



Multi-spectral and proteomic insights into the impact of proanthocyanidins on IgE binding capacity and functionality in soy 11S protein during alkali-heating treatment

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ABSTRACT

This study evaluated the impact of proanthocyanidins on immunoglobulin E (IgE) binding capacity, antioxidant, foaming and emulsifying properties in soy 11S protein following alkali treatment at 80 °C for 20 min. The formation of >180 kDa polymer was observed in the combined heating and proanthocyanidins-conjugation treatment sample (11S-80PC) rather than in the heating treated sample (11S-80) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The structural analyzes demonstrated that 11S-80PC exhibited more protein unfolding than 11S-80. Heatmap analysis revealed that 11S-80PC had more alteration of peptide and epitope profiles in 11S than in 11S-80. Molecular docking showed that PC could well react with soy protein 11S. Liquid chromatography tandem MS analysis (LC/MS-MS) demonstrated that there was a 35.6 % increase in 11S-80, but a 14.5 % decrease in 11S-80PC for the abundance of total linear epitopes. As a result, 11S-80PC exhibited more reduction in IgE binding capacities than 11S-80 owing to more obscuring and disruption of linear and conformational epitopes induced by structural changes. Moreover, 11S-80PC exhibited higher antioxidant capacities, foaming properties and emulsifying activity than 11S-80. Therefore, the addition of proanthocyanidins could decrease allergenic activity and enhance the functional properties of the heated soy 11S protein.

1. Introduction

Soy is valued nutritionally due to its high protein content (approximately 40 %) and well-balanced amino acids [1,2], and soy protein is divided into four major globulins (15S, 11S, 7S, and 2S) based on sedimentation coefficients [3]. According to WHO/IUIS Allergen Nomenclature Sub-Committee, 11S, 7S and 2S fractions were identified as soy allergens Gly m 6, Gly m 5 and Gly m 8, respectively, whereas the 15S fraction was not identified as soy allergens [4]. Gly m 8 is a minor soy allergen, whereas Gly m 5 and Gly m 6 are the two main soybean allergens responsible for soy allergy [5]. The 11S glycinin fraction (Gly m 6) accounts for 30 % of the soy protein, which is considered the major protein in soy [2]. Gly m 6 is a hexameric structure built up of five subunits (G1, 56 kDa; G2, 54 kDa; G3, 54 kDa; G4, 64 kDa; G5, 59 kDa) [6–8]. Each subunit contains a basic polypeptide (~20 kDa) and an

acidic polypeptide (~40 kDa) [5]. It was reported that IgE reactivity to subunit G5 was detected in sera from 20 of 22 children, whereas IgE reactivity to G1, G3 and G4 was observed in sera from 16 or 17 of the children, and IgE reactivity to G2 was seen in sera from 7 of the children [7]. Therefore, it is necessary to seek an effective method to reduce allergenicity in soy 11S protein, contributing to developing soy protein products with low allergenicity and desired functionality.

It was reported that polyphenols have successfully used to decrease the IgE binding capacities and simultaneously improve functional properties such as antioxidant activity and interface properties (e.g., emulsifying and foaming properties) in many foods such as peanut, soybean, egg, milk [9–12]. However, the formation of polyphenol-protein conjugates was achieved after reaction at 25 °C for 24 h in these studies, which was unsuitable for food processing. Our previous study has shown that the alkali-heating treatment (pH 9.0, 80 °C, 20

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min) could accelerate the formation of soy protein-proanthocyanidins conjugates, which was accompanied by the reduced IgE binding capacities and improved functional properties in the total soy protein [13]. Moreover, conjugation of proanthocyanidins using the alkali-heating treatment (pH 9.0, 80 °C, 20 min) did not reduce the IgE binding capacities in soy 7S protein (data not shown). Therefore, the study was used to investigate the impacts of proanthocyanidins on the allergenicity, antioxidant, foaming and emulsifying of soybean 11S proteins following the alkali-heating treatment (pH 9.0, 80 °C, 20 min). The alteration in protein structure was measured by scanning electron microscope, circular dichroism spectroscopies, ultraviolet, surface hydrophobicity and free sulfhydryl groups. The protein and peptide composition were measured by SDS-PAGE and proteomic analyses. The modification sites and epitope alteration were identified by molecular docking and LC/MS-MS. These results will provide new insights into the effect of proanthocyanidin on the allergenicity and functionality of soybean 11S proteins.

2. Materials and methods

2.1. Materials

Five patient sera (specific IgE to soybean: 5.484 to 72.333 kU/I) were purchased from Chongqing Manuik Technology Co., Ltd. (Chongqing, China) (Table S1). The sera were mixed together to use for ELISA and Western-blot experiments. Proanthocyanidins (purity $\geq 95\%$), 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH, purity 98 %) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, purity 98 %) were obtained from yuanye Bio-Technology Co., Ltd. (Shanghai, China). All other chemicals were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Extraction of soy 11S proteins

The soy 11S proteins were obtained from soybean seeds, as previously described in the study of Liu, et al. [14]. Soy was crushed by the grinder, defatted three times with n-hexane, and then air-dried overnight. The resulting sample was dispersed in distilled water at a ratio of 1:15 (w/v), adjusted to pH 8.0, and then stirred for 4 h. After conducting the centrifugation for 20 min at 10000 $\times g$, the supernatant was obtained, added with NaHSO₃ (0.98 g/L), and then adjusted to pH 6.4. After overnight at 4 °C, centrifugation (10,000 $\times g$, 20 min) was conducted to obtain the precipitate. The resulting sample was dissolved in distilled water, dialyzed with the dialysis bag (8000–14,000 Da), and then lyophilized to obtain the lyophilized 11S.

2.3. Formation of 11S-80PC conjugate

The 11S–80PC conjugate was formed by the alkali-heating treatment [13]. In brief, 100 mL distilled H₂O was added with 1.0 g 11S and 200 mg PC and then adjust to pH 9.0. Next, the resulting solutions were stirred at 80 °C with free exposure to air for 20 min. Finally, the resulting solutions were dialyzed with the dialysis bag (8000–14,000 Da) for 48 h at 4 °C for the removal of free PC, and then freeze-dried to obtain the 11S-PC conjugates (11S-80PC). 11S without treatment by PC and heating was used as a control. 11S without treatment by PC and with treatment by heating was named 11S-80.

2.4. SDS-PAGE

The SDS-PAGE was performed, as previously described in the study of Pi, et al. [12] with only changing the sample solution concentration to 2 mg/mL.

2.5. Structural analysis

2.5.1. Scanning electron microscope (SEM)

SEM was performed in accordance with the study of Pi, Sun, et al. [15]. In brief, the freeze-dried sample was sprayed with gold and captured at $\times 2000$ magnification.

2.5.2. Circular dichroism (CD) spectroscopy

The CD was performed at 0.2 mg/mL sample concentration and 100 nm/min wave speed from 190 nm to 260 nm by a CD spectrometer (MOS-4SO, BIO-LOGIC, France), as previously described in the study of Lin, et al. [3].

2.5.3. Ultraviolet (UV) absorption spectroscopy

The UV was performed for 200–420 nm at 0.2 mg/mL sample concentration by a T9 UV-visible spectrophotometer (Puxi Analytical Instrument Co. Ltd., Beijing, China), as previously described in the study of Pi, et al. [12].

2.5.4. Surface hydrophobicity

The 8-aniline-1-naphthalene sulfonate (ANS) method was conducted to assess the surface hydrophobicity in accordance with the study of Zhou, et al. [16]. In brief, 8 mM ANS solution (pH 7.4, 10 μ L) was added to the sample solution (0.2 mg/mL, 4 mL). After reaction for 2 h, the sample solution was detected under 420–600 nm by the cary eclipse fluorescence spectrometer (F-7100, HITACHI, Japan).

2.5.5. Free sulfhydryl (SH) group content

The Ellman method was conducted to evaluate the free SH group content, as previously reported by Meng & Li [17]. In brief, the sample solution (0.2 mg/mL, 200 μ L) was reacted with Ellman's reagent (10 μ L) for 20 min at 37 °C, and then measured the absorbance value at 412 nm (OD₄₁₂) by a microplate reader (SpectraMax reg iD3, Beckman Coulter, US). The content of free SH groups was computed by the Eq. (1):

$$\text{Free SH group content } (\mu\text{mol/g of protein}) = 73.5 \times \text{OD}_{412} \times D/C \quad (1)$$

Here, D was the dilution factor and C was the concentration (mg/mL) of samples.

2.6. LC/MS-MS

The EASY-nLC 1200 coupled with Q Exactive Plus-Orbitrap MS was used to conduct the LC/MS-MS in accordance with the study of Pi, et al. [12]. Briefly, the sample (150 μ g) was reduced by dithiothreitol, alkylated by iodoacetamide and then digested with trypsin at 37 °C overnight. After the digestion was stopped by adding C₂HF₃O₂ solution (10 %, v/v), the resulting sample was desalted, vacuum dried, and then dissolved with 20 μ L of 0.1 % formic acid solution for LC/MS-MS detection.

The Proteome Discoverer 24 Software (Thermo Fisher Scientific, Waltham, USA) was used to analyze the LC-MS/MS data under the soybean database (<https://www.uniprot.org/>, 2021.12.12). The abundance of the peptide was measured by the label free quantification (LFQ) method with the control 11S as the standard, as previously reported by Pi, et al. [12]. Heatmap was drawn on <https://biit.cs.ut.ee/clustvis/>. The detected peptide was considered an epitope based on the method of Zhang, et al. [18] that the peptide contained the fragments of the reported epitope [19,20] (Table S2).

2.7. Molecular docking

Schrodinger 2019.01 (Schrodinger, Inc., State of California, USA) was used to perform a docking study between soybean protein 11S and PC. The 3D coordinates structure of soybean protein 11S (ID: AF-A0A445LCA8-F1) was acquired from <https://www.uniprot.org/uniprot/A0A445LCA8/>. The 3D coordinates structure of PC (CID:

107876) was obtained from <https://pubchem.ncbi.nlm.nih.gov/>. Substrate binding affinity was estimated by the docking score and the number of top hits was set at 50. Pymol2.1 (Schrodinger, Inc., State of California, USA) was used for visualization.

2.8. The IgE binding capacity

2.8.1. Western blot analysis

The Western blot analysis was performed, as previously described by Li, et al. [21]. Firstly, proteins on SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membranes using a membrane Semi-Dry Transblot system (Bio-Rad). The PVDF membrane was blocked by 3 % skim milk and then washed by TBST three times at 15 min/time. Next, the membrane was incubated with the diluted (1:70) sera at 4 °C for 12 h, with the diluted (1:3000) goat anti-human IgE HRP conjugates (Chongqing Manuik Technology Co., Ltd., Chongqing, China) at 25 °C for 1 h, and then with super ECL Western Blotting Substrate at 25 °C for 5 min. The membrane was washed before each procedure. Finally, the membrane was detected by Western-bolt Enhanced New Chemiluminescence Detector (Clinx Science Instruments, Shanghai, China).

2.8.2. Indirect ELISA experiments

The IgE binding capacity was furtherly measured by indirect ELISA, as previously described by Sun, et al. [22]. Firstly, the sample was diluted to 1 µg/mL using a carbonate buffer solution (pH 9.6), and then coated with 100 µL/well at 4 °C for 12 h. After the plate was washed three times for 3 min/time using 0.01 M PBS (pH 7.0) containing 0.05 % Tween-20 (PBST), the plate was blocked by the 5 % skim milk at 37 °C for 60 min. Next, the plate was coated with 100 µL diluted (1:5) sera for 60 min. Then, the plate was coated with 100 µL diluted (1:2000) goat anti-human IgE HRP conjugates for 60 min. The plate was washed before each procedure. Finally, the plate was coated with 100 µL TMB solution at 37 °C for 13 min, and then terminated by adding 100 µL of 0.2 M sulfuric acid. The absorbance at 450 nm was immediately measured. The IgE binding capacity of the treated sample was computed by the Eq. (2) with the control 11S protein as 100 %.

The IgE binding capacities of other samples

$$= \frac{OD_{450}(\text{treated samples})}{OD_{450}(\text{control 11S})} \times 100\% \quad (2)$$

2.9. Antioxidant activity

The antioxidant activity was assessed by measuring the DPPH and ABTS free radical scavenging capacities [10]. Briefly, sample solutions (0.2 mg/mL, 100 µL) were reacted at a volume ratio (1:1) with the DPPH or ABTS working solution in the dark for 30 min or 10 min, and then measured the absorbance value of the sample (A_{sample}) at 517 nm or 734 nm, respectively. 100 µL distilled H₂O was treated with the same condition and then measured its absorbance value (A_0). The DPPH and ABTS free radical scavenging rates were computed by Eqs. (3) and (4), respectively.

$$\text{The DPPH radical scavenging rate (\%)} = \frac{A_0 - A_{\text{sample}}}{A_0} \times 100\%. \quad (3)$$

$$\text{The ABTS radical scavenging rate (\%)} = \frac{A_0 - A_{\text{sample}}}{A_0} \times 100\%. \quad (4)$$

2.10. Foaming properties

The foaming capacity (FC) and stability (FS) were measured according to the study of Yang, et al. [23]. After 2 mg/mL of sample solution (volume, $V = 10$ mL) was whipped for 1 min at 8000 rpm, the volume of the whipped sample after 0 min (V_0) and 10 min (V_{10}) was recorded. FC and FS were computed by Eqs. (5) and (6), respectively:

$$\text{FC (\%)} = \frac{V_0 - V}{V} \times 100\%. \quad (5)$$

$$\text{FS (\%)} = \frac{V_{10} - V}{V} \times 100\% \quad (6)$$

2.11. Emulsifying properties

The emulsifying activity (EAI) and emulsifying stability (ESI) were measured according to the study of Yang, et al. [24]. In brief, sample solutions (2 mg/mL) were blended with soybean oil, homogenized at 10000 rpm for 2 min, and then the emulsion was obtained from the bottom at 0 and 10 min. After mixing at a ratio of 1:4 (v/v) with SDS solution (0.1 %, w/v), a microplate reader was used to measure the absorbance at 500 nm for the bottom emulsion at 0 (A_0) and 10 min (A_{10}). EAI and ESI were computed by Eqs. (7) and (8), respectively

$$\text{EAI (m}^2/\text{g)} = \frac{2 \times 2.303 \times A_0 \times D}{N \times C \times 10000} \quad (7)$$

$$\text{ESI (min)} = \frac{A_{10}}{A_0} \times 100 \quad (8)$$

D, C, and N was the dilution factor, protein content (g/mL) and volumetric oil fraction, respectively.

3. Results and discussion

3.1. SDS-PAGE

The alteration of molecular weight (MW) in soy 11S proteins was assessed by SDS-PAGE (Fig. 1). Heating caused a slight diminution in the band denseness (Fig. 1, land 2), whereas conjugation of PC using alkali-heating treatment induced significant diminution in the band denseness, with some polymers (>180 kDa) being found (Fig. 1, land 3). Heating was reported to cause the denaturation of proteins and generate several peptides, resulting in the diminution in the denseness of protein bands based on SDS-PAGE [15,25]. Conjugation of polyphenols was also reported to induce the formation of protein-polyphenol conjugates and protein-crosslinks, which was responsible for the weaker bands in 7S-

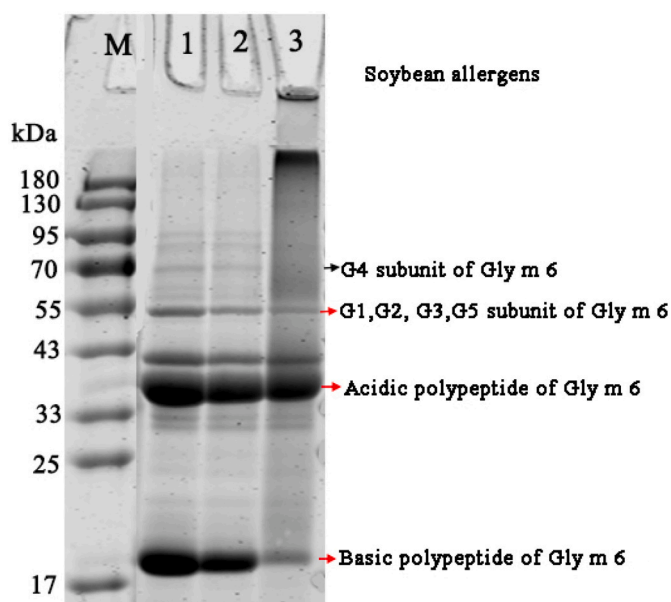


Fig. 1. The changes in protein profiles after soybean protein 11S was treated with heating and proanthocyanidins. M, marker; land 1, control 11S; land 2, 11S-80; land 3, 11S-80PC conjugates.

EGCG conjugates than the native 7S protein, with protein aggregations being found [3]. Therefore, 11S-80 exhibited a slight loss of band denseness due to the denaturation of proteins, whereas 11S-80PC exhibited extreme loss of band denseness and the formation of polymers (>180 kDa) owing to the formation of protein-polyphenol conjugates and the crosslinking of protein. Similar results were shown in Ara h 1-EGCG and soybean protein 11S-EGCG prepared by alkaline method at 25 °C for 24 h [3,9].

3.2. Changes in structure in 11S-80PC conjugates

3.2.1. Microstructure

The alteration of microstructure was assessed by SEM. As illustrated in Fig. 2A, 11S-80 exhibited a slightly rough surface structure with few

aggregates, whereas 11S-80PC showed an extremely rough surface structure with many aggregates. Thus, conjugation of PC using alkali-heating treatment caused more changes in microstructure than heating, which was attributed to the entry of proanthocyanidins into the “hydrophobic pocket” induced by the interaction of PC with 11S and the protein-unfolding induced by heating [26]. Specifically, the heating treatment might cause the soybean protein 11S to unfold and loosen for interacting with PC more easily, and conjugation of PC was mainly responsible for the changes in microstructure in 11S-80PC. Considering that the microstructure alteration is related to the conformational structure changes [15], it was suggested that conjugation of PC using alkali-heating treatment might induce more changes in conformational structure than heating.

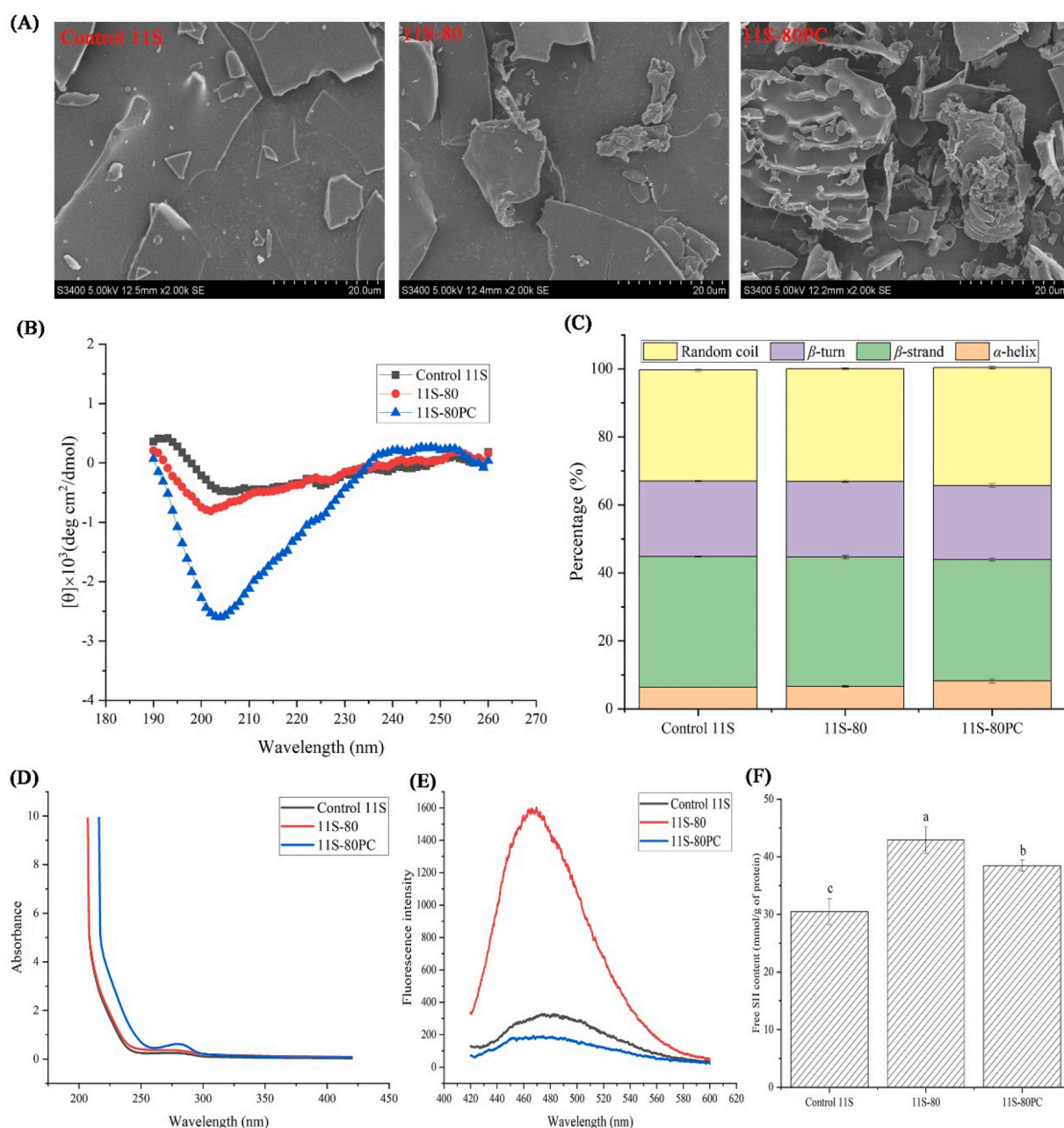


Fig. 2. The changes in the structure after soybean protein 11S was treated with heating and proanthocyanidins. A, SEM; B, Circular dichroism spectra; C, the secondary structure analyses based on Circular dichroism spectra, α -helix, β -sheet, β -turn and random coil was shown in orange, green, purple and yellow respectively; D, ultraviolet absorption spectroscopy; E, surface hydrophobicity. F, the content of free SH groups. Means with different letters (a-c) in the bars indicated significant differences ($P < 0.05$).

3.2.2. Secondary structures

The alteration of the secondary structure was assessed by CD spectroscopy. As illustrated in Fig. 2B, a negative band at about 208 nm represents the α -helical in proteins [12]. The samples 11S-80 and 11S-80PC showed a high absolute θ value at 208 nm than the control 11S, indicating an increase in α -helical after treatment. In addition, 11S-80PC showed a higher absolute θ value than 11S-80, implying a higher content of α -helical in 11S-80PC than in 11S-80. The random coil was reported to be related to negative bands at about 195 nm [27], implying a higher content of random coil in 11S-80PC than in 11S-80 because 11S-80PC showed a higher absolute θ value than 11S-80. Other second structures such as β -sheet or β -turn also correspond to negative bands at 218 nm and positive bands at 195 nm or positive bands at 206 nm and negative bands at 180–190 nm, respectively [27]. It was observed that the absolute θ value of these spectrum bands was changed, suggesting the alteration of secondary structure in 11S-80 and 11S-80PC, compared to the control 11S. The content of the secondary structure of the control 11S, 11S-80 and 11S-80PC was calculated on <http://dichroweb.cryst.bbk.ac.uk/html/process.shtml>. As shown in Fig. 2C (Table S3), the control 11S contained 6.40 % α -helical, 38.4 % β -sheet, 22.2 % β -turn and 32.7 % random coil, whereas 11S-80 comprised 6.63 % α -helical, 38.0 % β -sheet, 22.3 % β -turn, 33.1 % random coil and 11S-80PC contained 8.23 % α -helical, 35.7 % β -sheet, 21.8 % β -turn, 34.6 % random coil. The content of α -helical was increased by 3.6 % and 28.6 % in 11S-80 and 11S-80PC, respectively, whereas the content of random coil was enhanced by 1.2 % and 5.8 % in 11S-80 and 11S-80PC, which was consistent with results of Fig. 2B. 11S-80PC exhibited more alteration of α -helical and random coil than 11S-80, demonstrating that conjugation of PC using alkali-heating treatment caused more changes in secondary structure than heating treatment. In addition, the increase of random coil was reported to be responsible for the protein-unfolding [11,12], implying that conjugation of PC using alkali-heating treatment induced more protein-unfolding than heating treatment. Similar results were illustrated in Ara h 1-EGCG conjugates, showing the enhancement of random coil and the protein-unfolding [9].

3.2.3. Tertiary structure

The tertiary structure alteration was evaluated by UV spectra, surface hydrophobicity and free sulfhydryl groups [12].

UV spectra (Fig. 2D) showed that the absorption intensity was increased in 11S-80 and 11S-80PC, and 11S-80PC showed higher UV absorption intensity than 11S-80. UV absorption spectra at 280 nm are related to tryptophan and tyrosine residues [16]. The increased UV absorption intensity was reported to indicate the exposure of tryptophan and tyrosine residues and the unfolding of protein [16,28]. Thus, 11S-80PC exhibited more exposure of tryptophan and tyrosine residues and the unfolding of protein than 11S-80. In addition, an obvious red shift of the absorption peak was also observed in 11S-80PC rather than in 11S-80, which showed the alteration in polarity of the microenvironment of Tyr and Trp, and destruction of peptide bonds [16], thus probably changing the primary structure in 11S after conjugation of PC using alkali-heating treatment.

The ANS fluorescence intensity was increased in 11S-80 but decreased in 11S-80PC (Fig. 2E). The ANS fluorescence intensity represented the hydrophobic group and its increased intensity was reported to indicate the exposure of hydrophobic groups [29]. Thus, heating caused the exposure of hydrophobic groups, probably owing to the unfolding of the protein. Conjugation of PC using alkali-heating treatment induced the masking or destruction of hydrophobic groups, which was probably because of the hydrophobic interaction between 11S and proanthocyanidins and the entry of proanthocyanidins into the “hydrophobic pocket” in 11S protein [26,30]. Moreover, heating treatment might cause the soybean protein 11S to unfold and loosen for interacting with PC more easily, improving the accessibility of PC to the exposed hydrophobic groups, thus masking and destroying the hydrophobic groups for a decrease in ANS the fluorescence intensity in 11S-80PC.

As shown in Fig. 2F, there was a 0.8 % and 26.2 % increase in free SH content in 11S-80 and 11S-80PC, respectively, implying the exposure of free SH, which was attributed to the protein unfolding [24] and the cleavage or rearrangements of disulfide bonds [31]. On the one hand, heating treatment might cause the soybean protein 11S to unfold and loosen for interacting with PC more easily, improving the accessibility of PC to the exposed free SH groups, thus masking and destroying the free SH groups for the lower content in free SH groups in 11S-80PC than in 11S-80. On the other hand, conjugation of PC could induce the oxidation reaction, resulting in the oxidation of the free SH groups [28], thus resulting in a lower content in free SH groups in 11S-80PC than in 11S-80.

Overall, 11S-80PC conjugates exhibited more protein unfolding than 11S-80 compared to the control 11S, which was consistent with Fig. 2B and C. Similar results were illustrated in Ara h 1-EGCG conjugates, showing the protein-unfolding [9].

3.3. Changes in peptide and epitope profiles

As shown in Fig. 3A, the alteration in peptide fingerprints of soybean 11S protein was observed after heating and PC treatment. Heatmap analysis put control 11S and 11S-80 in the same category, demonstrating the similarity of peptide profiles in control 11S and 11S-80. Thus, conjugation of PC using alkali-heating treatment caused more modification of soybean 11S protein than heating, probably because the 11S-80PC sample formed >180 kDa polymers and exhibited more protein-unfolding than 11S-80. In these peptides, 10 peptides were identified as epitopes because they comprised the fragments of the reported epitope [19,20], which was shown in Table 1. Overall, there was a 35.6 % increase in 11S-80, but a 14.5 % decrease in 11S-80PC for the abundance of total linear epitopes. The increased abundance of epitopes represents the exposure of epitopes [12]. Thus, heating caused the exposure of many linear epitopes, whereas conjugation of PC using alkali-heating treatment led to the masking or destruction of many linear epitopes. The different changes in linear epitopes might result in different allergenicity between 11S-80 and 11S-80PC.

These identified linear epitopes are labeled in Fig. 4A. It was observed that these linear epitopes were located on α -helical, β -sheet and random coil in soybean 11S protein. 11S-80PC showed a decrease in total linear epitopes, demonstrating that PC could react with nucleophilic protein residues on α -helical, β -sheet and random coil to destroy and mask linear epitopes. As shown in Fig. 3B, heatmap analysis put control 11S and 11S-80 in the same category, demonstrating the similarity of linear epitope profiles in control 11S and 11S-80. Thus, conjugation of PC using alkali-heating treatment caused more modification of linear epitopes in soybean 11S protein than heating, probably because the 11S-80PC sample formed >180 kDa polymers and exhibited more protein-unfolding than 11S-80.

In addition to the reaction with nucleophilic protein residues (e.g., cysteine, methionine, histidine, lysine, tryptophan, and tyrosine) to form covalent bonds [12,32], molecular docking results (Fig. 4B–D) demonstrated that PC bound well to soybean 11S proteins with a binding energy of -7.853 kcal/mol. As shown in Fig. 4D, PC can also react with GLU-272, ASN-94, GLU-135, GLN-317, GLU-273, and ARG-134 in soybean 11S protein, in which ASN-94 and GLN-317 were involved in the epitope AA78-117 and AA313-246, implying that the two linear epitopes were also affected by hydrogen bond interactions. As shown in Fig. 4C, PC could be well matched to the surface of the protein site, implying that PC could mask the epitopes located on the surface of soybean 11S protein [20]. Therefore, conjugation of PC using alkali-heating treatment resulted in more changes in peptide and epitope profiles in soybean 11S protein than in heating treatment.

3.4. Changes in the IgE binding capacity

The IgE binding capacity was assessed by ELISA (Fig. 5A) and

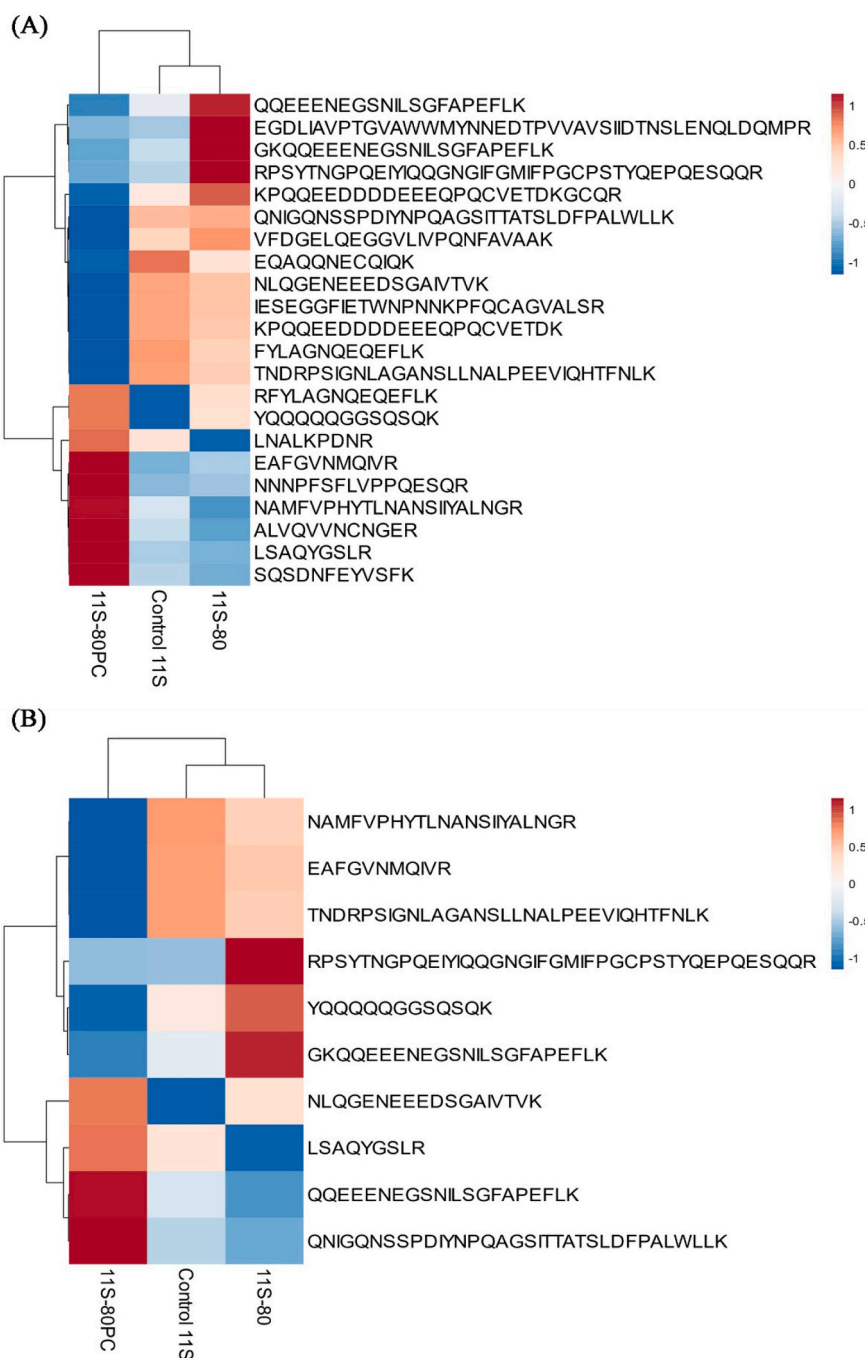


Fig. 3. Heatmap analysis of peptide (A) and epitopes (B) profiles after soybean protein 11S was treated with heating and proanthocyanidins.

western-blot (Fig. 5B). As shown in ELISA, a significant reduction in the IgE binding capacity was observed in 11S-80 and 11S-80PC, compared to the control 11S. The IgE binding capacity was reduced by 33.2 % in 11S-80, whereas it was reduced by 56.77 % in 11S-80PC. As shown in western-blot, the intensity of all bands was diminished in 11S-80 (Fig. 5B, land 2) and 11S-80PC (Fig. 5B, land 3), compared to the control 11S (Fig. 5B, land 1), implying a reduction in the IgE binding capacity. Additionally, 11S-80PC exhibited more diminution of all bands than 11S-80, demonstrating a lower IgE binding capacity in 11S-80PC than 11S-80, which was consistent with ELISA. Therefore, heating and conjugation of PC using alkali-heating treatment both decreased the IgE binding capacity of soybean 11S protein. And conjugation of PC using alkali-heating treatment resulted more decrease in IgE binding capacity than heating. Similar results were illustrated in heated (80 °C for 5–60

min) ovotransferrin and soy protein 7S-EGCG conjugates, showing a reduced IgE binding capacity [3,31]. Moreover, considering that the western-blot under reducing conditions aims to evaluate the IgE binding capacity of the denatured and dissociated samples, it was implied that the treated sample might exhibit a low IgE binding capacity after gastrointestinal digestion. Similar results were shown in protein-polyphenol conjugates, showing a low IgE binding capacity by Western-blot or ELISA experiments, which was accompanied by a lower IgE binding capacity after digestion [3,9,11,33,34].

The alteration in allergenicity is usually related to the changes in protein structure [31]. As shown in Fig. 2, heating and heating-PC treatment both caused the unfolding of 11S protein, which was reflected by the changes in SEM, CD spectroscopy, UV spectroscopy, surface hydrophobicity and free SH groups. Protein unfolding was reported

Table 1
Determination of allergic peptides in soybean 11S protein.

Sequence	Position	Abundance		
		Control 11S	11S-80	11S-80PC
RPSYTN <u>GPQEIYIQ</u> QNGIFGMIFPGCPSTYQEPQESQQR	AA78-117	3.7	295.2	1
<u>YQQQQGG</u> SQSQK	AA192-204	104.9	125.9	69.3
GKQQEEENEGSNILSGFAPEFLK	AA205-227	86	194.8	19.2
QEEENEGSNILSGFAPEFLK	AA207-227	89.1	69.7	141.3
EAFGVNMQIVR	AA228-238	224.9	217.2	157.9
NLQGENEEDSGAIVTVK	AA239-256	60.3	110.7	129
QNIGQNSSPDYINPQAGSITTATSLDFPALWLLK	AA313-346	85	77.2	137.7
<u>LSAQY</u> GSLR	AA347-355	108.6	61.4	130
NAMFVPHYTLNANSIYALNGR	AA357-378	123.1	113.9	63.1
TNDRPSIGNLAGANSLNALPEEVQHHTFNLK	AA426-457	141.9	127.7	30.4
Total		1027.5	1393.7	878.9

Note: The underline indicates that this segment of amino acids is the same as a segment of the reported epitope.

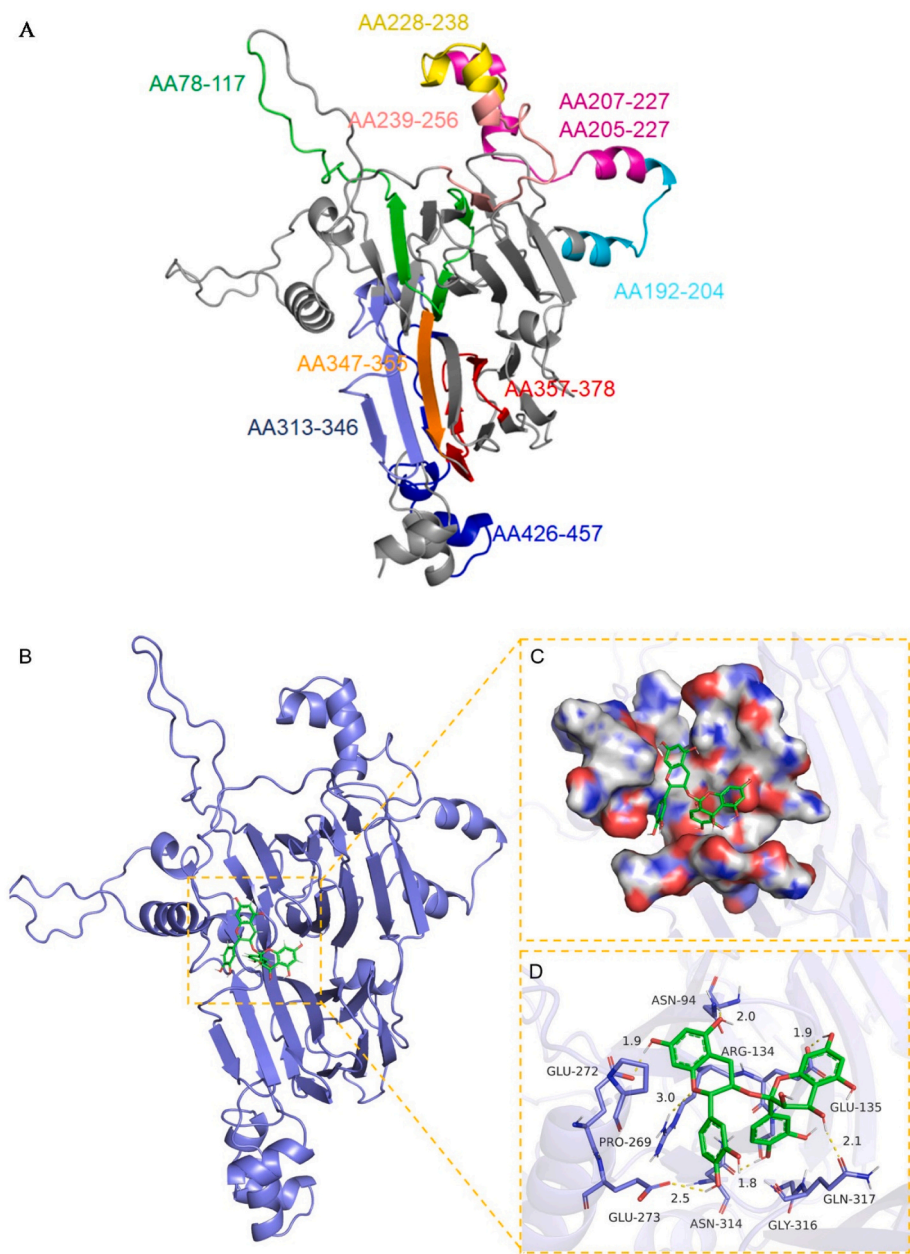


Fig. 4. The analysis of conjugation of proanthocyanidins with soybean 11S protein based on molecular model technology. A, Distribution of the identified linear epitopes; B, The 3D structure of conjugates; C, The electrostatic surface of soybean 11S protein active site; D, The detail binding mode of proanthocyanidins with soybean11S protein.

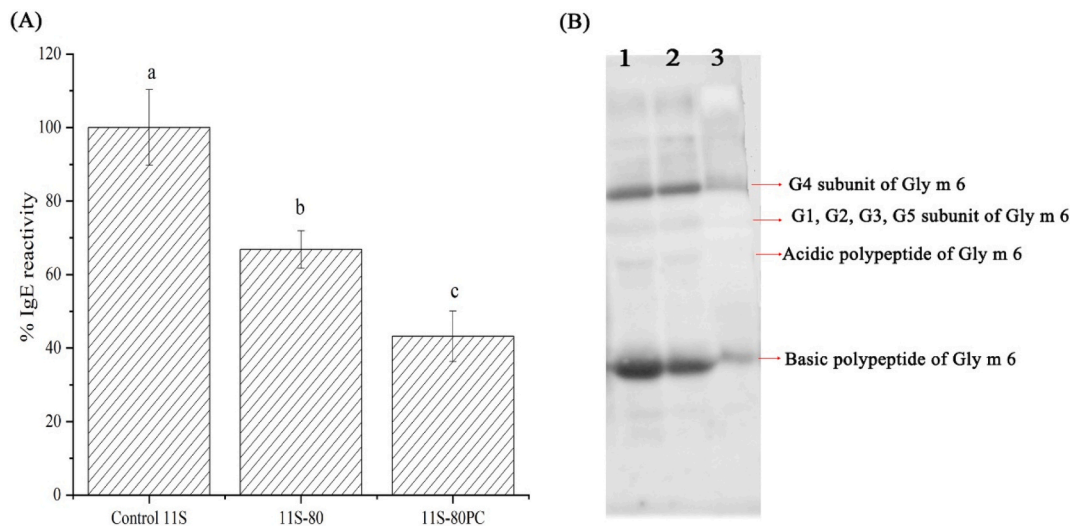


Fig. 5. The changes in IgE-binding capacity after soybean 11S protein was treated with heating and proanthocyanidins. A, ELISA. B, Western-blot, land 1, control 11S; land 2, 11S-80; land 3, 11S-80PC conjugates. Means with different letters (a–c) in the bars indicated significant differences ($P < 0.05$).

to obscure and disrupt conformational epitopes, resulting in the reduction of allergenicity in heated (60–100 °C for 20 min) silkworm pupa protein extract [35] and ovalbumin–EGCG [11]. Therefore, 11S-80PC and 11S-80 exhibited a reduction of IgE binding capacity, probably resulting from the obscuring and disruption of conformational epitopes. Ahmed, et al. [36] found a decreased IgE binding capacity in tropomyosin-caffeic acid conjugates because the unfolding of tropomyosin caused the exposure of antigen epitopes to improve the accessibility of caffeic acid for obscuring and disrupting more epitopes. Therefore, 11S-80PC showed more protein-unfolding (e.g., more random coil content and UV spectroscopy intensity) than 11S-80, probably implying more obscuring and disruption of conformational epitopes in 11S-80PC than 11S-80, which was responsible for the lower IgE binding capacity in 11S-80PC than 11S-80 (Fig. 5A and B). What's more, disulfide bonds are related to the integrity of the IgE-binding epitopes [31]. Fig. 2F showed an increase in the content of free SH, demonstrating the cleavage of disulfide bonds, thus disrupting the integrity of the IgE-binding epitopes and reducing the IgE binding capacity. In addition, considering that most epitopes in 11S were located on the surface of proteins [20], PC could mask the conformational epitopes to reduce the IgE binding capacity.

Besides conformational epitopes, the IgE binding capacity is also depended on linear epitopes [11,12]. As shown in Table 1, 11S-80PC exhibited a 14.5 % reduction in total linear epitopes, whereas 11S-80 showed a 35.6 % increase in total linear epitopes. Therefore, besides probably more obscuring and disruption of conformational epitopes in 11S-80PC than in 11S-80, the obscuring and disruption of total linear epitopes was responsible for the lower IgE binding capacity in 11S-80PC than 11S-80. In addition, PC could react with amino or sulfhydryl side chains of 11S, probably resulting in the disruption of linear epitopes for a decrease in IgE binding capacity, which did not exist in heating. Of note, conjugation of PC using alkali-heating treatment did not completely decrease the IgE binding capacity of soybean 11S proteins, probably resulting from the persistence of linear epitopes [31]. What's more, conjugation of PC using alkali-heating treatment induced the reduction of IgE binding capacity in soybean 11S proteins, which was responsible for the reduction of IgE binding capacity in total soybean protein after conjugation of PC using alkali-heating treatment.

3.5. Antioxidant activity

Certain food with desired antioxidant capacity contributes to the relief of oxidative damaged diseases such as atherosclerosis and aging

[37]. The antioxidant capacity is usually assessed by measuring DPPH and ABTS radical scavenging activities. As shown in Fig. 6A, 11S-80PC showed a significant ($P < 0.05$) increased (26.23-fold) DPPH radical scavenging activity, although 11S-80 had no significant ($P > 0.05$) changes. As shown in Fig. 6B, 11S-80 only showed a 2.02-fold increased ABTS radical scavenging activity, in comparison to the control 11S, whereas a 12.88-fold increase was observed in 11S–80P. Although heating slightly increased antioxidant capacity, probably resulting from the production of peptides [25], conjugation of PC using alkali-heating treatment significantly increased antioxidant capacity, which was mainly because hydroxyl groups in PC were introduced to the 11S protein [11]. Similar results were observed by Lin, et al. [3], who found the increased DPPH and ABTS radical scavenging activity in soybean protein 7S after alkaline treatment with EGCG at 25 °C for 24 h.

3.6. Foaming properties

The foaming properties are closely related to the production of some foods such as cake and ice cream. The foaming properties are usually assessed by measuring FC and FS. FC is the foam amount after whipping the protein solution and FS is the ability of the foam to resist collapsing during storage [23]. As shown in Fig. 6C, there was a significant ($P < 0.05$) increase in FC after treatment. And 11S-80 and 11S-80PC showed a 75 % and 50 % increase in FC, respectively. It was reported that surface activity, flexibility, and protein interactions played an important role in FC [23]. The FC was increased in 11S-80, probably resulting from the increase in surface hydrophobicity (Fig. 2E) [38], whereas 11S-80PC showed an increase in FC, probably due to the increased molecular flexibility (reflected by the increase of random coil, Fig. 2B) [12], although it showed a low surface hydrophobicity. As shown in Fig. 6D, the FS was significantly ($P < 0.05$) increased after treatment, and 11S-80 and 11S-80PC showed a 2.12- and 3.48-fold increase in FC, respectively. The FC was increased in 11S-80, probably because the formation of few aggregates increased the resistance of the foam to collapse, whereas 11S-80PC showed an increase in FC, probably because hydroxyl groups in proanthocyanidins were introduced on the 11S protein to enhance the ability of the foam to resist collapsing [12]. Similar results were observed in Lin, et al. [3], who found that FC and FS of soybean protein 7S were increased after alkaline treatment with EGCG at 25 °C for 24 h.

3.7. Emulsifying properties

The emulsifying properties are important for many food applications

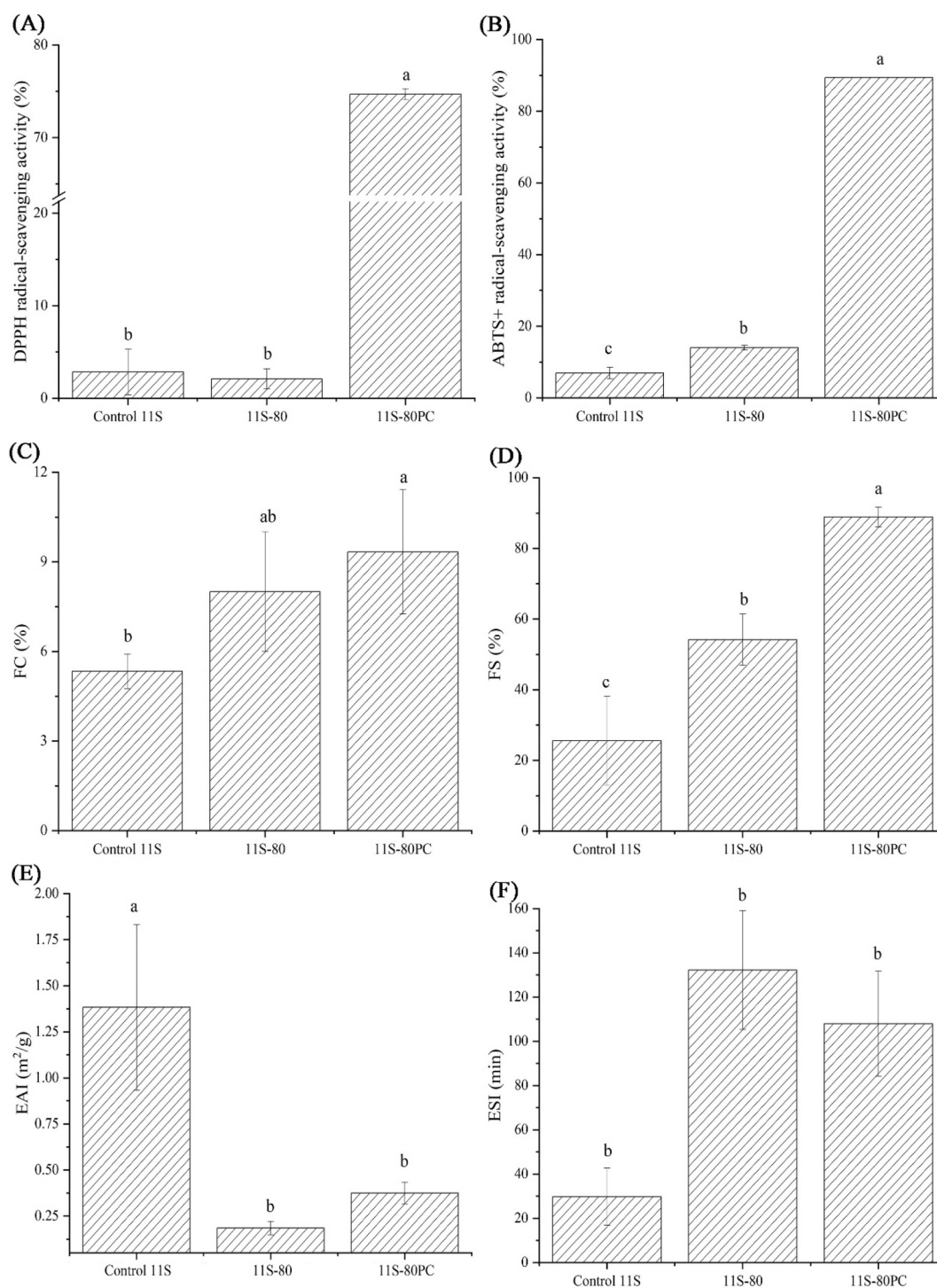


Fig. 6. The changes in functional properties after soybean 11S protein was treated with heating and proanthocyanidins. A, DPPH radical scavenging capacity; B, ABTS radical scavenging capacity; C, foaming capacity (FC); D, foaming stability (FS); E, emulsifying activity (EAI); F, emulsification stability (ESI). Means with different letters (a–c) in the bars indicated significant differences ($P < 0.05$).

such as sauces and condiments. The emulsifying properties are usually assessed by measuring EAI and ESI. The EAI refers to the ability of proteins to rapidly adsorb to the surfaces of oil droplets and form small droplets, whereas ESI refers to the ability of the emulsions to resist aggregation and phase separation [39]. As shown in Fig. 6E, there was a significant ($P < 0.05$) decreased EAI after treatment. And 11S-80PC showed a higher EAI than 11S-80, probably resulting from more unfolding in 11S-80PC than 11S-80 [33]. As shown in Fig. 6F, The ESI was significantly ($P < 0.05$) increased after treatment, and 11S-80 and

11S-80PC showed a 3.48- and 2.12-folds increase in ESI, respectively, probably resulting from the formation of aggregates to form a thick protein membrane, thus enhancing ESI for the oil droplets [40]. 11S-80PC showed a slight lower ESI than 11S due to the formation of more aggregates in 11S-80PC, decreasing the ability of the emulsions to resist aggregation and phase separation. A similar result was illustrated in ovalbumin-EGCG conjugates, showing an increased ESI [11].

4. Conclusion

The study assessed the alteration of the IgE binding capacity, anti-oxidant, emulsifying and foaming properties of 11S-80PC conjugates formed by alkali-heating treatment (pH 9.0 and 80 °C) for 20 min. During the alkali-heating treatment, heating accelerated the process of PC to form 11S-80PC conjugates and protein-crosslinks. What's more, 11S-80PC exhibited more protein-unfolding to increase the accessibility of PC for modifying the 11S protein, which was responsible for the significant differences in peptides and epitopes between the control 11S and 11S-80PC. Protein-unfolding caused the obscuring and disrupting of conformational epitopes. LC/MS-MS further showed that the obscuring and disruption of linear epitopes were observed in 11S-80PC rather than in 11S-80. Therefore, 11S-80PC showed more reduction of IgE binding capacity than 11S-80, probably resulting from more obscuring and disruption of linear and conformational epitopes induced by more protein unfolding. What's more, 11S-80PC exhibited higher antioxidant activity and foaming properties than the control 11S and 11S-80. The emulsifying activity was also slightly increased in 11S-80PC, compared to 11S-80. These results demonstrated that the conjugation of proanthocyanidins using alkali-heating treatment was an effective way to prepare hypoallergenic soy protein products with great functional properties. Future research should further optimize the reaction conditions for producing products with less allergenicity and more functionality, and conduct animal and human trials for ensuring hypoallergenic and functional effects.

CRedit authorship contribution statement

Xiaowen Pi designed the manuscript, performed preliminary data analysis, and wrote the initial manuscript. Ph. D Yuxue Sun, Shuxia Liang, Zengyu peng modified manuscripts. Jiafei Liu provided some supports for Xiaowen Pi. Professor Jianjun Cheng supervised the project and edited the final version of the manuscript.

Declaration of competing interest

All authors declare no conflict of interest.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2022.12.067>.

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