Effects of ultrasound on the structural and emulsifying properties and interfacial properties of oxidized soybean protein aggregates

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ABSTRACT
Oxidative attack leads to the oxidative aggregation and structural and functional feature weakening of soybean protein. We aimed to investigate the impact of ultrasonic treatment (UT) with different intensities on the structure, emulsifying features and interfacial features of oxidized soybean protein aggregates (OSPI). The results showed that oxidative treatment could disrupt the native soy protein (SPI) structure by promoting the formation of oxidized aggregates with β-sheet structures through hydrophobic interactions. These changes led to a decrease in the solubility, emulsification ability and interfacial activity of soybean protein. After low-power ultrasound (100 W, 200 W) treatment, the relative contents of β-sheets, β2-sheets, random coils, and disulfide bond of the OSPI increased while the surface hydrophobicity, absolute ζ-potential value and free sulfhydryl content decreased. Moreover, protein aggregates with larger particle sizes and poor solubility were formed. The emulsions prepared using the OSPI showed bridging flocculation and decreased protein adsorption and interfacial tension. After applying medium-power ultrasound (300 W, 400 W, and 500 W) treatments, the OSPI solubility increased and particle size decreased. The α-helix and β-turn contents, surface hydrophobicity and absolute ζ-potential value increased, the structure unfolded, and the disulfide bond content decreased. These changes improved the emulsification activity and emulsion state of the OSPI and increased the protein adsorption capacity and interfacial tension of the emulsion. However, after a high-power ultrasound (600 W) treatment, the OSPI showed a tendency to reaggregate, which had a certain negative effect on the emulsification activity and interfacial activity. The results showed that UT at an appropriate power could depolymerize OSPI and improve the emulsification and interfacial activity of soybean protein.

1. Introduction

Soy protein is a commonly utilized oilseed bean protein in the food sector because of its good functional qualities and physiological activity, high nutritional value and inexpensive cost [51]. Soy protein emulsification and interfacial activity are used to produce drinks, sauces, ice cream, sweets, coffee, etc [52]. The production and consumption of soybean protein in the food industry continue to increase, which has led to huge pressure in terms of its storage and transportation. However, in the production process of soybean protein, nearly 1% of lipids and highly active lipoxygenase (LOX) remain, resulting in a consistent oxidative environment during the storage and transportation of soybean protein. Under the catalysis of lipoxygenase, linoleic acid can produce a large number of reactive oxygen free radicals and active secondary oxidation products, which can react with protein in food and cause soybean protein oxidation [6]. Soy protein undergoes a conformational transition after being continuously attacked by oxidation, and it forms oxidative aggregates with a high degree of aggregation and reduction in emulsification activity. In the food industry, oxidized soybean protein is difficult to use [15]. To increase its emulsification and interfacial
activity, it is critical to investigate methods of modulating the oxidative aggregates of soybean protein isolates.

The physical control of protein aggregation is now being researched intensively. To enhance the functional characteristics of proteins, ultrasonic waves, pulsed electric fields, and high-voltage homogenization are employed to modify the spatial structure of protein aggregates [33]. The research shows that the protein aggregates can be effectively depolymerized only when the pressure exceeds 400 Mpa [10]. However, high-pressure HPH has large energy consumption and small workload, so there are difficulties in industrial use. Pulsed electric field treatment technology has been widely used, although it is difficult to apply for food processing due to the high environmental requirements, such as pH and temperature [22]. As a nonthermophysical treatment technology, ultrasound is more suitable for application in food plants owing to its advantages of greenness, high efficiency, and simple operation [30]. Ultrasonic physical field changes the spatial structure of protein through the shear force and turbulence effect produced by cavitation, so as to enhance the functional activity of protein. O’Sullivan’s research shows that the underlying mechanism of ultrasonic modification is mainly attributed to the conformational changes of protein molecules. The high-frequency oscillations generated by ultrasonic waves act on the liquid to cavitate the protein macromolecules [16]. Moreover, ultrasound generates strong turbulent flow and high-energy shear waves in an instant, which can change the structure of proteins. And the properties and functions of the protein will be affected [14]. The effect of ultrasonic treatment on the structure and properties of proteins depends on the ultrasonic intensity (ultrasonic time, temperature, power, etc.). O’Sullivan et al. found that using a certain intensity of ultrasonic pretreatment (20 kHz, ~34 W cm², 2 min) of wheat protein and soybean protein isolate will improve their solubility and emulsifying properties, and the low intensity sound energy is not enough to destroy the covalent interaction in the protein peptide chain [29]. When chandrapala et al. treated 10% sunflower seed oil-water system, the lotion formed by ultrasonic treatment at 20 kHz was the best. When the frequency increased to 211 kHz, the lotion could not be formed [4]. QZ et al investigated the effect of ultrasonic treatment on the structural and functional properties of Perilla protein isolate (PPI), and found that ultrasonic treatment reduced the particle size, induced the exposure of hydrophobic groups and the changes of secondary structure and tertiary conformation of PPI. In addition, ultrasonic treatment also improved the solubility, water/oil holding capacity, foaming and emulsifying capacity of PPI [31]. Related studies also show that this is related to the fact that ultrasound can destroy the chemical bonds that maintain the protein structure, such as hydrogen bonds and disulfide bonds [19]. The above studies show that ultrasound can enhance the functional activity of protein through the structure of protein. In addition, studies have shown that ultrasonic physical fields can also achieve directional regulation and improved functions of protein aggregates [52]. Ultimately, the aggregation degree of the protein oxidative aggregates is changed and the protein presents good functional features. Cao et al. found that ultrasound could modulate the aggregation and conformation of oxidized quinoa proteins by altering intermolecular forces, thereby enhancing the functional features of quinoa proteins [12]. Boora et al. found that ultrasoundation can improve the emulsification and physicochemical features of whey protein soluble aggregates [2]. Zheng et al. [40] noted that sonication altered the subordinate and tertiary structures of all SPI aggregates, thereby reducing the particle size and improving their solubility and surface hydrophobicity [40]. This finding suggests that turbulent flow and high-energy shear waves possessed by physical ultrasound fields can induce dissociation and aggregation of subunits by disrupting disulfide bonds and non-covalent interactions, ultimately leading to changes in the functional properties of protein aggregates. However, the above research is limited to the regulation of protein structure, function and activity by ultrasonic treatment. There are few studies on the effect of protein structure changes induced by ultrasound on the interface stability mechanism. Moreover, limited information is available on the structural modifications and functional changes of protein oxidative aggregates induced after sonication.

In this experiment, a soybean protein oxidation model was constructed by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) to study the impacts of different intensities of UT on the structure, emulsification features and interface features of oxidized soybean protein aggregates. Based on the impact of UT on the structural features of proteins, the associations among the structure, function and interface features of oxidized soybean protein aggregates were explored. This study can provide a theoretical basis for the use of ultrasonic physics in the regulation of protein aggregates and the development, modification, storage, and transportation of soybean protein products.

2. Materials and methods

2.1. Materials

Soybean protein isolate (92.4% protein) was obtained from Shandong Yuyang Ecological Food Industry Company Incomplete (Shandong, China). 8-Anilino-1-naphthalene sulfonate (ANS), 2,2′-dithiobis (5-nitro-2-pyridine) (DTNP) and other elements were acquired from Beijing Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China).

2.2. Formulation of oxidized soybean protein aggregates

The oxidized aggregates of soybean protein (OSPI) were obtained according to a previous study [44]. Soybean protein was softened in a phosphate buffer mixture (0.01 mol/L) at pH 7.2 (reached using 0.5 mg/mL Na2SO3) to prepare a 10 mg/mL soybean protein solution. AAPH was added to a final concentration of 0.5 mmol/L AAPH. After applying the oxidation treatment for 12 h at 37 °C in the dark, dialysis was carried out at 4 °C for 72 h with a 14000 kDa dialysis bag and H2O was altered every 6 h. Finally, an oxidized soybean protein aggregate solution with 12 h of oxidation time was obtained. After spray drying, the samples were obtained and named OSPI.

2.3. Ultrasound treatment

Eight different samples were liquified phosphate buffered saline (PBS) (0.01 mol/L) at pH 7.0 (reached using Na2SO3, 0.5 mg/mL) to fabricate a 10 mg/mL protein mix. After adding AAPH (1 mmol/L) to the protein solutions of different samples and treating them at 37 °C for 24 h in the dark, the solutions were placed in a 14000 kDa dialysis bag at 4 °C, and the deionized water was altered every 6 h. After 72 h of dialysis, 100 mL was collected and placed in an ultrasonic cell disruptor, ultrasonicated for 5 min under ultrasonic power (UP, 100 W, 200 W, 300 W, 400 W, 500 W, and 600 W), with a working time of 5 s and intermittent recovery of 5 s. The ultrasonication process was carried out under ice bath conditions. The sonicated solution was centrifuged at 4 °C and 9000 r/min for 20 min. After centrifugation, the supernatant was poured into the plate and freeze-dried to obtain 8 kinds of soluble ultrasonic soy protein isolate aggregates, including soy protein isolation (SPI) and oxidized soy protein isolation (OSPI). A total of 8 samples were obtained and named SPI, OSPI, OSPI-100, OSPI-200, OSPI-300, OSPI-400, OSPI-500, and OSPI-600.

2.4. Particle size distribution

The particle size distribution (PSD) was assessed using a laser scattering Mastersizer S (Malvern, UK) and a 300 reverse Fourier lens with the aid of a He–Ne laser λ = 633 nm according to the assay defined by Ma et al. The quantity was determined at RT (25 ± 2 °C), and the protein refractive index was 1.33. The samples were thinned with the deionized water to 50 mg/mL prior to detection, and the particle widths ranged from 0 to 10,000 μm.
2.5. Turbidity

The assay of Martini, Potter, & Walsh [24] was performed. Briefly, the OSp1 mix (0.1%, w/v) was agitated for 60 min at 25 °C, after which the adsorption level was detected at 600 nm with an Eppendorf Bio-Spectrometer fluorescence (Eppendorf Co., Germany) [24].

2.6. Protein solubility

The protein solubility determination was carried out based on the method of Shimada & Chetel [34]. First, the samples were dissolved in distilled water (pH 8.0, using 0.01 M NaOH to adjust pH) at a concentration of 2 mg/mL. After centrifugation at 10,000 g for 30 min, the protein content of the supernatant was determined using BCA Protein Assay Kit to calculate the protein solubility (%) according to the following equation.

\[
\text{Protein solubility(%) = \frac{\text{supernatant protein content}}{\text{total protein content before centrifugation}} \times 100}
\]

2.7. Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectra of fresh and oxidized soy protein were measured by a Nicolet-6700 FT-IR Spectrometer [41]. The lyophilized sample (1 mg) was methodically blended with KBr (100 mg) in an agate mortar. All the samples were assessed at RT (25 ± 2 °C) over a wave number range of 400-4000 cm⁻¹. All 64 scans had a resolution of 4 cm⁻¹ and an accuracy of 0.01 cm⁻¹. The software “Peakfit Version 4.1.2” was used to analyze the secondary structure of protein, and “Gaussian peak fitting” was the algorithm used [49].

2.8. Inherent fluorescence

The fluorescence emission ranges of the samples were observed via fluorescence spectroscopy (Hitachi, Japan) according to the assay of Jiang et al. [9]. Using 0.01 mol/L phosphate buffer, the soy samples were diluted to reach a protein concentration of 0.4 mg/mL to obtain fluorescence spectroscopy (Hitachi, Japan) according to the assay of Jiang et al. [9]. Using 0.01 mol/L phosphate buffer, the soy samples were diluted to reach a protein concentration of 0.4 mg/mL to obtain fluorescence spectroscopy (Hitachi, Japan) according to the assay of Jiang et al. [9]. Using 0.01 mol/L phosphate buffer, the soy samples were diluted to reach a protein concentration of 0.4 mg/mL to obtain fluorescence spectroscopy (Hitachi, Japan) according to the assay of Jiang et al. [9].

2.9. Surface hydrophobicity (HO)

The surface hydrophobicity (Hₒ) of the samples was examined via titration with ANS using the assay of Jiang et al. [17]. Four-milliliter aliquots of the cured sample (0.06–0.5 mg/mL) were fabricated in 10 mM PBS at pH 7.0 and blended with 25 μL of 8 mM ANS mix for 20 min at RT (25 ± 2 °C). The comparative fluorescence intensity (FI) was determined using with fluorescence spectroscopy, with a 374 nm excitation wavelength and 485 nm emission wavelength (F4500 fluorescence, Hitachi, Japan). The Hₒ index was assessed according to the original slope factor for successive protein waterings.

2.10. ζ-potential

The ζ-potential of the samples was detected in triplicate at 25 °C using a laser diffraclorimeter (Zetazizer NanoZS 90, Malvern Ltd., UK) based on the assay of Jiang et al. [17]. The protein sample was placed in 50 mmol/L PBS (pH 7.0) to confirm that the physical fraction was 0.2%, and the ζ-potential was expressed using ZetaSizer software.

2.11. Sulfhydryl (SH) content

The disulfide bond and free sulfhydryl (SH) group contents were measured using the assay of Wu [43]. The SH group levels in the sample were measured based on changes in Ellman’s assay and the retention of DTNP. The SH levels were calculated as nmol/mg protein via a molar extinction constant (13,600 M⁻¹ cm⁻¹).

2.12. Measurement the emulsifying activity index (ESI) and emulsion constancy index (EAI)

The EAI and ESI were measured using the assay of Pearce et al. [20]. A 15 mL sample of 0.1% (w/v) protein mix was blended with 5 mL of sunflower oil at 10 000 rpm for 10 min via a high-speed homogenizer (T-25 homogenizer, IKA, Germany) to obtain an emulsion. Aliquots (50 mL) of the emulsion were taken from the bottom of the chamber immediately (time 0) and at subsequent time intervals (time 30 min), and diluted into 5 mL (100-fold) 0.1% SDS (sodium dodecyl sulfate) solution. The absorbance of the diluted solutions was measured at 500 nm (Beckman DU 500, USA). All determinations were conducted in triplicate. The EAI and ESI were calculated as follows.

\[
\text{EAI(m²/g) = } \frac{2 \times 2.303 \times DF \times A₀ \times C \times \phi \times 10000}{1 - \theta} \times \frac{A₀}{A₀ - A₉₀} \times 10
\]

where A₀ is the absorbance at 0 min of the thinned suspension, DF is the thinning factor (×100), C is the sample level (g/mL), ϕ is the visual path, θ is the portion of oil (0.25), and A₉₀ is the absorbance after 30 min.

2.13. Confocal laser scanning microscope

The emulsion microstructure was evaluated using a Leica TCS SP2 CLSM (Leica Microsystems, Wetzlar, Germany). A total of 15 mL of 0.1% (w/v) protein mix was blended with 5 mL of corn oil at 900r/min for 30 min to obtain an emulsion. Dye containing 0.02% Nile red (40 μL) and 0.1% Nile blue (45 μL) was blended with 1 mL of the suspension. The staining time was 30 min. Then, the tinted emulsion was placed in the slide center and enclosed with a coverslip. Silicone oil was mounted to the coverslip to avoid water evaporation. The slide was viewed using a laser confocal microscope. The CLSM images were obtained by orderly scanning for protein excitation line at 633 nm and oil at 488 nm. A fluorescence copy was generated with a pixel density of 1024 × 1024 [48].

2.14. Percentage of adsorbed proteins (AP%)

The adsorbed protein ratios were evaluated based on the assay of Ma et al. [23]. Briefly, 10 mL emulsions were centrifuged at 13000 × g for 15 min at RT. After centrifugation, the emulsion cream sheet was at the highest position of the pipe while the aqueous stage was at the lowest position of the pipe. The aqueous stage was composed with a needle and the aqueous stage was at the lowest position of the pipe. The level of the proteins in the aqueous stage was examined based on the bicinchoninic acid (BCA) assay. The proportion of adsorbed proteins was determined based on Equation (3):

\[
\text{AP(%) } = \frac{C₀ - Cₐ}{C₀} \times 100
\]

where C₀ is the original protein level of the protein mixes and Cₐ is the unabsorbed protein level of the protein mixes.

2.15. Interfacial tension

The interfacial tension between the aqueous and oil stages was evaluated using a tensiometer K100 (Krüss, Germany) and the Wilhelmy plate assay. The Wilhelmy plate is composed of platinum, and it has a length, width and height of 19.9 mm, 10 mm, and 0.2 mm, respectively.
The Wilhelmy plate was submerged in 20 g of the aqueous solution to a depth of 3 mm, and surface exposure occurred at a rate of 15 mm min\(^{-1}\). The exterior discovery is the rapidity of the container drive utilized for the recognition of the liquid surface. Once the superficial has been noticed by the microbalance in the tensiometer, the container transfers at the selected surface recognition rapidity to the location stated by the absorption intensity (3 mm). Next, the edge between the oil and aqueous phases was shaped by carefully pipetting 50 g of the oil stage across the aqueous stage. The test was performed over 3600 s, and the temperature was maintained at 20 °C. The interfacial tension levels and error bars represent the average and standard deviation of 3 repeats, respectively.

2.16. Statistical analysis

Statistical analyses were performed using SPSS 20.0. The results were assessed using Duncan’s manifold series and ANOVA tests and are presented as the mean ± SD of triplicate analyses. A p-value ≤ 0.05 was considered significant.

3. Results and discussion

3.1. Particle size distribution

Protein particle size distribution can directly illustrate protein aggregation or degradation [13]. Fig. 1 shows that the particle size distribution of the SPI was unimodal at approximately 60 ~ 400 nm. After the oxidation treatment, the OSPI turbidity increased and the particle size distribution was trimodal, with a main peak at 60 ~ 400 nm and two slight peaks at 20 ~ 60 nm and 4000 ~ 8000 nm. On the one hand, oxidation led to the incomplete transformation of protein forms; therefore, additional hydrophobic clusters were visible. The increase in hydrophobic interactions increased the production of oxidized aggregates and then converted soybean protein into larger aggregates. Therefore, a large particle size peak of 4000 ~ 8000 nm was formed. On the other hand, some free radicals in the oxidation process attacked the amino acid side chains of proteins, thus causing the cleavage of peptide bonds to form molecules with small particle size, and a small particle size peak of 20 ~ 60 nm appeared. A slight rightward shift of the particle size distribution was observed after low-power ultrasound (100 W, 200 W) treatment, and the OSPI of the medium-power treatment showed the opposite trend. The physical field of low-power ultrasound induced protein unfolding and internal group exposure and increased the rate and probability of molecular collision. However, this intensity of UT is insufficient to break the intermolecular interactions and chemical bonds, thus resulting in an increase in particle size and the formation of large particles. However, the cavitation effect caused by medium-power ultrasound (300–500 W) produced high shear energy and turbulence and thus provided sufficient energy for the collision and dissociation of protein molecules. Therefore, protein molecules were dissociated into small molecules by destroying the intermolecular interactions and chemical bonds. However, when the treatment power increased to 600 W, a left shift in the observed peaks of the particle size distribution was apparent, which may be related to the increased likelihood of attack by the ultrasound energy and the sufficient energy provided for the collision and dissociation of protein molecules. Subsequently, the aggregates that were broken could reassemble into large particles under intermolecular interactions [35].

3.2. Turbidity

Turbidity can directly reflect the dispersion state and aggregation state of oxidized soybean protein aggregates in solution after UT. The impact of UT on the turbidity of the OSPI samples is shown in Fig. 2. Soybean protein has a globulin structure and can scatter visible light; therefore, SPI has a certain turbidity value without the oxidation treatment. After the oxidation treatment, the turbidity of the OSPI showed a significant increasing trend. The oxidation treatment could cause the SPI aggregation reaction, resulting in an increase in the content and particle size of macromolecular oxidation aggregates in the protein solution. As a result, the turbidity of the OSPI increased. With the increase in ultrasound power from 100 W to 500 W, the turbidity of the OSPI initially increased and then decreased. Low-power UT (100 W, 200 W, 300 W, 400 W, 500 W, and 600 W).

![Graph](image-url)

Fig. 1. Particle size distribution of unoxidized, oxidized treated, and ultrasound treated after oxidized treatment soybean protein aggregates at different time (100 W, 200 W, 300 W, 400 W, 500 W, and 600 W).
200 W) increased the probability of molecular collision, resulting in the further aggregation of hydrophobic regions of the OSPI molecules under intermolecular interactions to form macromolecular aggregates. Therefore, an upward turbidity trend was observed. As the UP increased (300–500 W), the turbidity of the OSPI decreased significantly. This finding indicated that moderate UT power could lead to the depolymerization of oxidized aggregates of soybean protein. This led to a downward trend in OSPI turbidity. However, when the UP increased to 600 W, the ultrasonic physical field cleaved the OSPI to produce small particle size protein molecules. An ultrasonic physical field would also lead to some cracked protein molecules colliding with each other and then further aggregating to form macromolecular aggregates through intermolecular interactions. As a result, the turbidity of the UOSPI-600 sample was lower than that of the OSPI but higher than that of the UOSPI-500. Gülseren et al. also noted that high-intensity sonication of innate bovine serum albumin resulted in the creation of aggregates and an increase in particle size, which was possibly due to the promotion of reaggregation of some aggregate molecules[3].
3.3. Solubility

Solubility is one of the most useful measures of protein denaturation and accumulation. As portrayed in Fig. 3, the soy protein solubility decreased after oxidation. AAPH could oxidize the key peptide chain and side sequence groups of soybean protein, thereby promoting the expansion of the protein structure and the exposure of internal groups. Oxidized aggregates were formed, resulting in a decline in solubility. Similar findings were reported by Sun et al., who noted that oxidation-induced protein structure unfolding hindered covalent and noncovalent binding of the oxidized protein and indicated that extra hydrophobic units were visible. After hydrophobic binding surpassed a serious level, proteins and led to lower solubility of the solution. The medium-power ultrasound treatment (300–500 W) UT can cause the folded structure of protein aggregates to break and transform from oxidized aggregates of macromolecules to oxidized aggregates of small molecules. This results in a reduction in the β1, β2 and random coil content. However, when the UP

Fig. 4. FTIR spectra of unoxidized, oxidized treated, and ultrasound treated after oxidized treatment soybean protein aggregates at different time (100 W, 200 W, 300 W, 400 W, 500 W, and 600 W).
increased to 600 W, the contents of β1 and β2 in OSPI showed a gradually increasing trend. These changes may be due to the thermal effect and high-speed turbulence caused by excessive ultrasound treatment, which led to the formation of large-sized oxidized protein aggregates through β1 and β2 folding of the broken oxidized aggregates. This finding indicated that ultrasound could dynamically regulate the depolymerization and reaggregation of oxidized aggregates of soybean protein.

3.5. Intrinsic fluorescence

Intrinsic fluorescence was utilized to imitate the conformational alterations in the tertiary construction of oxidized soy protein through ultrasonic treatment. The intrinsic fluorescence range of unoxidized, oxidation-treated, and ultrasound-treated soy protein is presented in Fig. 5. A slight blueshift was observed at the peak position of the intrinsic fluorescence spectrum (from 338 nm to 335 nm) after soybean protein oxidation. The results showed that under the action of oxidation, free radicals induced intermolecular crosslinking and aggregation of the protein so that the exposed tryptophan residues were rewrapped in the protein structure. This led to a decrease in the polarity of the microenvironment where the fluorescent emitter was located, resulting in the blueshift phenomenon. At the same time, the inherent fluorescence intensity of soybean protein decreased, indicating that the oxidative modification of alkylated free radicals can quench the fluorescence of aromatic amino acid residues of soybean protein. It may be that free radicals could change the hydroxyl position of tryptophan after oxidative attack to generate 3-hydroxykynurenine. A blueshift and decreased fluorescence intensity were observed in OSPI treated with low-power ultrasound (100 W, 200 W), indicating that the microenvironmental hydrophobicity of aromatic amino acids was enhanced. The low-power ultrasound treatment led to the further accumulation of protein aggregates, resulting in a denser structure of the protein aggregates. By increasing the UP (300–500 W), the fluorescence intensity of UOSPI-300, UOSPI-400 and UOSPI-500 increased and λmax showed a red shift. This result indicated that the high-speed shearing and molecular collision effects produced by medium-power sonication could cause structural fragmentation and subunit dissociation of OSPI. Therefore, due to oxidative aggregation, the fluorescent emitting groups buried
inside the protein structure are gradually exposed to the external polar environment, resulting in an increase in the polarity of the microenvironment where the aromatic amino acid residues are located. A study by Wang et al. also noted that UT can lead to protein molecule unfolding and contact of Trp/Tyr adducts, resulting in an increase in fluorescence strength (Y. [42]. As the processing power increased from 500 W to 600 W, there was a blue shift in \( \lambda_{\text{max}} \) and a decrease in the fluorescence strength. However, compared with the OSPI, the fluorescence strength of the UOSPI-600 increased and \( \lambda_{\text{max}} \) shifted red. Sonication at 600 W can also open the protein structure and transfer the aromatic amino acid adducts of protein aggregates from a hydrophilic state to a hydrophobic state, although to a lower degree than that at sonication at 500 W. This finding is essentially consistent with the particle size and turbidity data (Fig. 1 and Fig. 2), indicating that high-power ultrasound caused some protein molecules to aggregate to form aggregates of large particles, resulting in a decrease in fluorescence intensity. Tao et al. also revealed that UT can adjust the tertiary structure of proteins by disturbing the chemical bonds and binding between protein particles, thereby changing the longitudinal structure of protein aggregation particles, although these changes are related to the UP [38].

3.6. Protein surface hydrophobicity

The surface hydrophobicity (H_0) of a protein is carefully connected to the functional features, for example, emulsification and foaming features [8]. The impact of UT on the surface hydrophobicity of the OSPI samples is portrayed in Fig. 6. Compared with the SPI, the superficial hydrophobicity of the OSPI decreased significantly, indicating that oxidation significantly reduced the surface hydrophobicity of native soy protein. This may be because free radical attack can induce oxidative aggregation of SPI through anti-parallel folding of intermolecular \( \beta_1 \)-sheet, thus forming protein aggregates with large particle sizes, which results in buried hydrophobic groups and reduced surface hydrophobicity. The superficial hydrophobicity of the ultrasonically treated OSPI initially decreased and then increased with increasing treatment power (100–500 W), and the H_0 of UOSPI-200 was the lowest while that of UOSPI-500 was the highest. Low-power UT (100 W, 200 W) could promote covalent and noncovalent crosslinking of protein molecules that have undergone oxidative aggregation by increasing the movement rate and collision probability of the OSPI. Such changes led to the regrouping of the OSPI in the system and greater embedding of hydrophobic groups in the protein, thus reducing the superficial hydrophobicity. As the UP increased from 300 W to 500 W, the surface hydrophobicity showed an increasing trend. Combined with the decrease in particle size and \( \beta_1 \) sheet content of soybean protein after medium-power sonication (Fig. 1 and Table 1), H_0 may have increased because the cavitation produced by the ultrasonic physical field can reduce the degree of intermolecular binding within the OSPI, which leads to the dissociation of the OSPI aggregates, resulting in the contact of buried hydrophobic clusters with the solvent and hence the rise of H_0. The study by Hu et al. also showed that the increase in H_0 may be due to ultrasound-induced protein denaturation, which exposes hydrophobic areas of soybean protein from the interior to the exterior [11]. When the treatment power was increased to 600 W, the observed drop in H_0 could be attributed to soybean protein reaggregation. Another study showed that excessive sonication may alter the polar groups of protein molecules and cause reaggregation [36]. Therefore, compared with UOSPI-500, the structure of UOSPI-600 was further damaged under the action of high shear and cavitation physical fields, and the hydrophobic region was also damaged at this time. Moreover, aggregates were formed through the formation of a \( \beta_1 \)-sheet structure, resulting in a decrease in the H_0 of the OSPI.

3.7. \( \zeta \)-potential

The \( \zeta \)-potential was linked to the charge strength on the surface of the protein molecule in the protein solution system and reproduced the strength of electrostatic repulsion or attraction [46]. Therefore, the \( \zeta \)-potential amount is an important indicator of the strength of protein solution systems [47]. The effect of UT on the \( \zeta \)-potential of the OSPI samples is shown in Fig. 7. The absolute value of the \( \zeta \)-potential of the OSPI decreased significantly compared with that of the SPI. Oxidative stress may lead to an increase in the molecular interactions of soybean protein in the solution system, resulting in aggregation. The reduction of charged amino acids on the protein surface caused by aggregation led to an increase in the absolute \( \zeta \)-potential level of the protein. With the increase in UT power (100–500 W), the absolute \( \zeta \)-potential value presented an initially declining and then gradually increasing trend, which
would induce the fragmentation of the protein skeleton and increase the intermolecular force. Combining the results of secondary structure and surface hydrophobicity, low-power UT promoted further aggregation of the OSPI, causing charged amino acids to curl up inside the protein surface. Therefore, the surface charge of the OSPI was reduced, resulting in a decrease in the absolute level of the solution ζ-potential. With the increase in UP (300–500 W), soy protein oxidative aggregates were deteriorated by ultrasound, which induced the formation of small-sized protein aggregates with a loose structure and high molecular flexibility. These changes led to an increase in the surface charged amino acids of the soluble aggregates in solution and an increase in the electrostatic repulsion between the molecules, which resulted in an increase in the absolute ζ-potential. However, when the processing power was too high (600 W), the absolute ζ-potential decreased, which may be related to further aggregation of the OSPI caused by the thermal effect and high-speed turbulence caused by excessive sonication. This resulted in charge neutralization and a reduction in the net charge content of certain charged amino acids. In addition, this may also be related to the free radical effect induced by high-power ultrasound. The study by Guo et al. found that free radical attack induces unfolding of protein hydrogen bonds and destruction of higher-order structures, leading to the loss of certain charged amino acids and the reduction of absolute potential [49].

### 3.8. Sulphydryl content

Free sulphydryl (-SH) and disulfide bonds (-S-S-) are important functional groups in proteins that can control the spatial conformation and exert functional properties through mutual conversion between sulphydryl and disulfide bonds. The effects of ultrasound on the total sulphydryl, free sulphydryl and disulfide bonds of oxidized soy protein aggregates are given in Table 2. The content of total SH and free SH in the OSPI was lower than that in the SPI, whereas the content of disulfide bonds was larger than that in the SPI. Free radicals in the oxidizing environment could attack the sulphydryl group on the protein peptide bond and induce the mutual transformation between the sulphydryl group and disulfide bond, resulting in an increase in the disulfide bond content. This resulted in OSPI forming a tighter spatial structure and larger particle size protein molecules with antiparallel folds of β1. With the increase of UP from 100 W to 500 W, the free sulphydryl group of the OSPI initially decreased and then increased, the total sulphydryl content decreased significantly, and the disulfide bond values initially increased and then decreased. It could be inferred that low-power (100, 200 W) sonication can increase the free SH contact and -SH/-SS exchange reaction between OSPI molecules by inducing further collisions of the OSPI molecules, thereby promoting the occurrence of cross-linking reactions and the formation of disulfide bonds. In addition, the OSPI further formed oxidized aggregates with larger particle sizes and tighter structures, which may also be one of the reasons for the reduction of the hydrophobic groups of the low-power sonicated OSPI. With the increase of UP (300–500 W), the protein molecules in the OSPI solution system were impacted by a larger energy field. Therefore, the disulfide bond that promoted the formation of the OSPI was cleaved and then converted into a free sulphydryl group, which would expose the sulphydryl group.

![Fig. 7. ζ-potential of unoxidized, oxidized treated, and ultrasound treated after oxidized treatment soybean protein aggregates at different time (100 W, 200 W, 300 W, 400 W, 500 W, and 600 W).](image)

| Sample     | free sulphydryl (nmol/mg) | total sulphydryl (nmol/mg) | disulfide bond (nmol/mg) |
|------------|--------------------------|---------------------------|--------------------------|
| SPI        | 4.21 ± 0.08a             | 5.57 ± 0.13a              | 0.68 ± 0.09f             |
| OSPI       | 3.55 ± 0.11d             | 5.51 ± 0.11b              | 0.98 ± 0.14b             |
| UOSPI-100  | 3.96 ± 0.14c             | 6.02 ± 0.23d              | 1.03 ± 0.11b             |
| UOSPI-200  | 3.37 ± 0.11d             | 6.03 ± 0.21c              | 1.33 ± 0.13c             |
| UOSPI-300  | 4.18 ± 0.17b             | 5.6 ± 0.16b               | 0.71 ± 0.12c             |
| UOSPI-400  | 4.55 ± 0.16a             | 5.23 ± 0.14d              | 0.34 ± 0.15d             |
| UOSPI-500  | 4.72 ± 0.17a             | 5.18 ± 0.27c              | 0.23 ± 0.07c             |
| UOSPI-600  | 4.10 ± 0.13ac            | 5.40 ± 0.32bc             | 0.65 ± 0.12c             |

Note: Comparisons were carried out between values of the same row; values with different letter(s) indicate a significant difference at p ≤ 0.05.
inside the molecule, resulting in an increase in the free sulfhydryl cluster content and a decrease in the disulfide bond content. These changes also contributed to the transformation of the OSPI soluble aggregates and the exposure of active groups and promoted the enhancement of molecular flexibility [18]. However, after high-power ultrasound (600 W), the content of free sulfhydryl clusters increased and the level of disulfide bonds decreased compared with that of the OSPI. Compared with UOSPI-500, UOSPI-600 showed reduced free sulfhydryl content and increased disulfide bond content. This indicated that excessive ultrasonic intensity would lead to the formation of nondisulfide-bonded sulfur-containing compounds after breaking the disulfide bonds, and it would also promote the dissociation of protein aggregates and their reaggregation through the formation of disulfide bonds [1]. As a result, the content of free sulfhydryl clusters increased and the level of disulfide bridges decreased. Overall, ultrasound can affect the polymerization and depolymerization of oxidative aggregates by modulating the interconversion between protein sulfhydryl and disulfide bonds and the generation of sulfur-containing compounds. So as to realize the regulation of the functional properties of proteins.

3.9. Emulsion steadiness index and emulsifying activity index

Proteins have emulsifying activity because they contain hydrophilic and hydrophobic groups, and they represent an important emulsifier in food processing [54]. The effect of UT on the EAI and ESI of the OSPI samples is shown in Fig. 8. Compared with the SPI, the EAI and ESI of the OSPI were significantly lower. Oxidation led to the formation of highly ordered intermolecular β-sheet structures between protein molecules, thereby reducing the molecular flexibility. Protein aggregation led to the embedding of hydrophobic clusters and a decrease in surface hydrophobicity. Such changes inhibited SPI from adsorbing and rearranging at the oil–water border, and it formed unsolvable oxidized aggregates that were difficult to relax in structure, resulting in a significant decrease in the emulsifying effect and emulsifying steadiness of soybean protein. The solubility of protein molecules decreases after oxidation, thereby preventing rapid swelling at the oil–water border and decreasing the emulsification performance of the OSPI. As the UP increased from 100 W to 500 W, the EAI and ESI of the OSPI initially decreased and then increased. The protein aggregates treated with low-power ultrasound (100 W, 200 W) had a high degree of rewinding and showed reduced flexibility in the protein secondary structure. At the same time, the particle size of the OSPI increased and hydrophobic clusters and free sulfhydryl clusters were entrenched in the protein. Such changes were not conducive to its diffusion and rearrangement at the water–oil interface, resulting in reduced protein emulsification. High pressure, turbulence, and shear forces generated by medium-power ultrasound (300–500 W) led to the depolymerization and unfolding of oxidized soybean protein aggregates. The protein structure gradually changed from ordered to disordered, the molecular flexibility was enhanced, and the amphiphilicity of the protein was improved. Therefore, the surface tension of the water–oil border was reduced, which enhanced the emulsification features of the protein [26]. In addition, the shearing power generated by ultrasonic waves condensed the particle size of the protein and increased the precision of the superficial part of the protein, thereby improving the interfacial adsorption features of the OSPI and also enhancing the emulsification features of proteins. However, when the sonication power was increased to 600 W, both the EAI and ESI of UOSPI-600 decreased compared with those of UOSPI-500. Ultrasound-induced dissociated protein aggregates reaggregate through disulfide bond formation, which reduces molecular flexibility and interfacial membrane stability. Therefore, the EAI and ESI showed a downward trend.

3.10. Confocal laser scanning microscopy

To further investigate the correlation between particle size dispersal and emulsion microstructure, the interfacial framework and microstructure of the OSPI emulsion treated by ultrasound were observed by CLSM (Fig. 9). In the CLSM images of emulsions made from natural soybean protein, Nile blue staining of the water phase complex (red) encircles the Nile red-stained oil (green), which is indicative of typical oil/water emulsions. The interface layer was solid and orderly, indicating that the SPI had good amphiphilic and interface characteristics, could be adsorbed at the oil–water border, and formed a dense interface.
skeleton. However, droplet flocculation (aggregation of two or more emulsion droplets to form a floc) and union (assimilation of drops) were noted among the emulsion droplets prepared by OSPI after oxidation. Oxidation treatment could cause a decrease in the aggregation and solubility of protein molecules, resulting in the formation of a stable interfacial film during the emulsification process. Therefore, a large accumulation area of red oil droplets was observed. The results show that fewer and unchanging drops were acquired in the emulsion created by OSPI cured with ultrasound (300–500 W), and flocculation or union may have occurred in these emulsions formed using OSPI treated with low- or high-power ultrasound (100–200 W & 600 W). After low-power ultrasound (100 W, 200 W) treatment of the OSPI, the protein particles were unevenly distributed, indicating that the interfacial activity of the protein decreased and could not form a stable emulsion. The reason might be that the structural flexibility and surface hydrophobicity of protein aggregates decreased and the specific surface area decreased after low-power ultrasound treatment, which was not conducive to diffusion to the border and expansion on the superficial oil drops. This led to the sharing of a layer of protein adsorption between adjacent droplets, resulting in emulsion bridging flocculation. After the OSPI was processed by medium-power ultrasound (300–500 W), the surface of the UOSPI-300, UOSPI-400 and UOSPI-500 emulsion droplets showed a multilayer structure that was completely wrapped on the surface of the oil drops and produced a dense interfacial film. The distance between emulsion droplets increased, the volume decreased, and the distribution was relatively uniform. The medium-power ultrasound treatment could open the spatial structure of the OSPI and significantly improve the flexibility and amphiphilic features of the protein molecules. Therefore, the ability of interface adsorption and interface rearrangement was improved, which was conducive to inhibiting the occurrence of bridging flocculation and promoted the formation of smaller droplets with smaller particle sizes to improve emulsion stability [28]. However, after high-power ultrasound (600 W) treatment of the OSPI, the volume of UOSPI-600 protein increased and the flocculation and aggregation emulsion phenomena appeared again. The reason may be that the high-power UT led to the involution of the hydrophobic group of the protein and the reduction of lipophilicity and molecular flexibility. As a result, a stable protein interface film could not be formed to encapsulate the oil droplet molecules [32]. In addition, the state and degree of droplet flocculation were highly impacted by the employed protein level and/or oil portion [7].

3.11. Percentage of adsorbed proteins

The content of interfacial adsorbed protein (AP%) has a significant impact on the reliability of the emulsion [25]. A higher amount of interfacial protein adsorption corresponds to a stronger protein adsorption ability at the oil–water border. Fig. 10 shows the amount of interfacial protein adsorption of the sonicated soluble soybean protein isolate oxidized aggregate emulsion. It can be noted from Fig. 10 that the
amount of interfacial protein adsorption of the OSPI emulsion decreased. The reason may be that oxidation induces protein aggregation, and the involution of hydrophobic groups occurred inside the structure. At the same time, the solubility and structural flexibility were reduced and adsorption mainly occurred in the form of particles at the interface. This was not conducive to relaxation and adsorption at the interface, resulting in a reduction in the protein adsorption amount at the interface. With the increase of UP from 100 W to 500 W, protein adsorption initially decreased and then increased. Low-power UT (100 W, 200 W) promoted the formation of soluble aggregates with large particle sizes and complex structures so that more hydrophobic groups were embedded into the proteins. This phenomenon reduced the affinity of the protein to the interface and the rate of protein molecular rearrangement, resulting in a decrease in the protein adsorption capacity at the interface. However, UT with medium power (300–500 W) could depolymerize insoluble oxidized aggregates to form soluble aggregates, and it also exposed the hydrophobic and hydrophilic groups of the OSPI, improved protein solubility and promoted the interaction between the protein and oil phases. Therefore, the protein content at the oil–water interface was enhanced. Additionally, Morales et al. noted that the protein particle size, among other factors (protein assembly, form, charge, hydrophobicity, free sulfhydryl level, and amino acid arrangement), could be used to determine the protein adsorption ratio at the border, with smaller particle sizes corresponding to increased speed when crossing the liquid border and prolonged retention of protein at the border [27]. Therefore, the increase in AP% might also be linked to the reduced particle size. When the UP increased to 600 W, the percentage of adsorbed proteins of UOSPI-600 decreased compared with that of UOSPI-500. The reason might be that the shear force of the high-intensity ultrasound led to the uneven size of protein particles and the steric hindrance of large-sized protein aggregates weakened the diffusion rate of small-sized proteins. Such changes would reduce the overall diffusion rate, which is not conducive to protein adsorption to the border, resulting in a reduction in the adsorption capacity of interface proteins.

3.12. Interfacial tension

The time evolution of emulsion interfacial tension ($\pi$) of ultrasonically treated OSPI samples is shown in Fig. 11. In general, a non-linear $\pi$–t $1/2$ curves (with $t > 10$ mN/m at the beginning) was observed for the surface tension of all the samples. Moreover, with the extension of adsorption time, the surface tension of all samples increased rapidly and then tended to equilibrium. This may be because when too many proteins were adsorbed on the interface, the interfacial film would produce a steric repulsion effect due to the energy barrier in the adsorption and rearrangement process, resulting in a stable interfacial tension trend. After oxidation treatment, the surface tension of OSPI decreased compared to SPI. The reason may be hydrophobic groups and free sulphydryl groups were embedded in the protein, and the rigid structure in the protein molecule increased, which made the aggregate structure adsorbed at the oil–water interface difficult to unfold. Moreover, the existence of protein aggregates would form steric hindrance at the oil–water interface and improve the adsorption energy barrier, which was not conducive to the subsequent adsorption of proteins. As the ultrasonic power increased from 100 W to 500 W, the interfacial tension of OSPI first decreased and then increased. After low-power ultrasound (100 W, 200 W) treatment, the spatial structure of OSPI was opened and loosened, and the degree of aggregation was higher, resulting in decreased affinity with the interface. This reduced the diffusion rate and took longer to rearrange at the interface, resulting in a drop in interfacial tension. After medium power treatment (300–500 W), protein aggregates unfolded and stretched, exposing hydrophobic groups and free sulphydryl groups embedded in the protein. This enhanced the molecular flexibility of oxidized aggregates and facilitated interactions between protein molecules. Therefore, the adsorption energy barrier at the interface was lowered and the adsorption efficiency was improved, resulting in a decrease in the interfacial tension. However, when the ultrasonic power increased to 600 W, the interfacial tension of OSPI decreased. On the one hand, the flexible structure in protein molecules was transformed into rigid structure, which was not conducive to the rearrangement of protein molecules at the interface. On the other hand, the partially unfolded and denatured proteins would be more easily
adsorbed and associated to form a viscoelastic film on the oil–water interfaces. Extensive denaturation may result in poor interfacial mechanical properties, which would be detrimental to long-term stability of emulsions [21].

4. Conclusion

Ultrasound treatment, as a green non-thermal processing method, can effectively induce conformational changes of soybean protein and its oxidized aggregates by generating free radicals, larger aggregates and/or smaller particles. This structural modification exposes free -SH and buried hydrophobic groups on the protein surface and can improve their solubility, emulsifying properties and interfacial properties. However, the effects of sonication on these traits varied with the amount of sonication power. After applying medium-power ultrasound (300 W, 400 W, and 500 W) treatments, the OSI solubility increased and particle size decreased. The α-helix and β-turn contents, surface hydrophobicity and absolute ζ-potential value increased, the structure unfolded, and the disulfide bond content decreased. These changes improved the emulsification activity and emulsion state of the OSI and increased the protein adsorption capacity and interfacial tension. While low power (100, 200 W) and high power (600 W) have negative effects on the structure, emulsification properties and interfacial properties of OSI. In conclusion, the improvement effect of ultrasonic technology on the physicochemical and functional properties of oxidized soybean protein cannot be ignored, and further efforts should be made to optimize the operating parameters involved in the ultrasonic process to maximize the function and quality of oxidized soybean protein and prolong the shelf life of the product.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 11. Interfacial tension of unoxidized, oxidized treated, and ultrasound treated after oxidized treatment soybean protein aggregates at different time (100 W, 200 W, 300 W, 400 W, 500 W, and 600 W).
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