



Changes in IgE binding capacity, structure, physicochemical properties of peanuts through fermentation with *Bacillus natto* and *Lactobacillus plantarum* along with autoclave pretreatment

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ABSTRACT

This study investigated the immunoglobulin E (IgE) binding capacity, structure, and physicochemical properties of raw crushed peanut (RCP) after fermentation with *Lactobacillus plantarum* and *Bacillus natto* along with autoclaved pretreatment. SDS-PAGE showed the disappearance of partial protein (>45 kDa) in autoclaved peanuts (ACP) and fermented autoclaved peanuts with *L. plantarum* (LP), and of majority protein (>14.4 kDa) in fermented autoclaved peanuts with *B. natto* (BN) or a mixture of *L. plantarum* and *B. natto* (LPBN). Structural analysis revealed protein-aggregation and protein-unfolding in autoclaved and fermented peanuts, respectively. Indirect ELISA demonstrated that the IgE binding capacities in ACP, LP, BN and LPBN were reduced by 11.3%, 20.6%, 78.7% and 90.2%, respectively, compared to RCP. LPBN showed the lowest IgE binding capacity due to the highest masking and destruction of epitopes and exhibited the desirable physicochemical properties simultaneously. Mixed strain fermentation has the potential to produce hypoallergenic peanut products.

1. Introduction

Peanuts contain 44%–45% fat, 24%–36% protein, 20% sugar, and many vitamins, fiber, and minerals, providing many health functions, such as preventing cardiovascular disease and adiposity (Pi, et al., 2019). However, peanuts are one of the “big eight” food allergens that account for 90% of food allergic reactions (Chizoba Ekezie, Cheng, & Sun, 2018; Pi, et al., 2019; Rui, et al., 2019). Peanut allergies are the first most common in food allergy, affecting 1%–2% of the world’s population (Pi, et al., 2019; Pi, Yang, Sun, Wang, et al., 2021). The prevalence of peanut allergies among children is approximately 2%, and it continues to increase (Savage & Johns, 2015), probably due to the lifetime persistence of peanut allergies and the ubiquitous use of peanuts (Pi, Fu et al., 2021; Pi, Yang, Sun, Wang, et al., 2021). In addition, peanut allergies account for approximately 20% of food allergies (Iweala, Choudhary, & Commings, 2018) and could cause anaphylactic deaths which account for 59% of death induced by total food allergy (Pi, et al., 2019; Pi, Yang, Sun, Wang, et al., 2021).

Many studies have shown that the allergenicity of peanuts was effectively reduced through various technologies, including thermal

treatment (Cabanillas, et al., 2015; Cabanillasa et al., 2012), irradiation (Luo & Hu, 2019; Luo, et al., 2013) high-pressure treatment (Hu et al., 2011), and enzyme catalysis (Wu, et al., 2015), etc. However, thermal treatment may generate un-controllable by-products harmful to human health. Meanwhile, consumer acceptance of food irradiation remains controversial, and the cost of high-pressure treatment is quite high (Pi, et al., 2019; Pi, Yang, Sun, Wang, et al., 2021). In addition, bitter peptides could be produced during enzyme catalysis, thus limiting its application (Pi, et al., 2019). Therefore, these methods are usually limited due to unwanted effects on food quality, poor safety, and high cost. Interestingly, fermentation could significantly reduce the allergenicity of foods and improve nutritional value and physicochemical properties under low cost and no-additional chemical additives (Pi, Fu et al., 2021; Pi, et al., 2019; Pi, Yang, Sun, Cui, et al., 2021). Fermentation with *Neurospora crassa* induced a reduction in allergenicity of soybean meal, accompanied by the increase in total free amino acid contents, digestibility, and antioxidant activity in soybean meal (Li, et al., 2019). Rui, et al. (2019) demonstrated that fermentation with *Lactobacillus plantarum* induced 83.8%–94.8% reduction in IgE immunoglobulin E (IgE) reactivity of soybean proteins based on ELISA results

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due to the alternation in the primary and higher structure of soybean proteins. In addition, fermentation strains and metabolites could decrease human sensitization to food allergens by increasing the generation of Treg cells, regulating the balance of the Th1/Th2 response, enhancing the epithelial barrier, and increasing the levels of IgA and bacterial diversity (Pi, Yang, Sun, Cui, et al., 2021). Oral administration of *Lactobacillus casei* Zhang attenuated allergenic symptoms in tropomyosin-sensitized BALB/c mice, owing to the decrease in intestinal epithelial damage and the changes in the development and function of dendritic, T and B cells (Fu, et al., 2020). Similar results were shown in ovalbumin-sensitized BALB/c mouse model experiments, which demonstrated that oral intervention of *Lactobacillus* suppressed allergic responses via modulating enteric flora, intestinal CD11c⁺ cell, Treg cell functionality, and the Th1/Th2 immune balance (Huang, et al., 2016; Majumder, et al., 2020). Fermentation metabolites (e.g., acetate, propionate, and acidic polysaccharide APS-I) also alleviated allergic responses by increasing the induction of Tregs, inhibiting Th2 generation, and increasing the level of IgA (Cao, et al., 2019; Hirata & Kuni-sawa, 2017). However, few studies about the effects of fermentation on the allergenicity of peanuts have been reported, and only the previous study of the authors reported that fermentation reduced 69.3%–77.3% of the IgE reactivity in peanuts through liquid fermentation with *Bacillus natto* (Pi, Fu et al., 2021). Many studies have investigated the effects of fermentation on the allergenicity and physicochemical properties of soybeans and milk simultaneously (Li, et al., 2019; Meinschmidt, et al., 2016; Seo & Cho, 2016; Shi, et al., 2014; Yao, et al., 2013; Yao, et al., 2015). The present study aimed to investigate the changes in IgE binding capacity and physicochemical properties of peanuts after solid-state fermentation with *L. plantarum* and *B. natto*, along with autoclave pre-treatment. Structural changes in peanut protein were examined through multiple biophysical methods. The results established a link among the structural characteristics, IgE binding capacity, and physicochemical properties of peanut protein, laying the foundation for the development of high-quality and hypoallergenic peanut fermented products.

2. Materials and methods

2.1. Experimental materials

Raw peanuts were purchased from Tianhong Supermarket (Nanchang, China). Strains *B. natto* and *L. plantarum* were offered by BeNa Culture Collection (Beijing, China) and Nanchang University (Nanchang, China), respectively. Sera from 10 patients allergic to peanuts was provided by the First Affiliated Hospital of Nanchang University, and the IgE levels of these sera were 72.9, 70.0, 173.5, 184.2, 71.2, 55.6, 140.6, 99.5, 84.3 and 100.0 kU/I. Rabbit anti-human IgE HRP conjugate and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from Chongqing Manuik Technology Co., Ltd. (Chongqing, China). All chemicals and reagents were from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Preparation of fermented peanuts

Raw peanuts were soaked in distilled water at a ratio of 1:20 (m/v) for 12 h and drained for 5 min. After their red coat was removed, the peanuts were cut into pieces with a knife to obtain the raw crushed peanut (RCP). Next, RCPs (200 g) were autoclaved in a high-pressure steam sterilizer (ZDX-35BI, ShenAn Medical, China) at the autoclaving process [heating up (30 °C–121 °C) for 35 min, holding temperature at 121 °C for 20 min, cooling down (121 °C–90 °C) for 25 min] to obtain autoclaved crushed peanut (ACP). Finally, ACP was cooled to room temperature and then inoculated with *L. plantarum* (9.8×10^6 cfu/g), *B. natto* (9.8×10^6 cfu/g) and their cocultivation (4.9×10^6 cfu/g of *B. natto* and 4.9×10^6 cfu/g of *L. plantarum*) at 37 °C for 36 h to obtain fermented peanuts, respectively; these fermented samples were labeled as LP, BN and LPBN, respectively.

2.3. Extraction of peanut protein

Peanut protein was extracted in accordance with Luo, et al. (2013). Each sample was ground into peanut powder and defatted by acetone at a 1:10 (w/v) ratio for 2 h and then centrifuged at 6000 g. The defatted process was repeated twice to obtain defatted peanut powder. Air-dried defatted peanut powder was mixed with Tris-HCl solution (50 mM, pH 8.0) for 4 h, and centrifuged at 8000 g. Afterward, the supernatant was collected (peanut protein extraction). The precipitate was then resuspended in Tris-HCl solution, stirred, and centrifuged another two times to collect the supernatants. Three extractions were mixed and then analyzed. The protein content of peanut protein extraction was detected by Bradford assay with a standard of bovine serum albumin (Rui, et al., 2019). The results demonstrated that the protein concentration in the RCP, ACP, LP, BN, and LPBN extractions were 19.4, 5.3, 5.6, 8.3, and 8.6 mg/mL, respectively.

2.4. Sds-PAGE

SDS-PAGE was performed in accordance with Laemmli, (1970). Each sample (20 µg protein per lane) was mixed with Laemmli sample buffer and β-mercaptoethanol, heated at 100 °C for 3 min, and electrophoresed in 5%–12% Tris-HCl linear gradient precast gel. Proteins were visualized with Coomassie brilliant R-250 solution and then destained overnight.

2.5. Scanning electron microscopy (SEM)

The freeze-dried peanut protein was fixed on a glass slide, sprayed with gold at 150 Å, and examined by SEM (JSM 6701F, JEOL, Japan) to observe its microstructure.

2.6. Fourier transform infrared spectroscopy (FTIR)

FTIR was used to assess the secondary structure of the protein. Lyophilized-purified protein samples were mixed and ground with KBr thoroughly. After tablet pressing was performed, the secondary structure of the protein was determined by FTIR (Bio-Rad FTS-65A, Thermo Fisher Nicolet, USA). The number of scans was 32, and the resolution was 2 cm⁻¹. The spectroscopy results were analyzed by peakfit (version 4.12, AISN Software Inc.).

2.7. Ultraviolet (UV) absorption spectroscopy

UV absorption spectra analysis of purified protein solutions (section 2.3) was performed by a UV spectrophotometer (TU-1900, Pursee Corporation, China). The content of protein in each sample was adjusted to 0.1 mg/mL, and the samples were then scanned from 200 to 400 nm at a rate of 100 nm/min.

2.8. Surface hydrophobicity

Surface hydrophobicity was detected by 1-anilinonaphthalene-8-sulfonate (ANS) method (Matulis & Lovrien, 1998). After each protein solution (0.1 mg/mL protein) was mixed in a 200:1(v/v) ratio with ANS solution (50 mM in PBS, pH 7.4), the relative fluorescence of the mixed solution was measured using a spectrofluorometer (F-4500, HITACHI, Japan). The excitation wavelength was 390 nm, and the emission wavelength was from 450 nm to 650 nm at a scanning speed of 60 nm/min.

2.9. Free sulfhydryl groups

The content of free sulfhydryl groups was assessed in accordance with Luo, et al. (2013). The purified protein solutions (section 2.3) were diluted to 0.4 mg/mL, and reacted with Ellman solutions at a 1:4 (v/v)

ratio for 20 min at 25 °C. The absorbance at 412 nm was measured.

$$\text{Content of free sulfhydryl groups} = 73.5 \times A_{412} \times D/C \quad (1)$$

Where D is the dilution factor and C is 0.4 mg/mL (protein concentration).

2.10. IgE binding capacity of peanut protein

The IgE binding capacity was considered as the allergenicity of samples, and it was determined by indirect ELISA (Cabanillas, et al., 2015). A 96-well microtiter plate was coated with 100 µL/well of the protein extractions (0.75 µg/mL, pH 9.6), left overnight at 4 °C, and subsequently blocked with skim milk (50 mL/L, pH 7.2) for 1 h at 37 °C. After the wells were washed by PBS containing 0.5 mL/L Tween 20, each well was incubated with the serum pool for 1 h, washed, and incubated with HRP-conjugated rabbit anti-human antibody for 1 h. After the wells were washed, the peroxidase reaction was developed with the addition of tetramethylbenzidine solutions. The reaction was terminated with 2 mol/L H₂SO₄, and the OD was measured at 450 nm by a microporous plate fluorescence reader (epoch, BioTek, USA). OD₄₅₀ reflected the IgE binding capacity.

2.11. Physicochemical properties in peanut proteins

2.11.1. Foaming properties

The foaming activity and stability were determined in accordance with Xu, et al., (2019) with slight modification. Two g of freeze-dried peanut protein was fully dissolved in distilled water at a ratio of 1:45 (m/v) and stirred at 10000 rpm for 1 min. After the stirring process, the solution was poured into a measuring cylinder, and the volume at 0 min (V₀) and 30 (V₃₀) min was recorded.

$$\text{Foaming activity} = (V_0 - 90)/90 \quad (2)$$

$$\text{Foaming stability} = (V_{30} - 90)/(V_0 - 90) \quad (3)$$

2.11.2. Emulsion properties

The emulsion activity and stability were determined in accordance with Xu, et al., (2019) with slight modification. Freeze-dried peanut protein was fully dissolved in distilled water, with shaking to produce peanut protein solutions (1 mg/mL). The protein solution was mixed with soybean oil at a ratio of 3:1(v/v) and homogenized at 8000 rpm for 3 min. A 100 µL aliquot of the emulsion was immediately removed from the bottom of the beaker and added to 10 mL of 0.1% sodium dodecyl sulfate. The absorbances of the emulsion at 0 and 10 min were measured at 500 nm by a UV spectrophotometer (TU-1900, Pursee Corporation, China).

$$\text{Emulsion activity} = A_0 \quad (4)$$

$$\text{Emulsion stability} = A_0 \times \Delta t / \Delta A \quad (5)$$

Where A₀ is the absorbance value at 0 min, Δt is the time difference (min), and ΔA is the absorbance difference within Δt.

2.11.3. Grain size

The protein concentration of each sample was adjusted to 0.1 mg/mL by 0.01 M phosphate buffer (pH7.2), and the grain size of the protein in each sample was measured using a particle size analyzer (NanoZSP + MPT-2, Malvern, Britain) to evaluate the grain size.

2.12. Evaluation of fermented peanuts products

2.12.1. Content of soluble protein

Peanut protein extraction (section 2.3) was detected by Bradford assay with a standard of bovine serum albumin (Rui, et al., 2019). In brief, after the peanut protein extraction was reacted with Bradford

reagent at a 1:5 (v/v) ratio for 10 min at 37 °C, the OD₅₉₅ of the mixture was evaluated by a TU-1900 spectrophotometer (Persee Instruments, China).

2.12.2. Content of peptides

The content of peptides in fermented peanut products was determined with a standard of casein in accordance with Liu & Zeng, (2012). Two g of the fermented peanut products were ground, mixed, and stirred with 0.4 mol/L trichloroacetic acid solution for 3 h. After centrifugation was conducted at 8000 g for 20 min, 1 mL supernatant was mixed with 4 mL biuret reagent, and the OD value of the mixed solution was measured at 540 nm.

2.12.3. Water holding capacity (WHC)

The WHC of fermented peanut products was determined in accordance with He, et al. (2014). Two g of the fermented peanut products were ground, mixed, and stirred with distilled water at a ratio of 1:10 (m/v) for 30 min. After centrifugation was performed at 3000 g for 10 min, the supernatant was removed and weighed.

$$\text{WHC} = (W_2 - W_1)/W_0 \quad (6)$$

Where W₀ is the weight of the sample, W₁ is the weight of the sample and the centrifuge tube before centrifugation, and W₂ is the weight of the sample and the centrifuge tube after centrifugation.

2.12.4. Oil absorption capacity (OBC)

The OBC of fermented peanuts products was determined in accordance with He, et al. (2014). Two g of the fermented peanuts products were ground, mixed, and stirred with soybean oil at a ratio of 1:10 (m/v) for 30 min. After centrifugation at 3000 g for 10 min, the supernatant was removed and weighed.

$$\text{WHC} = (W_2 - W_1)/W_0 \quad (7)$$

Where W₀ is the weight of the sample, W₁ is the weight of the sample and the centrifuge tube before centrifugation, and W₂ is the weight of the sample and the centrifuge tube after centrifugation.

2.12.5. Sensory evaluation

Ten people with a normal sense of smell and taste took the sensory evaluation, and their information was shown in Supplementary Materials (Table S1). The evaluation score was 0–10 points, and the evaluation indicators were hardness, viscosity, chewiness, flavor, and preference. A high score in hardness, viscosity, chewiness, and preference indicated high corresponding index properties. In addition, a high score in flavor indicated a high concentration of the unique flavor of *B. natto* products.

3. Results and discussion

3.1. Characteristics of peanut protein isolate

3.1.1. Protein profile on SDS-PAGE

The electrophoretic patterns of RCP, ACP, LP, BN, and LPBN proteins were analyzed by SDS-PAGE (Fig. 1). Compared with RCP protein bands (lane 1, Fig. 1), ACP protein bands (lane 2, Fig. 2) with molecular weight > 45 kDa disappeared, whereas the intensity of other protein bands faded to varying extents. Similar studies on autoclaved (121 °C and 138 °C, 15 and 30 min) raw and roasted peanut seeds also showed that the protein bands with molecular weight > 50 kDa almost disappeared and the intensity of other protein bands faded (Cabanillas, et al., 2015; Cabanillas et al., 2012). Heat treatment could induce protein denaturation and -aggregation, resulting in the loss of protein solubility (Cabanillas et al., 2012; Kasera, et al., 2012). Therefore, the concentration of the protein in extracted samples was reduced due to the decrease in protein solubility, probably resulting in the disappearance of some protein bands. Many studies have found an increase in the content

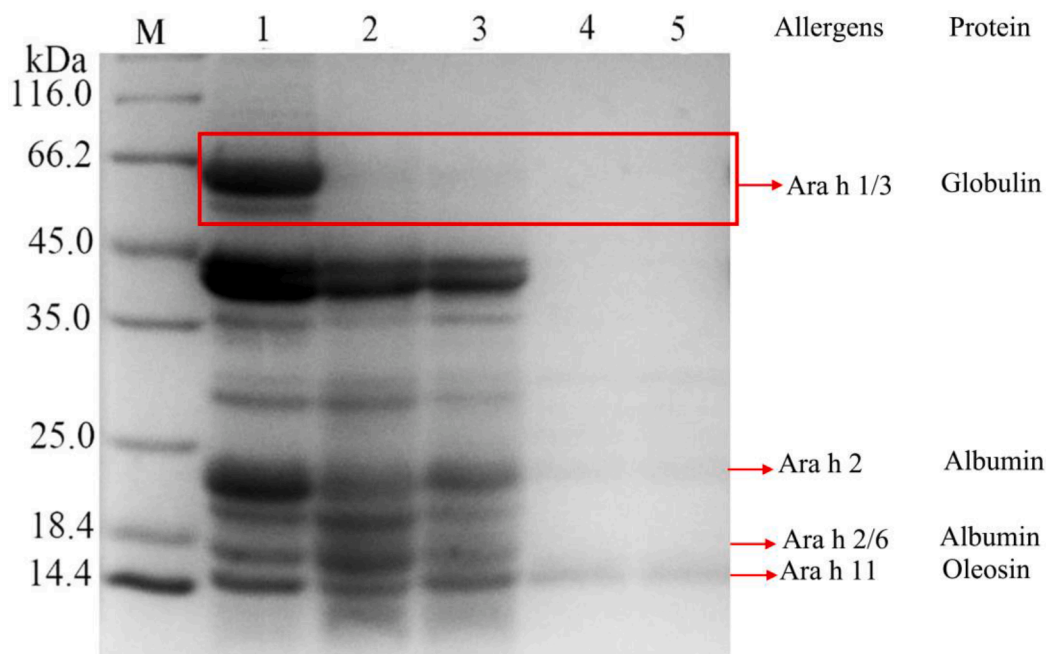


Fig. 1. The relative molecular mass of untreated and treated peanut protein. M, labeled protein; 1–5 represented protein extracted from RCP, ACP, LP, BN and LPBN.

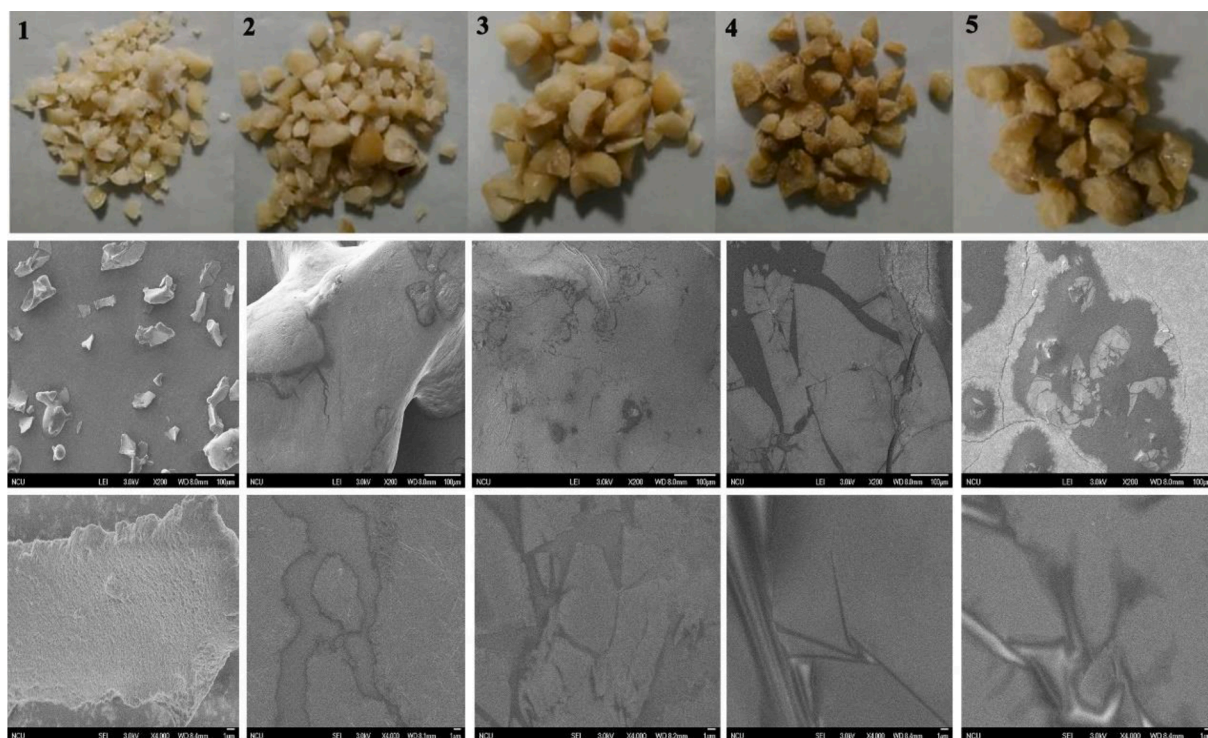


Fig. 2. Appearance of peanut products (first row) and their SEM of protein (magnification factor of second and third rows were 200 and 4000 respectively). 1–5 represented peanut products and their protein of RCP, ACP, LP, BN and BNLP.

of peptides and the degree of hydrolysis in the thermal treatment of bitter beans, peanut seeds, and sweet potato proteins (Muhialdin, Abdul Rani, & Meor Hussin, 2020; Pi, et al., 2020; Zhang, Huang, & Mu, 2019), suggesting the existence of protein-degradation. Therefore, the change in protein bands was related to the protein-denaturation, -aggregation, and -degradation in ACP. As shown in Fig. 1 (lane 3) fermentation with *L. plantarum* further reduced the intensity of certain protein bands (≤ 45

kDa) but did not induce the disappearance of the protein bands, suggesting the slight degradation of protein in LP. The results in agreement with those of fermented soybean protein with *L. plantarum*, showing slight degradation of proteins (Rui, et al., 2019). However, there was almost disappearance of total protein bands (≥ 14.4 kDa) in fermented autoclaved peanuts with *B. natto* (BN, lane 4, Fig. 1), and fermented autoclaved peanuts with *L. plantarum* and *B. natto* (LPBN, lane 5, Fig. 1),

suggesting the extensive degradation of protein in BN and LPBN. The proteolytic action of proteases produced by *B.s natto* was mainly responsible for the extensive degradation of protein in BN and LPBN. After all, compared to *L. plantarum*, *B. natto* has a strong ability to produce protease, and its production of protease is dozens of times higher than *Bacillus subtilis* (Pi, Fu et al., 2021). Similar results were also shown by Zhou (2014), who found the protein bands (≥ 17 kDa) gradually

disappeared with fermentation time in fermented peanut protein powder with *B. subtilis*.

3.1.2. Microstructure on SEM

Fig. 2 demonstrated that the structure of ACP protein became compact, suggesting the protein-aggregation induced by autoclaving. However, fermentation with *Lactobacillus plantarum* made the structure

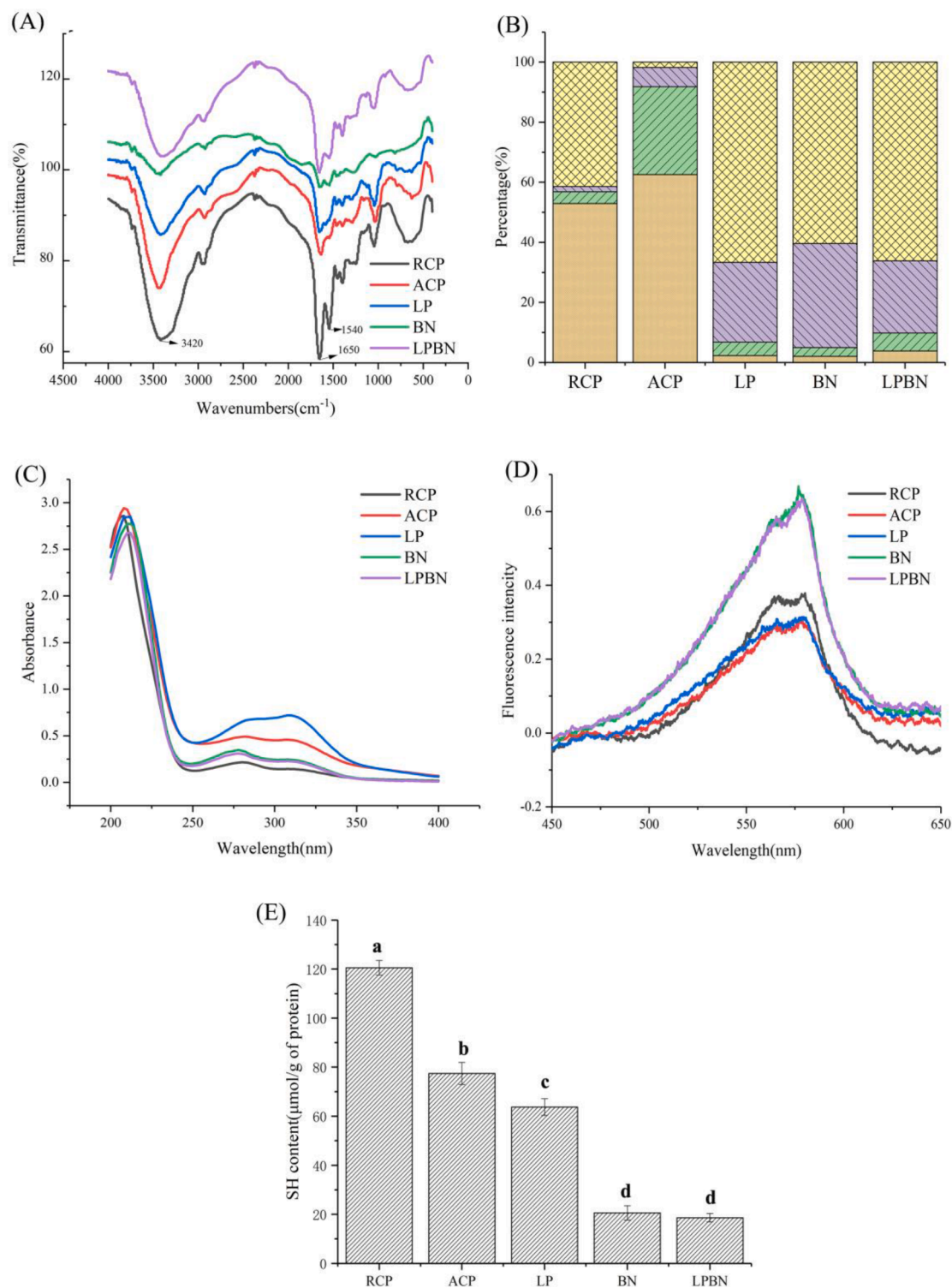


Fig. 3. The changes of structure in protein extracted from RCP, ACP, LP, BN and LPBN. **A.** Fourier infrared spectroscopy; **B.** corresponding secondary structure based on Fourier infrared spectroscopy, α -helix, β -sheet, β -turn and random coil was shown in $\square$, $\square$, $\square$, $\square$; **C.** UV absorption spectra; **D.** Fluorescence emission spectra; **E.** The content of free sulfhydryl group, means with different letters (a–d) indicated significantly different ($P < 0.05$).

of protein loose slightly with the appearance of tiny holes, suggesting slight protein-degradation. Looser structures and bigger holes were found in BN and LPBN than in LP, indicating the extensive protein degradation in BN and LPBN. In addition, LPBN showed the loosest structure and biggest holes, suggesting that the effect of protein-degradation in LPBN was the strongest.

3.1.3. Secondary structure on FTIR

FTIR is an effective tool to assess changes in protein structure, which is reflected by changes in spectral peak intensity and peak position (Jia, et al., 2016; Yan, et al., 2021). Different changes in spectral peak intensity and peak position were observed among all samples (Fig. 3A). In the spectrum of RCP, three strong bands were observed at 3420, 1650, and 1540 cm^{-1} , corresponding to the vibrations for the amide A, amide I, and amide II of protein, respectively. Compared to the spectrum of RCP, the intensity and position of these strong bands were changed in ACP, BN, and LPBN. The amide I band represents the vibration of C=O stretching of the peptide bond, whereas the amide II band is primarily due to the vibrations of N—H bending and C—N stretching (Jia, et al., 2016), and amide I and amide II bands are closely related to the secondary structure of proteins (He, et al., 2019). Therefore, the secondary structure of proteins was changed after peanuts were autoclaved and fermented. The corresponding secondary structure (Fig. 3B) was calculated by peakfit (version 4.12, AISN Software Inc.). The relative content of α -helix was increased in ACP compared with that in RCP (Fig. 3B). However, a reduction in α -helix was observed in autoclaved (121 °C, 20 min) raw peanut pulp (Pi, Fu et al., 2021) and autoclaved (121 °C, 15 min)-roasted peanut seeds (Cabanillasa et al., 2012). The different results were probably due to the different existing states and types in peanuts, resulting in different changes in the secondary structure induced by heat absorption. Autoclaving (138 °C, 30 min) was reported to decrease the reduction extent of α -helix in autoclaved roasted peanut seeds, compared with autoclaving at 121 °C for 30 min (Cabanillasa et al., 2012). Fig. 3B also demonstrated that each fermented autoclaved peanut showed an increase in α -helix. A similar result was shown by Rui, et al. (2019), who found that fermentation of soybean protein with *L. plantarum* increased the content of α -helix. Thus, autoclaving and fermentation induced extensive changes in the secondary structure of peanut proteins.

3.1.4. Changes in the conformational structure

UV absorption spectra could reflect the change in protein structure, depending on the UV absorption of tryptophan and tyrosine residues located at nearly 280 nm and on that of peptide bonds located at approximately 200–220 nm (Hu, et al., 2011; Pi, Fu et al., 2021). Fig. 3C showed that autoclaving of RCP resulted in increased UV absorption intensity at 208 and 280 nm, indicating the exposure of peptide bonds, tryptophan and tyrosine. The UV absorption intensity at 208 nm was gradually decreased in LP, BN, and LPBN probably due to the breaking of peptide bonds. The lowest UV absorption intensity at 208 nm in LPBN suggested the biggest breaking of peptide bonds and protein degradation, and this finding was consistent with that in SDS-PAGE (Figs. 1 and 2). However, an increase in the UV absorption intensity at 280 nm was observed in LP, BN, and LPBN, suggesting the exposure of tryptophan and tyrosine induced by protein unfolding (Luo, et al., 2013). The changes in UV absorption intensity at 208 and 280 nm reflected the changes in primary and conformational structure (Hu, et al., 2011). Thus, autoclaving and fermentation changed the primary and conformational structure of peanut protein.

The change in the structure of proteins was also reflected by surface hydrophobicity (Hu, et al., 2011). Fig. 3D showed that the surface hydrophobicity of RCP decreased after autoclaving, resulting in the masking or destruction of the hydrophobic group induced by protein-aggregation. However, surface hydrophobicity increased subsequently after fermentation, probably due to protein unfolding (Hu, et al., 2011). ANS fluorescence is enhanced in more hydrophobic environments, and

fluorescence-intensity enhancements provide strong evidence of protein unfolding (Yan, et al., 2021). In addition, BN and LPBN showed higher surface hydrophobicity than LP, suggesting higher protein unfolding in BN and LPBN.

Disulfide bonds play an important role in protein structure and changes in free sulfhydryl groups could reflect the alternation of protein structure (He, et al., 2014; Hu, et al., 2011; Sen, et al., 2002). Fig. 3E demonstrated that the content of free sulfhydryl groups was reduced by 35.8%, 47.2%, 82.9% and 84.6% after raw crushed peanuts were autoclaved, fermented with *L. plantarum*, *B. natto* and a mixture of *L. plantarum* and *B. natto*, respectively. Autoclaving induced protein-aggregation, probably resulting in the conversion of free sulfhydryl groups to disulfide bonds (He, et al., 2014; Pi, Fu et al., 2021; Zhao & Yang, 2012) to reduce the content of free sulfhydryl groups in ACP. Fermentation reduced the content of the free sulfhydryl group due to the hiding or oxidation of free sulfhydryl groups induced by protein-unfolding or peroxide-production (Pi, Fu et al., 2021; Yang, et al., 2017). Besides, BN and LPBN showed a lower content of the free sulfhydryl group than LP, suggesting the higher protein unfolding in BN and LPBN, which was consistent with the results of surface hydrophobicity (Fig. 3D).

3.1.5. Changes in IgE binding capacity of peanut protein

The IgE binding capacity is usually detected by ELISA, in which high OD₄₅₀ value represents high potential IgE binding capacity (Pi, Fu et al., 2021; Rui, et al., 2019; Xu, et al., 2019). Fig. 4 demonstrated that the IgE binding capacity of raw crushed peanut was reduced by 11.3%, 20.6%, 78.7% and 90.2% after autoclaving, fermentation with *L. plantarum*, *B. natto*, and their cocultivation (*L. plantarum* and *B. natto*), respectively. The reduction in allergenicity might be due to the masking and destruction of epitopes induced by the degradation of protein and changes in protein structure (Pi, et al., 2019; Pi, Yang, Sun, Cui, et al., 2021). The allergenicity of raw, fried, and roasted peanuts was reported to be reduced after autoclaving (121 °C, 15 or 30 min; 138 °C, 15 or 30 min) due to the degradation of protein, resulting in the reduction of content in Ara h 1, Ara h 2 and Ara h 6 (Cabanillas et al., 2015). The reduction in the content of Ara h 1-Ara h 3 induced by protein-degradation was also responsible for the reduction in allergenicity of the autoclaved roasted peanuts (Cabanillas, et al., 2012). In addition, autoclaving induced protein-denaturation and -aggregation to cause the loss of protein solubility (Cabanillasa et al., 2012), probably resulting in the loss of allergenic proteins in extracted peanut proteins. Therefore,

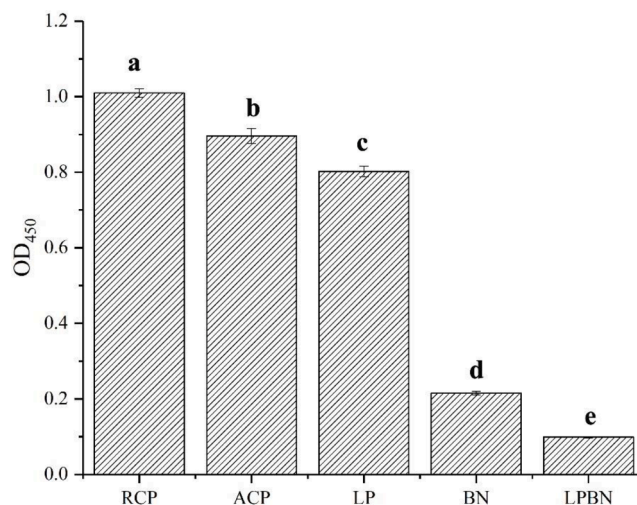


Fig. 4. The IgE binding capacity of protein extracted from RCP, ACP, LP, BN and LPBN. Means with different letters (a-e) in the bars indicate significantly different ($P < 0.05$). Percentages of reduction in allergenicity were shown in the table annexed to Fig. 4.

protein-denaturation, -aggregation, and -degradation were responsible for the reduction in peanut allergens (e.g., Ara h 1-Ara h 3, Ara h 6 and Ara h 11) in ACP (Fig. 1), resulting in reduced IgE binding capacity (Fig. 4). Similar results were shown in fermented peanut protein, which showed 52% reduction in allergenicity due to the reduction in peanut allergens (e.g., Ara h 1 and Ara h 2) induced by protein-degradation (Zhou, 2014). Protein-degradation usually causes the conversion of allergenic proteins to low-molecular-weight peptides so that serum did not recognize allergenic proteins (Kim, et al., 2011). As shown in Fig. 1, the reduction in peanut allergens (e.g., Ara h 1-Ara h 3, Ara h 6 and Ara h 11) induced by protein-degradation was an important reason for the reduction in the IgE binding capacity of autoclaved and fermented crushed peanuts (Fig. 4). No differences could be observed in the SDS-PAGE results between BN and LPBN. However, LPBN showed a higher reduction in IgE binding capacity than BN, indicating that the masking and destruction of epitopes were stronger in LPBN than in BN.

Alternation in protein structure could induce the masking and destruction of epitopes, thereby playing an important role in the reduction of allergenicity (Cabanillas, et al., 2012; Luo, et al., 2013; Rui, et al., 2019). The reduction and increase in α -helix were reported to have led to reduced allergenicity in autoclaved roasted peanut and fermented soybean protein, respectively (Cabanillas, et al., 2012; Rui, et al., 2019). Changes in the conformational structure were also responsible for the reduction in high-pressure-microfluid Ara h 2 and gamma-irradiated Ara h 6 (Luo, et al., 2013; Hu et al., 2011). Thus, autoclaving induced protein-denaturation, -aggregation, and -degradation resulting in the structural changes (e.g., increase in α -helix and UV absorption spectra and reduction in surface hydrophobicity and free sulfhydryl group), which caused the masking and destruction of epitopes to induce a reduction in IgE binding capacity. Structural changes were also observed in LP, BN, and LPBN, and these changes induced protein-unfolding to make exposed epitopes more susceptible to degradation through enzymes and acids secreted by *B.* and *L. plantarum*. No significant differences were observed in the UV absorption spectra (280 nm), surface hydrophobicity and free sulfhydryl group between BN and LPBN. However, significant differences ($P < 0.05$) were found in the IgE binding capacity of BN and LPBN, suggesting the differences in the masking and destruction of epitopes. Proteins are known to be allergic due to the existence of complete allergenic peptides or epitopes (Pi, Sun, et al., 2021; Pi, et al., 2019). More types of enzymes could be found in LPBN than in BN, resulting in more sites for enzymatic hydrolysis in LPBN proteins, which, in turn, led to a greater potential for the destruction of epitopes in LPBN proteins. Furthermore, a binding reaction between proteins and fermentation metabolites may exist during the fermentation processing (Pi, Fu et al., 2021), probably resulting in the differences in the masking of epitopes. LPBN had more types of microorganisms than BN, resulting in more types of fermentation metabolites, which, in turn, led to a greater potential for the masking of epitopes in LPBN proteins. Therefore, LPBN showed the lowest IgE binding capacity among the fermented peanuts due to the highest masking and destruction of epitopes, probably resulting from the more types of enzymes and fermentation metabolites in LPBN. However, fermentation with *B.* and *L. plantarum* did not remove the IgE binding capacity of peanuts, probably because the small molecules still retained IgE binding sites and characteristics (Guo, et al., 2012).

3.1.6. Physicochemical properties in peanut protein

Figs. 5A and 5B demonstrated that the foaming activity and stability of peanut proteins were decreased by 30% and 6% after peanuts were autoclaved, respectively, and then were increased by 64%–73% and 27.4%–36.6% after fermentation. Foaming activity and stability are usually affected by many factors such as protein type, pH, processing methods, viscosity and surface tension (Sadh, Chawla, & Duhan, 2018). The foaming activity and stability in ACP were reduced due to protein-aggregation, which caused a reduction in surface hydrophobicity and different formations of an elastic foam network at the air–water

interface (He, et al., 2019; Jia, et al., 2016). On the contrary, protein-unfolding and the increase in surface hydrophobicity resulted in the increase of protein from fermented peanuts (LP, BN, and LPBN). Similar results were shown by Sadh, Chawla, & Duhan (2018), who found an increase in foaming activity and stability of fermented peanut press cakes.

Emulsion activity refers to the assessment of the ability of a protein to form an oil–water interface and emulsion stability is indicative of the strain resistance of the emulsion droplets formed by the protein (Yan, et al., 2021), both are very important in food systems (Sadh, Chawla, & Duhan, 2018). Fig. 5C showed that the emulsion activity was reduced and then increased after autoclaving and fermentation, respectively. However, the emulsion stability was reduced after autoclaving and fermentation (Fig. 5D). The changes in emulsion properties are related to the changes in protein structure (Jia, et al., 2016). The emulsion activity and stability in ACP were reduced due to protein-aggregation, which caused the masking of the hydrophobic amino acid (Yan, et al., 2021). On the contrary, the emulsion activity of fermented peanuts was increased, probably resulting from protein-unfolding and an increase in surface hydrophobicity (Xu, et al., 2019; Yan, et al., 2021). Besides, the generation of low molecular-weight peptides in fermented peanuts was responsible for the increase in emulsifying activity (Sadh, Chawla, & Duhan, 2018). The emulsion stability was related to the extending of protein-unfolding and -rearrangement at the interface (Jia, et al., 2016). Thus, the reduction of emulsifying stability in fermented peanuts was due to the large extent of protein-unfolding.

Fig. 6 showed that the particle sizes in RCP, ACP, LP, BN and LPBN were mainly distributed at 188–343, 625–788, 226–432 285–376, and 197–226 nm respectively. The increase in particle sizes of autoclaved peanuts was due to protein-aggregation, consistent with the results of SEM (Fig. 3). Fermentation induced protein-degradation to generate low molecular-weight peptides, resulting in the reduction in particle sizes of fermented peanuts. Moreover, the lowest particle sizes were shown in LPBN, suggesting the strongest protein degradation in this sample.

3.2. Characterization of fermented peanut products

The physicochemical properties of final peanut products directly affect people's preferences and determine the success or failure of product development. Fig. 6 showed the content of soluble protein and peptides, WHC, and OBC. Fig. 6A showed that the content of soluble protein in ACP was reduced by 72.7% due to protein-aggregation or the information of protein-carbohydrate or -lipid complex, resulting in reduced protein solubility. On the contrary, fermentation induced protein-unfolding and -degradation to increase protein solubility. Protein degradation was also responsible for the increase in peptides of autoclaved and fermented peanuts (Fig. 6B). Besides, protein aggregation or the information of protein-carbohydrate or -lipid complex in autoclaved peanuts made the structure compact to make oil or water difficult to enter the peanuts, resulting in reduced WHC and OBC (Fig. 6C and Fig. 6D). However, fermentation made the structure loose to increase the capacity of water holding and oil absorption, consistent with SEM. Similar results were also revealed by Sadh, Chawla, & Duhan, (2018), who found that fermentation with *Aspergillus awamori* increased the WHC and OBC in peanut oil cake. Fig. 6E further demonstrated that fermented autoclaved peanuts with *L. plantarum* and *B. natto* are preferred by people due to great hardness, viscosity, chewiness, and flavor (low unpleasant smell induced by *Bacillus natto*). Thus, *L. plantarum* inhibited the production of *B. natto*-induced unpleasant smell, which was a serious problem for fermented food products with *B. natto*.

4. Conclusion

Fermentation with *B. natto* and *L. plantarum* and autoclave pre-treatment could significantly decrease the IgE binding capacity in

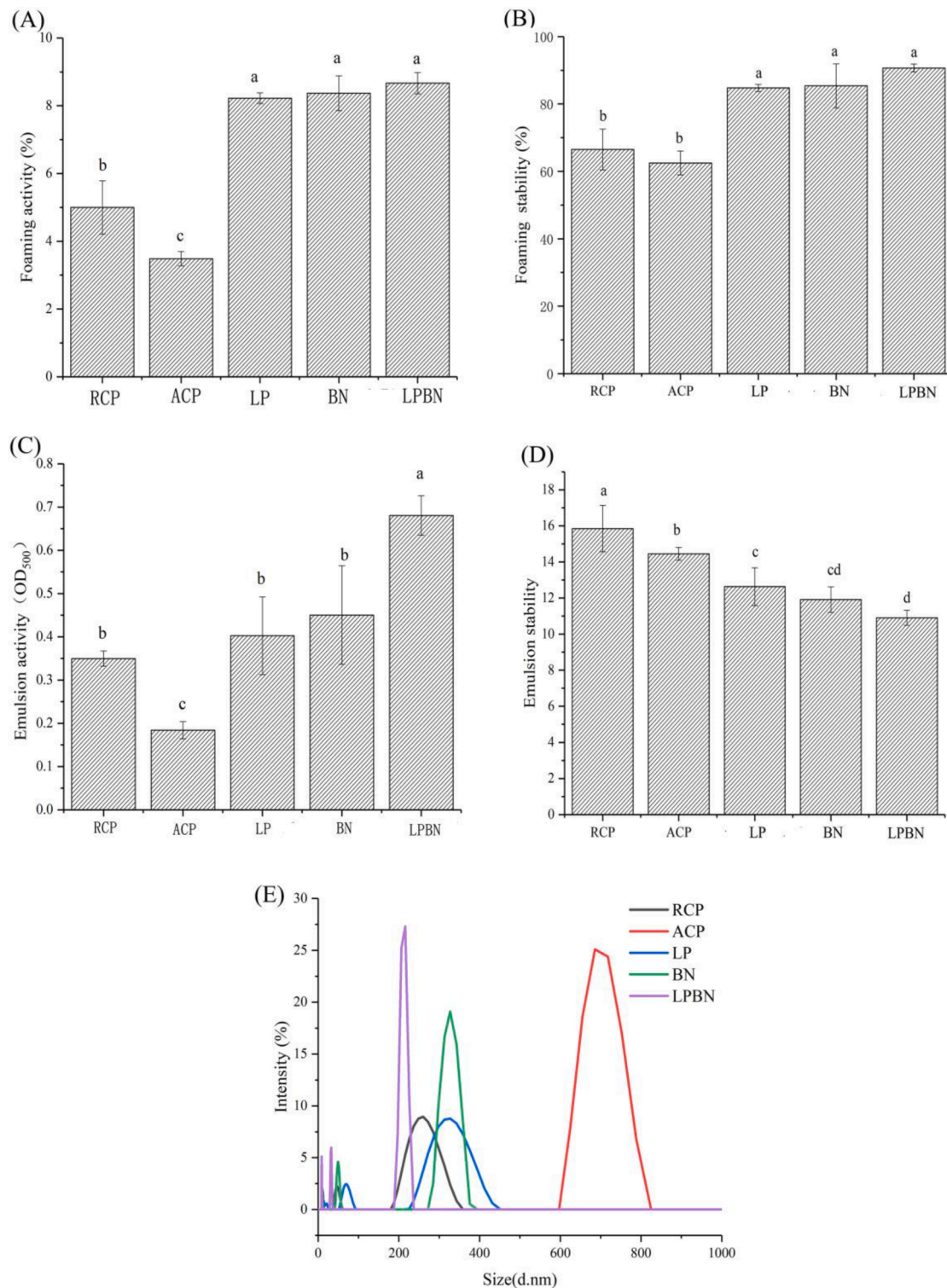


Fig. 5. The foaming activity (A), foaming stability (B), emulsion activity (C), emulsion stability (D) and grain size distribution (E) of protein extracted from RCP, ACP, LP, BN and LPBN. Means with different letters (a-d) in the bars indicate significantly different ($P < 0.05$).

peanuts. Autoclaving could induce protein-denaturation, -aggregation, and -degradation to cause the loss of allergens and structural changes, resulting in the reduction of IgE binding capacity. Fermentation could also induce protein-degradation and -unfolding to result in the masking and destruction of epitopes. Fermentation with a mixture of *L. plantarum* and *B. natto* showed a lower reduction in the IgE binding capacity of peanuts than fermentation with a single strain due to the higher destruction and masking of epitopes, probably resulting from the more

types of enzymes and fermentation metabolites available. Besides, the desired physicochemical properties (e.g., high foaming properties, emulsion activity, WHC and OBC, high contents of soluble protein and peptides, suitable hardness, viscosity, chewiness, and low unpleasant smell) were observed in the fermented peanuts with a mixture of *L. plantarum* and *B. natto*. Therefore, fermentation with a mixture of *L. plantarum* and *B. natto*, along with autoclave pretreatment, has considerable potential to produce hypoallergenic fermented peanut

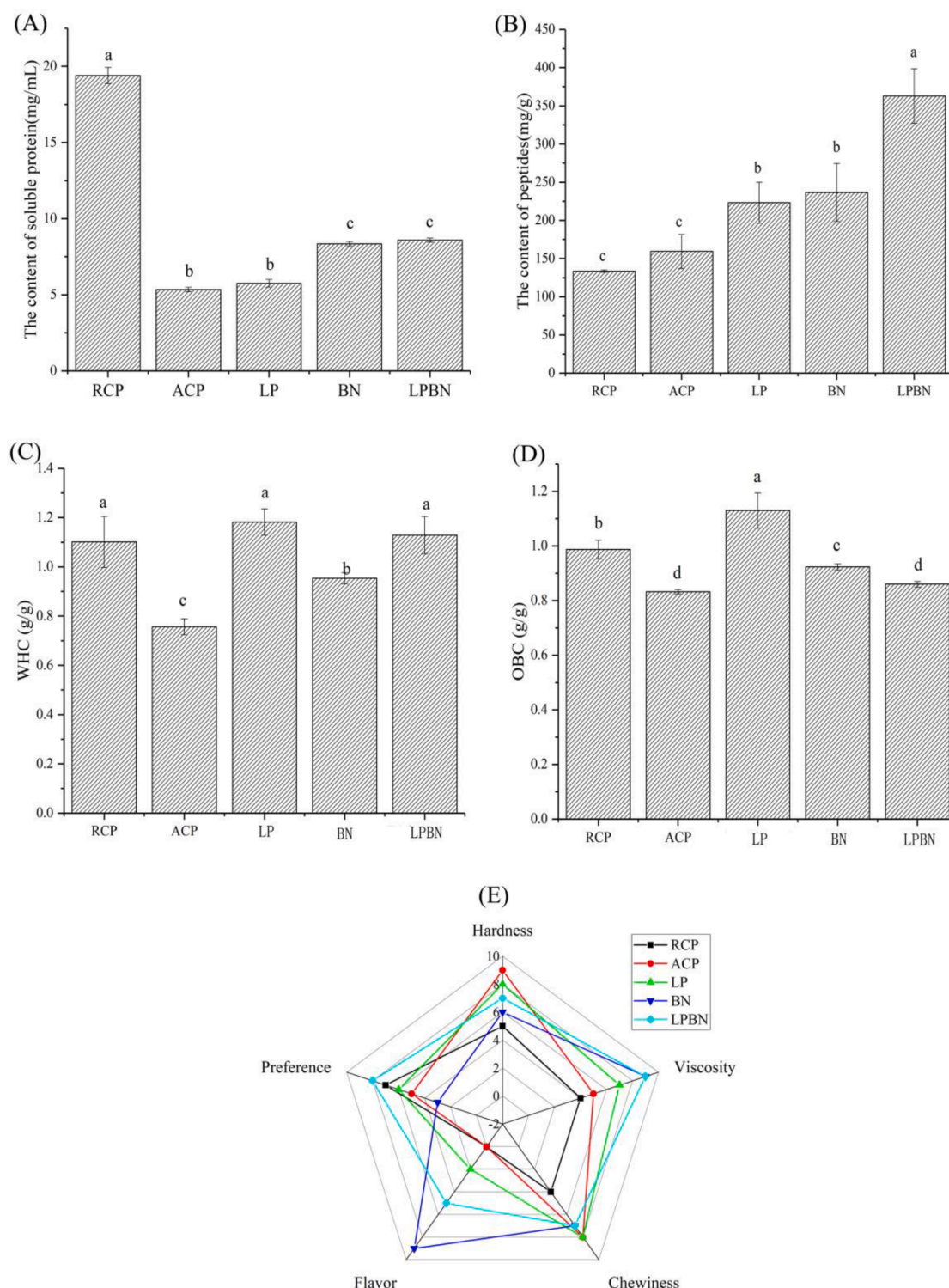


Fig. 6. The assessments of soluble protein content (A), peptides content (B), water holding capacity (WHC) (C), oil absorption capacity (OBC) (D) and sensory evaluation (E) for RCP, ACP, LP, BN and LPBN products. Means with different letters (a-d) in the bars indicate significantly different ($P < 0.05$).

products with desired quality. Further studies should explore the optimization of fermentation conditions and the confirmation of reduced allergenicity on the basis of cell and animal experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.133208>.

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