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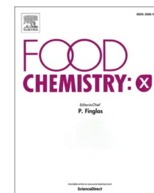

Identification of digestion-resistant peptides in various processed peanut reveals their distinct allergenicity

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A R T I C L E I N F O

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A B S T R A C T

Peanut protein is a significant food allergen that can trigger severe reactions. The allergenicity of peanut protein may be affected by the thermal processing method and matrices, and its anti-digestibility may also change accordingly. This study investigated how three heat treatment techniques affect the allergenicity and digestibility of peanut proteins and compared the differences in anti-digestive peptide segments by Mass spectrometry. Results showed that boiling and frying reduced sensitization, while roasting potentially increased it. After gastric digestion, allergenicity of Ara h 1 decreases due to breakdown of allergenic peptide segments. Hydrophobic regions of Ara h 1 where monomers interact resist degradation. Compared to boiling and frying, roasting can retain more allergenic peptides containing PGQFEDFF, YLQGFSRN, QEERGQRR, HRIFLAGDKD, and KDLAFPGSGE allergenic epitopes even after prolonged digestion. Meanwhile, digestion-resistant epitopes were affected by matrix and thermal treatments. These findings underscore the potential implications for food processing and allergy management strategies.

Introduction

The World Health Organization and the International Federation of Immunological Societies have identified peanuts as one of the eight major food allergens (Lopez-Pedrouso et al., 2020), and have included 18 peanut allergens named Ara h 1 to Ara h 18. Including major allergens such as Ara h1, Ara h2, Ara h3, and Ara h6, these four allergens can react with IgE antibodies in the serum of over 90 % of peanut allergy patients. This recognition underscores the substantial health risks associated with peanut consumption, given that even low doses of peanut allergens can result in severe allergic symptoms (Chizoba Ekezie et al., 2018). Such symptoms may manifest as decreased blood pressure, facial and throat swelling, shock, and potentially life-threatening conditions (Sindher et al., 2022).

As a result, the impact of peanut allergies on public health is considerable. The incidence rate of peanut allergy in the general population is approximately 1.5 %(Greenhawt et al., 2020), yet this prevalence varies significantly based on age, race, and geographic region. For instance, in North America and Europe, around 1 % to 2 % of children are sensitized to peanuts (Lieberman et al., 2018; Roberts et al., 2023). In contrast, a survey conducted by Feng et al. (2022) among Chinese university students found that the prevalence of peanut allergy is approximately 0.6 %. This disparity indicates that regional differences, including variations in peanut processing methods, may significantly influence allergy rates.

Given the potential impact of food processing methods, it is crucial to consider their role in food allergenicity. Thermal processing is one of the most commonly utilized methods, significantly altering food proteins. While much research exploring the effects of processing and sensitization predominantly utilizes isolated, purified allergens (Chang et al., 2022; Li et al., 2021), this singular focus overlooks the multifaceted chemical reactions occurring during processing, such as the Maillard reaction in roasted peanuts. In addition, it fails to account for the inhibitory or stimulatory effects of other matrix components on allergenicity. For instance, lipids may influence immune cell function, potentially leading to the activation and enhancement of protein allergenicity (Li et al., 2021). Water can interact with polar groups on the surface of proteins, such as carboxyl and amino groups, allowing

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proteins to dissolve in water (Tao et al., 2016). Therefore, exploring the structural changes of allergenic proteins within complex matrices—considering multiple factors such as matrix components and processing parameters—is crucial to accurately capturing the ways in which processing alters food allergenicity.

Following heat treatment, proteins may undergo various changes, such as denaturation, peptide bond hydrolysis, aggregation of non-covalent and disulfide bonds, and interactions with other food constituents like lipids and carbohydrates (Shah et al., 2019). These changes can have varying impacts on allergenicity; for example, heat treatment might either diminish protein allergenic sites, thereby reducing allergenicity, or conversely, expose new allergenic sites and enhance allergenicity (Vanga et al., 2016). Cabanillas et al. (2015) showed that boiling peanuts in water can effectively reduce Ara h 1 levels, a major allergen. Similarly, Tao et al. (2016) attributed reduced allergenicity in peanut protein under humid and hot conditions primarily to the depletion of allergenic proteins during boiling. On a different note, Zhang et al. (Zhang et al., 2016)observed a significant reduction in Ara h 1 and Ara h 2 levels in fried peanuts compared to raw peanuts. Comstock et al. (2016) reported extensive alterations in both the physical structure and chemical characteristics of Ara h 1 after frying, rendering it less soluble in water and thus less allergenic. Contrasting findings from Shi et al. (2020a, 2020b) highlighted that roasting, through the Maillard reaction in conjunction with sugar in the peanut matrix, can change the structural integrity of Ara h 1 and potentially enhance its allergenic potential. It can be seen that different heat processing methods have different effects on the allergenicity of peanuts, which may be due to differences in the anti-digestive ability of allergenic proteins in different peanut matrices.

In light of these complexities, evaluating the allergenicity of proteins through simulated *in vitro* digestion has emerged as a valuable approach. Despite their experimental simplicity, *in vitro* models are frequently used to predict *in vivo* digestive outcomes by aligning simulated results with actual *in vivo* data (Bohn et al., 2018). For instance, Di Stasio et al. (2020) observed that the digestibility of walnuts and hazelnuts increased post-roasting, while almonds demonstrated an increase in anti-digestive peptides after roasting. This comparison emphasizes how processing affects both digestibility and allergenicity. Moreover, Dupont and Mackie (2015) revealed that the interaction between allergic proteins and other food components or digestive enzymes can significantly alter the hydrolysis of allergic proteins in the gastrointestinal tract. This finding highlights the importance of the protein's environment in determining its stability and allergenic potential during digestion. In summary, the structural changes in allergens caused by processing, the specific action sites of digestive enzymes, and digestive resistance are critical factors in determining their impact on food allergenicity. Thus, clarifying the regulatory effects of processing methods and digestive systems on allergenicity can guide the appropriate strategies for allergen desensitization processing.

In this study, peanuts were prepared through boiling, roasting, and frying methods. Subsequently, each treated set of samples underwent simulated *in vitro* gastrointestinal digestion experiments. The digestibility of each heat treatment group was compared, and mass spectrometry technology was utilized to characterize the Ara h 1 enzyme cleavage sites and epitopes present in the digestion products. In addition, interactions between the food matrix and allergenic proteins during thermal processing were investigated, changes in allergenicity were examined, characteristic allergenic digestive peptides were identified. Our research findings hope to provide some assistance in elucidating the gastrointestinal sensitization mechanism of peanuts at the molecular level.

Materials and methods

Materials

The digestive enzymes used in simulated digestion include human salivary amylase, porcine pepsin, porcine trypsin, porcine pancreatic alpha amylase, porcine pancreatic lipase, and bovine pancreatic alpha chymotrypsin, which were purchased from Sigma Aldrich (Shanghai, China). SDS-PAGE gel kit, tris glycine running buffer, coomassie brilliant blue staining reagent, SDS-PAGE loading buffer were purchased from Solarbio (Beijing, China). pAb Rabbit anti Ara h 1 was purchased from Indoor Biotech (Beijing, China). Goat Anti Rabbit IgG-AP and Goat Anti Human IgE (ε - chain specific) - HRP were purchased from Sigma Aldrich (Shanghai, China). The serum of peanut allergy patients was provided by the First Hospital of Hebei Medical University. TMB Two-Component Substrate solution and BCIP/NBT kit were purchased from Solarbio (Beijing, China). LC-MS grade ultrapure water, LC-MS grade acetonitrile, and LC-MS grade formic acid were purchased from Thermo Fisher Scientific (Shanghai, China). The EASY-nLC™ 1200 NA Upgraded UHPLC and Q Exactive™ HF-X Mass spectrometer was purchased from Thermo Scientific (California, US). Peanuts and goldfish sunflower seed oil were purchased from local supermarkets (Shijiazhuang, China). Unless otherwise specified, all reagents and chemicals are analytical grade.

Sample preparation

According to common cooking methods for peanuts, divide them into four groups. Unprocessed group, do not perform any heat treatment. Boiling group, wait for the water temperature to reach 100 °C to put the peanuts in, and remove the peanuts from the 100 °C water after 25 min. Roasting group, wait for the oven temperature to reach 180 °C to put the peanuts in, and remove the peanuts from the 180 °C oven after 5 min. Fried group, wait for the oil temperature to reach 170 °C to put the peanuts in, and remove the peanuts from the 170 °C oil after 2 min. Peel the peanuts from the above four groups, freeze them in liquid nitrogen, and grind them into powder. Determine the total protein content of each group using the Kjeldahl method with a nitrogen conversion coefficient of 5.46. Each group of sample powders is stored at −20 °C.

Simulate digestion

Refer to Rao et al. (2020) experimental method and make slight modifications. Salivary amylase (80.29 U/g carbohydrate) was dissolved in Simulated Saliva Fluid (SSF, prepare a 0.15 M NaCl, 3 mM urea solution. Adjust to pH 6.9) and preheated at 37 °C. Weigh 0.5 g of the sample and place it in a stoppered glass tube, then add SSF and water in a ratio of food: SSF: water (total amount = "chew") 1:0.32:0.7. Prepare a total of 12 test tubes according to the above method. The "chew" was then stirred in a 37 °C water bath for 1 min, after which the digestion product was transferred to a centrifuge tube and centrifuged at 13000*g* for 10 min to obtain the supernatant for storage at −20 °C and the remaining 11 test tubes are ready for subsequent gastrointestinal digestion experiments.

Pepsin (16.1 U/mg substrate protein) was dissolved in Simulated Gastric Fluid (SGF, prepare a 0.1 M HCl; 0.15 M NaCl; 3 mM $CaCl_2$; 0.9 mM NaH_2PO_4; 16 mM KCl solution.) and preheated to 37 °C. Add SGF to the remaining 11 test tubes after Oral digestion, with a ratio of "Chew": SGF (total = "chyme") 1:1. Adjust the pH to 2.0 and shake in a water bath. And the pH was adjusted to 2.0 before shaking in a water bath. Then take out 1 test tube at time points of 0, 5, 15, 30, 60, and 120 min, adjust the pH to 7 using a 0.5 M $NaHCO_3$ solution to terminate digestion. The products at different digestion time points were transferred to centrifuge tubes, centrifuged at 13000*g* for 10 min, and the supernatant was stored at −20 °C for further analysis. The remaining 5 test tubes were subjected to intestinal digestion experiments after 120 min of

gastric digestion.

Alpha-amylase 0.49 U/mg carbohydrate substrate; trypsin 34.5 U/mg protein substrate and chymotrypsin 11.8 U/mg protein substrate were dissolved in Pancreatic Mix Solution (PMS, prepare a 0.15 M NaCl, 3 mM urea solution. Adjust to pH 6.9) and preheated at 37 °C. Following 120 min of digestion in the stomach, PMS and Hepatic Mix Solution (HMS, prepare a 12.5 mM sodium taurocholate, 12.5 mM sodium glycodeoxycholate, 146 mM NaCl, 2.6 mM $CaCl_2$, 4.8 mM KCl, 4 mM Cholesterol solution) were added to the product at a ratio of "Chyme": PMS: HMS 1: 0.5: 0.17, and the product was agitated in a water bath at 37 °C. To terminate digestion, Phenylmethanesulfonyl fluoride solution (PMSF, 0.1 M in ethanol) was added at time points of 5, 15, 30, 60 and 120 min and the PMSF addition amount is 4 μL/ mg protein substrate. The products at different digestion time points were transferred to a centrifuge tube, centrifuged at 13000*g* for 10 min, and the supernatant was stored at −20 °C for further analysis.

SDS-page

Adjust the concentration of peanut protein solution obtained at different time points during the digestion process to 4 μg/μL. Subsequently, protein loading buffer was added to the protein solution in a 4:1 ratio, ensuring thorough mixing, and then the mixture was incubated in boiling water for 5 min. A 12 % separation gel and a 5 % concentrated gel were prepared for SDS-PAGE electrophoresis. After the sample is cooled, load it onto the gel, 12.5 μL per lane. After the concentration gel electrophoresis voltage was 80 V for 20 min, the protein entered the separation gel, and the voltage rose to 120 V for 60 min before the electrophoresis ended. Then, the gel was stained in Coomassie brilliant blue R-250 staining solution for 30 min before being transferred to a decolorization solution until the background was clear. Finally, photographs were captured for documentation purposes.

Immunoblotting

After completing SDS-PAGE electrophoresis, proceed with immunoblotting. Closely adhere the protein gel to the NC membrane, assemble it into the transfer cassette, place the cassette into the transfer tank, set the current to 300 mA, immerse the transfer tank in an ice water bath, and transfer for 90 min. After the transfer is completed, the membrane was sealed with 5 % skim milk powder for 2 h, then washed three times with TBS-T (Tris Buffered Saline-Tween-20, Dissolve 2.42 g Tris base and 8 g NaCl in 1 L deionized water, adjust the pH to 7.6 with concentrated HCl and add 0.5 mL Tween-20 to mix evenly.), each time for 15 min. The primary antibody (pAb Rabbit anti Ara h 1) was diluted with TBS (Tris Buffered Saline, Dissolve 2.42 g Tris base and 8 g NaCl in 1 L deionized water, adjust the pH to 7.6 with concentrated HCl.) at a ratio of 1:1000, and the membrane was immersed in the primary antibody solution for overnight incubation at 4 °C. After the primary antibody incubation, the membrane was washed four times with TBS-T, each time for 15 min. Following the washing of the membrane, the secondary antibody (Goat Anti-Rabbit IgG-AP) was diluted with TBS at a ratio of 1:5000, and the membrane was immersed in the secondary antibody solution for an hour at room temperature. After the secondary antibody incubation, the membrane was washed four times with TBS-T, each time for 15 min. Following the washing of the membrane, it was repeatedly rinsed with BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate) /NBT (Nitroblue tetrazolium chloride) ALP Color Development Kit until the bands were clearly visible, then photos were taken for record.

Determination of specific IgE responses to the whole peanut protein extracts

This experiment employed indirect ELISA to assess the sensitizing capacity of digestion products subjected to various treatments. Protein solutions from each group were diluted to 0.2 mg/mL with a coating solution (15 mM sodium carbonate and 35 mM sodium bicarbonate), then added to a 96-well ELISA plate at 100 μL per well and incubated overnight at 4 °C. Following the coating process, the well contents were discarded and each well was filled with 300 μL of a sealing solution (5 % skim milk powder) and sealed at 37 °C for 2 h. After sealing, the well contents were discarded again and the wells were washed three times with PBS-T (Phosphate Buffered Saline - Tween-20, weigh 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4, and dissolve them in 1000 mL of deionized water. Adjust the solution to pH 7.4 with concentrated HCl and add 0.5 mL Tween-20 to mix evenly.) for 5 min each time. A serum pool was created from nine patients with peanut allergies by diluting their serum with PBS (Phosphate Buffered Saline, weigh 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4, and dissolve them in 1000 mL of deionized water. Adjust the solution to pH 7.4 with concentrated HCl.) at a ratio of 1:10. The pooled human serum was prepared by combining equal proportions of individual sera. This diluted serum was then added to the 96-well plate at 100 μL per well and incubated at 37 °C for 2 h. After the incubation, the plate was washed four times, each time for 5 min. The secondary antibody (Goat Anti-Human IgE (ε-chain specific)-HRP) was diluted with PBS at a ratio of 1:5000 and added to the plate at 100 μL per well, followed by an incubation at 37 °C for 1 h. Following the incubation, the plate was washed four times, each time for 5 min. Each well received 100 μL of TMB chromogenic reagent and was incubated in the dark at 37 °C for 15 min. Once the time was up, each well was treated with 50 μL of a stop solution (2 M sulfuric acid) and the OD_{450} of each well was measured using an ELISA reader. All experiments were approved by the Hebei University of Science and Technology. The human ethical approval certificate No. HEBUST-2020032001.

LC-MS/MS analysis

LC analysis

The digestive products of each group at 5, 30, 60 and 120 min of gastric digestion were slowly loaded to the C18 desalting column, washed with washing buffer (0.1 % formic acid, 3 % acetonitrile) 3 times, then added elution buffer (0.1 % formic acid, 70 % acetonitrile). The eluents of each sample were collected and lyophilized.

Mobile phase A (100 % water, 0.1 % formic acid) and B solution (80 % acetonitrile, 0.1 % formic acid) were prepared. The lyophilized powder was dissolved in 10 μL of solution A, centrifuged at 14000*g* for 20 min at 4 °C, and 1 μg of sample was injected into EASY-nLCTM 1200 nano-upgraded UHPLE system with a home-made C18 Nano-Trap column (4.5 cm × 75 μm, 3 μm). Peptides were separated in a home-made analytical column (15 cm × 150 μm, 1.9 μm), using a linear gradient elution as listed in supplementary information Table S-1.

MS analysis

The separated peptides were analyzed by Q ExactiveTM HF-X mass spectrometer, with ion source of Nanospray Flex™ (ESI), spray voltage of 2.1 kV and ion transport capillary temperature of 320 °C. Full scan ranges from *m/z* 350 to 1500 with resolution of 60,000 (at m/z 200), an automatic gain control (AGC) target value was 3×10^6 and a maximum ion injection time was 20 ms. The top 40 precursors of the highest abundant in the full scan were selected and fragmented by higher energy collisional dissociation (HCD) and analyzed in MS/MS, where resolution was 15,000 (at m/z 200), the AGC target value was 1×10^5, the maximum ion injection time was 45 ms, a normalized collision energy of 27 %, an intensity threshold of 2.2×10^4, and the dynamic exclusion parameter of 20 s.

Statistical analysis

GraphPad Prism 9.5 was used for mapping, and SPSS 26 was utilized for significance analysis. The search results from 1,841,512-Arachis_hypogaea. fasta (1131 sequences) in the Uniprot and PDB protein databases formed the basis of this work. The 3D protein structure diagram

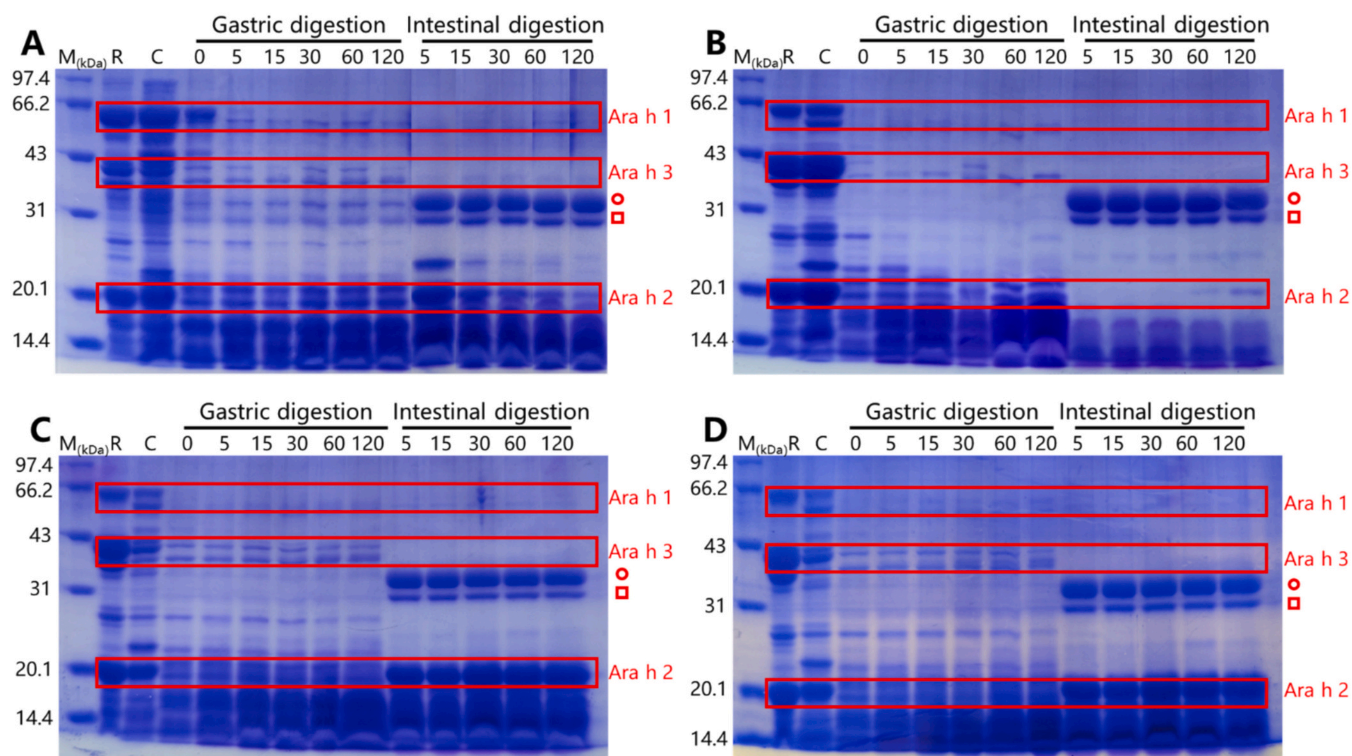

Fig. 1. Changes in the content of peanut protein during *in vitro* digestion. (A) Unprocessed group; (B) Boiling group; (C) Roasting group; (D) Fried group. Lane M is the Marker; Lane R is the undigested peanut whole protein control group; Lane C is a sample for oral chewing; The numbers for gastric digestion and intestinal digestion in the lane represent the digestion time of the corresponding area, measured in min. ○ chymotrypsin, □ trypsin.

was drawn using PyMol. IEDB and Expasy PeptideCutter were used to predict allergenic epitopes and cleavage sites.

Results and discussion

The effect of thermal processing on the digestibility of peanut protein

Simulated *in vitro* digestion experiments were conducted on peanut substrates under various treatment conditions, and the resulting digestion products at different time points were collected and analyzed using SDS-PAGE. The experimental findings are depicted in Fig. 1. The untreated peanut sample (Fig. 1A, Lane R) exhibited a diverse array of proteins with varying molecular weights, primarily distributed within the range of 66–30 kDa and below 20 kDa. In contrast, the protein bands observed in the thermal processing group (Fig. 1B-D, Lane R) were notably fewer compared to those in the untreated group. Tian et al. (2018) believed that during the boiling process of peanuts, proteins might leach into the cooking water leading to a reduction in protein content within the peanut matrix. Mills et al. (2009), on the other hand, propose that roasting and frying can induce protein aggregation or modification within food matrices; these alterations can result in decreased protein solubility and subsequently lead to reduced protein content within extraction solutions.

As shown in Fig. 1, during the process of digestion, peanut protein undergoes gradual degradation. Following oral mastication (Fig. 1A-D, Lane C), the Ara h 1 band will split in half and the large molecular

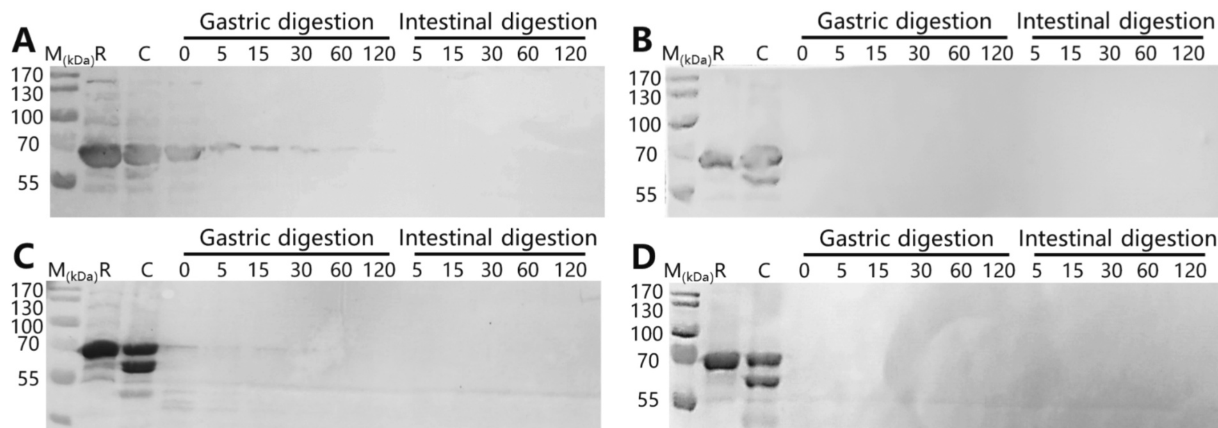

Fig. 2. Changes in sensitization of Ara h 1 during *in vitro* digestion. (A) Unprocessed group; (B) Boiling group; (C) Roasting group; (D) Fried group. Lane M represents the Marker, Lane R denotes the undigested peanut whole protein control group. Lane C is a sample for oral chewing. The numbers for gastric digestion and intestinal digestion in the lane represent the digestion time of the corresponding area, measured in min.

protein band will decrease, resulting in the appearance of smaller molecular protein bands. This phenomenon may be attributed to the breakdown of the starch network structure within the peanut matrix by salivary amylase, leading to the dissociation of originally polymerized proteins into independent entities. As shown in Fig. 1A, after mastication, the peanut matrices are introduced into the stomach. Upon exposure to gastric protease, Ara h 1 within the peanut matrix undergoes rapid hydrolysis, resulting in a noticeable lightening of color bands (Fig. 1A, Gastric digestion Lane 0–120). However, complete decomposition by gastric protease remains unattainable. Bavaro et al. (2018) posit that this phenomenon may be attributed to the trimeric structure of Ara h 1, which impedes its full degradation within the stomach. The digestion rate of Ara h 1 was observed to be faster in the boiled group compared to the remaining three groups. The infrared spectrum scanning results (supplementary information Table S-2) indicate an increase in the content of α - helices in the boiled sample. Since the content of alpha helices positively correlates with the *in vitro* digestibility of proteins, the proteins become more digestible after boiling (X. Zhang & Yu, 2012). In contrast, Ara h 2 and Ara h 3 exhibited strong resistance to digestion in the roasting and frying groups. Upon entering the intestine, gastric digestion products were found to further decompose Ara h 3 while maintaining high content of Ara h 2.

Due to the strong allergenicity of Ara h 1, it is capable of binding to IgE in 55–95 % of allergic patients and eliciting allergic reactions (Warmenhoven et al., 2023). However, owing to its trimeric structure, Ara h 1 retains a certain degree of resistance to gastric digestion (Bavaro et al., 2018), potentially augmenting its allergenic potential. In addition, after 120 min of gastric digestion, the Ara h 1 protein bands appeared lighter in each group (Fig. 1A-D, Gastric digestion Lane 120). Upon entering the intestine for further digestion (Fig. 1A-D, Intestinal digestion Lane 5), the disappearance of the Ara h 1 protein bands in each group indicates a significant reduction in Ara h 1 levels compared to the initial stages of both gastric and intestinal digestion processes. Therefore, in the subsequent experiments, we performed immunoblotting analyses on Ara h 1 within the digested samples from each group, with a focus on delineating changes in its allergenic properties throughout the digestive process.

Identification of allergenicity of Ara h 1 after digestion

Immunoblotting experiments were conducted on digestion products at various time points, and the findings are depicted in Fig. 2. The untreated group (Fig. 2 A, Lane R) exhibited a greater presence of Ara h 1 immune bands within the peanut matrix, displaying a broader molecular weight distribution. The 64 kDa band emerged as the primary sensitization band for Ara h 1. Subsequent to heat treatment, there was a reduction in the number of Ara h 1 bands in each group (Fig. 2 B—D, Lane R) compared to the untreated group. Notably, the boiled group displayed the fewest bands and appeared with a lighter coloration. This observation can be attributed to boiling inducing structural changes in Ara h 1 leading to its formation into rod-shaped branching aggregates which diminish its IgE binding capacity and weaken its sensitization potential (Blanc et al., 2011). On the other hand, the content of the main allergen Arah 1 in boiled peanuts was significantly reduced, and allergens were detected in the cooking water. This research result indicates that allergenic proteins in peanuts can be dissolved by boiling water, thereby reducing the allergenicity of peanuts (Cabanillas et al., 2015; Tian et al., 2018). The Ara h 1 band in the baking group showed the darkest color, indicating increased sensitivity. On the one hand, the Maillard reaction between allergenic proteins and reducing sugars during the baking process can alter their enzyme binding sites, thereby affecting their degradation rate during digestion (Y. F. Shi et al. 2020). Moreover, certain amino acids in Ara h 1 undergo chemical modifications and disulfide bond rearrangements, leading to local structural changes and enhancing their allergenicity (Y. Zhang et al., 2023). In each treatment group, two distinct immune bands of Ara h 1 were observed in lane C, with molecular weights approximately 64 kDa and 50 kDa. A new immune band near 50 kDa emerged due to the breakdown of the starch network structure by salivary amylase during SDS-PAGE, causing polymerized proteins to dissociate into individual entities. Wang et al. (2022) propose that the stable trimeric complex structure of Ara h 1 allows large fragments containing multiple intact antibody binding epitopes to maintain their original allergenicity, resulting in the appearance of new Ara h 1 immune bands around 50 kDa.

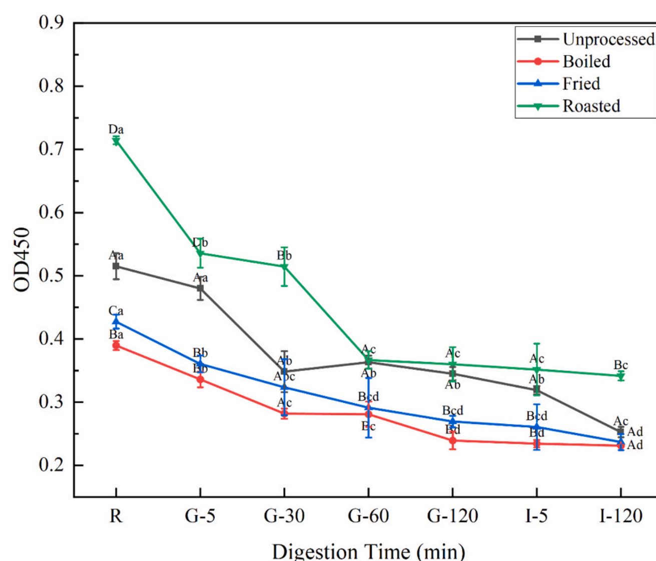

Fig. 3. Effect of *in vitro* digestion on the binding ability of peanut sensitizing protein IgE. The X-axis represents different digestion times, R represents undigested, G-5 represents gastric digestion for 5 min, G-30 represents gastric digestion for 30 min, G-60 represents gastric digestion for 60 min, G-120 represents gastric digestion for 120 min, I-5 represents intestinal digestion for 5 min, and I-120 represents intestinal digestion for 120 min. Different letters represent significant differences ($p < 0.05$), with lowercase letters indicating within groups and uppercase letters indicating between groups.

As depicted in Fig. 2A, the number of immune bands of Ara h 1 gradually decreases and the color becomes lighter with prolonged digestion time. However, immune bands are still observable after 120 min of gastric digestion. Upon completion of gastric digestion, no immune bands of Ara h 1 were detected in the intestine, indicating that Ara h 1 can maintain a certain degree of sensitization under prolonged action of gastric protease and is only digested and decomposed in the intestine, losing its sensitization ability. Following heat treatment, Ara h 1 is easily decomposed by gastric protease. The roasted group showed no detectable immune bands after 60 min of gastric digestion, while the boiled and fried groups exhibited minimal detection when entering the stomach post-chewing. This suggests that both boiling and frying treatments render Ara h 1 more easily digestible. During the boiling process, water molecules enter between protein molecules, causing them to lose their higher-order structures and gradually unfold highly folded regions, exposing more enzyme cleavage sites and thereby reducing their digestive resistance (Mattison et al., 2014). Beyer et al. (2001) found that the trimeric structure of Ara h 1 was almost undetectable in cooked and fried peanuts, while the quaternary structure of allergenic proteins can effectively protect their allergenic epitopes from being broken down by digestive enzymes. This indicates that frying and boiling can prevent the aggregation of Ara h 1 protein monomers to form stable polymers, thereby reducing their digestive resistance and allergenicity. Research demonstrated that boiling peanuts in water exposes more enzyme cleavage sites on proteins, thereby reducing their anti-digestion ability.

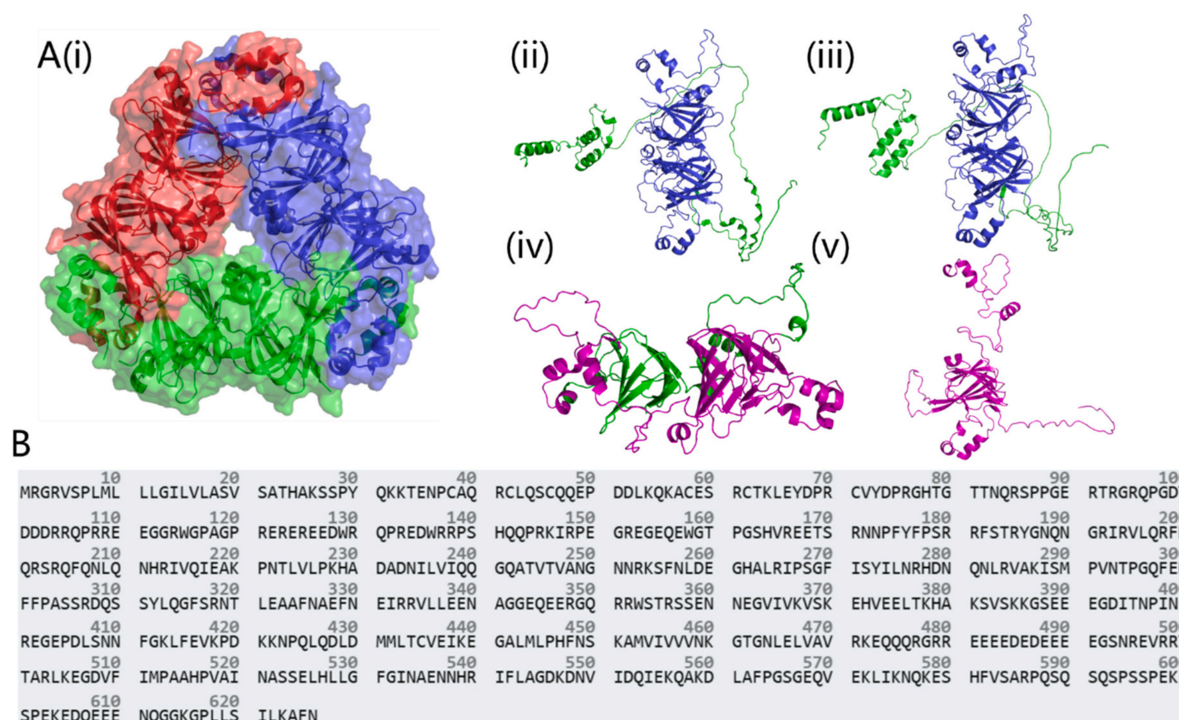

Fig. 4. 3D structure (A) and amino acid sequence (B) of peanut allergenic protein Ara h 1. (i) The 3D structural diagram of Ara h 1 trimer (Uniprot ID: P43238) and (ii ~ v) are the 3D structural diagrams of the four subtypes of Ara h 1 detected in the experimental samples (ii, Uniprot ID: E5G076; iii, Uniprot ID: B3IXL2; iv, Uniprot ID: Q6PSU4; v, Uniprot ID: Q6PSU5).

Effects of different thermal processing on the binding ability of sensitizing protein IgE in peanut matrix

ELISA is the predominant method for detecting and quantifying food allergens (Peng et al., 2015). In this study, a serum pool was formed using serum from nine peanut allergy patients, and the allergenicity of peanut total protein was assessed under different treatment methods and digestion times. The results are presented in Fig. 3. The OD value of the roasted group exceeded that of the other three groups, and even after gastrointestinal digestion, its sensitization capacity remained higher than that of the other treatment groups. Sensitization levels were lower in the fried and boiled groups compared to the unprocessed group; however, after gastrointestinal digestion, sensitization levels became comparable to those of the unprocessed group. This phenomenon can be attributed to factors such as damage to secondary and tertiary structures of peanut protein following heat processing, formation of protein aggregates during heating, protein degradation post-gastrointestinal digestion, and alterations in linear epitopes. During the high-temperature processing of peanuts, allergenic proteins undergo Maillard reactions with sugars or cross-linking polymerization between different allergenic proteins, leading to the formation of complex polymers. These polymers often exhibit heightened allergenicity and, due to increased steric hindrance, further conceal the cleavage sites of digestive enzymes, resulting in enhanced resistance to digestion. Following gastrointestinal digestion, they retain a certain degree of allergenic potential (Xi & Shi, 2016). The boiling and frying processes involve lower temperatures than roasting and use different heating mediums. During thermal processing, some proteins dissolve in water or oil, thereby reducing their sensitizing capacity. T. Zhang et al. (2018) found that during the boiling process, the α - helix and irregular curl content of peanut allergenic protein increased, while the β - fold content decreased, which is consistent with our experimental results (Supplementary Information Table S-2). This indicates that boiling water can alter the secondary structure of proteins, exposing or degrading some amino acid residues, thereby reducing the IgE binding ability of allergenic proteins.

Table 1
IgE binding linear epitope of Ara h 1.

serial number	linear epitope	position
1	KSSPYQKK	26–33
2	QEPDDLKQKA	48–57
3	EYDPRCVY	66–73
4	ERTRGRQP	90–97
5	GDYDDDRR	98–105
6	RREEGGRW	108–115
7	EREEDWRQ	124–131
8	EDWRRPSHQQ	134–143
9	PRKIRPEG	144–151
10	PGQFEDFF	295–302
11	YLQGFSRN	312–319
12	FNAEFNEIRR	325–334
13	QEERGQRR	345–352
14	DITNPINLRE	392–402
15	NNFGKLFEVK	409–418
16	GNLELV	463–468
17	RRYTARLKEG	498–507
18	ELHLLGFGIN	525–534
19	HRIFLAGDKD	539–548
20	IDQIEKQAKD	551–560
21	KDLAFPGSGE	559–568
22	KESHFVSARP	578–587
23	EKESPEKE	598–605

Frying is a high-temperature, short-duration thermal processing method. In terms of microstructure, frying can cause damage to the cell walls of peanut kernels, leading to the leakage of intracellular substances (Meng et al., 2019). Within a short frying period (170 °C, 2 min), deep frying can induce peanut protein cross-linking, forming high-molecular-weight aggregates, thereby reducing the content of the allergic protein, and reduce the binding rate of its IgE linear epitope (Shi et al., 2017).

Due to the substantial disparity in allergenicity between the boiling and roasting groups, the digestion products from these two groups were

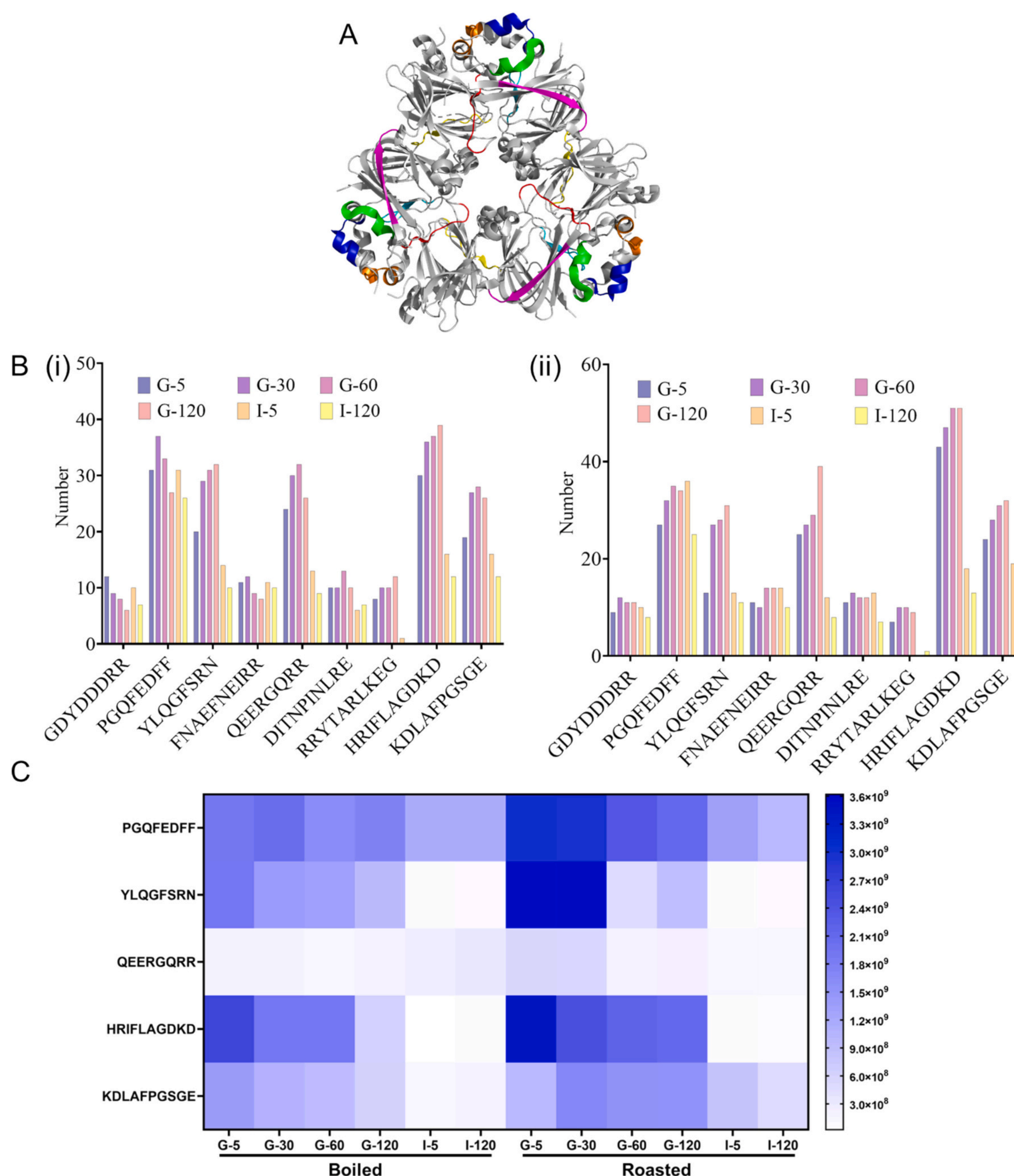

Fig. 5. Changes of Ara h 1's anti-digestive sensitizing peptides during gastrointestinal digestion. (A) is allergenic epitope for Ara h 1 were mapped on to the 3D structure. (B) is the quantity change of different epitope sensitizing peptide segments during digestion, (i) boiling group, (ii) roasting group; C is the abundance change of different epitope sensitizing peptide segments during digestion, and the darker the color, the higher the abundance.

chosen for mass spectrometry analysis in order to investigate alterations in Ara h 1 allergenic peptide segments within the peanut matrix following distinct heat treatments during digestion.

Effect of thermal processing on Ara h 1 sensitized epitopes

Mass spectrometry data compared with the Uniprot database revealed four subtypes of Ara h 1 detected in this experiment (Fig. 4A ii ~ v). The three-dimensional structure (Fig. 4A) and amino acid sequence (Fig. 4B) were obtained by searching the PDB protein database.

Upon comparing the mass spectrometry results in Table 1, a total of 9 complete IgE binding epitopes were identified in this experiment: ^{98}GDYDDDRR^{105}, ^{292}PGQFEDFF^{299}, ^{309}YLQGFSRN^{316}, ^{322}FNAEFNEIRR^{331}, ^{342}QEERGQRR^{349}, ^{390}DITNPINLRE^{399}, ^{498}RRYTARLKEG^{507}, ^{539}HRIFLAGDKD^{548}, and ^{559}KDLAFPGSGE^{568}. The specific positions of these sensitizing epitopes within Ara h 1 are illustrated in Fig. 5A. It is evident from the figure. That these sensitizing epitopes are predominantly concentrated at the subunit binding sites of the trimer and primarily manifest as alpha helix and beta fold structures. However, thermal processing may induce varying degrees of alteration to their secondary and tertiary structures. Y. Shi et al. (2020) conducted an analysis on the secondary structure of Ara h 1 using circular dichroism and observed changes in the content of conformational units following dry heat treatment. As dry heat time increases, there is a significant decrease in alpha helix content alongside a notable increase in beta fold

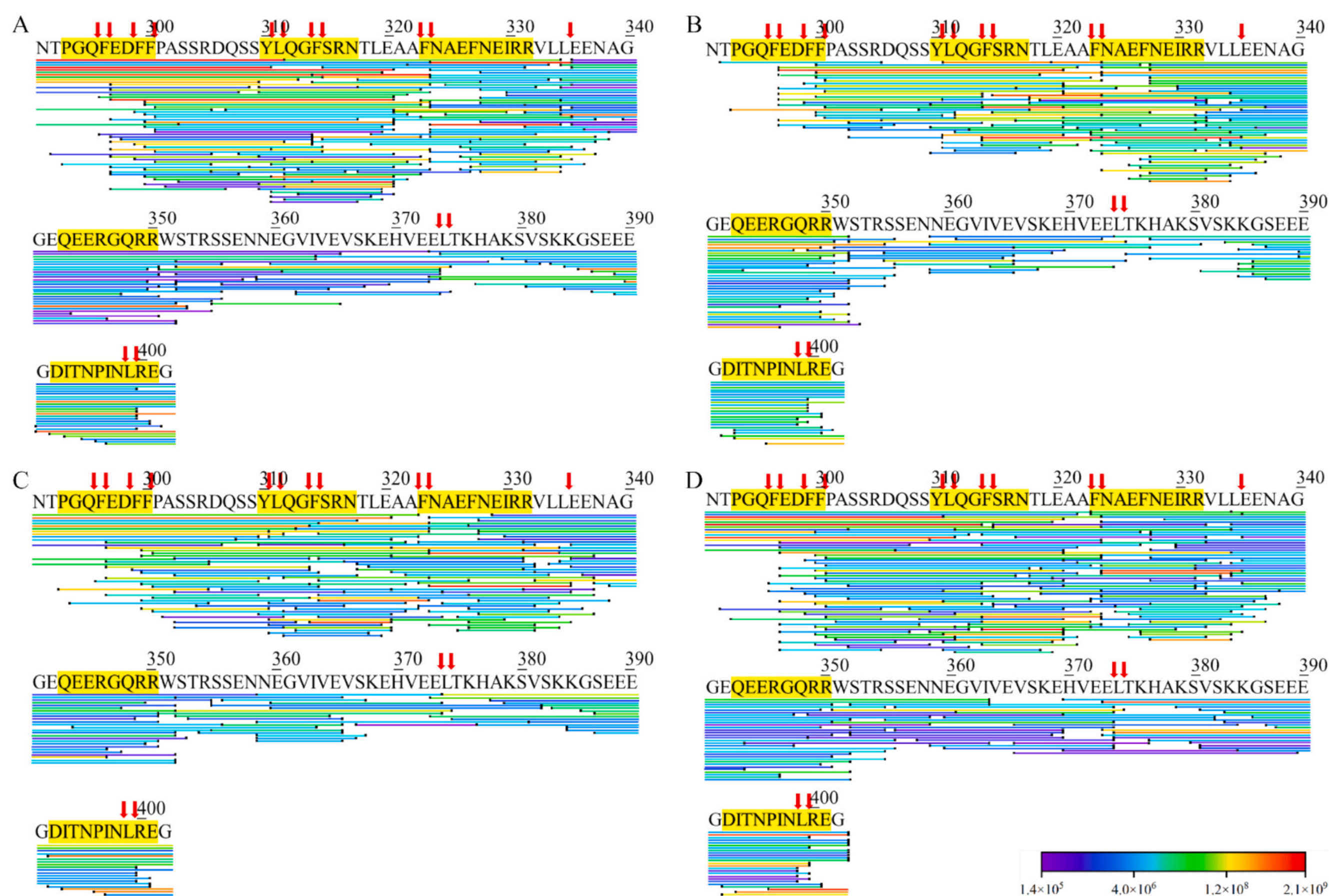

Fig. 6. Partial peptide profile of Ara h 1 after gastric digestion. A and B represent 5 and 60 min of gastric digestion in the boiling group, while C and D represent 5 and 60 min of gastric digestion in the roasting group. The linear IgE epitopes are highlighted in yellow. Red arrow: Predicted gastric protease cleavage site. The line segment represents the relative abundance of peptides calculated using spectral counting, and the gradient from blue to red indicates an increase in abundance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

content. Additionally, Y. Zhang et al. (2024) investigated structural changes within Ara h 1 through UV and fluorescence spectra analyses as well as molecular dynamics simulations. Their findings revealed an increase in UV absorbance post-roasting treatment which suggests exposure of hidden amino acids within the protein leading to alterations in its tertiary structure. Roasting induced diverse modifications to the overall allergen structure thereby impacting both the structure and electrostatic potential of IgE epitopes.

Based on the data presented in Fig. 5B, it is evident that there is a higher abundance of peptide segments containing the epitopes ^{292}PGQFEDFF^{299}, ^{309}YLQGFSRN^{316}, ^{342}QEERGQRR^{349}, ^{539}HRIFLAGDKD^{548}, and ^{559}KDLAFPGSGE^{568}. Analysis of the boiling group (Fig. 5Bi) reveals an initial increase followed by a decrease in the number of these peptide segments as digestion time progresses. The observed increase suggests that longer peptide segments are initially hydrolyzed into shorter peptides during early stages of digestion, leading to an overall rise in their abundance. However, prolonged gastric protease activity results in further decomposition and digestion of these short peptides containing complete allergenic epitopes, ultimately leading to a reduction in their abundance. The number of allergenic peptide segments in the roasting group (Fig. 5Bii) was consistently higher than that in the boiling group, and increased progressively with prolonged digestion time. Fig. 5C presents an abundance heatmap containing allergenic epitope peptide segments. It is evident from the figure that the abundance of peptide segments decreases gradually with digestion time, while the roasting group exhibits generally higher abundance compared to the boiling group. This suggests an enhanced anti-digestion ability of Ara h 1 after roasting treatment, leading to reduced hydrolysis by gastric protease and consequently increasing its sensitization potential. Di Stasio et al. (2017) conducted LC-MS/MS analysis of the gastrointestinal digestive products of raw peanuts *in vitro*, and the results showed that the number of peptides matching Ara h1 was small, indicating that Ara h1 in raw peanuts would be completely degraded after gastrointestinal digestion *in vitro*. It can be seen that in the process of hot processing, the food substrate interacts with the protein and reacts chemically, which greatly changes the original digestibility and sensitization of the sensitized protein.

As depicted in Fig. 5, the allergenic peptide segments are predominantly concentrated within the range of ^{290}N ~ G^{402}. Consequently, we have computed the relative abundance of all peptide segments falling within this range as a function of digestion time, as illustrated in Fig. 6. The theoretical restriction sites of pepsin were found in the Expasy PeptideCutter website and marked with red arrows in the figure. By comparing the theoretical cleavage sites of pepsin with the actual cleavage sites, we identified specific cleavage sites. For instance, peptides broken at theoretical cleavage sites such as Q^{294}, D^{298} and N^{398} were rarely observed in the actual digestion process, while actual cleavage sites such as A^{321} and N^{328} did not align with the theoretical predictions. The alterations in these enzyme cleavage sites suggest that various thermal processing methods can influence the digestibility of allergenic proteins, subsequently modifying the types and abundance of anti-digestive allergenic peptides. Furthermore, our analysis revealed that peptide breakage patterns in the boiling group (Fig. 6A, B) exhibited greater disorder compared to those in the roasting group (Fig. 6C, D). Additionally, there were more peptide breakage events within the IgE linear epitope in the boiling group than in the roasting group - for

example F^{314} and S^{315} - suggesting that proteins sensitized in the boiling group were more susceptible to digestion and decomposition, ultimately reducing sensitization levels.

From the graph, it is evident that with prolonged digestion time, there is a varying decrease in both the number and abundance of peptide segments, including those containing complete allergenic epitopes. This suggests that after gastric protease digestion, the allergenicity of Ara h 1 diminishes. After 5 min of gastric digestion, the water boiling group exhibited a lower number and abundance of allergenic peptide segments containing complete allergenic epitopes compared to the roasting group. Following 60 min of digestion, it was observed that despite varying degrees of decrease in the number and abundance of peptide segments across different treatment groups, the roasting group still displayed higher levels of allergenic peptide segments containing complete allergenic epitopes than the water boiling group. This suggests that roasting treatment enhances the resistance of Ara h 1 to digestion, allowing it to maintain a certain degree of allergenic ability during prolonged digestion. These experimental findings are consistent with previous results obtained from immunoblotting and ELISA assays.

Conclusion

We conducted an investigation into the impact of common thermal processing methods (boiling, roasting, and frying) on the allergenicity of peanut allergen Ara h 1. Boiling resulted in a reduction of protein content within the peanut matrix, while roasting and frying led to a decrease in the rate of protein extraction from the peanut matrix. Following boiling and frying treatments, Ara h 1 demonstrated increased susceptibility to enzymatic digestion, resulting in a decrease in its allergenicity. Conversely, roasting treatment enhanced the resistance of Ara h 1 to digestion, thereby increasing its allergenic potential by enabling it to withstand prolonged gastric protease action. Mass spectrometry revealed that most of the IgE epitopes of Ara h 1 are located in the hydrophobic region of monomer interactions, making it resistant to enzymatic and thermal degradation. After gastric digestion, the sensitization of Ara h 1 is decreased due to the breakdown of sensitizing peptide segments. However, the abundance and number of anti-digestive allergy peptides containing allergenic epitopes (PGQFEDFF, YLQGFSRN, QEERGQRR, HRIFLAGDKD, and KDLAFPGSGE) in roasted peanuts were higher than in boiled peanuts, even after prolonged digestion. This article only discusses Ara h 1, which has a high content in peanuts, and does not involve other allergenic proteins with high allergenicity and strong anti-digestion ability. The exploration of the changes in the advanced structure of Ara h 1 during the processing is called superficial. In the future, we will continue to conduct more in-depth and comprehensive research and discussions on this issue. Moving forward, our research will continue to explore other allergenic proteins within the peanut matrix with the aim of offering new perspectives on understanding changes in allergens during food processing and improving allergy risk management and detection methods.

CRediT authorship contribution statement

Tianyu Hou: Writing – original draft, Methodology, Investigation. **Xiaoluan Li:** Resources. **Li Niu:** Methodology. **Huan Rao:** Supervision, Project administration. **Jianxiong Hao:** Supervision. **Dandan Zhao:** Writing – review & editing. **Xueqiang Lui:** Writing – review & editing. **Wenhui Fu:** Resources.

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.

The data that has been used is confidential.

Data availability

No data was used for the research described in the article.

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Supplementary data

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