Effect mechanism of ultrasound pretreatment on fibrillation Kinetics, physicochemical properties and structure characteristics of soy protein isolate nanofibrils

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ABSTRACT

Self-assembly of soy proteins into nanofibrils is gradually considered as an effective method to improve their technical and functional properties. Ultrasound is a non-thermal, non-toxic and environmentally friendly technology that can modulate the formation of protein nanofibrils through controlled structural modification. In this research, the effect of ultrasound pretreatment on soy protein isolate nanofibrils (SPIN) was evaluated by fibrillation kinetics, physicochemical properties and structure characteristics. The results showed that the optimum ultrasound condition (20% amplitude, 15 min, 5 s on-time and 5 s off-time) could increase the formation rate of SPIN by 38.66%. Ultrasound reduced the average particle size of SPIN from 191.90 ± 5.40 nm to 151.83 ± 3.27 nm. Ultrasound could increase the surface hydrophobicity to 1547.67 in the initial stage of nanofibrils formation, and extend the duration of surface hydrophobicity increased, indicating ultrasound could expose more binding sites, creating more beneficial conditions for nanofibrils formation. Ultrasound could change the secondary and tertiary structure of SPIN. The reduction of α-helix content of ultrasound-pretreated soy protein isolate nanofibrils (USPIN) was 12.1% (versus 5.3% for SPIN) and the increase of β-sheet content was 5.9% (versus 3.5% for SPIN) during fibrillation. Ultrasound could accelerate the formation of SPIN by promoting the unfolding of SPI, exposure of hydrophobic groups and formation of β-sheets. Microscopic images revealed that USPIN generated a curlier and looser shape. And ultrasound reduced the zeta potential, free sulfhydryl groups content and viscosity of SPIN. SDS-PAGE results showed that ultrasound could promote the conversion of SPI into low molecular weight peptides, providing building blocks for the nanofibrils formation. The results indicated that ultrasound pretreatment could be a promising technology to accelerate SPIN formation and promote its application in food industry, but further research is needed for the improvement of the functional properties of SPIN.

1. Introduction

Soy protein has been widely used in food industry due to its high nutritional quality, health benefits, especially physicochemical and functional properties such as foaming capacity, emulsification, gelation, water-holding, oil-binding, and viscosity [1–3]. Currently, the properties of soy protein have been improved by soy protein aggregates such as ordered fibrils, irregular or spherical particulates, among which nanofibrils have attracted increasing attention in various research fields such as food, medicine and nanotechnology due to their unique functional properties. In the process of protein fibrillation, proteins in free state change to highly ordered self-assembled fibril aggregates. Many food proteins had shown the ability to form nanofibrils, such as ovalbumin [4], whey protein [5], bovine serum albumin (BSA) [6], soy protein [7] and lysozyme [8]. The proteins can form nanofibrils with 1–10 μm in length, 1–10 nm in diameter and multistranded twisted like structure when heated for a long time under the conditions of above their denaturation temperature, low ionic strength and pH values far away from the isoelectric point (pI) [9]. Soy protein nanofibrils had been used as thickeners, emulsifier, gelling agents, foaming stabilizers, surface-active ingredients, etc. [10,11]. In addition, soy protein nanofibrils had been used as carriers for delivering nutrients or drugs, and they also showed unique metal-binding ability [12,13]. Many varieties of factors including protein concentration [14], pH value [15], ionic strength [16], heating time and temperature [17], seeding [18], and stirring [19] had been reported to affect protein fibrillation and the characteristics of...
resulting fibrils. Many technologies had been used to improve the excellent functional properties of protein nanofibrils by modifying and changing the structure and aggregation of proteins, such as high-pressure treatment [20], proteolysis [21] and ultrasound treatment [22]. Ultrasound is a non-thermal, green technique that induces conformational changes in proteins and affects their physicochemical properties. The application of ultrasound in food industry is attracting more and more attention recently.

Ultrasound refers to a kind of sound with a frequency higher than 20 kHz, which cannot be heard by the human ear [23]. Ultrasound technology has been utilized to facilitate the extraction of bioactive components, enhance the solubility and water holding capacity of proteins, and modify the functional properties of food proteins, such as emulsification, foaming, and gelation [24]. Ultrasound can induce acoustic cavitation caused by the generation, consecutive growth, and sudden collapse of bubbles, liberating a large quantity of energy [25]. Extreme temperatures and pressures generated by the rapid formation and violent collapse of cavitation bubbles result in high shear stress waves and turbulence in the cavitation region, leading to physicochemical effects in the liquid such as micro-streaming, agitation, shock waves, generation of radicals [26,27]. The effects of ultrasound treatment on some properties of protein had been reported, such as particle size, solubility, surface hydrophobicity, water holding capacity, emulsification, foaming properties, and gel properties. Ultrasound accelerates the fibrillation process by promoting the protein denaturation and unfolding behavior, and the surface hydrophobicity of β-lactoglobulin increased immediately after high-intensity ultrasound (20 kHz) treatment followed by the ThT binding fluorescence intensity and particle size increased [22]. So et al. [28] proposed that fibril formation was mostly limited by the high free-energy barrier, and ultrasound could reduce the high free-energy barrier effectively. Nakajima et al. [29] found the transient cavitation bubbles produced by ultrasound accelerated the fibrillation reaction. In summary, it can be referred that ultrasound could regulate protein fibrillation through some specific approaches. But the effect mechanism of ultrasound pretreatment on the soy protein isolate nanofibrils has been rarely reported.

In the present work, soy protein isolate nanofibrils (SPIN) and ultrasound-pretreated soy protein isolate nanofibrils (USPIN) were prepared, the fibrillation kinetics were analyzed, and the effect of ultrasound pretreatment on their physicochemical properties and structure characteristics were investigated. This research will provide a theoretical basis for understanding the effects and regulate mechanism of ultrasound pretreatment on nanofibrils formation.

2. Materials and methods

2.1. Materials

Soy protein isolate (protein content > 85%) and urea were purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China). 2-(4-(dimethylamino) phenyl)-3, 6-dimethylbenzothiazolium chloride (ThT) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). 5, 5’-Dithiobis-2-nitrobenzoic acid (DTNB) and 8-anilino-1-naphthalenesulfonate (ANS) were purchased from Aladdin Biotechnical Technology Co., Ltd. (Shanghai, China). Gel electrophoresis related reagents were purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China) and Beyotime Biotechnology Co., Ltd. (Shanghai, China). BCA protein assay kit was purchased from Nanjing Jiancheng Biotechnology Institute Co., Ltd. (Nanjing, China). All other chemicals were analytical grade, and deionized water was used throughout the experiment.

2.2. Methods

2.2.1. Preparation of soy protein isolate nanofibrils

The preparation of SPIN was carried out according to the method of Tang et al. [30]. SPI dispersion (4%, w/v) was adjusted to pH 2.0 with 6 mol/L, 2 mol/L and 0.1 mol/L HCl and stirred for 2 h at 25 °C, followed by storing at 4 °C overnight for fully hydrated. The dispersion was centrifuged (7500 g, 20 min) at 4 °C, and then the protein concentration in the supernatant was determined using the BCA method and diluted to 1% (w/v). Then precisely adjusting pH to 2.0 by using 2 mol/L and 0.1 mol/L HCl.

The supernatant was sonicated using an ultrasonic processor (model VCX750, Sonics & Materials Inc., USA) at 20 kHz frequency with a 13 mm (1/2 in.) titanium alloy probe. The sonication probe was immersed in the sample to a depth of 15 mm. Samples were treated at different amplitudes (20%, 40%, 60%, and 80%) for different time (2, 5, 10, and 15 min) with different pulse durations (5 s on /5 s off, 2 s on /5 s off, 5 s on /2 s off, and 2 s on /2 s off) in an ice-water bath. The ice-water bath was used to dissipate most of the heat generated by ultrasound treatment. The control is the supernatant without ultrasound pretreatment.

All samples were filtered with 0.22 μm membrane filters and then heated in a water bath at 85 °C for 8 h. The samples were placed in an ice bath immediately to stop the reaction after being completed, and soy protein isolate nanofibrils (SPIN) and ultrasound-pretreated soy protein isolate nanofibrils (USPIN) were obtained.

2.2.2. Fibrillation kinetics by ThT fluorescence

ThT fluorescence spectral was analyzed by the method of Zhao et al. [22] with slight modifications. ThT stock solution (8 mg ThT, 10 mL phosphate buffer (pH 7.0, 10 mmol/L), 150 mmol/L NaCl) was filtered with 0.22 μm membrane filters to remove undissolved ThT and then stored at 4 °C with tinfoil. The stock solution was diluted 50 times to generate the working solution. Then, 20 μL sample was mixed with 2 mL ThT working solution and reacted for 20 min. 200 μL mixed sample was added into a microtiter plate. ThT fluorescence intensity was measured using an Infinite M200 PRO (Tecan, Grödig, Austria).

The fibrillation kinetics were analyzed using a single-exponential equation fitted by Origin 2019, where q is the fluorescence intensity at equilibrium, A is the total increase in fluorescence during the exponential phase, t is the heating time, and k refers to the apparent rate constant [31].

\[ y = q + A \exp(-kt) \]  
\[ (1) \]

2.2.3. Particle size and zeta potential

Particle size and zeta potential were determined according to the method described by Xia et al. [21] with some modifications. The samples were diluted to 1 mg/mL with deionized water (pH 2.0). The particle size and zeta potential were measured by using a Zetasizer Nano ZS 90 (Malvern Instruments, UK).

2.2.4. Surface hydrophobicity (Hs)

The surface hydrophobicity (Hs) of the samples were measured with ANS as the hydrophobic fluorescence probe according to Mohammadian et al. [32] with some modifications. The protein concentration of samples was diluted to 0.02, 0.04, 0.08, 0.16 mg/mL by using 0.01 mol/L phosphate buffer (pH 7.0), and 30 μL ANS solution (8 mmol/L) was added to 5 mL diluted sample. The suspensions were vortexed and reacted for 15 min. The fluorescence intensity was determined using an F-7100 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The excitation wavelength and emission wavelength were 390 nm and 470 nm, respectively. The excitation and emission slits were both 5 nm. The initial slope of fluorescence intensity versus protein concentration (mg/mL) by linear regression analysis represents surface hydrophobicity (Hs).

2.2.5. Free sulfhydryl (SH) groups content

Free sulfhydryl groups content was determined according to Gao et al. [33] with a little modification. 4 mL Tris-Gly buffer (0.086 mol/L, Tris, 0.09 mol/L glycine, and 0.004 mol/L EDTA, pH 8.0) containing 8

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mol/L urea was mixed with 1 mL sample (10 mg/mL). Then, 0.02 mL Ellman’s reagent (4 mg/mL DTNB) was added. The mixture was vortexed and kept in the dark at 25 °C for 15 min, then the absorbance was read at 412 nm. Reagent blank was prepared using the buffer instead of the sample. Free sulphydryl groups (mmol SH/g) content was calculated as follows:

\[
\text{SH}(\mu\text{mol/g}) = \frac{(73.53 \times A_{412} \times D)}{C}
\]

Where \(A_{412}\) is the absorbance at 412 nm; C is the protein solution concentration (mg/mL); D is the dilution factor.

2.2.6. Viscosity
The viscosity was evaluated using a TA Discovery HR-1 rotating rheometer (TA Instruments Ltd., USA) at 25 °C according to the method described by Tang and Wang [7]. The samples were placed between two parallel plates with a gap set to 1.0 mm. The apparent viscosity was measured as the shear rate increased from 0.1 to 100 s⁻¹.

2.2.7. Circular dichroism spectroscopy
Circular dichroism (CD) spectroscopy was obtained using a MOS-450 spectropolarimeter (BioLogic Science Instrument, France) at room temperature according to Munia et al. [34]. The protein concentration of all samples was diluted to 0.01 mg/mL for far-UV CD and 1.0 mg/mL for near-UV CD by deionized water at pH 2.0. The measurement was performed in a quartz cuvette with an optical path of 0.1 cm for far-UV CD and 1 cm for near-UV CD. The samples were scanned to obtain far-UV and near-UV CD spectroscopy respectively over a wavelength range from 190 to 260 nm and from 250 to 320 nm. The secondary structure contents of samples were estimated using the CDpro.

2.2.8. Transmission electron microscopy
The microstructures were observed using TEM with negative staining, according to the method described by Tang et al. [30]. The samples were diluted to an appropriate concentration, and a drop of diluted samples was placed onto the carbon support film on a copper grid and allowed to dry completely at room temperature. The grid was then negatively stained with 1% uranyl acetate for 3 min, washed with an appropriate buffer, and finally dried with a stream of cold air. The micrographs were obtained at 100 kV using a Hitachi H-7650 (Hitachi, Ltd., Tokyo, Japan).

2.2.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
According to the method of Laemmli [35] and Khatkar et al. [36], SDS-PAGE was performed on a discontinuous buffer system using 10% separating gel and 5% stacking gel. 60 μL sample (2 mg/mL) was mixed with 120 μL 2 × loading buffer, and the mixture was treated in a boiling water bath for 5 min. 10 μL processed sample was loaded into each lane of the gel. After electrophoresis, the gel was stained with Coomassie blue R-250 aqueous solution for 1 h, then destained for 1 h, and finally dehydrated with absolute ethanol. The average particle size of SPIN was 243.0 ± 1.32 nm, while the average particle size of SPI pretreated by ultrasound was 182.4 ± 0.70 nm, indicating ultrasound could decrease particle size of SPI. From the particle size distribution (Fig. 3), it can be seen that the particle size of ultrasound-pretreated SPI was more homogeneous and had a larger proportion of small particles. The decrease of particle size of ultrasound-pretreated SPI might be due to the microstreaming, shear and turbulent forces resulting from cavitation caused by the ultrasound, which might disrupt noncovalent bonds between SPI molecules [39].

3. Results and discussion

3.1. Fibrillation kinetics of soy protein isolate nanofibrils
ThT fluorescence assay is commonly widely used to monitor the formation of highly ordered nanofibrils, because ThT can specifically bind to the cross-β structures of nanofibrils, resulting in a greatly enhanced fluorescence intensity [37,38]. The maximum ThT fluorescence intensity as a function of heating time of USPIN were shown in Fig. 1.

In Fig. 1, the ThT fluorescence intensity of all samples showed a gradually increasing trend with heating time, indicating that USPIN and SPIN were successfully formed in this research. The nanofibrils formation of all samples displayed single-exponential kinetics. Samples treated with different ultrasound amplitudes showed approximate fluorescence intensity change (Fig. 1a). In the initial heating stage, it showed a sharp increase in ThT fluorescence intensity. After about 1 h, the increase slowed down and finally leveled off, suggesting that a large amount of nanofibrils were formed within a short time [31]. The apparent rate constant obtained by exponential fitting were shown in Fig. 1a, the order of apparent rate constant was 20% amplitude > 60% amplitude > 40% amplitude > 80% amplitude, indicating that the lower ultrasound amplitude resulted in a larger rate of nanofibrils formation.

In Fig. 1b, 15 min ultrasound pretreatment showed the greatest apparent rate during nanofibrils formation. In this study, long-time ultrasound pretreatment of SPI significantly increased the formation rate of nanofibrils. Fig. 1c showed that the samples treated with the pulse durations of 5 s/5 s had the greatest nanofibrils formation rate. Appropriate ultrasound conditions significantly increased the rate of nanofibrils formation, and the sample treated by 20%-15 min-5 s/5 s ultrasound had the greatest apparent rate of nanofibrils formation, which was 38.66% higher than that of the untreated SPI. It might be because ultrasound could change the secondary structure of SPI and promote the generation of β-sheets, thereby increasing the nanofibrils formation rate. In view of the ThT fluorescence assay results, the sample 20%-15 min-5 s/5 s was selected to research physicochemical properties and structure characteristics of USPIN.

3.2. Particle size and zeta potential of soy protein isolate nanofibrils
The average particle size of SPIN and USPIN determined by dynamic light scattering were shown in Fig. 2a. At 0 h, the average particle size of SPI was 243.0 ± 1.32 nm, while the average particle size of SPI pretreated by ultrasound was 182.4 ± 0.70 nm, indicating ultrasound could decrease particle size of SPI. From the particle size distribution (Fig. 3), it can be seen that SPI showed a trimodal particle size distribution at 0 h, while SPI pretreated by ultrasound was more homogeneous and had a larger proportion of small particles. The decrease of particle size of ultrasound-pretreated SPI might be due to the microstreaming, shear and turbulent forces resulting from cavitation caused by the ultrasound, which might disrupt noncovalent bonds between SPI molecules [39]. Similarly, Arzeni et al. [40] reported that 20% amplitude and 20 min ultrasound pretreatment could cause a decrease in the particle size of SPI. The violent agitation and collision of SPI molecules during ultrasound pretreatment resulted in smaller particles and narrower size distribution. The distribution peaks of SPI pretreated by ultrasound shifted slightly towards smaller size at different heating time (Fig. 3). Fig. 2a showed the particle size of SPI pretreated by ultrasound increased first and then decreased as heating time increased. The increase in particle size at the initial stage of heating was attributed to the release of the building blocks required for fibrillation, while the heating time was extended, the large agglomerates disappeared because of acid hydrolysis, and the samples were mainly composed of smaller particles [41,42]. The increase of the average particle size of SPI pretreated by ultrasound was more significant than SPI at 0.5 h. It is speculated that ultrasound could promote the release of the building blocks during the initial heating stage, thereby affecting the nanofibrils formation process. The increase in average particle size at 8 h might be attributed to the agglomeration of peptides. The average particle size of the final formed USPIN was 151.83 ± 3.27 nm, while the average particle size of SPIN was 191.90 ± 5.40 nm.

Zeta potential is constantly regarded as an indication for protein solution stability. The higher absolute values of zeta potential, the larger
electrostatic repulsion, the greater separation distance between particles, and the more stable the solution [43]. Fig. 2b showed that ultrasound had no significant effect on the zeta potential at 0 h. With the extension of heating time, the zeta potential increased significantly first and then decreased. The zeta potential of SPI remained unchanged after heating for 2 h, while that of USPIN continuously decreased to 8 h. The decrease of zeta potential might be due to the formation of protein aggregates [44]. Zeta potential is related to the surface charge of particles, and the surface charge mainly determines their aggregation and dispersion [45]. The reduction of charge in USPIN would weaken the electrostatic repulsions between particles, thereby promoting the aggregation of proteins to form nanofibrils [46,47]. This was consistent with the results of particle size.

Fig. 1. Maximum ThT fluorescence intensity of USPIN. a, ultrasound amplitudes; b, ultrasound time; c, pulse durations.

Fig. 2. Average particle size (a) and zeta potential (b) of USPIN. Different letters indicate significantly different (P < 0.05).
3.3. Surface hydrophobicity and free sulfhydryl groups content of soy protein isolate nanofibrils

ANS is a hydrophobic fluorescent probe, which is widely used to detect exposed nonpolar surfaces in protein because it can prioritized bind to hydrophobic clusters [48]. Surface hydrophobicity and free sulfhydryl groups content of USPIN were shown in Fig. 4.

It can be seen from Fig. 4a, the H₀ value increased first and then decreased with the extension of heating time. The increase in surface hydrophobicity was due to the hydrolysis of proteins leading to low molecular weight polypeptides, exposing the hydrophobic groups inside the protein molecules which may be binding sites for protein aggregation [48,49]. The H₀ value of SPI reached the maximum when heated for 0.5 h, but ultrasound pretreatment prolonged the increase to 1 h (up to
After heating for a long time, due to protein aggregation, the exposed hydrophobic residues were buried in the aggregates, which might cause a slight decrease in surface hydrophobicity [22]. After heating for 2 h, the \( \text{H}_2 \) value of USPIN was less than that of SPIN. Ultrasound pretreatment induces a lot of aggregates, that is, more nanofibrils, so less hydrophobic groups were exposed to fibril surfaces [50].

The determination of free sulfhydryl (SH) groups content can reflect the disulfide bonds, and thereby reflecting the forces in the process of aggregation [51]. Free SH groups content of USPIN was shown in Fig. 4b. Ultrasound pretreatment did not cause obvious changes on free SH groups content when heating for 0–1 h, but caused a decrease after 1 h, while the free SH groups content of SPI always showed a downward trend. The decrease may be due to the fact that as the heating time increased, SPI fibrillation tends to toward longer fibril structures, resulting in some free SH groups being buried in the fibrillated structure [52]. In the process of fibrillation, the free SH groups content of USPIN was always lower than that of SPIN, because ultrasound promoted the transformation of free SH groups to disulfide bonds [53]. Ultrasound cavitation generates high temperature, high pressure and strong shear forces, which promotes protein molecule collisions, thereby promoting the binding of free SH groups on cysteines between different protein molecules to form disulfide bonds and leading to a decrease in free SH groups content. Similarly, Hu et al. [54] showed SH groups of 7 S and 11 S fractions decreased after 40 min high-intensity ultrasound treatments. Gülseren et al. [55] showed the amount of free SH groups in bovine serum albumin decreased with increasing ultrasound time. The hydrogen peroxide generated by ultrasound cavitation might oxidize free SH groups of SPI, resulting in a reduction of free SH groups.

3.4. Viscosity of soy protein isolate nanofibrils

The nanofibrils structure can affect the rheological properties of proteins, where viscosity represents fluid resistance to flow and is related to molecular interactions [56]. The apparent viscosity of USPIN was shown in Fig. 5. The apparent viscosity of all samples decreased and the shear stress increased as the shear rate increased, which was a typical shear-thinning phenomenon. The SPI pretreated by ultrasound had shown a much lower viscosity than SPI. The decrease in viscosity after ultrasound pretreatment might be related to the physical forces and the disruption of protein intermolecular interactions induced by the oscillation and collapse of the cavitation bubbles led to broken biopolymer backbone. The viscosity of a fibril dispersion depends highly on the volume fraction and the extent of entanglement of the fibrils [17]. In this study, the USPIN was looser compared with SPIN (TEM images in Fig. 7), which might result in a lower viscosity. Martinez-Velasco et al. [57] reported that high-intensity ultrasound treatment of faba bean protein isolate produced a decrease in viscosity. The decrease in viscosity after ultrasound pretreatment might also be due to the decrease in particle size [58], which was consistent with the particle size results above.

3.5. Structure of soy protein isolate nanofibrils

Far ultraviolet circular dichroism (Far-UV CD) spectroscopy used to characterize the secondary structure of SPIN and USPIN were shown in Fig. 6a, and the contents of \( \alpha \)-helix, \( \beta \)-sheet, \( \beta \)-turn, and random coil were shown in Table 1. Ultrasound pretreatment increased the content of \( \alpha \)-helix by 3.9%, decreased the content of \( \beta \)-sheet by 1.1%, and reduced the contents of \( \beta \)-turn and random coil at 0 h. Chandrapala et al. [50] reported that 60 min sonication of \( \beta \)-lactoglobulin caused a 10% increase in \( \alpha \)-helical, and a 6–9% decrease in \( \beta \)-sheet and \( \beta \)-turn, which was consistent with the results of this research. Ultrasound pretreatment may disrupt molecule interactions, resulting in secondary structure changes. During the nanofibrils formation, the \( \alpha \)-helix content of USPIN decreased by 12.1% and the \( \beta \)-sheet content increased by 5.9%, while the \( \alpha \)-helix content of SPI decreased by only 5.3% and the \( \beta \)-sheet content increased by 3.5%. Stathopoulos et al. [59] reported the aggregates formed after sonication had an increase in \( \beta \)-structure with a decrease in \( \alpha \)-helix structure. Nanofibrils are \( \beta \)-sheet rich structures, which are important in the formation and stability of nanofibrils [60]. The reduction in \( \alpha \)-helix and the increase in \( \beta \)-sheet are the apparent characteristics of nanofibrils formation. It can be inferred that ultrasound pretreatment induced the \( \alpha \)-helix structure of SPI to transform into \( \beta \)-sheet structure, which was beneficial to the nanofibrils formation.

As shown in Fig. 6b, the changes in tertiary conformations of USPIN were compared using near-UV CD spectroscopy. The dichroism bands at around 255–270 nm, 275–285 nm and 285–310 nm are the characteristics of Phe, Tyr and Trp residues of the proteins, respectively [56]. The Phe and Tyr dichroism intensity of USPIN and SPIN decreased with increasing heating time, that might be due to protein structural unfolding and polypeptide hydrolysis [34]. Ultrasound pretreatment induced an increase in Phe and Tyr dichroism intensity at 0 h. Combined with the particle size results, it is believed that ultrasound could depolymerize larger proteins aggregates into smaller protein particles through cavitation and mechanical effects, exposing more soy protein particles to the solution, thus increasing the soluble tertiary structure [61,62]. Ultrasound pretreatment slightly increased the Tyr and Trp dichroism intensity of SPI at 8 h, which indicated that ultrasound changed Tyr and Trp residues microenvironment in USPIN. Therefore, ultrasound could promote the unfolding of SPI and the progressive unfolding of the tertiary conformations.
3.6. Microstructure of soy protein isolate nanofibrils

At 0 h, ultrasound pretreated SPI showed a natural globular protein structure like SPI, without any linear aggregates, however, the sphere diameter was smaller after ultrasound pretreatment (Fig. 7a, e), consistent with the particle size distribution results (Fig. 3). When heated for 1 h, the USPIN was more orderly than SPIN. USPIN was more dispersed, while SPIN was tangled. As heating time prolonged, the globular protein structure gradually disappeared and the linear aggregates began to form. When heated for 8 h, both SPIN and USPIN were almost formed. Long straight nanofibrils and curly nanofibrils coexisted in the TEM image of SPIN, while USPIN were more fragmented and curlier.

3.7. Molecular weight of soy protein isolate nanofibrils

As shown in Fig. 8, the formation of SPIN and USPIN during heating at pH 2.0 and 85 °C were analyzed by SDS-PAGE. SPI pretreated by ultrasound did not show significant changes in protein electrophoresis profiles without heat treatment, indicating that ultrasound pretreatment did not change the molecular weight of SPI. Jiang et al. [63] had obtained similar results in the SDS-PAGE studies of black bean proteins after ultrasound treatment of different power and time. Hu et al. [64] also observed that ultrasound treatment did not modify the protein SDS-PAGE profiles of SPI at varying intensities and time. As heating time increased, the polypeptide components of SPI with molecular weights of about 80, 75 and 50 kDa were gradually hydrolyzed into low molecular weight peptide, which agreed with the results of Li et al. [56]. During the initial stage of heating, the α’α subunits were gradually degraded and completely disappeared at 2 h of heating, which may be related to the

| Secondary structure contents of USPIN. |
|---------------------------------------|
| Contents (%) | SPIN | | | | USPIN | | | |
| | 0 | 0.5 | 1 | 2 | 8 | 0 | 0.5 | 1 | 2 | 8 |
| α-Helix | 37.2 | 40.4 | 40.8 | 33.9 | 31.9 | 41.1 | 41.3 | 43.2 | 41.6 | 29.0 |
| β-Sheet | 17.1 | 15.3 | 15.0 | 21.6 | 20.6 | 16.0 | 15.8 | 14.3 | 15.5 | 21.9 |
| β-Turn | 19.0 | 17.3 | 17.0 | 18.4 | 19.3 | 17.9 | 20.0 | 17.7 | 17.5 | 17.7 |
| Random coil | 27.5 | 25.9 | 25.4 | 29.3 | 32.4 | 26.7 | 25.0 | 26.2 | 26.1 | 32.6 |

Fig. 6. Far-UV (a) and near-UV (b) circular dichroism spectroscopy of USPIN.

Fig. 7. TEM images of USPIN. a-d, SPIN at 0 h, 1 h, 2 h, 8 h; e-h, USPIN at 0 h, 1 h, 2 h, 8 h.
increase of $H_\beta$. The $\beta$ subunit of 7 S and the acidic subunit of 11 S were gradually degraded with the extension of heating time and largely disappeared after 4 h of heating, while the basic subunit was almost retained. The formation of protein nanofibrils is mainly related to protein hydrolysis into small peptides that are further assembled into nanofibrils at low pH, low ionic strength and high temperature [65]. After heating, the electrophoretic bands of the USPIN were more intense than that of SPIN. This might indicate that ultrasound promoted SPI converging into low molecular weight peptides, which could provide building blocks for the nanofibrils formation. 

4. Conclusion

In this study, the effect of ultrasound pretreatment on fibrillation kinetics, physicochemical and structural characteristics of soy protein isolate nanofibrils were assessed. The results showed ultrasound pretreatment increased the formation rate of nanofibrils by increasing the surface hydrophobicity, changing the secondary structure of soy protein isolate and promoting protein unfolding. The release of more hydrophobic residues and the prolonged exposure time of binding sites after ultrasound pretreatment provided more chance for self-assembly of soy protein isolate to nanofibrils. The production of $\beta$-sheets during the fibrillation process increased after ultrasound pretreatment, promoting the formation of nanofibrils. In addition, ultrasound also changed particle size, zeta potential and microstructure of soy protein isolate nanofibrils. Overall, this research provided useful information to understand the mechanism of ultrasound pretreatment on fibrillation kinetics of soy protein isolate nanofibrils. Ultrasound pretreatment is a green and promising physical approach to regulate the fibrillation process of soy protein isolate and promote the application of soy protein isolate nanofibrils in food industry.

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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