Effect of Ultrasonic Treatment on Freeze-thaw Stability of Soy Protein Isolate Gel

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Abstract: This study investigated the impact of ultrasonic modification at various ultrasonic power extents on the freeze-thaw stability of soy protein isolate (SPI) gel. The freeze-thaw stability of the gel was evaluated by examining the changes in texture, water holding capacity, microstructure and soluble protein content during the process of 5 freeze-thaw cycles. In addition, effects on particle size, surface hydrophobicity and structure were also explored. The results showed that within a certain range, the average particle size of the protein gradually decreased, and the particle size distribution was narrower with ultrasonic intensity, it may be due to the high shear and cavitation effects of sonication that reduce the degree of protein aggregation. Furthermore, we also detected that treated proteins had lower fluorescence intensity, higher surface hydrophobicity and more flexible molecular structure with the reduction of α-helical structure as well as the rise of random coil. In terms of gel freeze-thaw stability, moderate ultrasound treatment made the water holding capacity and soluble protein content of SPI gel reduce by 38.27% and 3.58%, whereas the hardness and elasticity increased by 510.23g and 0.06mm after 5 FTC. The corresponding changes of indexes of the control group were 75.05%, 51%, 1062.75g and 0.11mm, respectively. It can be observed that the change range of treated SPI gel properties was smaller than that of natural SPI gel, indicating that ultrasonic treatment can remarkably improve the freeze-thaw stability of the gel which might have something to do with changes of protein structure.

Key words: ultrasound treatment, protein gel, freeze-thaw stability, effect

1 Introduction

Ultrasound has a frequency within 20 ~ 1000 kHz, which can be categorized into low frequency high intensity (20–100 kHz, power 10–1000Wcm⁻²), and high frequency low intensity (100 ~ 1000 KHz, power < 1 W/cm²) based on the energy and ultrasound frequency².

Low frequency high intensity ultrasound is widely used for physical-chemical modifications of food ingredient. Ultrasonic exerts its effects on the solution system that is primarily related to the cavitation effect. Ultrasonic wave propagates through the medium molecules in the form of a series of compressions and rarefactions. Under the condition of high power, the sparse period exceeds the attraction of the solution molecules, and the gas nuclei existing in the solution form cavitation bubbles, which are distributed throughout the solution. After several cycles, the cavitation bubbles grow to a marginal size, and then become unstable and collapse violently. The violent collapse of cavitation bubbles contributes to energy accumulation and results in locally higher temperature and pressure, which eventually generate high-energy shear and shock in the cavitation area³. These phenomena facilitate the change of physical and chemical properties of food.

At present, the effect of ultrasonic treatment on protein modification has been extensively studied. O’sullivan et al.³ found that the size of emulsion droplets was reduced significantly by sonication milk protein isolates in comparison of untreated control, which could be attributed to the structural rearrangement of interfacial proteins. García et al.⁴ studied the effects of ultrasonic pretreatment on whey protein enzymatic hydrolysis, they pointed out that sonication caused protein rearrangement and aggregation, which changed the secondary structure of the protein, enhancing the enzymatic hydrolysis reaction. Kang et al.⁵ researched the impacts of ultrasonic treatment on beef protein oxidation and structure during processing, they reported that
the numbers of free sulfhydryl groups and surface hydrophobicity increased due to the unfolding of protein secondary structure after ultrasonic treatment, which accelerated the lipid oxidation. Jambrak et al.\(^8\) examined ultrasonic probe treatment resulted in sharp changes in protein conductivity, solubility, surface area and emulsifying, while the effects of ultrasonic bath on protein properties were not distinct.

Soy protein isolate (SPI), an important by-product of soybean oil production\(^7\), is recognized a high-quality plant protein with a protein content of at least 90%. Soy protein is a complete protein commercially, which contains essential amino acids. It also has excellent functionality such as emulsifying, holding water and forming gels\(^8\). Protein gel refers to a 3-dimensional cross-linking of protein molecules under various inducing physical chemical and enzymatic factors\(^9\). Because the network structure can bind or trap components such as water, fat and flavor substances, protein gelation is widely used in food industry as food functional additive. For example, Protein gel was applied to traditional soy products, dairy products and meat products\(^10\). However, protein gel might be subject to inevitable temperature fluctuation in the supply chain, leading to freeze-thaw cycles. Formation and disappearance of ice crystals over the freezing and thawing process, which resulted in the loss of gel water, the deterioration of texture and the occurrence of syneresis that impact overall quality of the product and make the application in frozen food is limited\(^11\). Therefore, it is necessary to raise its freeze-thaw stability. In addition, the previous research on freeze-thaw stability primarily focused on protein emulsion. Reports on improving freeze-thaw stability of gels are rarely found. It has been reported that ultrasonic treatment\(^12\) could unfold the molecular second structure to expose the internal lysyl residue, increasing the binding site of transglutaminase, which could contribute to a better cross-linking in gelling. Moreover, a cut of studies had showed that TG enzyme can improve the protein freezing resistance, so the goal of this study was to investigate the effect of ultrasonic modification on the freeze-thaw stability of TG-induced protein gels.

2 Materials and Methods

2.1 Materials

Defatted soybean powder was supplied by Yu Wang co., Ltd. (Shandong, China). TG Enzyme was purchased from Yi Ming biological products Co., Ltd. All other chemicals were of analytical grade.

2.2 Preparation of SPI

SPI was prepared based on the method described by Sorgentini et al.\(^13\). The processed soybean powder was dissolved in deionized water at the weight ratio of 1:10, and adjusted to pH 8.5 using 2M NaOH solution, and stirred for 2 h at room temperature. The mixture was centrifuged at 4000 r/min for 20 min, the supernatant was adjusted to pH 4.5 utilizing 2 M HCl solution and stored overnight at 4°C. Then, the supernatant was discarded and centrifuged the residue at 4000r/min for 20 minutes. Collected precipitate and washed twice using deionized water. Subsequently, the precipitate was dissolved as well as the solution was adjusted to pH 7 with NaOH, which was lyophilized to obtain SPI powder contained 90.03% protein, stored at −20°C until use.

2.3 Ultrasound treatment

SPI was dissolved in a phosphate buffer at a final concentration of 40 mg/mL. Sodium azide was added to prevent microbial growth, then refrigerated to hydrate adequately in the refrigerator. Protein samples were treated with different intensity of ultrasound (0 w, 120 w, 240 w, 360 w, 480 w) for 30 minutes (both work time and interval were 1s) via operating a JY92-IIIDN Ultrasonic cell disruptor (Scientz Biotechnology Co. Ltd., Ningbo, China). Ultrasound treatment was conducted according to the method described by Hu et al.\(^14\). The protein solution was divided into 100 mL conical flask and the titanium probe with a diameter of 0.636 cm was inserted in the sample solution, subsequently, all samples were sonicated at different ultrasonic intensities in ice water bath. Then stored samples at room temperature until used.

2.4 Protein gel preparation

The ultrasonicated samples were lyophilized to prepare a 10 mg/mL protein solution. TG enzyme was added to the solution at a mass ratio of TG enzyme to protein solution of 3:100 and stirred for 15s by a magnetic stirrer to form a homogeneous system, which was dispensed into a 25 mL small beaker, and incubated in a 45°C water bath for 2 h. Enzyme was inactivated by heating at 90°C for 10 min. Finally, the gels were cooled and stored overnight at 4°C prior to subsequent analyses.

2.5 Freeze-thaw treatment

Freeze-thaw cycle consists of the freezing process and the thawing process. Gel samples were placed in −20°C for 22 h followed by thawing via keeping at room temperature for 2 h\(^11\), which was called one freeze-thaw cycle. A batch of samples were randomly selected for experimental analyses. The freeze-thaw treatment was repeated up to 5 times.

2.6 Particle size analysis

In this experiment, a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK) was used to measure the particle size distribution, and average particle size. The
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2.7 Surface hydrophobicity
The protein solutions treated and untreated were diluted with phosphate buffer solution (0.01 M, pH 7.0) to form protein solutions with different concentrations that were transferred separately into new test tubes, then added 20 μL of ANS purchased from Sigma of USA that was used as a fluorescent probe and mixed well. After protected from light for 15 min, applying an F-4500 Fluorescence Spectrophotometer (Hitachi, Japan) measures fluorescence intensity at excitation and emission wavelengths of 390 nm and 470 nm respectively, with a slit of 5 nm. The surface hydrophobicity was determined by taking the measured fluorescence intensity as the ordinate and the protein concentration as the abscissa, the slope obtained was the surface hydrophobicity of the protein.

2.8 Fourier transform infrared spectroscopy
The sonicated protein solutions were freeze-dried, and 1 mg of the sample powder was mixed with 150 mg of KBr powder, then the mixture was subjected to press to form a solid thin sheet at room temperature for 30 min. We defined the gel strength by referring to the method of Liu with slight modification. The soluble protein content in the protein gel was determined using the method reported by Sun et al. with a little modification. The quantitative gel was cut and crushed in phosphate buffer (0.01 M, pH 7.0). When the liquid was constant volume, magnetic stirring was carried out for 0.5 h and centrifuged at 10000 r/min for 30 min. The content of protein in the supernatant was assessed by the Coomassie Brilliant Blue method.

2.10 Determination of texture profile analysis
Before analyzing the texture, it is key to take out gel stored in the refrigerator and equilibrated at room temperature for 30 min. We defined the gel strength by referring to the method of Jin et al. with minor modifications. The texture characteristics of the gel were performed using a TA-XT2i type texture analyzer (British SMS Company) equipped with a P/0.5 cylindrical probe. The parameters were as follows: the downward speed of the probe was 1 mm/s, the detection speed and the retraction speed were both 5 mm/s, the trigger force was 5 g, and the probe was pressed twice a time.

2.11 Determination of water holding capacity
We determined the water holding capacity of the gel by centrifuging. A certain amount of gel was placed in the centrifuge tube, weighed the gel mass m as well as the total weight of the gel and centrifuge tubes m1, respectively, then centrifuged at 1000 r/min for 10 min at room temperature, the moisture in the centrifuge tube was carefully removed, and measured the weight of the centrifuge tube and gel m2. The water holding capacity of the gel was calculated according to the following formula:

\[ \text{WHC} = \left( \frac{m - (m_1 - m_2)}{m} \right) \times 100\% \]

2.12 Scanning electron microscopy
We can clearly observe the microstructure of the gel by SEM. In this experiment, the method of Mandujano et al. was utilized to analyze the changes of gel microstructure under different processing conditions. Gels were cut into small pieces (2 mm × 5 mm), fixed with 2.5% glutaraldehyde (pH 6.8), and stored at 4°C overnight, then dehydrated in different concentrations of ethanol (50%, 70%, 90%), continued to dehydrate 3 times with 100% ethanol, next, they were replaced with a mixture (100% ethanol: tert-butanol = 1:1) and pure tert-butanol for 15 min respectively. After lyophilization epitaxial gold plating was performed. The samples were observed under a scanning electron microscope (Hitachi High-Tech Company).

2.13 Determination of soluble protein content
The soluble protein content in the protein gel was determined using the method reported by Sun et al. with a little modification. The quantitative gel was cut and crushed in phosphate buffer (0.01 M, pH 7.0). When the liquid was constant volume, magnetic stirring was carried out for 0.5 h and centrifuged at 10000 r/min for 30 min. The content of protein in the supernatant was assessed by the Coomassie Brilliant Blue method.

2.14 Statistical analysis
All experiments were executed in triplicate. The data were presented the average value of repeated experiments. Data processing and analysis of variance (ANOVA) were performed using SPSS 19.0. Figures were plotted using Origin 8.6 software, Peakfit 4.12 software was used to calculate the protein secondary structure. All results were expressed as mean ± error. Significant difference was indicated by lowercase letters (p < 0.05).
3 Results and Discussion

3.1 Effect of ultrasound treatment of the protein particle size

In this study, the effect of ultrasound on the particle size distribution of SPI was measured by using static light scattering. As seen in Fig. 1, compared with the control group, treated proteins showed a more compact size distribution. In addition, we can also observe from the figure that the average particle size of untreated protein was 495.2 nm, while the protein size of the modified proteins was obviously reduced, and the smallest particle size treated with 360w was 147.5 nm, indicating that the ultrasonic treatment could effectively improve the protein particle size. It might be attributed to the cavitation effects, micro-streaming, turbulent forces and vortex generated during sonication, which could disrupt non-covalent bonds such as hydrogen bonding and hydrophobic interaction between protein particles and alter the aggregation state of natural proteins. Similarly, Hu et al. pointed out that the average particle size of protein treated with 400 w ultrasound for 40 min was decreased apparently and the particle size distribution was more concentrated. The conclusions reached by Shen et al. were consistent with our current results. They found that ultrasound caused a decrease in whey protein particle size. Similarly, Gregersen et al. also obtained that sonication could reduce the size of milk fat globules, and speculated that this change was most likely related to the release of energy during sonication. However, the protein size increased to 148.6 nm after 480 w sonication, indicating the particle size became large after stronger sonication, which may be due to the fact that the increase in energy density of the solution resulted from high-intensity ultrasound, causing the solution violently oscillated and generated thermal effect that increased collision and aggregation between protein molecules with the formation of small aggregates that contributed to an increase in particle size. The results of this study indicated that ultrasonic treatment proteins can enhance the freeze-thaw stability of SPI gels and we speculated that this improvement was related to the reduction of protein particle size. This may be due to the fact that protein molecules with small particle size can better penetrate into the pores of the gel network in the form of fillers, which exerted a certain supporting effect on the gel network, enhancing the hardness and elasticity of the gel, and forming a stable network structure, which had good frost resistance. Therefore, in the frozen food industry, we can use the appropriate intensity of ultrasound (360 w) to process the protein to meet the consumer demand for the quality of frozen food.

3.2 Surface hydrophobicity

Surface hydrophobicity ($H_0$) is used to characterize the number of hydrophobic residues on the surface of proteins in polar environments, reflecting the conformational changes of proteins. Surface hydrophobicity is closely associated with gel properties. As shown in Fig. 2, the surface hydrophobicity of samples treated changed dramatically. As expected, the surface hydrophobicity increased significantly with ultrasonic intensity (from 0 w to 360 w). The surface hydrophobicity increased from 5017.4 to 7055.9 with 360 w ultrasonic treatment, reaching the peak value, which may be explained by the rapid formation and collapse of cavitation bubbles induced by ultrasound, leading to the temperature and pressure locally raised in the area around the bubble, which were responsible for the original dense structure of the protein became loose, accompanied by certain changes in structure and the hydrophobic groups hidden interior the SPI were gradually exposed to the external, therefore, ANS can easily combine with the hydrophobic groups initially buried inside the molecule, increasing the surface hydrophobicity. This finding was in accordance with the result obtained by Sun et al. They suggested that surface hydrophobicity was improved with ultrasound pretreatment of recombinant milk protein concentrate. Several studies had pointed out that during heat-induced gel formation, hydrophobic force might facilitate the formation of protein-protein aggregates, which were beneficial to form uniform and compact gel.

![Fig. 1](image1.png)  
**Fig. 1**  Size distribution and average particle size of soy protein isolate treated by different intensity sonication (0 w, 120 w, 240 w, 360 w, 480 w).

![Fig. 2](image2.png)
3.3 Fourier transform infrared spectroscopy

Figure 3 and Table 1 illustrated the changes in protein secondary structure after sonication by infrared spectroscopy. The infrared absorption of the amide I band is mainly related to the stretching vibration of C=O or C=N, where in the wavelength range of 1648-1664 cm⁻¹ is α-helical structure, 1615-1537 cm⁻¹ and 1682-1700 cm⁻¹ are β-Folded structure, 1664-1681 cm⁻¹ and 1637-1648 cm⁻¹ are β-turn and random coil structures, respectively. It can be seen from Table 1 that the secondary structure of SPI changed after ultrasonic treatment. The modified sample at 360W, the α-helical structure was decreased (from 22.13% to 17.04%), which might be associated with the formation of disulfide bonds and aggregates between protein molecules, and β-sheet was also slightly reduced (from 35.85% to 34.84%). Whereas there were increases in β-turn (from 23.98% to 26.51%) and random coil (from 18.04% to 21.61%), which indicated that SPI after ultrasound, the rigid structure was decreased, the content of flexible structure was increased, and the molecular structure was subjected to transform from order into disorder. This may be clarified by cavitation and mechanical effects of ultrasound, which can destroy original spatial structure of the protein, leading to protein unfolding. However, as the ultrasonic power increased (from 360 W to 480 W), the content of α-helix, β-fold and β-turn increased by 0.39%, 0.17% and 0.5% respectively, on the contrary, the content of irregular coil reduced by 1.06%, which was related to re-aggregation of proteins. However, Qin et al. reported that the α-helix and β-turn content were gradually declined as the ultrasound time, while the β-sheet and random coil content were thinly raised, the difference in ultrasound treatment may be considered as a possible reason for the disparities.

3.4 Fluorescence spectroscopy

Intrinsic fluorescence primarily derives from tryptophan and tyrosine residues, which are very sensitive to the change of micro environment changes. Changes in protein structure are reflected by observing fluorescence intensity. As can be seen from Figure 4, the fluorescence intensity of the control group was higher than those of the treated samples. As the ultrasonic power increased, the fluorescence intensity was decreased, and the 360 W ultrasonic treatment had the lowest fluorescence intensity. The reason might be that the ultrasonic treatment destroyed the protein structure, plentiful chromophores were exposed in the solvent, which resulted in the fluorescence intensity was reduced. Zhang et al. also obtained similar results by studying the effects of sonication on peanut protein. However, certain studies had shown that the fluorescence intensity of proteins after sonication was enhanced. This contradiction may be caused by different
stretches and folds of proteins. After 480 W treatment, the fluorescence intensity was increased slightly, probably because the ultrasonic treatment destroyed the molecular structure of the protein, the protein curled and folded, which weakened the solvent quenching effect. However, the maximum fluorescence intensity $\lambda_{\text{max}}$ did not shift apparently, indicating that the polarity of the microenvironment where the tryptophan residues are located did not change.

### 3.5 Determination of TPA of protein gel

In the gel texture evaluation indexes, the gel hardness reflects the ability of the gel to resist external force compression; gel elasticity reveals the capable of the gel system restoring deformation. From the Fig. 5, some critical information can be obtained, for example, we can observe that the hardness and elasticity of the ultrasonic SPI gel were significantly increased compared with the SPI gel, at 360 W, the hardness and elasticity of the gel reached the maximum value, which were 522.94 g and 0.96 mm, respectively, which were 1.78 and 1.14 times of control group. The soy protein gel properties were significantly improved after ultrasonic treatment, this may be due to the fact that with the increase of ultrasound intensity, protein denatured sufficiently, the evenly dispersed protein system and the soy protein subunits were intertwined to form a dense gel network, which enhanced the gel elasticity. Moreover, active groups inside the protein were exposed with sonication treatment, facilitating the formation of disulfide bond during heat-induced gel formation, and study had demonstrated that numerous disulfide bonds can withstand greater external pressure, which means that the gel had greater strength. Frydenberg et al. also reported that ultrasound treatment increased the hardness of whey protein gel, and they found that the gel hardness was significantly increased due to the increase in disulfide bond. However, excessive sonication had a negative impact on protein gel properties, it may be that the high energy released by excessive sonication caused protein degradation, which reduced the degree of cross-linking between the proteins, thereby forming a weak gel.

During freezing and thawing, the hardness and elasticity of all samples were increased significantly. With the increase of freeze-thaw times, protein freezing denaturation became severer and was susceptible to syneresis, which was characterized by a narrower distance between the three-dimensional network, a tighter colloidal structure, a smaller space between the gels, and a part of the water was eliminated, contributing to an increase in gel hardness. After 5 FTCs, the hardness and elasticity of the sample treated at 360 W increased by 510.21 g and 0.06 mm, respectively, while that of the control group increased by 1062.75 g and 0.11 mm, respectively, indicating that the ultrasonic treatment could relieve the deterioration degree of the gel in the freeze-thaw process, which confirmed that the ultrasonic treatment exerted a certain antifreeze and thaw effect. We hypothesized that the possible reason was that high-power ultrasound leaded to an increase in the sound energy density that was converted into more chemical and physical energy, which made SPI be subjected to

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**Fig. 4** Changes in protein fluorescence intensity under different sonication conditions.

**Fig. 5** Changes of gel properties of soy protein isolate under different treatment conditions.
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more intense mechanical shear, stirring and cavitation, hastening the destruction of SPI conformation, which caused more charge to be exposed, resulting in stronger electrostatic interactions that keep the protein stable, producing a greater spacing between protein gel junctions, reducing the tightness of aggregation between protein particles during freezing and thawing. Therefore, the freezing and thawing stability of the gel can be improved by preventing the gel from getting harder.

3.6 Determination of water holding capacity

The water holding capacity of the protein gel under different sonication conditions was shown in the figure. It can be seen from the Fig. 6 that the water holding capacity of treated protein gels was sharply enhanced. And the gel retaining water content after 360 w ultrasonic treatment reached 93.33%, which was 1.24 times of untreated. A possible explication for amelioration was that transient high temperatures generated by ultrasound, which induced partial protein denaturation was accompanied by structural expansion, particle size decrease, solubility increase, which caused binding between protein and water was enhanced. Another possible reason for this phenomenon was that the formation of disulfide bonds during ultrasound promoted gel cross-linking to form a dense network structure, allowing moisture to be firmly trapped in the network structure. Our current conclusion was similar to that obtained by Qin et al. Instead, the water holding capacity was decreased with stronger sonication, for example, when the protein was treated with ultrasonic 480w, the water retention of the gel was decreased from 93.33% to 91.95%, which may be caused by immoderate ultrasound leaded to a looser protein structure and the disulfide bonds were more likely to be destroyed resulting in a coarse network structure with large pores, finally, a large amount of water can be separated out by centrifugation. Jun et al. had proved that the uniform and dense gel structure can better retain water molecules, while the loose and irregular network structure is prone to throw out water during centrifugation.

After freeze-thaw treatment, the water holding capacity of all sample was dropped dramatically with the freeze-thaw cycles, which indicated that the fluctuation of temperature has an apparent influence on the water holding capacity of the gel. Xu et al. also came up with similar result that was the freeze-thaw process increased the hardness, elasticity, cohesion and gloss of the tofu, and the water retention was reduced.

During the first freeze-thaw treatment, the gel network structure was unstable and the water was precipitated by centrifugation. In the process of multiple freeze-thaw cycles, the network structure was strengthened to form a "water cage" structure, in which water was locked and the water released by the centrifugal was reduced. As mentioned above that after the first freezing-thawing cycle, the water holding capacity of the gel formed by 360 w ultrasound-treated protein decreased sharply by 32.66%; after the fifth freezing-thawing cycle, the water holding capacity of the gel reduced only by 0.31% compared with that of the third freezing-thawing cycle. Decrease in water holding capacity might be due to the continuous formation and disappearance of the ice crystal, which exerted a negative impact on the three-dimensional network structure resulted in a series of large holes, which reduce the ability to retain water. Moreover, the syneresis was another reason for the decrease of water holding capacity. Studies had shown that some of the non-flowable water was transformed into free water, and the protein-water bond was replaced by the protein-protein bond, resulting in a decrease in water holding capacity. Similar result was also found by Yuan et al., they pointed out that during the freeze-thaw process, the ice crystals were converted into liquid water, which was susceptible to escaped from the polymer network structure. After 5FTC, the ultrasonic SPI gel had higher water holding capacity and the gel had the highest value at 360 w, which was 33.53% lower than that of 0 FTC, while the untreated sample was reduced by 48.62% after 5FTC, indicating that the sonication had the potential to improve the freeze-thaw stability of the gel. We inferred that the improvement can be attributed to the following two reasons. First, the cavitation effect of the ultrasonic treatment enable the SPI particle size to become smaller, so that the protein particles can better penetrate into the gel network structure in the form of a filler, which was beneficial to form a more uniform and dense network structure, which can firmly capture water molecules. Another possible reason was that the mechanical effect of ultrasound caused the protein to unfold with more TG enzyme binding sites were exposed. The TG enzyme interacts with the ε-amino group of the lysine residue on the peptide chain to form a stronger ε-lysine isopeptide bond,
which weakened the destruction of the gel network structure caused by freeze-thaw, thereby water holding capacity was relatively increased. Similarly, Huang et al.\textsuperscript{40} studied the effect of TG on the frozen dough system and found that after five weeks of frozen storage, the dough with TG added had less damage to the gluten structure and the bread texture was softer.

3.7 Scanning electron microscopy

The microstructure of the gel under different treatment conditions can be observed in Fig. 7. SEM showed that the unsonicated gel was coarser with larger holes caused by loose distribution of protein particles. Compared with the untreated sample, the microstructures of treated protein gels were apparently improved. For example, the network structure of the sample after 120 w ultrasonic treatment had changed greatly. For example, the cross-linking phenomenon could be seen, the mesh became smaller, as well as a fine gel structure was formed initially. When 360 w power ultrasound was used, the gel had a uniform and delicate network structure, and the pores were evenly distributed, presenting a honeycomb micelle, indicating that the interaction between protein molecules achieved a better balance. Predecessors mentioned that a gel network with a “honeycomb” structure can accommodate more water, which was in agreement with our findings on water retention. This may be due to the fact that the network structure of the gel is related to the size and shape of the protein molecule. Ultrasound can generate cavitation in the medium by transmitting energy, which may generate shearing force on macromolecular proteins leading to protein particle size reduction. Hu et al.\textsuperscript{41} reported that smaller protein particles can make the binding stronger to form a denser network structure. When the protein was treated with a larger ultrasonic power, the gel structure became non-uniform, which may be due to the higher energy delivered by the excessively ultrasonic treatment contributed to the aggregation of the protein itself\textsuperscript{42} that make the interaction with the TG enzyme was reduced, in addition, ultrasound promotes the cross-linking effect of TG on proteins. However, excessive cross-linking might form ultra-long molecular proteins, which were not conducive to the formation of a uniform structure\textsuperscript{43}. When the degree of cross-linking exceeds the number of aggregates required by the gel, a rough network structure might be formed. Studies had manifested that fine structures were formed only when the degree of aggregation or the number of aggregates just reached the gel.

In each freeze-thaw cycle, the water distributed in the gel network changed into solid phase due to low temperature, and the formation of solid ice crystals was accompanied by an increase in volume which will damage the original gel network. During the freeze-thaw cycle, recrystallization led to an increase in the diameter of ice crystals, which further damaged the three-dimensional network structure\textsuperscript{44}. After five freeze-thaw cycles, the network structure of the pure protein gel produced larger pores and cracks, the structure became more chaotic and rough, and the network structure collapsed completely\textsuperscript{45}, which could be illustrated by repeated freezing and thawing weakened the ability of protein molecules to bind surrounding water molecules, resulting in the destruction of the microstructure, which also verified the above changes in water holding. The treated protein gels also were broken after 5 freeze-thaw cycles with visible broken cellulose, however, it was also noted that the these changes were not as obvious as the control group, which may be due to ultrasonic treatment reduced the particle size of the protein, more protein molecules with small particle size were involved in the formation of gel, and ultrasound also promoted the cross-linking reaction catalyzed by TG to form a high degree of cross-linking network structure, which can alleviate the damage of ice crystals to gel structure during freezing and thawing. Therefore, in the frozen food industry, we can make full use of ultrasonic technology to raise the freeze-thaw stability of some foods. For example, frozen tofu unavoidably underwent freezing and thawing process during storage and transportation, which caused a series of bad changes, for example, moisture was discharged from the network structure of the gel, and the taste was deteriorated. Fortunately, we have demonstrated in the above experiments, sonicated proteins can prevent the gel from becoming inferior during freezing and thawing, hence we might try our best to apply ultrasonic technology to enhance the texture of frozen food, expanding the application of ultra-
3.8 Determination of soluble protein content

The amount of protein involved in gel formation can be assessed by soluble protein content. In general, the content of soluble protein in the gel is inversely related to the gel hardness. It can be concluded from the Fig. 8 that the soluble protein content of SPI gel was up to 14.09%. Whereas, the soluble protein in the gel was obviously reduced after ultrasound treatment. The soluble protein content was as low as 13.41% at 360 w ultrasonic power, which was contrary to the change in hardness. Ultrasonic treatment reduced the average particle size of proteins and led to the increase of solubility, which were beneficial to the formation of a more uniform and dense network that requires the participation of a considerable proteins16, leading to the content of soluble proteins was reduced. However, at higher ultrasound intensity, the cavitation effect of ultrasound caused degradation of protein subunits and increased soluble protein content.

The content of soluble protein was decreased during the freeze-thaw cycle. After 5 FTCs, the soluble protein declined by 7.23% compared with no freeze-thaw. It might be associated with protein denaturation caused by freezing and the gel structure was rearranged, inducing partial originally free proteins were incorporated into the gel system, forming a rougher network structure, which contributed to gel hardness was increased and water holding capacity was decreased. The release of water during freezing and thawing boosted the re-aggregation of proteins into larger insoluble aggregates by the interaction of covalent and non-covalent bonds, which was also a possible cause of the decrease in soluble protein content. When the ultrasonic power was 360 W, the soluble protein content was 9.82%, which was 3.58% lower than that of 0 FTC, indicating that ultrasonic treatment had a positive effect on improving the freeze-thaw stability of TG enzyme-induced gel, which may be connected with the decrease in protein particle size and the increase in solubility caused by the cavitation effect, which make the protein more fully involved in the network structure of the gel.

4 Conclusions

In summary, through the above analyses, we concluded that sonicated protein can improve the freeze-thaw stability of the gel, and to a certain extent, the ultrasonic treatment is positively correlated with the freeze-thaw stability of the gel. With sonication, the molecular structure of the protein was unfolded, the γ-hydroxynamide group on the glutamine residue reacted with the ε-amino acid on the lysine residue on the peptide chain to form an ε-lysine iso-peptide bond, which was stronger than the hydrogen bond and other non-covalent bonds. The ε-lysine isopeptide bond reduced the destructive effect of crystallization on the gel. At the same time, ultrasound also led to an increase in the various forces forming a gel network structure, inducing a tight hard gel network structure was formed, enhancing the ability to resist the damage caused by freezing and thawing cycles.

In short, ultrasound can be widely used in the frozen food industry as a fresh technology to improve the freeze-thaw stability of gels.

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