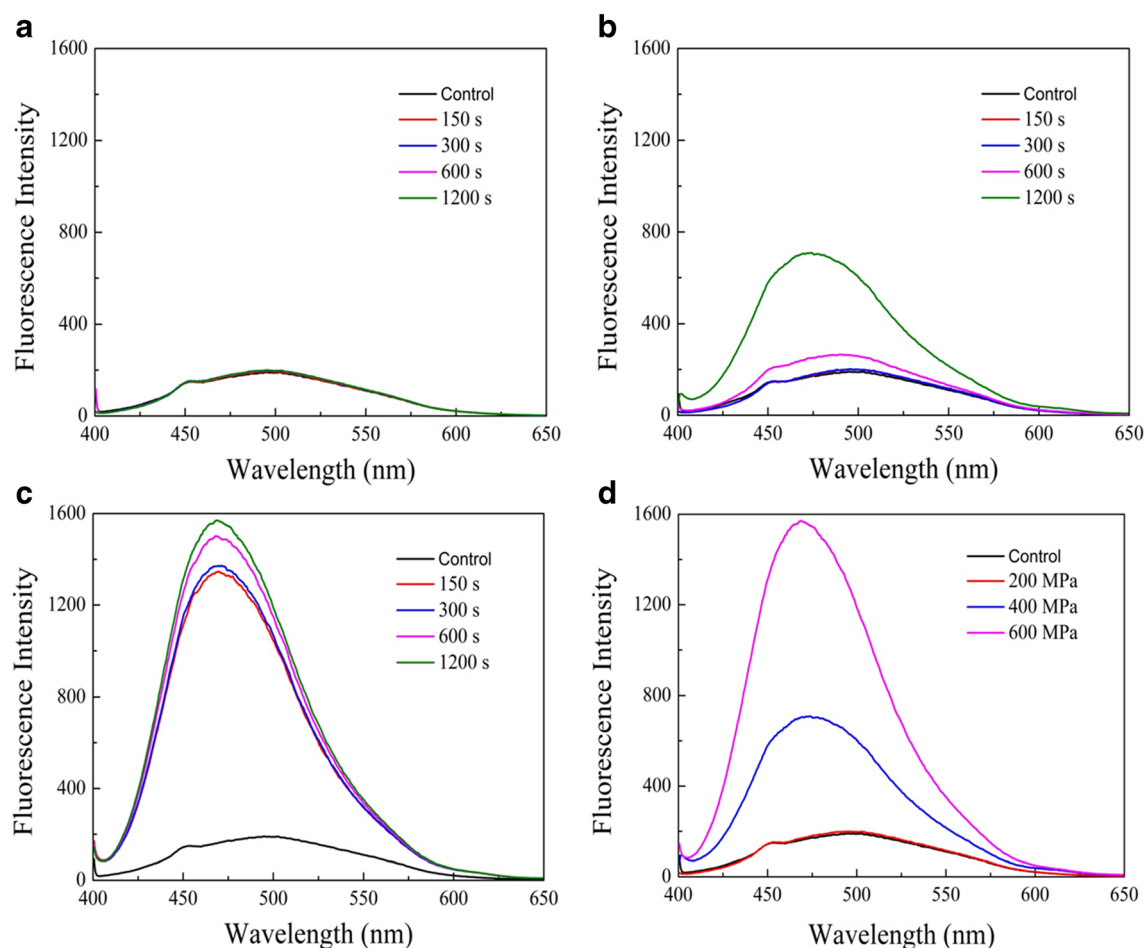


Fig. 8 Fluorescence emission spectra of untreated and HHP-treated Ara h 1. Untreated Ara h 1 as control. **a** Various holding times with 200 MPa. **b** Various holding times with 400 MPa. **c** Various holding times with 600 MPa. **d** Various pressure holding times for 1200 s

acids in the tertiary structure of Ara h 1 possibly exposed the buried immunoreactive sites, resulting in the obvious reduction of immunoreactivity at 600 MPa. In addition, concerning the result of UV absorption spectra in Fig. 4, a slight blue shift of the absorption peak was also observed as the pressure increased, which showed that the polarity of Trp and Tyr had changed after HHP.

The free -SH is a strong reducible group that affects the electrostatic interactions of molecules, and disulfide bonds play an important role in defining protein structure (Wang et al. 2017). As shown in Fig. 7, the free -SH content of the untreated protein was 5.28 $\mu\text{mol/g}$ protein, and it showed no change at 200 MPa ($P > 0.05$). By contrast, the free -SH content of the protein treated at 400 MPa and 600 MPa obviously increased ($P < 0.05$), with the content at 400 MPa being greater than that at 600 MPa for the same treatment time, except at 150 s. Moreover, the free -SH content continued to increase as the treatment time extended at 400 MPa, which attributed to the exposure of the internal -SH group during unfolding and extension induced by pressurization (De Maria et al. 2016). At 600 MPa, the content presented the highest value for a holding

time of 150 s and showed a slight decrease as the treatment time extended. This result illustrated that the HHP treatment could break disulfide bonds and cause proteins unfolded partially to make the free -SH exposed. The change of the tertiary structure caused the decrease of the immunoreactivity. Combined with the results of UV absorption spectra and the change of free -SH at 600 MPa and 400 MPa, it was suggested that higher pressure and longer pressure holding time could cause the exposed -SH groups to reoxidize into disulfide bonds. The reoxidation leads to the formation of new multimers, which could bury some immunoreactive sites and further reduce the immunoreactivity of Ara h 1.

Protein surface hydrophobicity helps to promote the stabilization of a protein's tertiary and quaternary structure. Relative to other forces, such as hydrogen bonds and salt bonds, protein surface hydrophobicity is thus considered to be the most important factor in maintaining the high-level structure of proteins. In this study, ANS was used as a fluorescence probe to measure protein surface hydrophobicity, which is positively correlated with fluorescence intensity. For the fluorescence intensity, no difference was observed

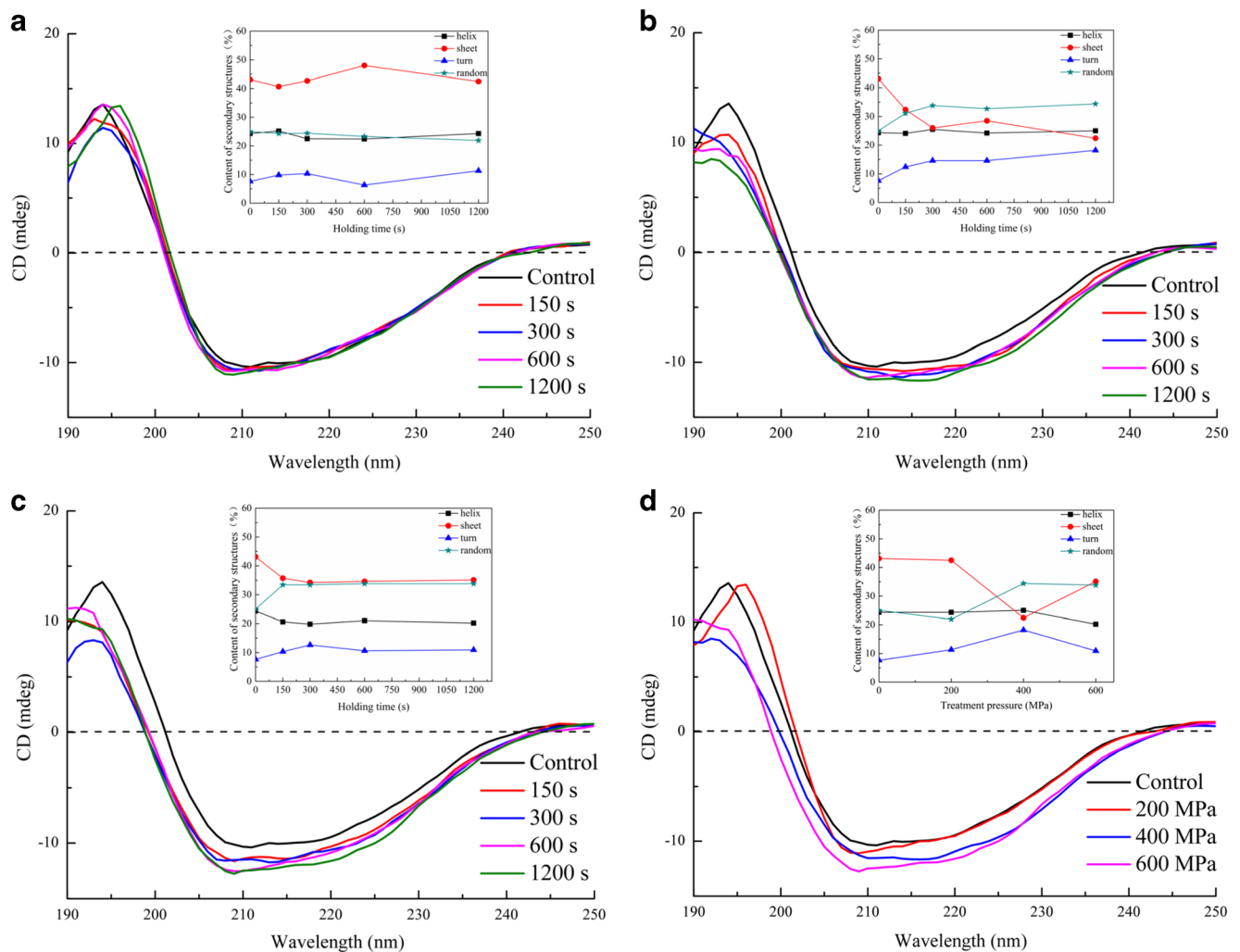


Fig. 9 Far-UV CD spectra and the content of each secondary structure in untreated and HHP-treated Ara h 1. Untreated Ara h 1 as control (the holding time was 0 s). **a** Various holding times with 200 MPa. **b** Various

holding times with 400 MPa. **c** Various holding times with 600 MPa. **d** Various pressure holding times for 1200 s

between the control group and the 200 MPa treatment group (Fig. 8a). At 400 MPa (Fig. 8b), the fluorescence intensity began to increase when the holding time reached 600 s. Meanwhile, the value significantly increased at the beginning of the 150-s treatment time when the pressure was adjusted to 600 MPa (Fig. 8c); this value was higher than that at 400 MPa for 1200 s. This value increased further as the holding time at 600 MPa extended. Pressure level clearly had a significant positive effect on the surface hydrophobicity of Ara h 1 (Fig. 8d). After the HHP treatment, the protein was unfolded, and the hydrophobic groups buried in its interior were exposed to the surface (Zhu et al. 2017). Therefore, the huge increase of the surface hydrophobicity of Ara h 1 under HHP (≥ 400 MPa) treatment reflected the considerable change in the tertiary structure of the protein.

The results of the UV absorption spectra, -SH contents, and fluorescence emission spectra indicated that the HHP treatment (≥ 400 MPa) caused obvious changes in the tertiary

structure of Ara h 1, and longer pressure holding time also makes the tertiary structure change a lot, which is closely related to its immunoreactivity. When the pressure reached 400 MPa, the immunoreactivity of Ara h 1 decreased by $\sim 50\%$, indicating that the HHP treatment destroyed the protein's natural structure and induced the spatial distribution of the immunoreactive sites on the surface of the native Ara h 1 conformation, which was also lower with the increase of treatment time; as a result, the immunoreactivity of the protein dropped (Rahaman et al. 2016; Beyer et al. 2001). What is more, compared to 400 MPa, the immunoreactivity of Ara h 1 at 600 MPa continuously declined and finally decreased by $\sim 75\%$. However, the free -SH contents and UV absorption at 600 MPa were lower than those at 400 MPa. The analysis showed that the excessive pressure could have caused the structural rearrangement of Ara h 1 to form new multimers. The formation possibly caused the outside amino acid residues and other immunoreactive sites to be buried again.

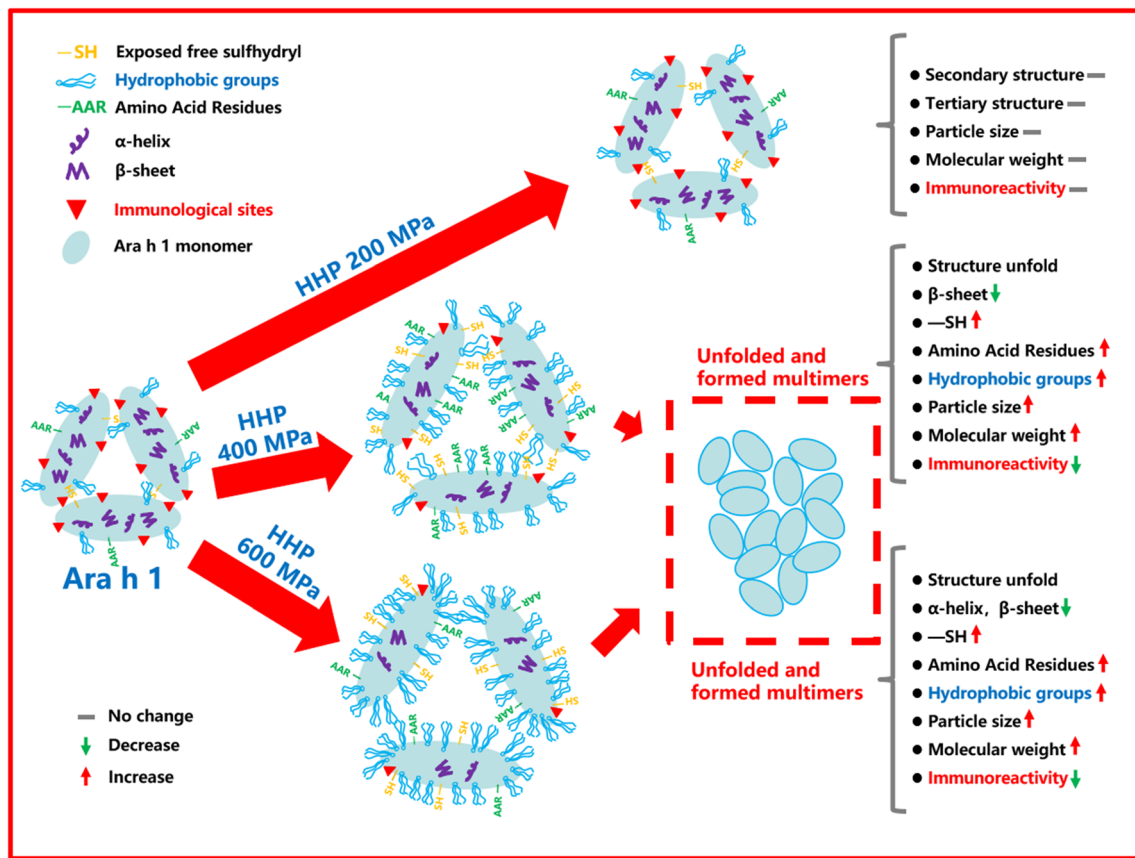


Fig. 10 The probable mechanisms of the changes in Ara h 1 structure and immunoreactivity after HHP treatment

Effects of HHP on the Secondary Structure of Ara h 1

The contents of different secondary structures were calculated by JASCO secondary structure software (Meng et al. 2016) according to the measured CD spectra. As shown in Fig. 9, the native Ara h 1 (control) comprised 24.3% α-helix, 43.1% β-sheet, 7.6% β-turn, and 25.0% random coil. In Fig. 9 a, there are no differences between curves (CD as a function of wavelength). Besides, in Fig. 9 b and c, the whole CD band is downward shifted which means the secondary structure of Ara h 1 has been changed. Basically, 200 MPa-treated Ara h 1 had the same content of each secondary structure as the control (Fig. 9a). When the pressure reached 400 MPa and the holding time extended to 1200 s (Fig. 9b), β-sheet decreased by 20.7%; β-turn and random coil increased by 10.6% and 9.4%, respectively; and α-helix remained stable. At 600 MPa (Fig. 9c), the change in each secondary structure showed a similar trend to that at 400 MPa for holding times of 150 s and 300 s, except for α-helix, which dropped by 4.1%. These alterations are clearly shown in Fig. 9 d. Similarly, Zhao et al. (2018) found that 250 MPa had no effect on the secondary structure of sweet potato protein, but the α-helix content was decreased at 550 MPa at pH 6.0. Thus, with increasing pressure, proteins unfolded, α-helix content decreased, and random coils content increased, which suggested that the

exchange of secondary structure component might lead to changes in protein functional properties under HHP treatment.

On Ara h 1, the IgE-binding epitopes 12, 20, and 21 are located on the α-helix, and epitopes 14–19 and 22 are located on the β-stand (Shin et al. 1998). Thus, the decrease of α-helix and β-sheet of Ara h 1 could cause the destruction of the immunoreactive sites, thereby contributing to the reduction of Ara h 1's immunoreactivity. Similar observations were reported by Zhou et al. (2016). When the pressure reached 500 MPa, α-helix and β-sheet of ginkgo seed protein significantly decreased. Meanwhile, the immunoreactivity was greatly reduced. Furthermore, except for the difference in the molecular weight and the tertiary structure, it should be pointed out that the reason why the immunoreactivity of Ara h 1 at 600 MPa was lower than 400 MPa is considered to be closely related with the sole decrease of α-helix at 600 MPa.

The Probable Mechanisms of Connecting Ara h 1 Structure Alterations with Its Immunoreactivity Change After HHP Treatment

The probable mechanisms of the changes in the structure and immunoreactivity of Ara h 1 after HHP treatment are summarized in Fig. 10. The lower pressure (≤ 200 MPa) could not destroy the native structure of Ara h 1 to affect its

immunoreactivity effectively. By contrast, the high pressure (≥ 400 MPa) could change the conformation and reduce the immunoreactivity of Ara h 1 significantly. We considered that the changes in the conformation of Ara h 1 may alter or hide the position of some immunoreactive sites in the protein and further change the binding degree of the immunoreactive sites to immunoglobulins. Thus, the decrease of immunoreactivity was mainly due to the changes in the protein's tertiary structure, which caused the formation of multimers. According to some of the changes observed at 400 MPa and 600 MPa, the considerable decrease of the immunoreactivity at 600 MPa was possibly due to the high pressure treatment destroying the important secondary structure of α -helix. On the other hand, the HHP (400 MPa) causes the unfolded protein to aggregate and form different multimers from the natural trimers. As a result, a large number of immunoreactive sites were buried, and the protein's immunoreactivity further decreased. Moreover, the immunoreactivity of Ara h 1 was decreased with the longer pressure holding time at 400 MPa, and the lowest immunoreactivity was treated at 600 MPa when the treatment time reached at 1200 s, so the treatment time also plays an important role in reducing the immunoreactivity of Ara h 1.

Conclusions

In conclusions, HHP treatment (≥ 400 MPa) combined with a long holding time considerably reduced the immunoreactivity of Ara h 1, which had the highest reduction of 74.7% at 600 MPa (1200 s) compared to the control. A close correlation was noted between the immunoreactivity and the secondary/tertiary structure changes of Ara h 1. HHP could modify the spatial conformation, which caused the formation of new multimers. Such changes, in turn, altered the positions of some immunoreactive sites in the protein and further decreased the immunoreactivity. These results imply that HHP treatment can reduce immunoreactivity effectively by altering the structures and multimer formation of Ara h 1. On the other hand, HHP is considered to be a promising emerging technology for improving food quality and safety. Therefore, it is potential to apply HHP technology in the production of hypoallergenic peanut products.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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