Comparison of soy protein isolate-(−)-epigallocatechin gallate complexes prepared by mixing, chemical polymerization, and ultrasound treatment

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ABSTRACT

The effects of the preparation method (mixing, chemical polymerization, or ultrasound treatment) on the structure and functional properties of soy protein isolate−(−)-epigallocatechin-3-gallate (SPI-EGCG) complexes were examined. The mixing treated SPI-EGCG samples (M–SE) were non-covalently linked, while the chemical polymerization and ultrasound treated SPI-EGCG samples (C–SE and U–SE, respectively) were bound covalently. The covalent binding of EGCG with protein improved the molecular weight and changed the structures of the SPI by decreasing the α-helix content. Moreover, U–SE samples had the lowest particle size (188.70 ± 33.40 nm), the highest zeta potential (−27.82 ± 0.53 mV), and the highest polyphenol binding rate (59.84 ± 2.34 %) compared with mixing and chemical polymerization-treated samples. Furthermore, adding EGCG enhanced the antioxidant activity of SPI and U–SE revealed the highest DPPH (84.84 ± 1.34 %) and ABTS (88.89 ± 1.23 %) values. In conclusion, the SPI-EGCG complexes prepared by ultrasound formed a more stable composite system with stronger antioxidant capacity, indicating that ultrasound technology may have potential applications in the preparation of protein-polyphenol complexes.

1. Introduction

Soy protein is a “high-quality plant protein” which contains amino acids closest to those found in the human body. Soy protein is environmentally sustainable, economically effective, and nutritionally complete [1]. However, its molecular conformation, aggregation state, and functional properties can change under different storage, packaging, and processing conditions. Additionally, natural protein does not usually meet the needs of practical complex food processing applications. For example, previous research has established that soy protein exists as a globular structure, and compact spherical structures result in poor flexibility and poor interface performance [2]. Meanwhile, the compact tertiary and quaternary structures of soy protein result in poor functional characteristics [3]. Furthermore, the foaming properties and emulsifying and antioxidant activities are influenced by the composition and structure of protein, which greatly limits its application [4–6]. Hence, several strategies including physical, chemical, and enzymatic have been used to enhance the properties of proteins [7–10]. Increasing attention has been paid to improving the physicochemical properties of proteins by adding natural food-grade biomolecules.

Polyphenols are known as the seventh nutrient and have powerful antioxidant effects, including scavenging free radicals, reducing inflammation, and inhibiting cancer cell growth [11]. Natural compounds combined with proteins and polyphenols have a wide range of health benefits, including nutritional and sensorial properties as well as disease prevention and biological functions [12–14]. Recent evidence suggests that whey protein isolate–polyphenol complexes are characterized by a higher binding rate, antioxidant capacity, and thermal stability [15]. Moreover, Li et al. [16] reported that lactoferrin and procyanidin increase the foaming performance of lactoferrin through hydrophobic bonding. EGCG, as the most potent polyphenol in tea inhibits most reactive oxygen species (ROS) [17]. Many studies have established that EGCG promotes health through its antioxidant, anti-inflammatory, anticaner, and antibacterial activities [17–19].

The preparation method significantly affects the physicochemical properties of protein-polyphenol complexes. The methods to prepare the complexes include mixing, alkaline conditions, enzymatic catalysis, and the free radical method [12,13,20]. Thereinto, protein–polyphenol complexes formed by mixing are compounds non-covalently bound, which are prone to dissociation. Unlike non-covalent interactions, the covalent interactions are irreversible forces that stabilize food systems. The alkaline, enzymatic, and free radical methods are used to prepare
protein–polyphenol covalent complexes. The free radical method reaction features mild reaction conditions, environmental friendliness, and no production of toxic by-products [21,22]. Two main ways have been proposed to prepare protein–polyphenol covalent complexes using the free radical method. One way to produce hydroxyl free radicals is to use a hydrogen peroxide-ascorbic acid redox reaction system, which is as safe, minimally toxic, and inexpensive [20,23]. Another way is to promote the covalent reaction between proteins and polyphenols by ultrasound [24]. Ultrasound is a non-thermal processing technology which has been extensively utilized in food industry and the cavitation, microstreaming, and mechanical effects generated by sonication could improve the physical and chemical properties of materials [25,26]. Among them, the cavitation effect causes the droplets to break up and produce a large number of hydroxyl radicals in liquid [27,28]. At the same time, the protein structure is unfolded mechanically, and the exposed amino and sulfhydryl groups react with hydroxyl radicals to generate activated protein groups; thus, covalently bonding with the polyphenols [24,29].

Thus, different treatment methods can affect protein–polyphenol complex interactions. However, few studies are available on the structural and functional properties of protein–polyphenol complexes prepared using different methods. In the current study, we choose three typical approaches (physical mixing, chemical polymerization, and ultrasound treatment) and investigated the effects of the different methods on the polyphenol binding rate, sulfhydryl content, secondary structure, and antioxidant activity. Our study will provide important insight into applying the SPI-EGCG complex in food industry.

2. Materials and methods

2.1. Materials

The SPI was purchased from Hupu Industrial Co., Ltd. (Shanghai, China). EGCG was obtained from Tongze Biotechnology company (Xian, China). Ascorbic acid, urea, and sodium dodecyl sulfate (SDS) were bought from Beijing Solarbio Industrial Co., Ltd. Hydrogen peroxide (30%) was provided by Sinopharm Chemical Reagent Co., Ltd. [30]. Polyphenol binding rate of the complexes was calculated as [31]:

\[
\text{Polyphenol binding} = \frac{\text{total polyphenols - free polyphenols}}{\text{total polyphenols}}
\]

2.2. Preparation of the SPI-EGCG complexes

Preparation of the mixing-treated SPI-EGCG complexes (M–SE): SPI powder was dissolved in deionized water at a concentration of 10 mg/mL. After stirring for 2 h, the solution was hydrated overnight at 4°C. EGCG was added to SPI solution to make its final concentration of 40 mmol/L with stirring for 24 h. The reacted samples were dialyzed (48 h, 4°C) and then freeze-dried into a powder.

Preparation of the chemical polymerization-treated SPI-EGCG complexes (C–SE): The SPI solution was prepared as described for M–SE. Then, 1 mL hydrogen peroxide was combined with 0.25 g ascorbic acid and this mixture was added to the protein solution with stirring magnetically for 2 h. EGCG was added as described in the M–SE sample with reacting for 24 h, then the mixture was dialyzed (48 h, 4°C) and then lyophilized.

Preparation of the ultrasound-treated SPI-EGCG complexes (U–SE): The SPI-EGCG mixture was prepared as described above for the M–SE samples. The mixture was placed in an ultrasonic device (Ningbo Scientz Bio-Technology company) at 300 W for 10 min (2 s on/off), and the frequency was set at 20 kHz equipped with a 0.636 cm diameter probe. Also, an ice-water bath was used to prevent heating of the solution during sonication. Then, the treated solution was dialyzed (48 h, 4°C) and then lyophilized.

2.3. Particle size and zeta-potential

Zetasizer Nano ZS90 instrument (Malvern Instruments, Worcestershire, UK) was employed for particle size and zeta-potential measurement. Samples were diluted to 1 mg/mL protein concentration. The refractive index of the continuous phase was 1.46, and that of dispersed phase was 1.33.

2.4. Polyphenol binding

The SPI-EGCG lyophilized powder was dissolved in deionized water or SDS /urea solution (0.5 % SDS and 6 M urea) to a 1 mg/mL concentration. A dialysis bag (molecular weight 12–14 kDa) was utilized to obtain free EGCG. The EGCG content was determined by the forint phenol method at a 760 nm absorption value (UV-2700, Shimadzu Co., Ltd) [30]. Polyphenol binding rate of the complexes was calculated as [31]:

\[
\text{Polyphenol binding} = \frac{\text{total polyphenols - free polyphenols}}{\text{total polyphenols}}
\]

2.5. Amino and sulfhydryl group contents

The ortho-phthalaldehyde (OPA) approach was used to test amino group content [32]. A 50 mL aliquot of OPA reagent contained 40 mg of phthalic acid (dissolved in 1 mL methanol), 2.5 mL of 20% (w/v) SDS solution, 25 mL of 0.1 mol/L borax solution, and 100 mL of β-mercaptoethanol. A 200 μL aliquot of the 2.5 mg/mL sample solution was reacted with 4 mL of OPA reagent at 35°C for 2 min using a UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan) at OD 340 nm. The free amino group content was analyzed based on the lysine standard curve.

The sulfhydryl (SH) group content was assayed by Ellman’s reagent method as previously reported by Shamida et al. [33], and the calculation formula is as follows:

\[
\text{SH} = \frac{73.53 \times A_{12} \times D}{C}
\]

Where, 73.53, D, and C represent the conversion factor, dilution factor, and sample concentration (mg/mL), respectively.

2.6. SDS-PAGE analysis

SDS-PAGE was carried out referred to Laemmli et al. [34]. The sample was mixed in a buffer solution and heated at 95°C for 5 min to denatured the protein. The acrylamide gel consisted of a 12% separating gel and a 4% concentrating gel. Electrophoresis was performed at 80 mV in a laminated gel and then at 120 mV separation gel. The gel was stained with Coomassie Bright Blue G-250 solution for 2 h and then decolorized. The image was photographed and analyzed with an HP1000 scanner.

2.7. Fourier transform infrared spectroscopy (FTIR)

The method used for the FTIR analysis (Nicolet is50, Thermo Fischer Scientific, USA) was referred from Mantsch et al. [35]. A 2 mg sample was added to 100 mg potassium bromide and measured after squashing. The scanning ranges, wavenumber accuracy, and scan times were 4,000–400 cm−1, 0.01 cm−1, and 64, respectively.

2.8. Fluorescence spectroscopy

A RF-6000 fluorescence spectrometer (Shimadzu Co., Ltd., Kyoto, Japan) was used to perform the fluorescence of SPI-EGCG complexes. The sample was dissolved in phosphate buffer and the excitation spectrum, emission wavelength, and slit width were 290 nm, 300–450 nm, and 5.0 nm, respectively.

2.9. Antioxidant activity

DPPH assay [36]: DPPH was dissolved in absolute ethanol to prepare
the DPPH ethanol solution at a concentration of $2 \times 10^{-4}$ mol/L. The sample was combined with a 1:1 mix (v/v) of DPPH-ethanol diluent, and the absorbance was measured at 517 nm after 30 min in the dark. The DPPH was computed as following equations:

$$\text{DPPH free radical scavenging rate (\%)} = \frac{A_0 - A}{A_0} \times 100$$

Where $A_0$ is the absorption value of the control group, and $A$ is the absorption value of the SPI-EGCG complex.

ABTS assay \[37\]: ABTS stock fluid was produced using the mixture of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate fluid (1:1; v/v) and then 12 h-dark treatment. The ABTS stock solution was diluted with PBS (pH 7.4, 5 mM) until its absorbance value was 0.70 ± 0.02. A 2.5 mL aliquot of the sample solution was mixed with 2.5 mL of ABTS diluted solution, and absorbance was measured at 734 nm after reacting for 18 min.

$$\text{ABTS free radical scavenging rate (\%)} = \frac{A_0 - A}{A_0} \times 100$$

Where $A_0$ is the absorption value of the control group, and $A$ is the absorption value of the SPI-EGCG complex.

2.10. Statistical analysis

Graphics were prepared using OriginPro8.5 software (Origin Laboratories, Northampton, MA, USA), and SPSS 19.0 software was used to detect differences using analysis of variance. A $p$-value < 0.05 was considered significant.

3. Results and discussion

3.1. Particle size and zeta-potential

Figure 1 illustrates the particle size and zeta-potential of the SPI-EGCG complexes. The M–SE and C-SE samples exhibited a larger particle size than SPI alone (Fig. 1b). The M–SE samples formed non-covalent complexes, and the interactions between SPI and EGCG were hydrophobic and hydrogen bonding. Polyphenols acted as bridges to polymerize the proteins (as shown in Fig. 1a); thus, increasing particle size \[38\]. Furthermore, the chemical treatment led to a larger particle size, possibly because the hydroxyl radicals induced by ascorbic acid and hydrogen peroxide reacted with the amino and sulphydryl groups in SPI, which activated the proteins into free radicals; therefore, covalent grafting of EGCG occurred and the binding capacity of the protein-polyphenols was enhanced \[39\] (Fig. 1a). The U-SE samples possessed the smallest particle size with a unimodal distribution and the lowest PDI values (Fig. 1b-c). The cavitation and mechanical effects of ultrasound unfolded the internal structure and reduced the SPI consistency index \[40,41\]; therefore, reducing the aggregation of SPI and improving the stability of the SPI-EGCG complexes. Meanwhile, the hydroxyl radicals (H$_2$O $\rightarrow$ H$^+$+OH$^-$) generated by ultrasound reacted with SPI to generate more protein radical binding sites, which promoted the covalent binding of SPI with EGCG.

The influence of the different treatment approaches on zeta-potential of the SPI-EGCG complexes are depicted in Fig. 1d. The absolute zeta-potential values for SPI exhibited a lower charge, whereas combinations of SPI and EGCG exhibited higher absolute zeta-potential values, particularly in the U-SE samples. The reason was that adding EGCG changed the protein’s surface charge, and the negatively charged EGCG neutralized the positive charge of SPI, which, in turn, reduced the number of exposed sites of positively charged groups in the protein. The highest zeta-potential produced by ultrasound was from the shear forces and cavitation effects. These effects forced protein unfolding, and SPI was separated into smaller particles, thereby increasing the surface area to expose more charged groups, which was evidenced by the particle size results \[42\] (Fig. 1a).

![Fig. 1.](image-url)

Fig. 1. (a) Schematic diagram, (b) particle size and PDI values, (c) particle size distribution, (d) zeta-potential of the SPI-EGCG complex. Different small letters indicate significantly different means ($p < 0.05$).
3.2. Polyphenol binding

The polyphenol binding is an important index reflecting the bond between SPI and EGCG and provides proportional information about the functional groups in the modified products [43]. Fig. 2a presents the binding rates of polyphenols in the SPI-EGCG complexes treated by the three methods. For the same treatment method, the EGCG binding of the SPI-EGCG complexes dispersed in deionized water was higher than that in the SDS/urea solution, indicating the destruction of the hydrophobic SPI-EGCG interactions in the latter solution [44]. Moreover, the EGCG binding rate of the C-SE and U-SE samples was higher than that of the M–SE samples, suggesting different mechanisms of the SPI-EGCG complexes. The M–SE samples were bound primarily by hydrophobic interactions, which is a reversible non-covalent complex [45]. Nevertheless, the protein was activated into protein-free radicals after the chemical polymerization and ultrasound treatments. Covalent bonding of SPI-EGCG was induced through the hydrogen peroxide-ascorbic acid redox reaction system and the ultrasonic cavitation effect. Furthermore, the binding rates of the U-SE samples in deionized water and the SDS/urea solution were 59.84% and 41.89%, respectively, and their EGCG binding was significantly higher than that of the M–SE and C-SE samples. This occurred because of the cavitation and mechanical effects of ultrasound generating a large number of hydroxyl radicals, which reacted with hydrogen atoms at sites, such as amino or sulfhydryl groups in the protein so that the benzene ring of EGCG and the protein formed C–N, or the C–S covalent bonds induced a more stable covalent bond between SPI and EGCG. Meanwhile, the microstreaming generated by ultrasound led to high-speed collisions between SPI and EGCG, and the reduction in particle size and the expansion of the SPI structure to a larger specific surface area improved the binding efficiency of SPI and EGCG.

3.3. Amino and sulfhydryl group contents

As shown in Fig. 2b, the contents of amino and sulfhydryl groups in the SPI-EGCG complexes decreased significantly comparing the results of SPI alone, indicating that the phenolic hydroxyl groups provided by EGCG combined with the amino and sulfhydryl groups of SPI [46]. Their contents in the C-SE and U-SE samples were lower than those in the M–SE samples, suggesting that the decrease in the covalent complexes was more pronounced than with the non-covalent samples. The benzene ring in polyphenols interacts with amino or sulfhydryl groups on protein molecules and eventually forms C–N or C–S covalent bonds [15,22]. Moreover, the greatest reduction in free amino and sulfhydryl groups was achieved by the ultrasound treatment because the cavitation effect and mechanical shear force unfolded the internal structure of the protein and extended the peptide chain [47], which exposed more amino and thiol groups on the surface. This “exposed” protein structure provided more binding sites for covalent grafting of EGCG, which promoted the reaction of hydroxyl radicals with hydrogen atoms at the amino and sulfhydryl groups in the proteins to generate protein radicals that formed stable C–N or C–S covalent bonds, as evidenced by the polyphenol binding (Fig. 2a). In addition, the free sulfhydryl groups were easily oxidized to disulfide bonds. Adding SDS and urea inhibited the conversion of free sulfhydryl groups to disulfide bonds. Hence, the reduction of free sulfhydryl group content also indicates that EGCG covalently reacted with the SPI free sulfhydryl groups to form C–S covalent bonds [48].

3.4. SDS-PAGE

As expected, SPI is a complex that can be divided into 2S, 7S, 11S, and 15S components [49]. About 65–80% of SPI is storage proteins consisting of 7S and 11S globulins [49]. Fig. 3 shows that the 7S protein has three subunit structures, such as the α’, α, and β subunits. The 11S protein is a hexamer, and the subunit is composed of acidic and basic peptides, represented as A and B subunits. No difference was observed between the band patterns of SPI alone and the M–SE samples, indicating no effect of mixing on the protein band patterns. These results may have occurred because the M–SE samples were bound by weak non-covalent interactions that were easily broken, resulting in only a trace amount of EGCG binding to the protein molecular chain in the final product; thus, the molecular weight did not increase significantly. Interestingly, although the C-SE complexes were covalently linked, the band position did not change significantly. The electropherogram of the U-SE sample not only appeared to be high molecular weight aggregates with a darker color, but the intensity of the α’, α, and β subunits decreased and the upward migration of the band. Ultrasound led to a higher degree of covalent bonding between SPI and EGCG, resulting in the formation of higher relative molecular weight substances.

3.5. FTIR analysis

As illustrated in Fig. 4, the peak at 1700–1600 cm\(^{-1}\) (C=O stretching vibrations) was defined as the amide I band, and that at 1600–1500 cm\(^{-1}\) represented the amide II band (CN stretching vibrations and NH bending vibrations), which reflected the protein secondary structure. Adding EGCG resulted in a red shift in the amide I band. The M–SE sample exhibited a more pronounced redshift (from 1647.9 cm\(^{-1}\) to 1654.6 cm\(^{-1}\)) than C-SE and U-SE samples (from 1647.9 cm\(^{-1}\) to 1654.1 cm\(^{-1}\)).

![Fig. 2. (a) Polyphenol binding, (b) amino and sulfhydryl group contents of the SPI-EGCG complex. Different small letters indicate significantly different means (p < 0.05).](image-url)
cm$^{-1}$) and SPI alone, possibly because EGCG bound to the C–O group of SPI through hydrophobic interactions [50]. The M–SE and C-SE samples observed a blueshift in amide II while a U-SE redshift was observed from 1583.3 cm$^{-1}$ to 1592.9 cm$^{-1}$, possibly because the C–N and N–H groups were involved in the reaction of EGCG with the amino groups in SPI, as evidenced by the amino acid content (Fig. 2b). Additionally, the amide I and II intensities decreased after EGCG was added, indicating that the binding reaction of EGCG to SPI resulted in the rearrangement of the protein-peptide chain and a change in the secondary structure. Moreover, the peak of the SPI-EGCG complexes at 3308.3 cm$^{-1}$ was located in the amide A band (3400–3300 cm$^{-1}$), which was mainly formed by N–H bond stretching vibrations. The SPI-EGCG complexes had a redshift in the amide A band and the intensity of the amide A band decreased, further supporting the reaction between EGCG and the amino groups of SPI.

The secondary structure of the SPI-EGCG complexes is presented in Table 1. Adding EGCG decreased α-helix and increased random coil in the SPI. As α-helices are more stable than random coils [49], we speculated that the secondary structure was altered by the interactions between SPI and EGCG, transforming it from an ordered to a disordered...
structure. Tang et al. [51] demonstrated that polyphenols decrease α-helix and β-sheet contents, and increase random coil content, indicating that the SPI-EGCG interaction contributes to the formation of a relatively loose protein structure. The α-helix, β-sheet, and β-turn contents decreased significantly in the U-SE samples, while random coil content increased. Such a change resulted from the generation of hydroxyl free radicals and the cavitation effect of ultrasound. The ordered structure was destroyed and it was gradually transformed into a disordered structure.

3.6. Fluorescence spectroscopy

Fluorescence spectroscopy is a useful method to reflect the microenvironmental polarity changes in aromatic amino acids which are widely used in protein conformational analysis and intermolecular interactions [52]. Fluorescence quenching occurred after adding EGCG (Fig. 5), and the magnitude of this effect was greater for the covalently bound samples. We expected that the SPI-tryptophan interactions would be stronger in covalent compounds. Only fluorescence quenching occurred in the M-SE and its absorption maximum did not change significantly, indicating that physical mixing barely affected the protein structure. Nevertheless, fluorescence intensity decreased in the C-SE and U-SE samples, and the maximum absorption wavelength was redshifted, suggesting that the microenvironmental polarity of tryptophan changed and surface hydrophobicity decreased when the tryptophan residues in the hydrophobic interior were exposed to the hydrophilic exterior [52,53]. The fluorescence intensity of the SPI-EGCG complexes treated with ultrasound decreased the most, indicating that ultrasound enhanced the interactions between EGCG and tryptophan.

3.7. Antioxidant activity

Fig. 6 shows the influences of the different treatment methods on the antioxidant capacity of the SPI-EGCG complexes. DPPH and ABTS were significantly enhanced after adding EGCG, due to the introduction of more hydroxyl groups into the SPI after combining with EGCG [53,54]. EGCG has strong antioxidant activity and effectively scavenges free radicals and delays the production of ROS [55]. Hence, adding EGCG increased the antioxidant capacity of the system. This result further demonstrates that the phenolic hydroxyl groups have antioxidant properties even though the polyphenols are bound to proteins. Here, the C-SE and U-SE samples had higher DPPH and ABTS values than the M-SE samples, demonstrating that free radicals were strongly scavenged after the covalent bonding of SPI-EGCG, which, in turn, formed a more stable product and terminated the radical chain reaction [46]. Notably, the highest antioxidant activity (DPPH: 84.84 ± 1.34 %; ABTS: 88.89 ± 1.23 %) was observed in the U-SE samples. One reason was the modified protein induced by ultrasound exhibited stronger protection due to the higher affinity for EGCG [56], as demonstrated by the higher EGCG binding (Fig. 2a). Another reason was the collapse of cavitation bubbles generated by ultrasound induced the formation of more hydroxyl radicals, thus improving the interaction of SPI and EGCG, eventually promoting the antioxidant activity [57].

4. Conclusion

In this study, three approaches (mixing, chemical polymerization, and ultrasound) were developed and applied to SPI-EGCG complexes. We concluded that hydroxyl radicals were produced by the hydrogen peroxide–ascorbic acid redox reaction and ultrasound treatment, which promoted the association between EGCG and the amino or sulfhydryl groups to generate C–N or C–S covalent bonds to form the SPI-EGCG covalent complex. Moreover, the U-SE samples exhibited smaller particle sizes, lower PDI values, a higher zeta-potential, and a higher EGCG binding than the M-SE and C-SE samples, indicating that covalent bonding induced by ultrasound produced a more stable SPI-EGCG complex. Meanwhile, substances with larger molecular weights were formed with the SPI-EGCG complex, the flexibility of the SPI structure increased, and the molecule changed from an ordered to a disordered structure. In addition, the antioxidant activity of the SPI-EGCG complex was enhanced compared to SPI alone, particularly ultrasound-treated samples. Overall, among the three treatment methods, ultrasound resulted in a higher degree of covalent bonding between SPI and EGCG. This study will contribute to a better understanding of the mechanisms involved in the formation of SPI-EGCG complexes and support further development of ultrasonic technology for preparing protein–polyphenol complexes. Future work will explore the optimal conditions of ultrasound treatments on the protein and polyphenol to expand its utilizations of such novel technology.

**CRediT authorship contribution statement**

Mengjie Geng: Conceptualization, Methodology, Investigation,
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

Data will be made available on request.

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