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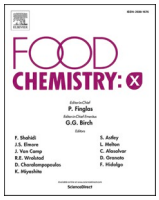

Investigation of the differences in the effect of (−)-epigallocatechin gallate and proanthocyanidins on the functionality and allergenicity of soybean protein isolate

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ABSTRACT

In this study, the differences in effects of (−)-epigallocatechin gallate (EGCG) and proanthocyanidins (PC) on the functionality and allergenicity of soybean protein isolate (SPI) were studied. SDS-PAGE demonstrated that SPI-PC conjugates exhibited more high-molecular-weight polymers (>180 kDa) than SPI-EGCG conjugates. Structural analysis showed that SPI-PC conjugates exhibited more disordered structures and protein-unfolding, improving the accessibility of PC to modify SPI, compared to SPI-EGCG conjugates. LC/MS-MS demonstrated that PC caused more modification of SPI and major soybean allergens than EGCG, resulting in a lower abundance of epitopes. The successful attachment of EGCG and PC to SPI significantly increased antioxidant capacity in conjugates. Furthermore, SPI-PC conjugates exhibited greater emulsifying activity and lower immunoglobulin E (IgE) binding capacity than SPI-EGCG conjugates, which was attributed to more disordered structure and protein-unfolding in SPI-PC conjugates. It is implied that proanthocyanidins may be promising compounds to interact with soybean proteins to produce functional and hypoallergenic foods.

1. Introduction

Soybean proteins, which account for about 43 %–48 % (w/w) in soybean, are rich in balanced amino acid composition (Li, Zhou, Ren, Fan, Hu, Zhuo, et al., 2019), making them a great source of protein supplements for animal and human consumption (Geng, Liu, Frazier, Shi, Bell, Glenn, et al., 2015; Sui, Zhang, & Jiang, 2015). Moreover, soybean proteins have the great potential for increasing many functional properties, such as emulsification, gelation, foaming, and water and fat absorption, which improves the broad range of food applications and the acceptance of food by consumers (Sui, Zhang, & Jiang, 2021). However, soybean proteins are major food allergens, containing at least 33 allergic ingredients (Pi, Sun, Fu, Wu, & Cheng, 2021). Soybean proteins can induce allergic reactions such as conjunctivitis, urticarial, vomiting, anaphylactic shock and death (Lin et al., 2022; Pi, Sun, Fu, Wu, & Cheng, 2021), which is mainly triggered by major soybean allergens such as Gly m 4, Gly m 5, Gly m 6, Gly m Bd 28 K, Gly m Bd 30 K and Kunitz trypsin inhibitor (Hanafusa, Murakami, Ueda, Yano, Zaima, & Moriyama, 2018; Pi, Sun, Fu, Wu, & Cheng, 2021). Therefore, it would be promising to develop effective methods of increasing the functional properties and decreasing the allergenicity of soybean proteins simultaneously.

Polyphenols are an effective and promising way to reduce the allergenicity, while improving the functional properties of proteins (Pi, Sun, Cheng, Fu, & Guo, 2022). There was a reduction of allergenicity in covalent EGCG-lactoferrin (Li et al., 2021), EGCG-ovalbumin (He et al., 2019) and chlorogenic acid-Ara h 1 conjugates (He et al., 2020), which was accompanied by the increase of antioxidant activity, digestibility, emulsifying and foaming properties (He, et al., 2019; He, et al., 2020; Li, et al., 2021). Similar results were shown in noncovalent caffeic acid- and EGCG-whey protein conjugates, showing the reduction of allergenicity and the increase of thermal stability (Pessato et al., 2018). Compared to noncovalent conjugates, covalent conjugates might be suitable for food processing (Pi, Sun, Cheng, Fu, & Guo, 2022).

EGCG and proanthocyanidins are both flavonoids and have powerful antioxidant activity, which is widely found in green tea and fruits, respectively. EGCG has been successfully used to reduce the

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allergenicity and improve functional properties in whey proteins (Pessato, et al., 2018), peanut protein (He, et al., 2020), β-lactoglobulin (Wu, et al., 2018), ovalbumin (He, et al., 2019), lactoferrin (Li, et al., 2021), soybean 7S protein (Lin, et al., 2022). Our previous study has also shown that conjugation of proanthocyanidins could reduce the allergenicity and increase the antioxidant activity, foaming properties and emulsion properties in soybean proteins simultaneously (Pi et al., 2023). It was reported that polyphenol types played important role in crosslinking reaction between protein and polyphenol, directly affecting the alteration of allergenicity and functional properties (Pi, Liu, Sun, Ban, Cheng, & Guo, 2023; Pi, Sun, Cheng, Fu, & Guo, 2022). At present, the differences in the effect of EGCG and proanthocyanidins on the functionality and allergenicity of soybean proteins are not available. Moreover, the relationship between allergenicity or functional properties and structural changes or protein modification is limited.

In this study, we evaluate the differences in emulsifying properties, antioxidant activity, and allergenic capacity of covalent SPI-EGCG and SPI-PC conjugates prepared by alkali treatment. The formation of the conjugates was determined by SDS-PAGE and Fourier transform infrared (FTIR) spectroscopy analyses. The structural changes were evaluated by circular dichroism, ultraviolet, and fluorescence spectroscopy. The detailed information for the modification of proteins, peptides and epitopes was determined by LC/MS-MS. This study will provide the foundation for the production of hypoallergenic SPI-based foods and the potential application of SPI-polyphenol conjugates as functional ingredients in foods.

2. Materials and methods

2.1. Materials

EGCG (purity $\geq$ 95 %) and proanthocyanidins (purity $\geq$ 95 %), 1,1-diphenyl-2-picryl hydrazine (DPPH, purity 98 %), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, purity 98 %) were purchased from yuanye Bio-Technology Co., ltd (Shanghai, China). Soybean oil was purchased from Zhongjun Supermarket (Harbin, China). Human sera from five patients allergic to soybeans (Table S1), goat anti-human IgE HRP conjugate and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) were obtained from Chongqing Manuik Technology Co., ltd. (Chongqing, China). The sera were pooled together as an IgE + serum for the enzyme-linked immunosorbent assay (ELISA). Other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Extraction of soybean protein isolate

The extraction of soybean protein isolate was prepared according to the method of Ju, Zhu, Huang, Shen, Zhang, Jiang, et al. (2020) with slight modifications. After the soybean was defatted three times with *n*-hexane at a ratio of 1:5 (m/v) for 3 h at 25 °C, defatted soybean flour was dispersed in distilled water (1:15, w/v) and the pH was adjusted to 8.0 using 2 M NaOH. Then the mixture was stirred for 4 h followed by centrifugation at 10000 × g for 20 min. Next, the supernatant was carefully obtained and the pH was adjusted to 4.5 using 2 M HCl. After centrifugation at 10000 × g for 20 min, the precipitated proteins were collected and then dispersed in distilled water. Finally, after dialysis with an 8000–14000 Da dialysis bag at 4 °C for 48 h, the protein solution was lyophilized to obtain soybean protein isolate (SPI).

2.3. Preparation of SPI–polyphenol conjugates

SPI–EGCG and SPI–PC conjugates were synthesized using the alkali method (He, et al., 2020). In brief, 0.15 g SPI was dispersed in distilled H_2O at a ratio of 1:200 (m/v), and then 0.15 g EGCG or PC was added. The pH of the mixture was adjusted to 9 followed by continuously stirring at 25 °C with free exposure to air for 24 h. Next, the samples were dialyzed with an 8000–14000 Da dialysis bag at 4 °C for 48 h to remove the free EGCG and PC. Finally, the dialyzed sample was lyophilized to obtain SPI–EGCG and SPI–PC conjugates for subsequent studies. Treatment SPI without polyphenol was used as a control. The content of polyphenols in conjugates was measured by the Folin − Ciocalteu method in accordance with Pi, et al. (2023).

2.4. Characterization of SPI–polyphenol conjugates

2.4.1. Sds-PAGE

The SDS-PAGE was conducted according to previous studies (Pi, Sun, Deng, Xin, Cheng & Guo, 2022), simply changing the sample concentration to 5 mg/mL.

2.4.2. Fourier transform infrared (FTIR) spectroscopy

The freeze-dried sample (10 mg) was mixed with potassium bromide (1.5 g) and then ground into fine uniform powders. After tableting the resulting sample, the spectrum was obtained for 400–4000 cm^{-1} at a resolution of 4 cm^{-1} (32 scans/sample) using a Fourier transform infrared spectrometer (Nicolet iS10, Thermo Fisher, US) (Li, et al., 2021).

2.4.3. Circular dichroism (CD) spectroscopy

The freeze-dried sample was diluted with distilled water to 0.125 mg/mL and then was detected under 190–260 nm at 100 nm/min by the CD spectrometer (MOS-4SO, BIO-LOGIC, France) (Pi, Fu, Dong, Yang, Wan, & Xie, 2021). The content of the secondary structures (e.g., α-helix, β-sheet, β-turn and random coil) was calculated using DichroWeb secondary structure software (https://dichroweb.cryst.bbk.ac.uk/html/process.shtml).

2.4.4. Ultraviolet (UV) absorption spectroscopy

The UV of the control SPI, SPI-EGCG and SPI-PC conjugates was examined as our previous studies (Pi, Sun, Guo, Chen, Cheng, & Guo, 2022). Each freeze-dried sample was diluted with distilled water to 0.125 mg/mL and then was scanned from 200 to 420 nm by a T9 UV–visible spectrophotometer (Puxi Analytical Instrument Co. ltd, Beijing, China).

2.4.5. Surface hydrophobicity

The surface hydrophobicity of the control SPI, SPI-EGCG and SPI-PC conjugates was estimated using the 8-aniline-1-naphthalene sulfonate (ANS) method (Liu, Song, Li, Chen, Liu, Zhu, et al., 2021). In brief, the sample solution (0.125 mg/mL, 4 mL) was mixed with ANS solution (10 μL, 8 mM, pH 7.4), followed by reacting in the dark for 2 h. The relative ANS fluorescence intensity was measured for 420–600 nm emission wavelengths at 390 nm excitation by a cary eclipse fluorescence spectrometer (F-7100, HITACHI, Japan).

2.4.6. The content of free sulfhydryl (SH) group

The content of free SH group in the control SPI and SPI-polyphenol conjugates were examined according to our previous study (Pi, Sun, Guo, Chen, Cheng, & Guo, 2022). In brief, 40 mg DTNB was dissolved in 10 mL Tris-Gly solution (pH 8.0, 0.086 M Tris, 0.09 M glycine, 4 mM EDTA) for Ellman's reagent. Each freeze-dried sample was diluted with distilled water to 0.25 mg/mL. Next, 0.4 mL of sample solution was mixed with 0.6 mL of phosphate buffer solution (0.1 M, pH 8.0), and then mixed with 10 μL Ellman's reagent, followed by incubation at 37 °C for 20 min. After taking 200 μL of the resulting sample in the microtiter plate, and the absorbance was measured at 412 nm using the microplate reader (SpectraMax reg iD3, Beckman Coulter, US). The content of free SH group was computed using the following equation:

$$The\ content\ of\ free\ SH\ group\ (\mu mol/g\ of\ protein\ ) = 73.5 \times OD_{412} \times D/C \quad (1)$$

OD_{412} represented the absorbance at 412 nm; D and C were the dilution factor and the content (mg/mL) of samples, respectively.

2.4.7. LC/MS-Ms

The procedures of LC/MS-MS were performed as previously described in Sun, Wang, Sun, Jiang, & Guo, (2020). The samples (150 μg) were dissolved with NH_4HCO_3 solution (50 mM, 100 μL), reduced by dithiothreitol (500 mM, 2 μL), and then alkylated by iodoacetamide (500 mM, 14 μL). The resulting samples were filtered by 10-kDa cutoff filter, followed by redissolving with NH_4HCO_3 solution (50 mM, 100 μL). Next, the solution was digested with trypsin at 37 °C for overnight and then was added in 8 μL of $C_2HF_3O_2$ solution (10 %, v/v) for terminating the digestion reaction. The resulting peptides were desalted by C18 tips and then were vacuum dried, followed by redissolving the dried samples with 20 μL of 0.1 % formic acid solution. The peptide mixture was separated with an EASY-nLC 1200 (Thermo Fisher Scientific) under 0.1 % formic acid in 80 % acetonitrile at 300 nL/min, followed by identifying with Q Exactive Plus-Orbitrap MS (Thermo Fisher Scientific) at a range of 350–2000 *m/z*, 1.8 kV spray voltage and 300 °C heater temperature. MS/MS was conducted at a range of 200–2000 *m/z*, 27 eV collision energy and 60 s dynamic exclusion.

2.5. The IgE binding capacity of conjugates

2.5.1. Western-bolt

The IgE binding capacity of the control SPI, SPI–EGCG, and SPI-PC conjugates was evaluated by western-bolt (Lin, et al., 2022). After electrophoresis, the sample in gels was transferred to a PVDF transfer membrane (0.45 mm) at 100 V for 1.5 h. The PVDF membrane was blocked with 5 % skim milk powder (w/v) in TBST at 37 ℃ for 60 min, washed by TBST three times for 15 min/each time, and incubated with human serum (diluted 1:70) at 4 ℃ overnight. After washing, the PVDF membrane was incubated with goat anti-human IgE HRP conjugates (diluted 1:3000) at 37 ℃ for 60 min. Finally, after washing, the PVDF membrane was incubated with super ECL Western Blotting Substrate at 25 ℃ for 10 min, and then detected using Western-bolt Enhanced New Chemiluminescence Detector (Clinx Science Instruments, Shanghai, China).

2.5.2. Elisa

The IgE binding capacity of the control SPI, SPI–EGCG, and SPI-PC conjugates were further evaluated by ELISA (Lv, Qu, Yang, Liu, & Wu, 2021) with slight modification. Firstly, each sample was diluted to 1 μg/ml using carbonate buffer solution (pH 9.6), and was incubated in a 96-wells microtiter plate for 100 μL/well. After incubation at 4 ℃ overnight and washing by PBS (0.01 M, pH7.0) with 0.05 % Tween-20 (PBST) three times, 5 % skim milk powder in PBS was used to block the plates. After washing the plates, the pooled patient serum was added in the well, followed by incubation at 37 ℃ for 1 h. Next, the well was incubated with goat anti-human IgE HRP conjugates at 37 ℃ for 1 h after washing again. Finally, TMB was added to each 100 μL/well after washing again and then incubated at 37 ℃ for 13 min, followed by terminating the reaction with 2 M sulfuric acid. The absorbance of each well was measured at 450 nm using the microplate reader (SpectraMax reg iD3, Beckman Coulter, US). The allergenic capacity (%) was computed using the following equation:

$$The\ allergenic\ capacity\ (\%) = \frac{OD_{450}(SPI - polyphenol\ conjugates)}{OD_{450}(the\ control\ soybean\ proteins)} \times 100\% \quad (2)$$

2.6. Functional properties

2.6.1. Antioxidant activity

Antioxidant capacity, DPPH• and ABTS•+ scavenging capacity, of the control SPI, SPI–EGCG, and SPI-PC conjugates were evaluated according to the previous study (Liu, et al., 2021).

For DPPH• scavenging capacity, the sample solution (0.25 mg/mL, 100 μL dissolved in deionized water) was mixed in DPPH• solution (0.1 mM, 100 μL, dissolved in absolute ethanol), followed by incubation at 25 °C for 30 min in the dark. The absorbance of mixtures was measured at 517 nm using the microplate reader (SpectraMax reg iD3, Beckman Coulter, US). The DPPH• radical scavenging rate (%) was calculated by the following equation:

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

Here A_0 is the absorbance of DPPH• at 517 nm with deionized water and A_{sample} is the absorbance of DPPH• at 517 nm with the sample solution.

For ABTS•+ scavenging capacity, the sample solution (0.25 mg/mL, 100 μL dissolved in deionized water) was mixed in ABTS•+ radical solution (1.225 mM potassium persulfate and 3.5 mM ABTS, 100 μL, dissolved in deionized water), followed by reaction at 25 °C for 10 min in the dark. The absorbance of mixtures was measured at 734 nm using the microplate reader (SpectraMax reg iD3, Beckman Coulter, US). The ABTS•+ radical scavenging rate (%) was computed using the following equation:

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

Where A_0 represented the absorbance of ABTS•+ at 734 nm with deionized water and A_{sample} represented the absorbance of ABTS•+ at 734 nm with sample addition.

2.6.2. Emulsifying properties

The emulsifying properties were determined according to the method of Yan, Xie, Zhang, Jiang, Qi, & Li, (2021). Briefly, the control SPI, SPI–EGCG conjugates, and SPI-PC conjugates solution (6 mL, 5 mg/mL) was mixed with soybean oil (2 mL), respectively, followed by homogenization 10000 rpm for 2 min. 50 μL aliquot from the bottom of the emulsion was mixed with an SDS solution (0.1 w/v%), followed by measuring the absorbance at 500 nm after 0 and 10 min. The emulsifying activity index (EAI) and emulsifying stability index (ESI) were computed using the following equation:

$$EAI = \frac{2 \times 2.303 \times A_0 \times D}{N \times C \times 10000} \quad (5)$$

$$ESI\ (\text{min}) = \frac{A_0 \times T}{A_0 - A_{10}} \times 100\% \quad (6)$$

where A_0 and A_{10} represented the absorbance at 412 nm after 0 and 10 min, respectively; T is the time (10 min); D is the dilution factor; C is the protein mass concentration (g/mL); and N is the volumetric oil fraction.

2.7. Statistical analysis

All experiments were performed three times, and the corresponding results were analyzed with SPSS version 22.0 (Chicago, IL, USA) and expressed as means ± standard deviation (SD). All LC-MS/MS data were analyzed by the soybean database (https://www.uniprot.org/,2021.12.12) using Proteome Discoverer 24 Software (Thermo Fisher Scientific). The abundance of proteins and peptides was evaluated through the label free quantification (LFQ) method with the control SPI as the standard according to our previous study (Pi, Sun, Deng, Xin, Cheng, & Guo, 2022). Venn diagram, Peptide map, and heat map were drawn through online tools (e.g., http://bioware.ucd.ie/peptigram/ and https://hiplot.com.cn/basic/density).

3. Results and discussion

3.1. The formation of SPI-polyphenol conjugates

As shown in Fig. 1A, the content of EGCG and PC in SPI–EGCG and

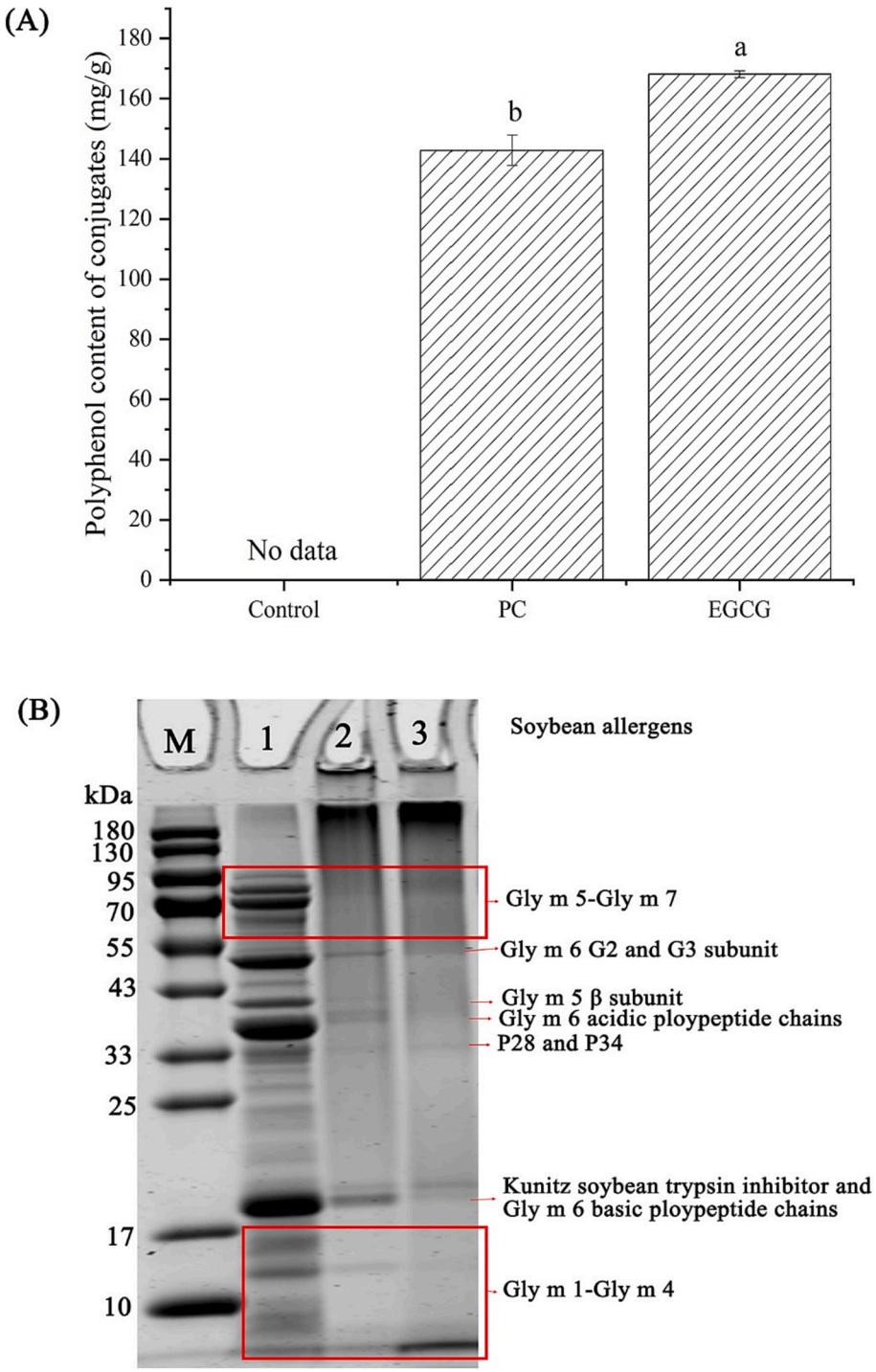

Fig. 1. The formation of conjugates. A, the content of polyphenols in conjugates**.** Means with different letters (a–b) in the bars indicated significant differences ($P < 0.05$). **B,** Change in SDS-PAGE profiles after soybean proteins were conjugated with EGCG and PC**.** M, marker; 1, untreated soybean proteins; 2, SPI-PC conjugates; 3, SPI-EGCG conjugate.

SPI–PC conjugates was 168.08 ± 1.12 mg/g and 142.71 ± 5.04 mg/g, respectively. The increase of polyphenols in conjugates indicated that polyphenol was successfully conjugated to SPI. The alteration of molecular weight in soybean proteins before and after covalent reaction with EGCG and PC was evaluated by SDS-PAGE under reducing conditions (Fig. 1B). Compared to the control SPI, SPI-EGCG and SPI-PC conjugates both featured higher molecular weights (>180 kDa), indicating the formation of covalent bonds between soybean proteins and EGCG as well as PC (Liu, et al., 2021; Yan, Xie, Zhang, Jiang, Qi, & Li, 2021). After all, the addition of SDS and β-mercaptoethanol resulted in the destruction of disulfide bonds and non-covalent interactions in proteins under SDS-PAGE. Similar results were shown by He, et al. (2019) and Yan, Xie, Zhang, Jiang, Qi, & Li, (2021), who found that the increase in molecular weights was observed in SPI-EGCG and ovalbumin-EGCG conjugates prepared by alkaline treatment, resulting from the formation of covalent bonds. Additionally, more high-molecular-weight polymers were observed in SPI-PC conjugates (Fig. 1B, land 2) than in SPI-EGCG conjugates (Fig. 1B, land 3). This phenomenon occurred because PC exhibited a stronger binding affinity to SPI than EGCG, which was attributed to a higher molecular weight

and phenolic hydroxyl content in PC than EGCG (Fig. S1) (Hasni, Bourassa, Hamdani, Samson, Carpentier, & Tajmir-Riahi, 2011; Liu, et al., 2021). Due to the relatively low molecular weight of EGCG (458.37 Da) and PC (594.52 Da), it was implied that the information of high-molecular-weight polymers was also induced by the protein cross-linking. It was reported that the reaction between protein and polyphenol under alkali treatment might induce protein cross-linking (Pi, Sun, Cheng, Fu, & Guo, 2022). Thus, PC caused more protein cross-linking under alkali treatment than EGCG. Compared to SPI-EGCG conjugates, SPI-PC conjugates showed low polyphenol contents but exhibited more formation of high-molecular-weight polymers, implying that many PC in conjugates used as cross-linkers during the formation of conjugates (Li, et al., 2021). These results were consistent with those reported by Li, et al., (2021), who demonstrated that the molecular weight of lactoferrin was increased after conjugation with EGCG through alkali treatment, resulting from the cross-linking of lactoferrins and the formation of covalent bonds between EGCG and lactoferrin.

3.2. Change in secondary structure

To evaluate changes in secondary structure, FTIR spectroscopy and CD spectroscopy were conducted. As shown in Fig. 2A, there were differences in the positions and intensity of the characteristic absorption peaks after SPI was conjugated with EGCG and PC. In the spectrum of the control soybean proteins, there were three strong bands at 3303.9 (amide A, representative of the N—H stretching coupled with hydrogen bonding), 1656.11 (amide I, representative of the vibration of C═O stretching of the peptide bond), and 1542.63 cm^{-1}(amide II, representative of C — N stretching coupled with N — H bending) (Jia, Zheng, Tao, Chen, Huang, & Jiang, 2016; Li, et al., 2021). After conjugation with EGCG and PC, the amide A band of soybean protein was moved from 3303.9 to 3304.4 and 3365.7 cm^{-1}, respectively, indicating the formation of H-bonding by connecting the carbonyl group with the peptide linkage of the protein (Ma, Li, Wu, Huang, Teng, & Li, 2022). Moreover, the formation of H-bonding in SPI-PC conjugates was more than in SPI-EGCG conjugates, which was attributed to a higher content of phenolic hydroxyl in PC than in EGCG (Fig. S1). Compared with control SPI, the amide I and amide II bands of SPI-EGCG moved from 1656.11 to 1659.17 cm^{-1} and 1542.63 to 1540.00 cm^{-1}, respectively; the amide I and amide II bands of SPI–PC moved from 1656.11 to 1656.5 cm^{-1} and 1542.63 to 1532.57 cm^{-1}. Therefore, the C═O, C—N and N—H were involved in the interaction between polyphenols and SPI. Moreover, more C—N and N—H were involved in the interaction between SPI and PC than in the interaction between SPI and EGCG, probably because PC exhibited a stronger binding affinity to SPI than EGCG. What's more, both amide I and amide II bands consist of overlapping bands at characteristic frequencies corresponding to different secondary structure elements (He, et al., 2019; Jia, Zheng, Tao, Chen, Huang, & Jiang, 2016). Therefore, changes in the peak positions of the amide I and II bands indicated the changes in the secondary structure and the peptide side-chain rearrangement in the conjugates (Yan, et al., 2021).

To further assess the changes in the secondary structure of SPI-EGCG and SPI-PC conjugates, CD spectroscopy was conducted. It was reported

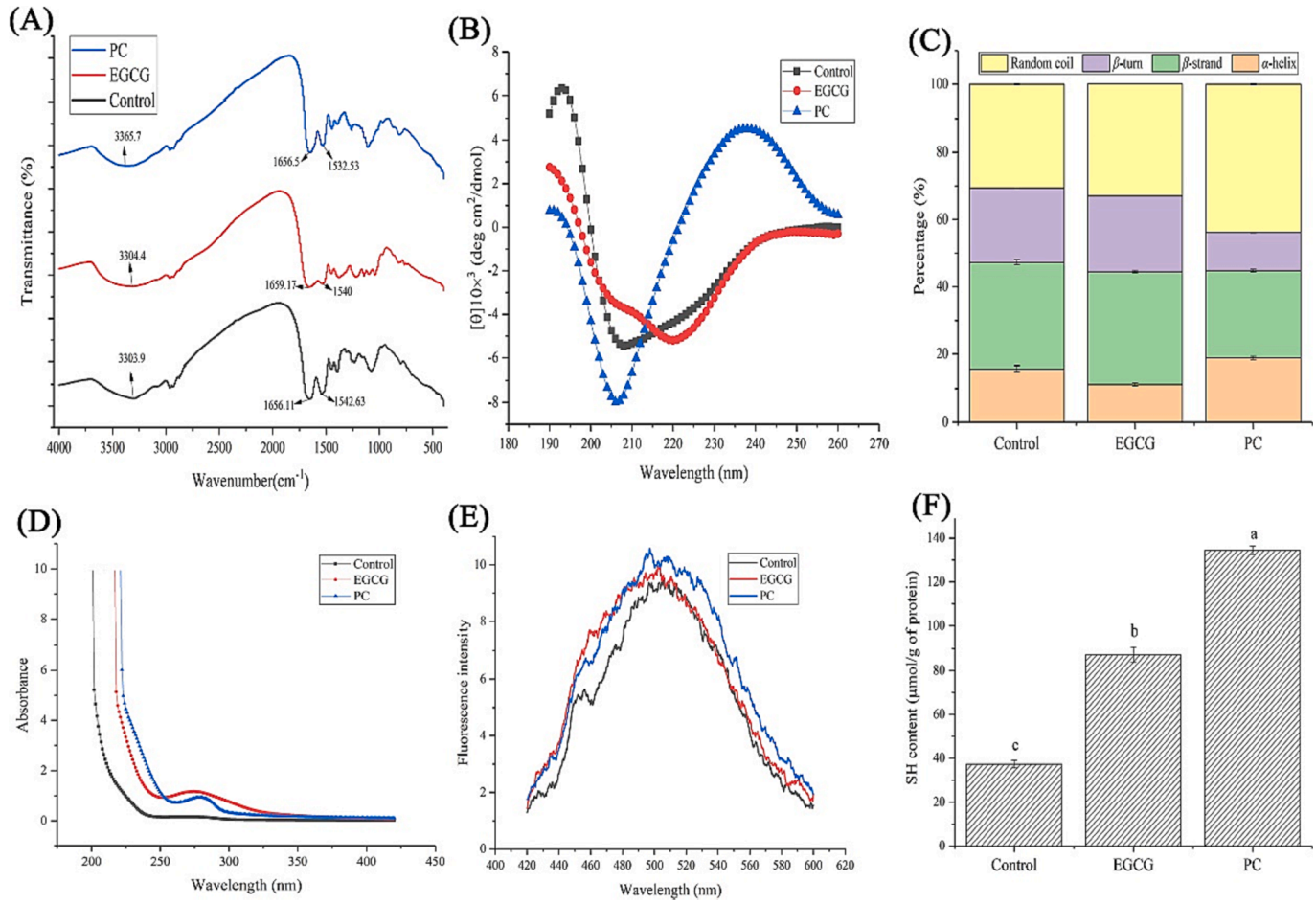

Fig. 2. Changes in the structure of SPI after conjugation with EGCG and PC. A. FTIR spectroscopy analyses; B. Circular dichroism spectra; C, the secondary structure analyses based on Circular dichroism spectra; D, ultraviolet absorption spectroscopy; E, surface hydrophobicity. F, The content of free SH groups. Means with different letters (a–c) in the bars indicated significant differences ($P < 0.05$).

that a negative band at about 208 nm in CD spectra was related to the characteristic of α-helical structures in proteins (Wu, et al., 2018). Compared to the control SPI, high absolute θ values were observed in SPI-PC conjugates, implying the increase of α-helical structures, while low absolute θ values were observed in SPI-EGCG conjugates, speculating the decrease of α-helical (Fig. 2B). As shown in Fig. 2C, the content of α-helical was reduced by 29 % and increased by 19 % after soybean protein was conjugated with EGCG and PC, respectively, whereas the content of random coil was increased by 8 % and 43.8 %. Therefore, different polyphenols caused different changes in secondary structure. This result was consistent with Liu, et al. (2021), who found a decrease and increase of α-helical in whey protein-EGCG and whey protein-naringenin prepared through free-radical grafting, respectively. He, et al. (2019) and Lv, Qu, Yang, Liu, & Wu, (2021) also found an increase of random coil in ovalbumin–EGCG conjugates prepared by a radical or alkaline method, and in tropomyosin-EGCG and tropomyosin-chlorogenic acid conjugates prepared by the free radical method. The increasing random coil of SPI after conjugation with EGCG and PC demonstrated that the SPI-EGCG and SPI-PC conjugates had high flexibility, probably resulting in the enhancement of some functional properties of proteins (He, et al., 2019). Moreover, the content of random coil was increased, which was probably attributed to the unfolding of soybean proteins, as previously described by He, et al. (2019) and Wu, et al. (2018). Thus, SPI-PC conjugates exhibited more random coil than SPI-EGCG conjugates, implying that more protein unfolding was observed in SPI-PC conjugates than SPI-EGCG conjugates.

3.3. Change in tertiary structure

To assess the alteration in the tertiary structure of proteins, ultraviolet absorption spectra, surface hydrophobicity and free sulfhydryl groups were measured.

As shown in Fig. 2D, there was an increase in UV absorption intensity after SPI was conjugated with EGCG and PC, suggesting the exposure of Tyr and Trp residues (Pi, Sun, Guo, Chen, Cheng, & Guo, 2022). Additionally, SPI-PC conjugates exhibited lower UV absorption intensity than SPI-EGCG conjugates, indicating a low exposure of Tyr and Trp residues in SPI-PC conjugates.

As shown in Fig. 2E, there was an increase in ANS-fluorescence intensities after SPI was conjugated with EGCG and PC, indicating the exposure of hydrophobic regions (Hu, Chen, Gao, Luo, Ma, & Tong, 2011). This phenomenon was also observed in tropomyosin-chlorogenic acid and tropomyosin-EGCG conjugates prepared by the free radical method, showing an increased surface hydrophobicity (Lv, Qu, Yang, Liu, & Wu, 2021). Moreover, the increase in ANS-fluorescence intensities suggested protein unfolding (Hu, Chen, Gao, Luo, Ma, & Tong, 2011). Thus, SPI-PC conjugates showed higher ANS-fluorescence intensities than SPI-EGCG conjugates, implying that more exposure of hydrophobic regions and unfolding of proteins were observed in SPI-PC conjugates than SPI-EGCG conjugates.

The disulfide bonds play important roles in the secondary and tertiary structure of proteins (Hu, Chen, Gao, Luo, Ma, & Tong, 2011; Pi, Fu, Dong, Yang, Wan, & Xie, 2021). The sulfhydryl group (SH) exists as free groups or forms disulfide bonds, and the changes in free SH reflect the structural change in proteins (Pi, Fu, Dong, Yang, Wan, & Xie, 2021). As shown in Fig. 2F, the contents of free SH were increased 2.33– and 3.6-fold after SPI was conjugated with EGCG and PC, which was attributed to the rupture of the S—S bond and the exposure of free SH in protein (Hu, Chen, Gao, Luo, Ma, & Tong, 2011; Pi, Fu, Dong, Yang, Wan, & Xie, 2021; Yan, et al., 2021). What's more, the increased content of free SH also suggested the unfolding of protein in SPI-EGCG and SPI-PC conjugates, which was consistent with Yan, et al. (2021). Thus, SPI-PC conjugates showed a higher free SH content than SPI-EGCG conjugates, suggesting that more rupture of the S—S bond, exposure of free SH, and unfolding of proteins were observed in SPI-PC conjugates than SPI-EGCG conjugates.

Overall, these results implied that the covalent conjugation of EGCG and PC caused changes in the tertiary structure and the unfolding of soybean proteins. Additionally, PC led to more structural changes and protein unfolding than EGCG. This phenomenon occurred because PC exhibited a stronger binding affinity to SPI than EGCG, causing more structural changes and protein unfolding than EGCG, which was attributed to a higher molecular weight and phenolic hydroxyl content in PC than EGCG.

3.4. Changes in protein and peptide composition

As shown in Fig. S1, Venn diagram analysis showed that there was a decrease in total and unique protein amounts of SPI-EGCG and SPI-PC conjugates, suggesting the modification of soybean protein through EGCG and PC. The formation of SPI-EGCG and SPI-PC conjugates might lead to the difficult degradation of trypsin for soybean proteins, causing the protein to be undetectable by LC/MS-MS. SPI-PC conjugates exhibited a higher decrease in total and unique protein amounts than SPI-EGCG conjugates, suggesting a highly difficult degradation of trypsin in SPI-PC conjugates. This might be due to the formation of more high-molecular-weight polymers in SPI-PC conjugates, which was proved by SDS-PAGE (Fig. 1B). A reduction in the amount of total and unique peptides was also observed in SPI-EGCG and SPI-PC conjugates (Fig. S2), probably resulting from the formation of more high-molecular-weight polymers. SPI-PC conjugates exhibited more polymers than SPI-EGCG conjugates (Fig. 1B), which was responsible for a lower amount of total and unique peptides in SPI-PC conjugates than SPI-EGCG conjugates. Overall, covalent conjugation of EGCG and PC caused changes in protein and peptide compositions. Additionally, more these changes were observed in SPI-PC conjugates than SPI-EGCG conjugates, implying more modification of SPI through PC. This might be because SPI-PC conjugates exhibited a more disordered structure and protein-unfolding, resulting in its convenience to modify soybean proteins.

3.5. Changes in major allergenic protein and their peptide composition

Based on Table S2, major soybean allergens were detected in SPI-polyphenols conjugates, including Gly m 4 (UniProtKB, A0A445K157), Gly m 5 (O22120, Q948X9, P11827, A0A445ISC9, O22121 and F7J077), Gly m 6 (A0A445LCA8 and A0A445IHI2), P28 (B2YDR0) and Kunitz soybean trypsin inhibitor (P25272, Q39898, A0A0B2NXT3, B1ACD5). As shown in Fig. 3A, the abundance of major allergenic proteins was decreased after conjugation with EGCG and PC, indicating that the major allergenic proteins were involved in the formation of high-molecular-weight polymers (Fig. 1B). SPI-PC conjugates showed a higher reduced abundance of major allergenic proteins than SPI-EGCG conjugates, suggesting that more modification of major allergenic proteins existed in SPI-PC conjugates, which was also proved by PCA (Fig. 3B) and heatmap analysis (Fig. 3C). SPI-PC conjugates exhibited a high modification of major allergenic proteins, contributing to a high reduction in peptide abundances (Fig. 3D) and total peptide amounts (Fig. 3E) of major allergenic proteins.

To further assess the changes in peptide profiles among these major allergenic soybean proteins, the peptide map and corresponding PCA analysis were conducted. As shown in Fig. 4A, SPI-PC conjugates exhibited a lower abundance of ALVTDADNVIPK (AA22-23) in Gly m 4 than SPI-EGCG conjugates. However, a new peptide (AVEAYLLAHP-DYN, AA146-158) was detected in SPI-polyphenol conjugates. Decreased abundance at a position means that the corresponding peptide is masked, and vice versa (Pi, Sun, Deng, Xin, Cheng, & Guo, 2022). Therefore, SPI-PC conjugates showed more masking of ALVTDADNVIPK and lower exposure of AVEAYLLAHPDYN than SPI-EGCG conjugates. Additionally, corresponding PCA analysis demonstrated that SPI-PC conjugates exhibited a greater distance in PC1 (60.4 %) from the control SPI than SPI-EGCG conjugates (Fig. 4A), suggesting a more modification of Gly m 4 in SPI-PC conjugates than SPI-EGCG conjugates. For

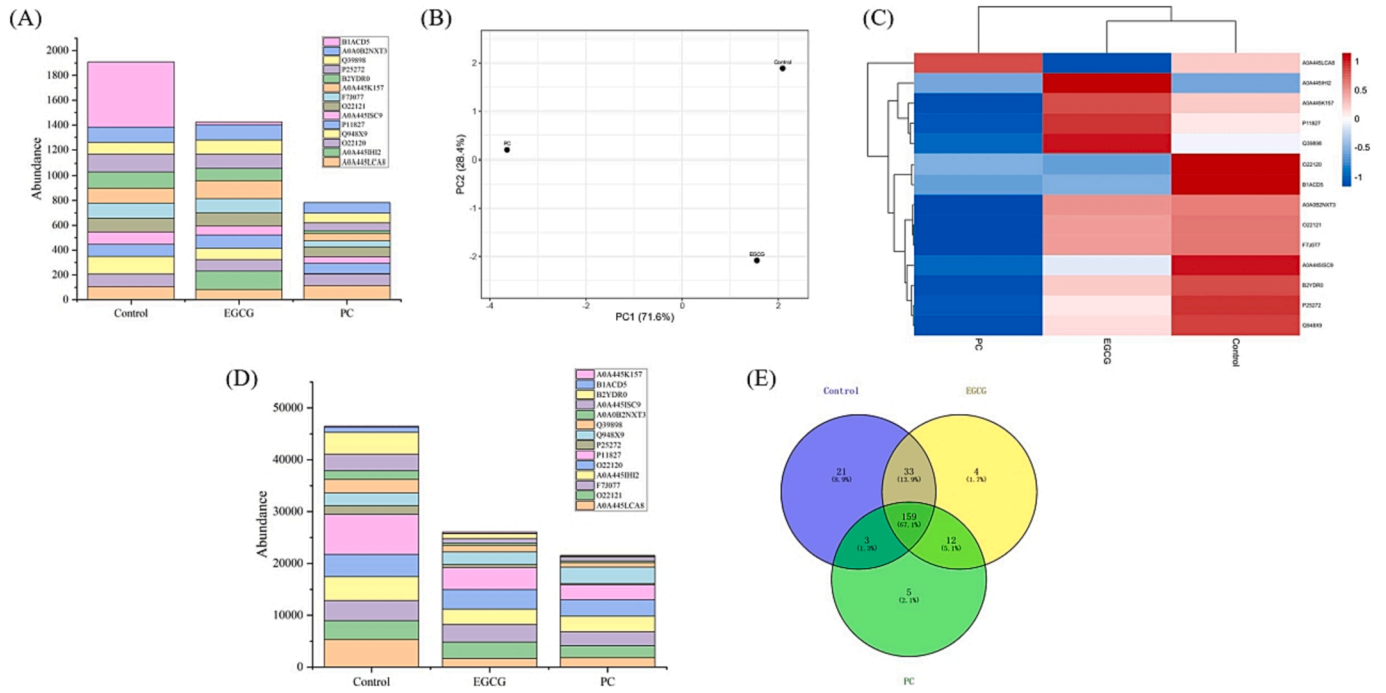

Fig. 3. Changes in major soybean allergens and their peptide composition after SPI was conjugated with EGCG and PC. A, the abundance of major soybean allergens in SPI-EGCG and SPI-PC conjugates. **B**. PCA analysis of major allergenic protein composition. **C**, heatmap analysis of major allergenic protein composition. **D**, the abundance of peptides in major soybean allergens. **E**, Venn diagram analysis of peptide compositions in major soybean allergens.

Gly m 5 (Fig. 4B), there were also differences in the peptide map between SPI-polyphenol conjugates and the control SPI. It was obvious that many reduced abundances were observed in NKNPFLFGSNR (AA130-140) and NPFLFGSNRFETLFK (AA132-146) of O22120, QPHQEEEHEQKEEHEWHR (110–127), QPHQEEEHEQKEEHEWHRK (110–128), GSEEEQDEREHPRPHQPHQK (136–155), GSEEEQDEREHPRPHQPHQKEEEK(136–159), EHPRPHQPHQKEEEKHEWQHK (145–165) of P11827, EQREEKEEEGQGSEDSHSK (AA169-187), EEKEEEGQGSEDSHSKR (AA172-188) of A0A445ISC9, NPIYSNNFGK (223–232), VREDENNPFYFR(3–14) and SRNPIYSNNFGK(221–232) of O22121, and NPIYSNNFGK(246–255) and SRNPIYSNNFGK (244–255) of F7J007 after soybean proteins were conjugated with EGCG and PC. Therefore, compared to the control SPI, the masking of these peptides was observed in SPI-EGCG and SPI-PC conjugates. However, the exposure of certain peptides was observed in SPI-polyphenol conjugates due to the increased abundance in NQRESYFVDAQPK (AA590-602) of Q948X9, ESYFVDAQPQQKEEGSKGR (AA409-427) of F7J077, and ESYFVDAQPQQKEEGSKGR (AA 386–404) of O22121. In general, most peptides in Gly m 5 were masked after conjugation with EGCG and PC (Fig. 4B). In addition, corresponding PCA analysis demonstrated that SPI-PC conjugates exhibited a greater distance in PC1 of O22120, Q948X9, A0A445ISC9, O22121 and F7J077 from the control soybean protein than SPI-EGCG conjugates (Fig. 4B), suggesting a more modification of Gly m 5 in SPI-PC conjugates than SPI-EGCG conjugates. Similar results were shown in the peptide map of Gly m 6 (Fig. 4C), P28 (Fig. 4D) and Kunitz soybean trypsin inhibitor (Fig. 4E), representing a more reduced abundance of most peptides in SPI-PC conjugates than SPI-EGCG conjugates. What's more, the corresponding PCA analysis heatmap (except for A0A0B2NXT3) (Fig. 4C-4E) suggested a more modification of Gly m 6, P28 and Kunitz soybean trypsin inhibitor in SPI-PC conjugates than SPI-EGCG conjugates. The reduction of abundance in most peptides among major allergenic proteins provided great potential for masking linear epitopes. Based on the method of Zhang, Wu, Li, Li, Yang, Tong, et al. (2019) that the detected peptide was identified as an epitope if it contained the fragments of reported linear epitopes (Table S3), the changes in linear epitopes of SPI-EGCG and SPI-PC conjugates were concluded in Table S4. It was found that the total abundance of epitopes was reduced by conjugation with PC and EGCG (Table S4), which demonstrated that most linear epitopes were masked or destroyed by conjugation with PC and EGCG. Additionally, SPI-PC conjugates exhibited a lower abundance of epitopes than SPI-EGCG, probably because PC caused more modification of major soybean allergens than EGCG (Fig. 4).

Overall, EGCG and PC conjugated with soybean proteins to induce the changes in the profiles of major allergenic proteins and their peptides. SPI-PC conjugates exhibited more modification of major allergenic proteins than SPI-EGCG conjugates, which was probably attributed to a more disordered structure and protein-unfolding in SPI-PC conjugates, resulting in its more convenience to modify major allergenic proteins.

3.6. *Changes in IgE binding capacity*

As shown in Fig. 5A, western-bolt demonstrated that strong IgE antibody binding occurred for all of the protein fractions in the control SPI (Fig. 5A, land 1) but the disappearance of many bands bound IgE antibody occurred for the SPI-EGCG (Fig. 5A, land 2) and SPI-PC conjugates (Fig. 5A, land 3). This result showed that conjugation of EGCG and PC inhibited the IgE binding to the allergens, suggesting the reduction of IgE binding capacity in SPI-EGCG and SPI-PC conjugates. Additionally, SPI-PC conjugates exhibited weaker binding than SPI-EGCG, implying a lower IgE binding capacity in SPI-PC conjugates than SPI-EGCG. The reduction of IgE binding capacity in conjugates was further measured by ELISA (Fig. 5B). SPI-EGCG and SPI-PC conjugates exhibited 54.63 % and 66.18 % reduction in the IgE binding capacity, indicating the reduction of allergenic potential in SPI-polyphenol conjugates. The allergenicity is mainly related to allergens and epitopes (conformational and linear epitopes) in foods (He, et al., 2019; Pi, Sun, Fu, Wu, & Cheng, 2021; Pi, Wan, Yang, Li, Wu, Xie, et al., 2019). The reduced abundance of allergenic protein and their peptides (Fig. 3A, 3D) might contribute to the reduction of allergens and epitopes, probably resulting in the reduction of IgE binding capacity in SPI-polyphenol conjugates. Similar results were shown by Pi, Sun, Deng, Xin, Cheng,

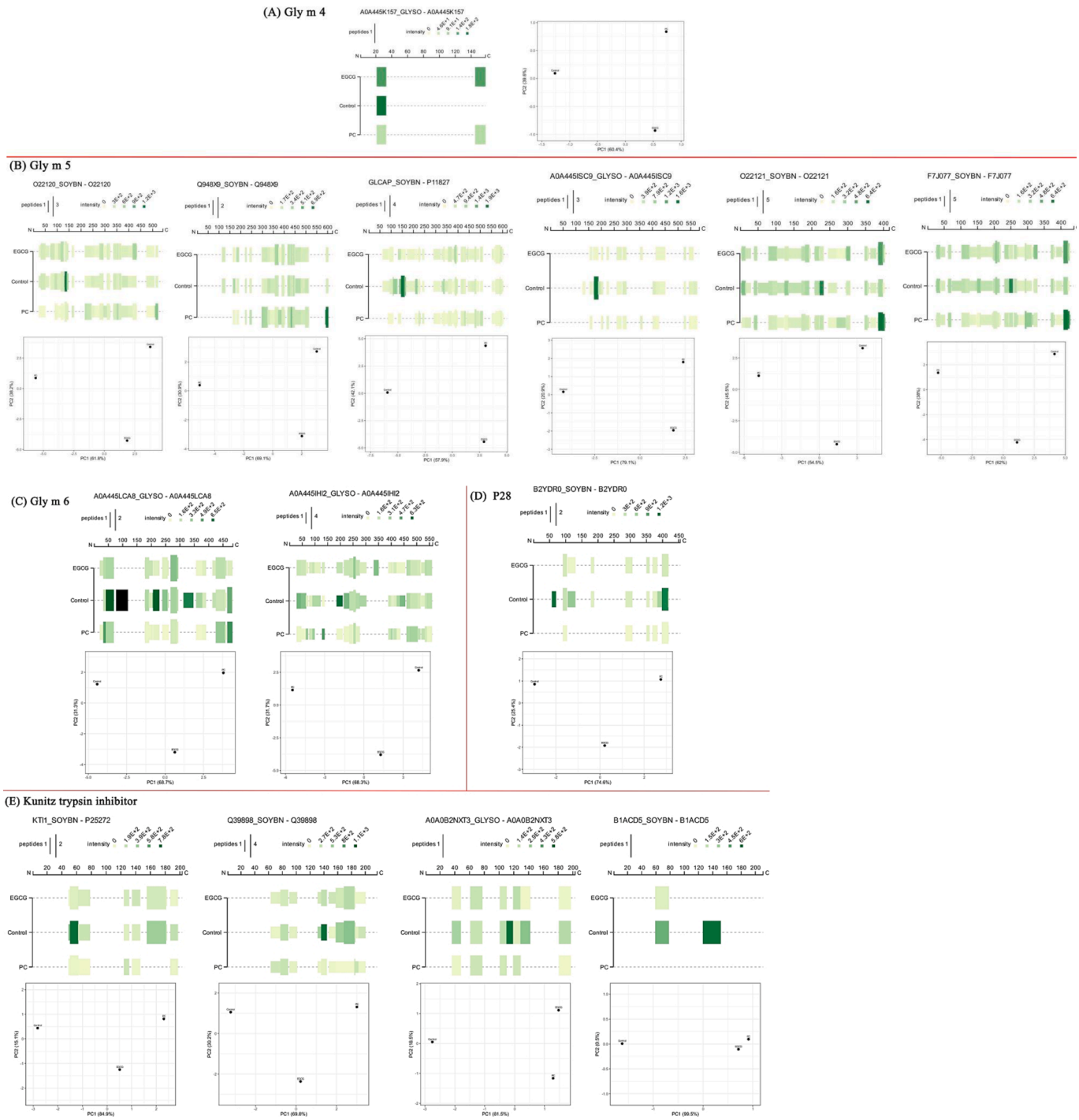

Fig. 4. Changes in peptide map and the corresponding PCA analysis of Gly m 4 (A), Gly m 5 (B), Gly m 6 (C), P28 (D), and Kunitz trypsin inhibitor (E) after SPI was conjugated with EGCG and PC.

& Guo, (2022), who found that boiled and autoclaved soybean proteins exhibited a low IgE binding capacity due to the low content of allergenic proteins. Thus, SPI-PC conjugates exhibited a lower IgE binding capacity than SPI-EGCG conjugates, probably resulting from its lower abundance of allergenic protein and their peptides.

The changes in IgE binding capacity are also related to the structural changes (Pi, Sun, Guo, Chen, Cheng, & Guo, 2022). It was reported that the reduction of IgE binding capacity was observed in ovalbumin-EGCG conjugates prepared by a radical or alkaline method because the conformational epitopes were masked and disrupted due to the protein unfolding (He, et al., 2019). Protein unfolding caused the exposure of conformational epitopes to increase the accessibility of polyphenols to mask and destroy conformational epitopes, resulting in the reduction of IgE binding capacity (Ahmed, Lv, Lin, Li, Ma, Guanzhi, et al., 2018). Therefore, conjugation to EGCG and PC induced the unfolding of soybean protein to mask and disrupt conformational epitopes, resulting in the reduction of IgE binding capacity. In addition, SPI-PC conjugates exhibited lower IgE binding capacity than SPI-EGCG conjugates, which was attributed to more disordered structure and protein-unfolding in SPI-PC conjugates, thereby increasing the accessibility of PC to mask and destroy conformational epitopes. Linear epitopes are also related to IgE binding capacity (He, et al., 2019). Table S4 showed that SPI-PC

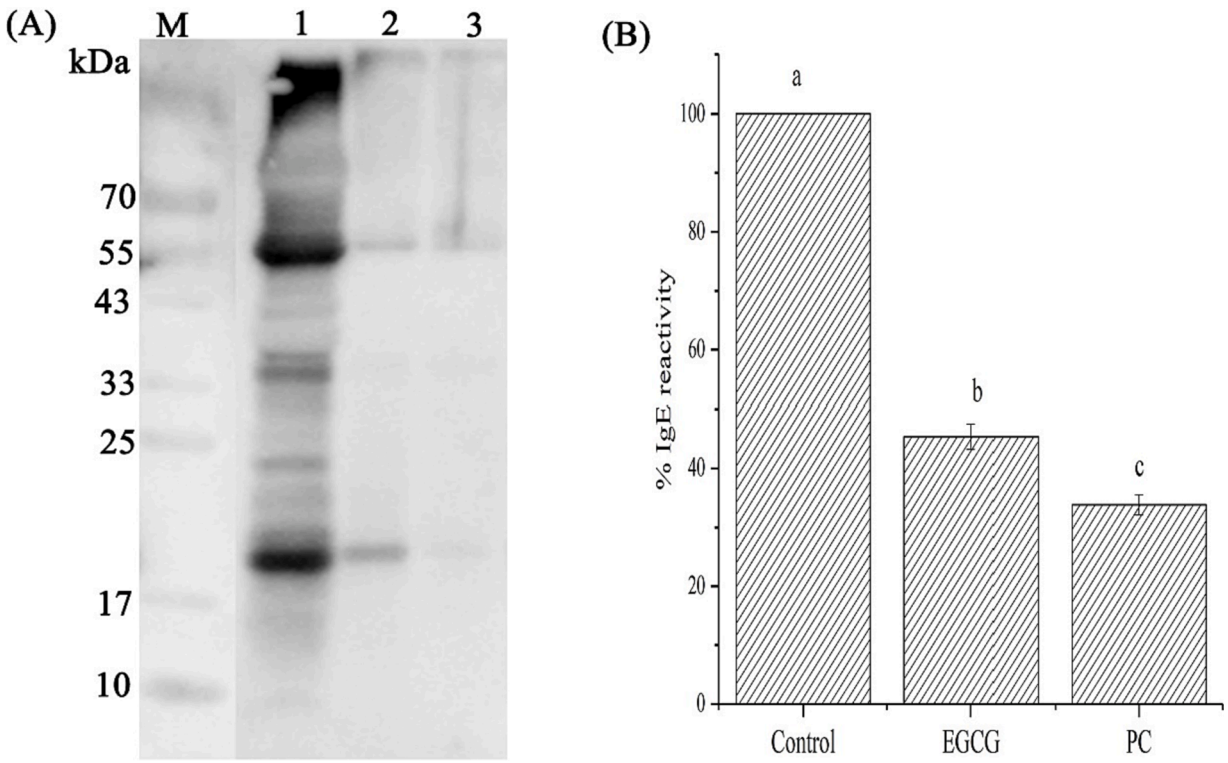

Fig. 5. Changes in IgE binding capacity of SPI after conjugation with EGCG and PC based on western- bolt (A) and ELISA (B). M, marker; 1, control SPI; 2, SPI-EGCG conjugates; 3, SPI-PC conjugate. Means with different letters (a–c) in the bars indicated significant differences ($P < 0.05$).

conjugates exhibited a lower abundance of epitopes than SPI-EGCG conjugates, which was also responsible for a low IgE binding capacity in SPI-PC conjugates. This phenomenon was consistent with previous studies, which observed the masking of the linear epitopes in β-lactoglobulin-EGCG or -chlorogenic acid conjugates prepared by the free radical method (Wu, et al., 2018) and ovalbumin-EGCG prepared by free radical method or alkaline method (He, et al., 2019).

Overall, the reduction of major soybean allergens and their epitopes was responsible for the decrease in binding IgE capacity after SPI were conjugated with EGCG and PC. In addition, SPI-PC conjugates exhibited a lower binding IgE capacity than SPI-EGCG conjugates due to a low abundance of major soybean allergens and their total epitopes. This phenomenon has occurred because PC exhibited a higher molecular weight and phenolic hydroxyl content to cause more protein-unfolding and disordered structure, resulting in more modification of major allergenic proteins in conjugates, compared to EGCG.

3.7. Changes in functional properties

DPPH• and ABTS•+ radical scavenging assays were used to evaluate the antioxidant properties of SPI-EGCG conjugates and SPI-PC conjugates. There was a significant ($P < 0.05$) increase in DPPH• and ABTS•+ radical scavenging activities after soybean proteins were conjugated with EGCG and PC (Fig. 6A and 6B). Similar results were shown by He, et al. (2019), who found an increase of ABTS•+ and DPPH• radical scavenging activities in ovalbumin-EGCG conjugates prepared by alkaline or free radical method. Although there were no significant differences in DPPH• and ABTS•+ radical scavenging activities between SPI-EGCG conjugates and SPI-PC conjugates, their increased antioxidant capacity indicated the successful attachment of EGCG and PC to SPI, as previously described in ovalbumin-quercetin prepared by the alkaline method or free radical method (Zhang et al., 2020).

Emulsifying activity (EAI) and emulsification stability (ESI) assays were used to evaluate the emulsifying properties of SPI-EGCG and SPI-PC conjugates. EAI is representative of the ability of a protein to form an oil–water interface and ESI is representative of the strain resistance of the emulsion droplets formed by the protein (Yan, et al., 2021). As shown in Fig. 8C, the EAI of SPI-EGCG and SPI-PC conjugates were increased by 3.65, and 6.9-fold, respectively, indicating the increased ability of soybean protein to form an oil–water interface through conjugation with EGCG and PC. This effect might occur due to structural changes in soybean protein. The EAI is usually related to the flexibility of protein and exposure of hydrophobic groups. The increase of random coil, the exposure of hydrophobic groups and the unfolding of proteins were reported to increase the EAI of the protein (He, et al., 2019; Hu, Zhao, Sun, Zhao, & Ren, 2011; Jia, Zheng, Tao, Chen, Huang, & Jiang, 2016; Xu, et al., 2019; Yang, Tu, Li, Kaltashov, & McClements, 2021). Therefore, the EAI of SPI-EGCG and SPI-PC conjugates was increased due to the increase of random coil, the exposure of hydrophobic groups and the unfolding of proteins. Additionally, SPI-EGCG conjugates exhibited a higher EAI than SPI-EGCG conjugates, probably resulting from its higher content of random coil (Fig. 2C) and more exposure of hydrophobic groups (Fig. 2E). Our results are consistent with Lin, et al. (2022), who reported that the increased EAI was observed in soybean 7S protein-chlorogenic acid and soybean 7S protein-EGCG conjugates prepared by the alkaline method. On the other hand, there were no significant ($P < 0.05$) differences in ESI among SPI and SPI-polyphenol conjugates. This suggested that the presence of EGCG and PC did not appear to affect the ability of protein-formed emulsion droplets against strain.

4. Conclusion

Conjugation to PC and EGCG significantly reduced the binding IgE capacity and improved the functional properties (antioxidant capacity, emulsifying properties) of SPI, which was accompanied by the protein modification and structural changes in SPI-PC and SPI-EGCG conjugates. SPI-PC conjugates exhibited more protein modification and structural changes than SPI-EGCG conjugates because PC exhibited a stronger binding affinity to SPI than EGCG, which was attributed to a higher

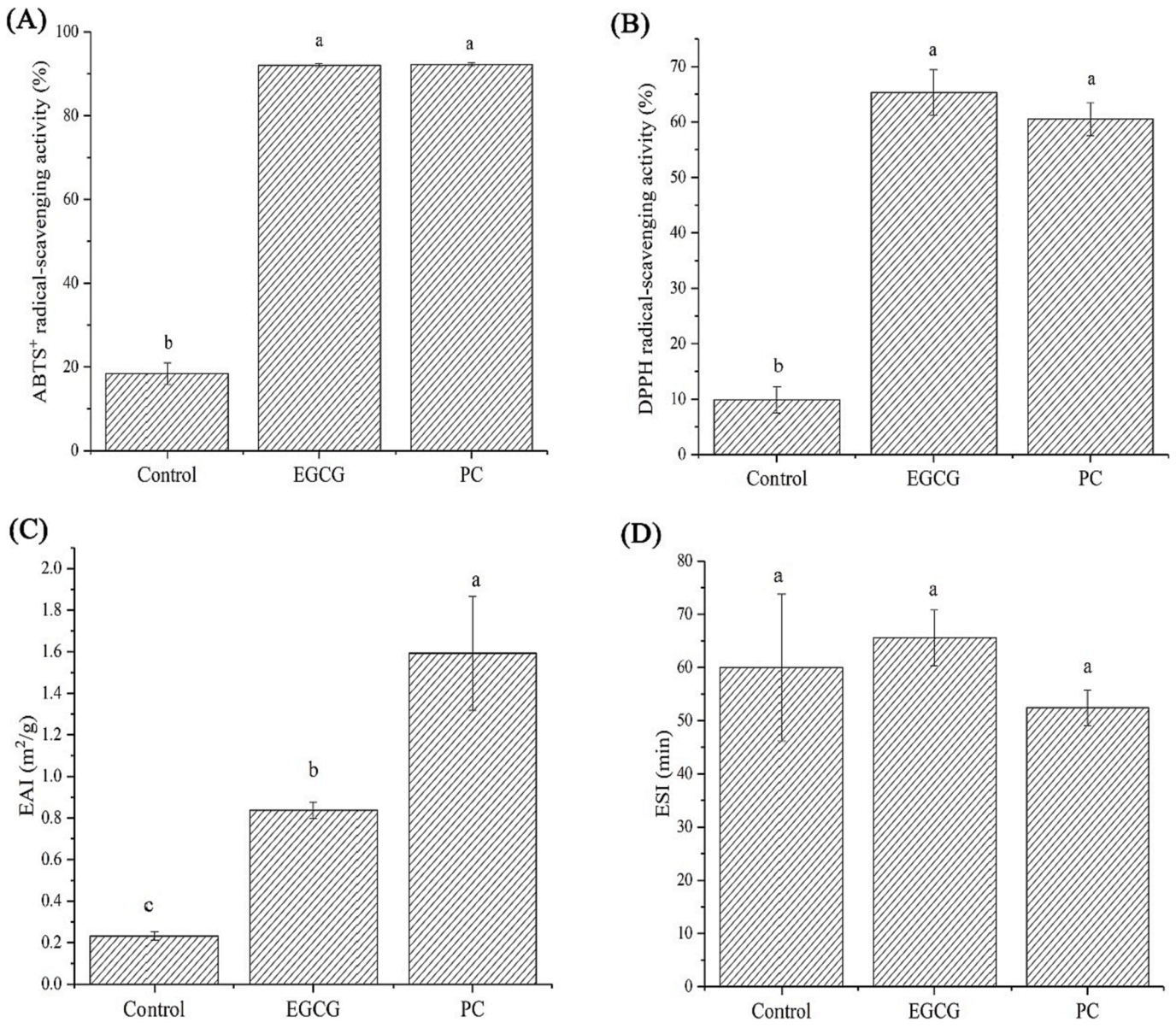

Fig. 6. Changes in functional properties of SPI after conjugation with EGCG and PC. A, DPPH• radical scavenging capacity; B, ABTS•+ radical scavenging capacity; C, emulsifying activity (EAI); D, emulsification stability (ESI); Means with different letters (a–c) in the bars indicated significant differences ($P < 0.05$).

molecular weight and phenolic hydroxyl content in PC than EGCG. The increase of DPPH• and ABTS•+ radical scavenging capacity was observed in SPI-PC and SPI-EGCG conjugates because PC and EGCG were bound to SPI. SPI-PC conjugates showed higher emulsifying activity than SPI-EGCG conjugates because SPI-PC conjugates contained a more disordered structure, greater protein unfolding and higher surface hydrophobicity than SPI-EGCG conjugates. What's more, a more reduction of binding IgE capacity was observed in SPI-PC conjugates than in SPI-EGCG conjugates, resulting from the lower abundance of allergenic proteins and the more masking or destruction of epitopes (linear and conformational epitopes) in SPI-PC conjugates. Overall, this study demonstrated that proanthocyanidins have more potential applications than EGCG for improving the functional properties of SPIs, while also reducing their allergic potential. Animal experiments and clinical trials should be used to evaluate and confirm the reduction of allergenicity of SPI-PC conjugates.

CRediT authorship contribution statement

Xiaowen Pi: data curation, writing-original draft, writing-review&editing; **Ph. D Yuxue Sun and Ph. D Xiaomeng Sun:** investigation. **Jiafei Liu and Zhigang Sun:** methodology. **Professor Jianjun Cheng and Mingruo Guo:** Project administration, supervision.

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.

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.fochx.2023.100566.

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