



Thermal processing effects on peanut allergen Ara h 2 allergenicity in mice and its antigenic epitope structure



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ABSTRACT

Ara h 2 was purified from peanuts that were thermally treated by various processes, including boiling, glycation, frying and roasting. The allergenicity of Ara h 2 in Balb/c mice and the influence of thermal processing on the structural characteristics, and binding capacity of three core antigenic epitopes were studied. The results demonstrated that boiling, glycation and frying induced the down-regulation of the allergenicity of Ara h 2 in Balb/c mice, the collapse of its tertiary/secondary structure, and a reduction in the core epitope binding capacity; roasting showed a comparable allergenicity and the weakest inhibitory effect on core epitope binding capacity. These results indicate that thermal processing causes alteration of the protein structure and core epitopes of Ara h 2, and may affect its allergenicity.

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1. Introduction

Peanuts (*Arachis hypogaea*) are a nutrient-rich and very popular food, which provide protein and fat to humans (Visser et al., 2011); However, they can also cause serious allergic reactions (Chruszcz et al., 2011; Zhang, Cai, Guan, & Chen, 2015), including acute vomiting, hives, shock and even death (Mueller et al., 2011; Stanley et al., 1997). Peanut allergy is a type I hypersensitivity reaction, which is mediated by the crosslinking of peanut allergens and specific IgE. Currently, 13 peanut allergens, designated Ara h 1–13, are recognized by the WHO/IUIS allergen nomenclature subcommittee. Ara h 2, for which the allergic response is mediated by IgE, is of major importance and can be recognized by more than 90% of sera from patients with peanut allergy (de Leon et al., 2007; Shen et al., 2015). Ara h 2.01 and Ara h 2.02 are two subtypes of Ara h 2 (Apostolovic et al., 2013) and are nearly identical except for the insertion of a DPYSPS sequence in the higher molecular weight isoform (Koppelman, Hefle, Taylor, & de Jong, 2010). Ten linear epitopes have been identified in Ara h 2, and among them, DRRCQSQCER (epitope 3), RDPYSP (epitope 6), and SQDPYSPS (epitope 7) were recognized by all patients tested, and bound more IgE than other epitopes, indicating that they are

the immunodominant epitopes of the Ara h 2 protein (Stanley et al., 1997).

Interestingly, peanut allergy is a generally persistent food allergy in North America and several European countries. However, it appears to be less prevalent in other parts of the world, such as China. Peanuts in China are generally boiled or fried in contrast to roasted peanuts that are generally consumed in western countries. Recently, it was shown that roasting may enhance the allergenic properties of peanuts (Davis, Smales, & James, 2001; Moghaddam et al., 2014) and it has been proposed that the differences in cooking practices may be responsible for the disparity in peanut allergy prevalence (Beyer et al., 2001). The allergenicity of peanut allergens can be altered through the modification of their protein structure by food processing procedures, which results in unfolded, aggregated proteins that interact with other constituents in the food (Mondoulet et al., 2005). Previous studies have reported that the IgE binding capacity and allergenicity of Ara h 2 decreased following heating in the presence or absence of glucose. Compared with roasted peanuts, there was significantly less IgE bound to the Ara h 2 of fried peanuts (Beyer et al., 2001; Mondoulet et al., 2005; Shen et al., 2015; Visser et al., 2011). However, there have been few studies discussing whether structural changes, particularly the alteration of epitope structure, would affect the allergenicity of Ara h 2. In view of this, the aim of the present study was to investigate the effect of thermal processing on the structure and epitope binding capacity of Ara h 2, and assess the allergenicity to Balb/c mice by measuring the level of specific antibodies and Th1/Th2 cytokines.

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2. Materials and methods

2.1. Materials and reagents

Peanuts (*A. hypogaea*) were purchased from a local supermarket and stored at -20°C . A DEAE-Sepharose Fast Flow column ($16 \times 250\text{ mm}$) was purchased from Bestchrom Biotechnology Co., Ltd (Shanghai, China). Monoclonal antibodies of core IgE-binding epitope 3 (DRRCQSQLER), epitope 6 (RDPYSP), and 7 (SQDPYSPS) were prepared by GL Biochem Co., Ltd (Shanghai, China). Polyvinylidene fluoride (PVDF) membranes ($0.45\text{ }\mu\text{m}$) were purchased from Merck Millipore. Goat anti-mouse IgG-HRP was purchased from Nanjing Key GEN BioTECH Co., Ltd. BeyoECL Plus and TMB horseradish peroxidase color development solution for the enzyme-linked immune sorbent assay (ELISA) were purchased from Beyotime Biotechnology Co., Ltd.

2.2. Protein preparation and thermal treatment

Peanut kernels were treated under one of the following four conditions: A) boiling for 15 min at 100°C ; B) boiling with 100 mmol/l glucose (100°C , 15 min); C) frying in peanut oil (280°C , 2 min), or D) roasting (170°C , 15 min), and subsequently ground until a homogeneous paste was obtained. Whole peanut extracts (WPE) were extracted by stirring in extraction buffer (5% w/v, 20 mmol/l Tris-HCl pH 8.2) at 4°C for 3 h. After centrifugation at $5000g$ for 5 min at 4°C , the supernatant was collected and centrifuged again at $25,000g$ for 30 min, the pooled supernatants of the different extracts were then filtered through a $0.45\text{ }\mu\text{m}$ membrane, and stored at -80°C . In addition, the cooking water was collected and lyophilized for use in later assays.

Ara h 2 was purified from different thermally-treated peanuts as previously described (Mondoulet et al., 2005), which involved anion exchange chromatography using a DEAE-Sepharose Fast Flow column ($16 \times 250\text{ mm}$), followed by $0\text{--}1\text{ mol/l}$ NaCl linear gradient elution, dialysis three times, concentration by 3KD Millipore, and then storage at -20°C .

2.3. SDS-PAGE and Western blotting

2.3.1. SDS-PAGE

Whole peanut protein extracts and purified Ara h 2 were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (5% stacking gel, 19% resolving gel) as described by Wu et al. (2015).

2.3.2. Western blotting

After electrophoresis, the contents of the gels were transferred to a PVDF membrane (300 mA , 1 h). After repeated rinsing in TBS-Tween (0.1% Tween 20 in TBS), the membrane was blocked using 5% skimmed milk powder for 1 h. The membrane was incubated with monoclonal antibodies ($1:2000$ dilution) of three core IgE-binding epitopes overnight at 4°C . After rinsing, the membrane was incubated with a goat anti-mouse IgG antibody labeled with peroxidase ($1:5000$ dilution) for 1 h. The membrane was then incubated with the Beyo ECL Plus visualization reagent for 1 min and revealed on ChemiDoc XRS film (Bio-rad, MA, USA).

2.4. Protein structure characteristic analysis

2.4.1. Ultraviolet spectrum

Ara h 2 proteins purified from crude, boiled, glycated, fried, and roasted peanuts were analyzed using a UV-vis spectrophotometer (UV759CRT) with a scanning wavelength of $250\text{--}420\text{ nm}$ and scanning rate of 100 nm/min .

2.4.2. Circular dichroism (CD) spectroscopy

A Jasco J-815 spectropolarimeter (Jasco, MA, Japan) was used for CD measurements. The concentration of Ara h 2 was $10\text{ }\mu\text{M}$ (dissolved in PBS) for the far-UV region ranging from 190 to 250 nm . The ellipticity was recorded at a scanning speed of 500 nm/min , an accumulations of 3, and a bandwidth of $0.199 \pm 0.1\text{ nm}$.

2.5. Epitopes binding capacity detection

The antigenic epitopes binding capacity of Ara h 2 was analyzed using the indirect ELISA as previously described (Cai, Zhang, Zhu, & Chen 2016). Native and thermal-treated Ara h 2 were diluted in carbonate buffer (pH 9.6, 0.05 mol/l). They were then coated onto microtiter plates, and stored overnight at 4°C . After repeated rinsing in PBS-Tween (0.05% Tween 20 in PBS), the plates were blocked using 5% skimmed milk powder ($200\text{ }\mu\text{l}$, 1 h, 37°C) and washed again ($5 \times 300\text{ }\mu\text{l}$ PBS-Tween). The plates were then incubated with monoclonal antibodies of three core IgE-binding epitopes diluted at $1:5000$ ($200\text{ }\mu\text{l}$, 2 h, 37°C). After rinsing, they were incubated with goat anti-mouse IgG-HRP, diluted at $1:5000$ ($200\text{ }\mu\text{l}$, 1 h, 37°C). The TMB solution was then added in the dark for about $10\text{--}15\text{ min}$, after which, $50\text{ }\mu\text{l}$ 2 mol/l H_2SO_4 was added as a stop solution. Absorbance was determined at 450 nm using a single-function optical absorption microplate measurement EMax Plus (Molecular Devices, MA, USA).

2.6. Allergenicity in Balb/c mice

Pathogen-free female Balb/c mice (4 weeks of age) were purchased from the animal experiment center of Fudan University (Shanghai, China) and housed under specific pathogen-free conditions within the animal care facility for about 10 days. One hundred micrograms of WPE of the differentially treated peanuts and isopyknic Freund's complete adjuvant were intraperitoneally injected in each mouse, and after 7 days, the mice were injected with 20 mg Ara h 2 plus isopyknic Freund's incomplete adjuvant. This was repeated every week for two weeks (exposure on days 14, and 21). Control groups received PBS in Freund's incomplete adjuvant. Mice received a challenge dose of 40 mg Ara h 2 on day 28, and then were bled and sacrificed after 30 min. The experiments were approved by the Animal Experiments Committee of the School of Life Science, Shanghai University, China.

The method of detecting the increase of vascular permeability, spleen swelling, and mast cell degranulation refers to Liu (Liu et al., 2012). Serum Ara h 2-specific IgE, and -IgG2a were obtained on day 28 and analyzed by indirect ELISA as described previously (Smit et al., 2011; van Wijk, Hartgring, Koppelman, Pieters, & Knippels, 2004). Histamine and cytokine levels were determined using an ELISA kit (Moredun Scientific Ltd.) and performed according to instructions of the manufacturer.

2.7. Statistical analysis

The data were expressed as means $\pm$ standard deviation (SD) of three replicates or means $\pm$ standard error (SE) of eight replicates using the Statistica 7.0 software (StaSoft Inc., OK, USA), followed by ANOVA and Tukey's studentised range test to verify significant differences.

3. Results

3.1. Effect of thermal processing on WPE and Western blotting of Ara h 2

Proteins from crude, boiled, glycated, fried and roasted peanuts were extracted and analyzed by SDS-PAGE (Fig. 1A). Almost all bands ranged from 11 to 65 kDa in molecular weight (MW); the

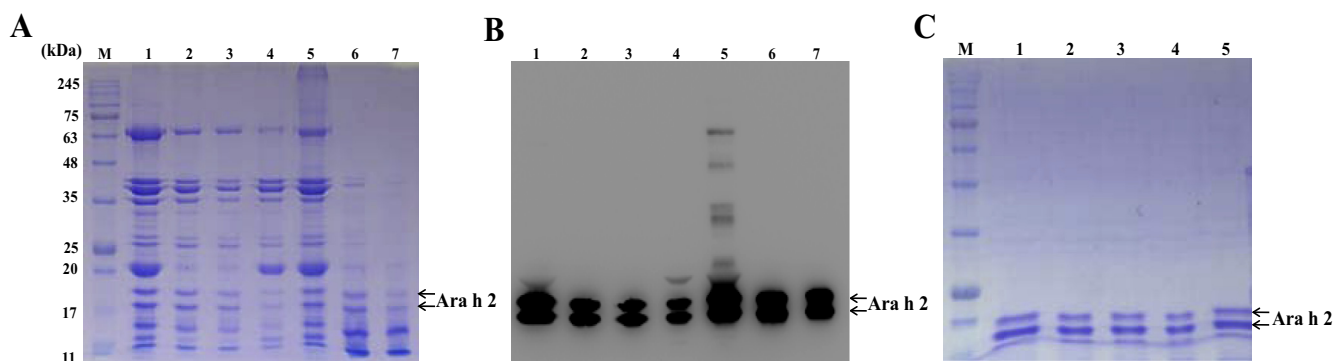


Fig. 1. Effect of thermal treatment on the WPE and purification of Ara h 2. (A) SDS-PAGE analysis of the different WPE; (B) Western blotting detection of the different WPE; (C) Purification of Ara h 2; M: Molecular weight markers; (A, B, 1–5): WPE of crude, boiled, glyated, fried, and roasted peanuts; (A, B, 6–7): Protein extracted from the boiling water of boiled and glyated peanuts; (C, 1–5): Ara h 2 of crude, boiled, glyated, fried, and roasted peanuts.

protein content of boiled and glyated peanut extracts were clearly reduced, especially in the 20 kDa position. The low MW protein bands, including that of Ara h 2, were observed in the cooking water. The monomeric form of Ara h 1 (65 kDa) and bands of Ara h 2 (17–20 kDa) were remarkably reduced in the fried peanuts compared with crude peanuts, whereas changes in the roasted peanuts were much less pronounced. However, a band of high MW (over 245 kDa) could be detected in roasted peanuts.

Western blotting was performed using monoclonal antibodies of core IgE-binding epitopes of Ara h 2. The results are shown in Fig. 1B. The two isoforms of Ara h 2, designated as Ara h 2.01 and 2.02 (hereafter collectively referred to as Ara h 2), were detected in all samples, which suggested significant specificity of antibodies. The band of Ara h 2 was reduced in boiled, glyated, and fried samples; however, Ara h 2 was detectable in the cooking water, indicating that the protein had dissolved into the water during the boiling process. Particularly, a few bands of high MW were present in roasted samples, which suggest that Ara h 2 had aggre-

gated with some other components of the peanuts. The high purity of thermal-treated Ara h 2 (about 95%) was obtained by using a DEAE-Sepharose FF column (Fig. 1C).

3.2. Effect of thermal processing on the structure of Ara h 2

The UV spectrum was applied to analyze the effect of thermal treatment on the tertiary structure of Ara h 2 (Fig. 2A). Absorbance values of Ara h 2 at 280 nm increased after all thermal treatments, with the fried Ara h 2 showing the most obvious increase, indicating that heating resulted in extensive denaturation, destruction of the tertiary structure, and possible exposure of parts of the amino acid residues embedded in Ara h 2.

Far-UV CD chromatography was used to analyze the secondary structure of Ara h 2. The result is shown in Fig. 2B. In crude Ara h 2, a positive maximum at 192 nm and two negative minimums at 208 and 222 nm were detected, indicating the existence of α -helices in Ara h 2 (Fig. 2B). The percentage of conformational units was

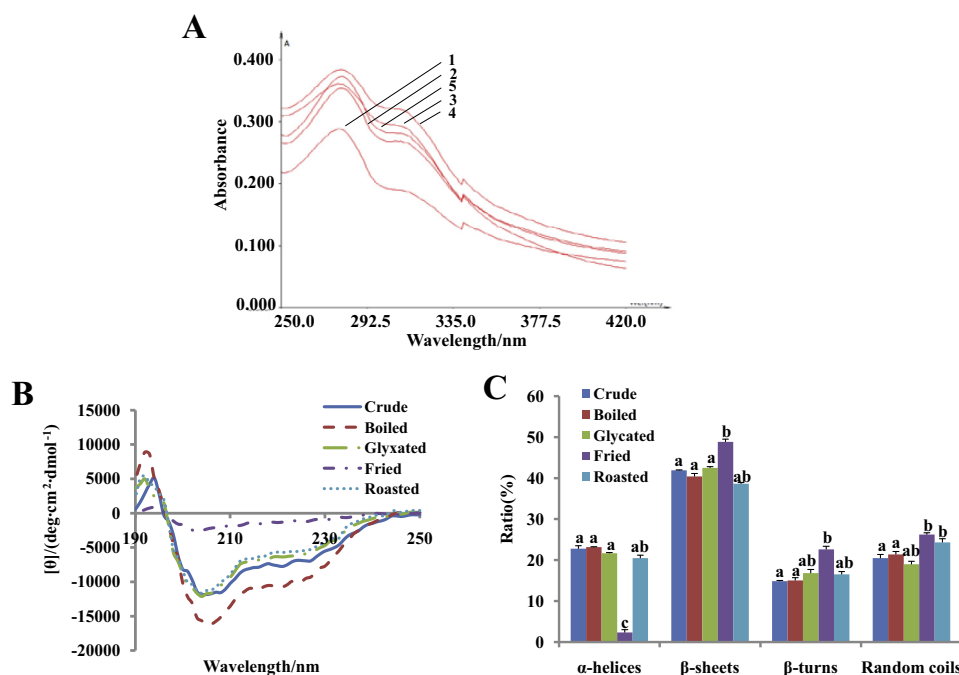


Fig. 2. Effect of thermal processing on structure of Ara h 2. (A) Absorbance values at 280 nm of Ara h 2. 1–5 represents Ara h 2 of crude, boiled, glyated, fried, and roasted Ara h 2, respectively; (B) CD spectra of Ara h 2; (C) The alteration of conformational units after thermal processing. Data are expressed as mean SD (n = 3). Different letters represent significant differences (P < 0.05).

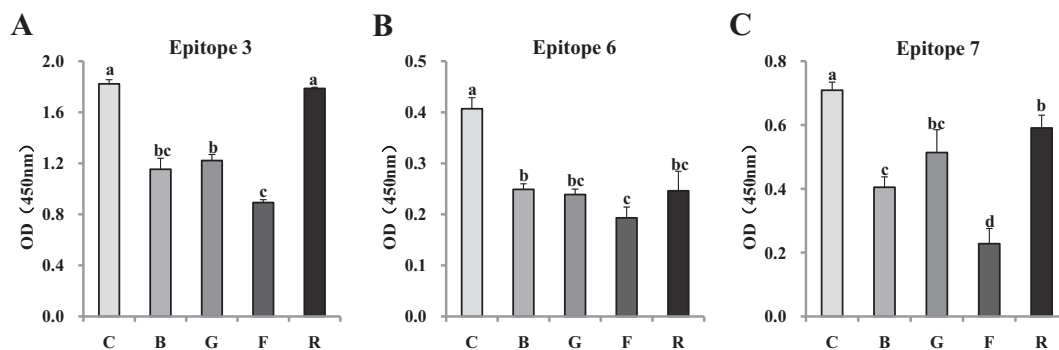


Fig. 3. Influence of thermal processing on epitopes binding capacity of Ara h 2. (A–C) Epitopes binding capacity of epitope 3, 6, 7; C, B, G, F, R stands for Ara h 2 protein of crude, boiled, glycated, fried, and roasted peanuts, respectively. Data are expressed as mean SD (n = 3). Different letters represent significant differences ($P < 0.05$).

calculated using CDPro software, and the results are shown in Fig. 2C. In contrast with the control, the content of conformational units changed after thermal treatment. A significant shifting was observed in fried Ara h 2: the content of the α -helices was sharply decreased from 20.8 to 2.3%, whereas the β -sheet, β -turn, and random coil content increased from 36.4–48.2%, 12.9–22.3%, and 17.8–25.9%, respectively. Moreover, there was no significant difference in α -helices, β -sheet and β -turn after boiling, glycating, and roasting.

3.3. Effect of thermal treatment on antigenic epitope binding capacity of Ara h 2

To further study the effect of thermal processing on the structure of Ara h 2 from the aspect of epitopes, antibodies of three core IgE-binding epitopes (epitopes 3, 6, and 7) were used to determine the binding capacity of Ara h 2 after thermal processing by indirect

ELISA. The results are shown in Fig. 3. The binding capacity for epitope 3, 6, and 7 decreased after boiling, glycating, and frying, and the fried Ara h 2 showed the weakest binding capacity, which was about 70%, 50%, and 37% of the control, respectively. However, the influence was different among the three epitopes after roasting; the binding capacity of epitope 3 was almost equivalent to that of crude Ara h 2, while that of epitope 6, and 7 were significantly decreased.

3.4. Effect of thermal processing on the allergenicity of Ara h 2 in Balb/c mice

Purified Ara h 2 from differentially thermal-treated peanuts was applied to study the impact of thermal processing on the allergenicity of Ara h 2 in mice. The immunization procedure is shown in Fig. 4A. Typical allergy symptoms, including increased vascular permeability, spleen swelling, and mast cell degranulation

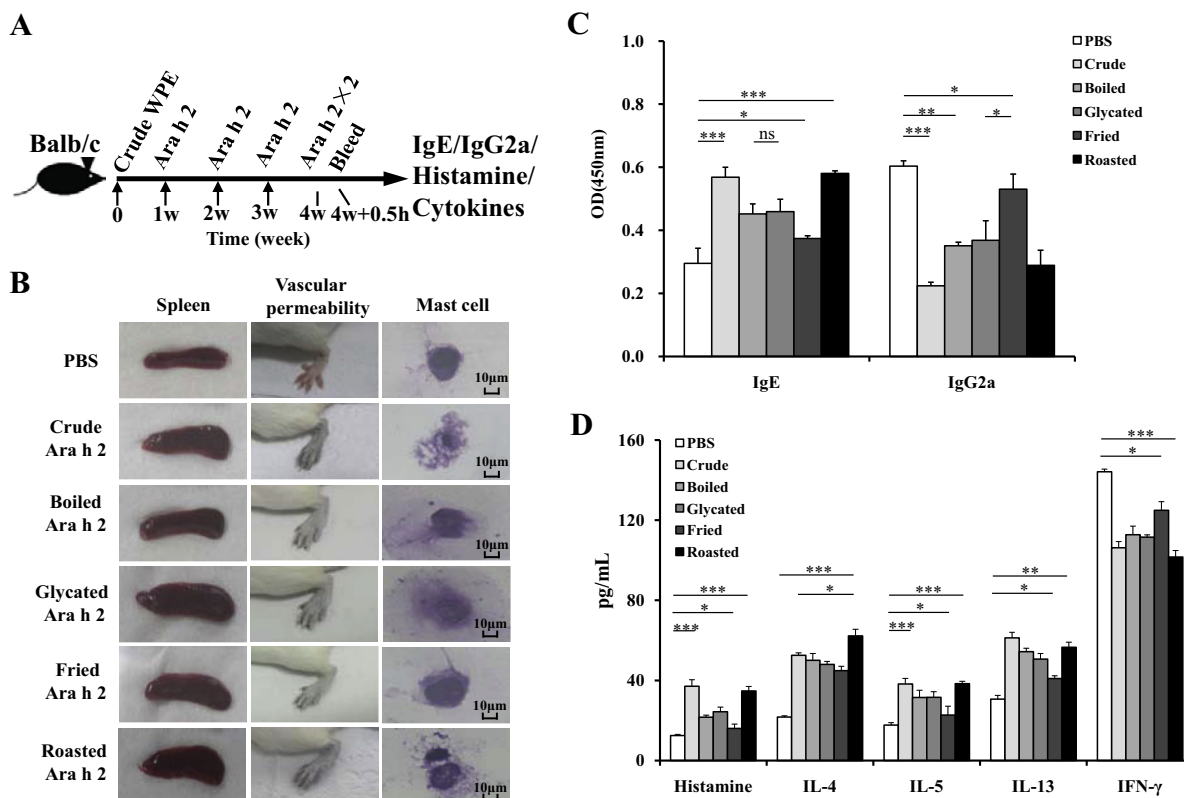


Fig. 4. Allergenicity of thermal-treated Ara h 2 in Balb/c mice. (A) Immunization procedure; (B) Anaphylaxis of Balb/c mice; (C) Levels of IgE and IgG2a in serum of Balb/c mice; (D) Levels of histamine, cytokines (IL-4, IL-5, IL-13 and IFN- γ) in serum of Balb/c mice. Data are represented as mean $\pm$ SE of 8 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Fig. 4B), were observed after intraperitoneal injection of crude Ara h 2, indicating that Balb/c mice were sensitized by the Ara h 2 allergen. We then injected the thermal-treated Ara h 2 into the mice. The content of specific IgE, histamine, and Th2 type cytokines, including IL-4, 5, and 13, were all up-regulated, whereas IgG2a and Th1 type cytokines, such as IFN- γ , were down-regulated (Fig. 4C and D), suggesting that thermal-treated Ara h 2 could trigger varying degrees of anaphylaxis in mice. Compared with crude Ara h 2, the roasted Ara h 2 showed a comparable allergenicity. However, boiled, glycated, and fried Ara h 2 exhibited a significantly reduced allergenicity, and the frying treatment had the strongest inhibition effect.

4. Discussion

Protein structure is the basis of the function, and changes of allergen conformation are responsible for the alteration of allergenicity (Achouri & Boye, 2013; Philipp, Durga, Krisztina, & Ferdinand, 2011). Typically, heating to temperatures (70–80 °C) able to denature protein, destroys tertiary structure, which is followed by the loss of secondary structure, formation of intra/inter-molecular interactions, and the rearrangement of disulfide bonds (Davis & Williams, 1998). At high temperatures, above 90 °C, chemical modifications, such as the Maillard reaction, which lead to the formation of advanced glycation end products (AGEs), may occur in the food matrix (Hebling, McFarland, Callahan, & Ross, 2013). When peanuts were boiled, low molecular weight proteins were hydrolyzed, and part of Ara h 2 was dissolved into the boiling water, which may result in further linear epitope degradation and masking. To simulate the Maillard reaction, glucose was added when boiling; the results were similar to that of boiling Ara h 2, which verified that there was no significant effect of the Maillard reaction on the characteristics of Ara h 2. The most significant changes of secondary structure were detected in fried Ara h 2, and the frying process showed the strongest inhibitory effect on allergenicity. These results indicate that secondary structure may be a dominant factor in influencing allergenicity, which is consistent with our previous study involving recombinant Ara h 2.02 (Mueller et al., 2011). In roasted peanuts, the formation of dimers and higher order oligomers was detected; however, no obvious changes were observed in the secondary structure and allergenicity of Ara h 2. Presumably, the key conformation of Ara h 2 was not affected by the roasting process.

Anaphylaxis is mediated by the recognition of key epitopes on the allergen and IgE in the serum of the allergic individuals (Burks, 2008), thus the structural integrity of epitopes is of major importance. Generally speaking, thermal-processing denatures proteins, and destroys the conformational epitopes of allergens, leaving behind linear epitopes (Liu, Teodorowicz, van Boekel, Wichers, & Hettinga, 2016). The linear epitopes might be destroyed or masked by protein degradation or modification caused by thermal-treatment. Thus, in the present study, antibodies against 3 core linear epitopes in Ara h 2 were obtained to assess the influence of thermal treatment on the epitope structure. All the epitope binding capacities were significantly reduced after thermal treatment, except epitope 3 which remained its binding capacity after roasting, suggesting that alteration of the protein structure resulted in the disruption of the key epitope structure after thermal treatment. The changes of epitope binding capacity may affect the allergenicity *in vivo*.

The Balb/c mice were primed to study the influence of thermal treatment on the allergenicity of Ara h 2. Under normal conditions, the Th1/Th2 cells are in a relatively balance state in mice (Shin et al., 2015). When body dysfunctions, such as anaphylaxis, occur, it is characterized by a shift in the balance to Th2 cells, thereby

resulting in a series of symptoms, including spleen swelling, increased vascular permeability, and mast cell degranulation. All of these symptoms were observed in Balb/c mice after injection of Ara h 2 in the present study. Meanwhile, in contrast to the PBS control group, the levels of specific IgE, histamines, and Th2-type cytokines were up-regulated, whereas the Th1-type cytokines were down-regulated upon injection with thermal-treated Ara h 2. Compared with crude Ara h 2, boiled, glycated, and fried Ara h 2 exhibited a reduced allergenicity, whereas comparable results were detected in roasted Ara h 2, which is consistent with the results of the epitope binding capacity in the present study, suggesting that the key epitopes were responsible for the allergenicity in mice. Our results were also consistent with the observations of previous studies (Beyer et al., 2001; Davis et al., 2001; Mondoulet et al., 2005), verifying that thermal processing affects the allergenicity of Ara h 2. Moghaddam and co-workers provided the evidence for the enhanced immunogenicity of whole roasted peanut extracts in Balb/c mice, which is probably mediated by the new epitopes formed during the roasting process (Moghaddam et al., 2014). However, roasted Ara h 2 displayed a similar allergenicity with the control in the current study. It is not contradictory but suggests that new epitopes may be formed on the other allergens in the roasting peanuts.

In conclusion, when peanuts were treated in various thermal conditions, modification of the structure of Ara h 2 leads to changes in epitope binding capacity, thereby affecting its allergenicity in Balb/c mice. Further research is needed to evaluate the molecular mechanism of these modifications on the allergens during thermal treatment.

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