Improving soaking efficiency of soybeans through sweeping frequency ultrasound assisted by parameters optimization

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\textbf{ABSTRACT}

Soybean soaking is important to the processing of bean products, however, restricted by the long soaking time. Herein, the soybean soaking was assisted by 60 kHz sweeping frequency ultrasound (SFU). Shortening mechanism of soaking time and physicochemical properties of soybeans were analyzed. Results showed that soaking temperature of 37 °C, ultrasonic power of 60% (144 W), and soaking time of 214 min were optimum SFU-assisted parameters. The soaking time was reduced by 45.13%, and soluble protein content increased by 14.27% after SFU. Based on analysis of acoustic signals, the maximum voltage amplitude of SFU increased with the increment of oscillation periods of cavitation bubbles, which enlarged the intercellular space and size of soybean, and cell membrane permeability was enhanced by 4.37%. Unpleasant beany flavor compounds were reduced by 16.37%–47.6%. Therefore, SFU could significantly improve the soaking efficiency of soybeans and provide a theoretical basis for the processing enterprises of soybean products.

1. Introduction

Soybean [\textit{Glycine max} (L.) Merr.] is one of the most important sources of edible protein in the world which contains up to 40% protein and a complete set of amino acids for human nutrition [1]. About 90% of soybean protein is water soluble [2], so the soluble protein content (SPC) is an important parameter for the soybean product industry. It plays a significant role in soy milk, tofu, yuba and other soybean products. Soybeans can be used to make all kinds of soybean products, but then, they must be soaked, which is mainly used to soften them and facilitate the subsequent cooking. Thus, the protein can be fully extracted after grinding and crushing of soybeans and protein-rich products are produced [3]. Soaking process affects soybean texture, which is related to soaking time and temperature. In the process of soy milk production in Illinois, soybeans need to be soaked for 12 h at room temperature [4]. If the soaking time is short, the protein or fiber cannot absorb enough water. So the soybean body becomes soft and is not fully swelled, and excessive stickiness may be caused easily during grinding, which makes the protein and fiber cannot to be effectively separated. Furthermore, part of the protein will be lost with the tofu residue during filtration [5]. However, too long soaking time will also reduce the quality and efficiency of cooking soybeans. A study found a high temperature of 85 °C could reduce soaking time [6], but high levels of protein denaturation destroyed the solubility of soybean protein, and denaturation temperatures of β-conglycinin (7S) and glycinin (11S) started from 65 to 75 °C and 85-95 °C, respectively [7]. Thus, the long soaking time and high soaking temperature will result in a significant loss of solids, such as proteins and isoflavones, into the aqueous medium [8]. Therefore, it is necessary to control the soaking time and temperature of soybean to obtain as much soybean protein as possible.

Ultrasound is a green, simple and fast physical method, and its effect comes from collapse of cavitation bubbles during the oscillation process, making the local temperature as high as 5000 K and the pressure as high as 50 MPa. The instantaneous high temperature and pressure generated by ultrasound have a short time and a small space, which can change the structure of soybean protein, but will not cause complete denaturation of protein. This instantaneous high temperature and pressure causes the destruction of cell wall, enhances the contact between the solvent and the target compound, and promotes the mass transfer of the material. It is shown that using fixed-frequency ultrasonic (FFU) pretreatment

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before soaking soybeans can reduce the soaking time by about 33%, which is helpful for economic and industrial use [9]. Moreover, The other study has shown that ultrasound-assisted extraction (UAE) is a "green" process compared with conventional extraction (CE) methods, and it consumes less energy and time. Higher yields of soluble proteins was produced [10]. Compared with FFU, Sweeping frequency ultrasound (SFU) has a wider vibration spectrum, and can realize the resonance of material composition to a greater extent. At the same time, SFU generates an acoustic field that is more conducive to the ultrasonic cavitation, and the stability of phenolic acid [11], the enzymolysis efficiency and activity of ACE inhibitory peptide were optimized [12]. The above two studies have shown that SFU has more advantages than FFU during application. Our previous research has applied SFU to improve the efficiency of the drying (dehydration) process and quality of okra [13]. However, SFU has not yet been applied to the rehydration (hydration) of agricultural products. Considering this, in this research work, SFU was utilized to assist soaking process of soybeans. Taking SPC as a characteristic indicator, response surface methodology (RSM) was utilized to optimize the soaking time and temperature of soybean soaking assisted by SFU (Fig. S1 in Supplementary), and then the soaking (rehydration) mechanism was comprehensively studied.

The micro jet and shear stress produced with instantaneous high temperature and pressure can decompose polymer, break chemical bonds, and generate free radicals, etc. In this way, mass transfer effect is promoted by ultrasonic waves, and protein molecules can be combined adequately with water molecules. Therefore, SFU treatment can not only increase the solubility of SPC, but also cause structural changes in soy protein. Ren et al. [14] showed that 550 W ultrasonic treatment reduced the particle size and viscosity of soybean protein solution, increased surface hydrophobicity and solubility, and improved emulsifying activity and foaming ability. Liang et al. [15] indicated that 300 W ultrasonic pretreatment can significantly reduce the particle size of soybean milk by breaking down larger droplets, and improve the water retention capacity and texture of soy protein isolate gel. Lin et al. [16] showed that ultrasound can fold protein structure, induce protein denaturation and polymerization, thus the functional properties and texture of tofu was affected. All these studies indicate that ultrasonic waves have a significant impact on soybean protein. Generally speaking, the effect of ultrasonic waves on the protein is mainly due to cavitation effect, inducing conformational and physicochemical properties changes of protein molecules, and functional properties are improved such as solubility, emulsification, foaming and gel properties of the protein.

In summary, SFU was used to assist soybean soaking process to reduce soaking time and improve SPC of soybeans. Through RSM, the optimal parameters of SFU-assisted soaking process were determined; and the real-time online monitoring system of ultrasonic field and Low-field nuclear magnetic resonance (LF-NMR) technology were introduced to evaluate the mechanism of SFU on soaking process. On this basis, rehydration, physicochemical properties and protein structure of soybeans were studied.

2. Materials and methods

2.1. Materials

Dried soybeans were purchased from the Soybean Food Store in Luliang City (Shanxi Province, China), and the initial moisture content was 3.59 ± 0.18 g/100 g. Plump soybeans with no mildew spots, bright color and uniform size were selected and stored in a dry condition at room temperature for soaking experiments.

2.2. SFU-assisted soaking process of soybeans and acoustic field monitoring

SFU coupling temperature control system was used to assist soybean soaking process. 50 g of soybeans were weighed and put into a gauze bag after washing, spread on the bottom of the SYC-15B thermostat water bath (Changzhou Henglong Instrument CO., LTD. China) with 8000 mL of water. The SFU plate transducer was fixed at about 5–6 cm directly above soybeans by an iron stand and the transducer was submerged in water, and then the temperature in water bath was heated to the set point (±1 °C). The temperature of the soaking solution was continuously checked through the temperature probe throughout the soaking process, and through the optimization of the ultrasonic intermittent ratio, the temperature is kept at ±1 °C during the whole soaking process to avoid the influence of ultrasonic heating effect. The center frequency of SFU was 60 kHz, the sweeping amplitude was ±1 kHz, the sweeping period was 100 ms, and the maximum power was 100% (240 W). Soybean is a relatively hard material, and it takes a long time to soak without the loss of nutrients. 60 kHz ultrasound has strong penetrating power, and is conducive to mass transfer. At the same time, it has little damage to the material surface with low noise, which is more suitable for long time soybean soaking. In addition, our group previously concluded that 60 kHz SFU can reduce the drying time of agricultural crops and promote the extraction of functional components, so it is a more effective method for mass transfer and nutrient extractions [17]. Therefore, 60 kHz SFU was used in this study to assist soybean soaking process, hoping to improve the efficiency of soybean soaking and promote the dissolution of SPC at the same time. In each single-factor test, the volume of water was kept constant, the intermittent ratio, soaking temperature, ultrasonic power and soaking time were changed and compared.

Real-time online monitoring of the whole SFU-assisted soaking process of soybeans with the optimal parameters was carried out. Polyvinylidene fluoride (PVDF) sensor was introduced to collect acoustic (noise) signals. The effective area of the PVDF sensor was 10 mm × 10 mm, its thickness was 30 μm, and the sensitivity was 2 × 10⁻⁸ V/Pa. It was oriented to the middle position of the transducer, and a 50 Ω resistor was connected in parallel to improve the stability of acoustic signal collection and avoid distortion. The collected instantaneous pressure of acoustic field was converted into electrical signals through the PVDF sensor, then recorded and stored in the form of voltage signals by the MDO3024 oscilloscope (Tektronix, Inc., USA). The schematic diagram is shown in Fig. 1a, the physical map is shown in Fig. 1b, and there were three groups of samples: dried soybeans (Dried), soaked soybeans without SFU (Control), and soaked soybeans with SFU by the optimum parameters (SFU), respectively.

2.3 Research methods of soybean soaking process

2.2.1. Kinetic model of water absorption

Dried soybeans (50 g of dry basis) and water were put into the water bath, and soybean soaking process in Control and SFU groups was performed. The weight of soybeans was measured every 10 min in the first half an hour, every 15 min in the middle hour, and every 30 min in the last two hours. The rehydration process began when soybeans were soaked in the water. Before weighing, the water on the surface of soybeans was drained with a sieve and wiped gently with absorbent paper. The moisture content (M_t) of soybeans was determined by Eq. (1) [18].

\[ M_t = \frac{(W_t - W_0)}{W_0} \]  

(1)

where \( W_t \) is soybean mass at the time \( t \); \( W_0 \) is soybean initial mass, g; \( M_t \) is moisture content at the time \( t \), %.

The coefficients \( K_1 \) and \( K_0 \) of Peleg model (Eq. (2)) were determined by the moisture content of soybeans during rehydration, which were usually used to describe the moisture content of grain [19].

\[ M_t = M_0 + t(K_1 + K_2t)^{-1} \]  

(2)

where \( M_0 \) is initial moisture content of soybeans, %; \( K_1 \) is Peleg rate, min⁻¹%; \( K_2 \) is the Peleg constant capacity, %; \( t \) is the rehydration time, min.
2.2.2. Low-field nuclear magnetic resonance (LF-NMR)

The LF-NMR imaging of soybeans in SFU group was performed by NMI20-060VJS-I analyzer (Niumai Technology Co., Ltd., China). The specifications of the multi-slice spin echo sequence proton density imaging were as follows: the repetition time TR was 5000 ms, and the echo time TE was 10 ms. The proton density image was 512 × 512pt. Each sample was measured 8 times repeatedly, and the scanning number was 256 every time. The center of the soybean longitudinal section was selected for imaging. The obtained images were transformed into pseudo-color ones by NMR image processing software V3.0.

The transverse relaxation time (T_2) of soybeans was determined by the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence according to the method of Li et al. [20]. A soybean sample was placed in a cylindrical glass tube with a diameter of 25 mm in the LF-NMR analyzer. The proton resonance frequency was 21 MHz, collected echo C_0 was 3000, repeated scanning time NS was 16, and half echo time TE was 0.1 ms. All measurements were made at 25 °C. The inversion data were analyzed by the software Ver4.0 to obtain the corresponding distribution of relaxation times from the multi-exponential decay curves.

2.2.3. Microstructure observation of soybeans

The soybeans in Dried, Control and SFU groups were cross-cut and sliced into thin slices, dyed with 0.1% (w/v) acid fuchsin for 2 min, and observed with an ECLIPSE Ni-U biological microscope (NIKON CORPORATION, Japan), while micrographs were taken by a Nikon DS-Qi2 camera (NIKON CORPORATION, Japan) [21].

The microstructure of soybeans was observed by a Hitachi S-3400 N scanning electron microscope (SEM) (Hitachi Ltd., Japan). Two different positions of soybeans were selected, namely the center of cross and longitudinal section, and cut with thickness of 0.8 ± 0.11 cm. They were
glued on the SEM stage with conductive adhesive after freeze-drying. The samples were sprayed with gold by ion sputtering apparatus. The spraying current was 10 mA, and the spraying time was 50 s for two times. Surface morphologies were imaged at the magnifications of 250 and 100× [22].

2.2.4. Measurement of cell membrane permeability

The conductivity of soybeans in Dried, Control and SFU groups was determined by a calibrated DDS-307A conductivity meter (YUEPING, China). The soybean milk was centrifuged at the rotating speed of 10,000 r/min for 10 min under room temperature. The supernatant was diluted 50 times with distilled water, and 20 mL of diluted supernatant was placed in the water bath at 25 °C. The conductivity was measured to demonstrate cell membrane permeability of soybeans, and the unit was ms/cm [23].

2.3. Determination of physicochemical properties of soybeans

2.3.1. Measurement of SPC

Coomassie brilliant blue colorimetry was used to determine SPC of soybeans, and carried out in triplicate. Soybeans were mashed with solid–liquid ratio of 1:40 in distilled water, passed through two layers of gauze to remove residues, and then centrifuged at 10,000 r/min for 10 min under room temperature. 1 mL of supernatant was collected and diluted 50 times with distilled water, and 20 mL of diluted supernatant was placed in the water bath at 25 °C. The conductivity was measured with a known volume of water. Here the quartz-powder displacement method was used, the difference in quartz-flour volume between the new and initial level in the measuring cylinder was recorded as the volume of soybeans, mm³ [25].

Ten soybeans were randomly selected from the bulk samples. A vernier caliper was used to measure the axial dimensions with an accuracy of 0.02 mm. The Dₐ of soybeans was evaluated using Eq. (3).

\[ D_a = \frac{(L + W + T)}{3} \]  

(3)

where \( D_a \) is the arithmetic mean diameter, mm; \( L, W \) and \( T \) are the length, width and thickness of soybeans, respectively, mm [26].

The hardness of soybeans was determined by puncture test of a texture analyzer (TAXT2i Ltd., UK). Values were measured when P/N° probe passed through soybeans. The speed of probe before test was 1 mm/s, the test speed was 1 mm/s, the speed after the test was 2 mm/s, the strain was 50%, and the trigger force was 5.00 g. The maximum peak height of the force–deformation curve was recorded and determined as the hardness. The hardness measurements were measured with six repetitions [9].

2.3.2. Volume arithmetic mean diameter and hardness

Three samples were weighed and immersed in a measuring cylinder with a known volume of water. Here the powder–powder displacement method was used, the difference in quartz-flour volume between the new and initial level in the measuring cylinder was recorded as the volume of soybeans, mm³ [25].

Ten soybeans were randomly selected from the bulk samples. A vernier caliper was used to measure the axial dimensions with an accuracy of 0.02 mm. The Dₐ of soybeans was evaluated using Eq. (3).

2.3.3. Electronic nose (E-nose) analysis

The E-nose was equipped with a sensor array with 10 kinds of semiconductor metal oxide chemical sensing elements. Each type of sensor element corresponded to a certain difference in the type of the main sensitive substances. 3.0 ± 0.1 g samples in Dried, Control and SFU groups were respectively weighed, put into every 20 mL glass bottle, and sealed at 25 °C for 30 min. Then, the inside headspace gas was allowed to equilibrate. The injection needle was directly inserted into the glass bottle and collected odor was measured by E-nose. The sampling time was 1 s/group, and self-cleaning time of the sensor was 180 s, and the time of sensor going back to zero was 5 s. The sample preparation time was 5 s, the injection flow rate was 400 mL/min and the sampling time for analysis was 120 s [27].

2.4. Research methods of soybean milk

2.4.1. Particle size

The litesizer 500 particle size analyzer (Anton Paar, Austria) (detection range from 0.3 to 10 μm) was used to measure particle size of soybean milk. The soybean milk in Dried, Control and SFU groups was prepared and diluted 200 times with deionized water to reduce the effect of multiple light scattering. The refractive index and absorption parameter of samples were 1.450 and 0.001, respectively; and the refractive index of aqueous solution was 1.330. Each sample was tested for 3 parallels at 25 °C [28].

2.4.2. Circular dichroism (CD)

The secondary structure of soybean milk was measured by J-815CD spectroscopy (JASCO, Japan). All the samples in Control and SFU groups were prepared with ultrapure water and centrifuged at 10,000 r/min to remove insoluble residues. The CD spectrum was recorded using a CD spectrometer in a 0.1 cm quartz cuvette at 25 °C, the wavelength in the far-ultraviolet ranged from 190 to 250 nm, and the interval was 1 nm. The scanning speed was 50 nm/min. The percentage of α-helix, β-sheet, β-turn and random coils of the samples was calculated on the Bestsel website [29].

2.4.3. Fluorescence spectrum

The fluorescence spectrum of soybean milk was measured by card-98 Cary Eclipse (VARIAN, USA). The protein suspension in Dried, Control and SFU groups was diluted 200 times with deionized water and measured to obtain the emission spectrum. The excitation wavelength was 280 nm, emission spectrum was 300–450 nm, and data interval was 1 nm. The scanning speed was 600 nm/min, and the bandwidth of excitation and emission was 5 nm [30].

2.5. Data processing

All the experiments were conducted in at least three replicates and the mean ± standard deviation (SD) was used in the analysis by Excel Version 2010 (Microsoft Corp., USA) and OriginPro Software Version 8.0 (OriginLab Corp., MA, USA). Statistical significance was evaluated by Duncan’s multiple-range test (p < 0.05) by SPSS 23.0 software (SPSS Inc., Chicago, USA). Design expert software version 11.1.0 was used to perform RSM.

3. Results and discussion

3.1. Parameter optimization of soybean soaking process assisted by SFU

3.1.1. Optimization of intermittent ratio during SFU-assisted soaking process

During SFU-assisted soaking process, the heating effect cannot be ignored for long time. However, it can be improved by the intermittent ratio to prevent the interference of ultrasonic heating effect on the temperature control process, and sufficient time will be left between each SFU pulse to improve the spatial distribution of the cavitation bubbles. As a result, bubbles have enough time to grow and collapse in a specific area, that is, oscillation, which improves the uniformity and intensity of the ultrasonic cavitation effect [31]. In recent years, researches on intermittent microwaves of dried foods are also emerging in the food industry, the drying unevenness can be prevented and energy consumption reduces [32]. Therefore, it is necessary to optimize the SFU intermittent ratio to achieve suitable cavitation characteristics. As shown in Fig. 2a, the soaking temperature, ultrasonic power and soaking time of soybeans are fixed at 30 °C, 60% (144 W) and 240 min, respectively, during SFU-assisted soaking process. It is found that as the intermittent ratio increases, the SPC reaches the maximum value of 15.70 mg/g at the intermittent ratio of 1:5, and then decreased to 14.22 mg/g. Compared with continuous ultrasound (intermittent ratio of 1:0,
SPC of 12.28 mg/g), the addition of intermittent ratio gives a better soaking effect of soybeans. Due to the intermittent ratio of 1:5, sufficient cavitation effect is generated by the oscillation of bubbles and the hydration and rehydration process is promoted [33]. If the interval time between two ultrasonic pulses is too small, there will not be enough time for the oscillation process of bubbles. Meanwhile, too long ultrasonic time will greatly consume bubble nuclei, and eventually there will not be enough bubbles to participate in the oscillation process [34]. When the interval time between two ultrasonic pulses is too long, such as 1:6, the real working time of SFU is reduced, and the ultrasonic intensity is not enough to promote the soluble protein into the solvent. Considering the soaking temperature and ultrasonic effect, the final ultrasonic intermittent ratio is 1:5.

3.1.2. Single factor test during SFU-assisted soaking process

3.1.2.1. Effect of soaking temperature on SPC of soybeans. The ultrasonic power and soaking time are fixed at 60% (144 W) and 240 min, respectively, and the effect of soaking temperature on SPC of soybeans is studied. In Fig. 2b, as the soaking temperature increases from 10 °C to 40 °C, SPC increases from 14.65 to 16.78 mg/g with a growth rate of 14.50%. It is known that most of the soluble proteins in plants are enzymes involved in various metabolisms. On one hand, SPC increases with the increment of temperature, and the contribution of temperature to the solubility of soluble protein is related to intermolecular and intramolecular interactions [35]; on the other hand, the lower the temperature, the slower the water absorption rate of soybeans (soaking mechanism in Section 3.2). Not enough water is absorbed to fully swell soybeans, resulting in a low SPC after soaking time of 240 min. Conversely, as the soaking temperature increases from 40 °C to 50 °C, SPC decreases to 13.89 mg/g with a decrement rate of 17.20%. It was found that as the temperature increased, denaturation and unfolding of proteins induced the exposure of active sites on the surface, the interaction between particles was promoted, leading to the formation of larger soluble and insoluble polymers, and the solubility was reduced [36]. In general, irreversible aggregation reaction will happen when the protein undergoes denaturation, when heated above 60 °C. This agrees with the findings of Miranda et al. [37] that when soaking temperature reached 45, 55, and 65 °C, the water was absorbed quickly during soaking process, tissue rupture and a large loss of soluble protein occurred, therefore soybeans were vulnerable to damage.

3.1.2.2. Effect of SFU power on SPC of soybeans. The effect of SFU power on SPC is studied when the soaking temperature and time are fixed at 30 °C and 240 min. In Fig. 2c, SPC increases significantly (p < 0.05) with the increment of ultrasonic power. It has been reported that the cavitation effect and shear force of ultrasound can depolymerize large aggregates into small ones, the hydrophobicity of the protein surface is improved and the exposure of fusion groups increases. Ultrasonic waves produce strong shear force and micro-turbulence on the surface of the material, and the cell wall can be fully affected; with the increment of ultrasonic power, the dense and uniform structure of soluble protein gradually is changed into loosened one, which is beneficial to dissolution. Karki et al. [38] found that ultrasonic pretreatment can increase the release rate of the protein and also improve its dissolution. However, when the SFU ultrasonic power exceeds 60% (144 W), SPC significantly reduces due to the excessively strong shear effect on the soybean protein structure for a long time (p < 0.05). It was reported that too high ultrasonic power adversely affected the microstructure and rehydration properties of soybean protein, which was not conducive for the dissolution of soluble protein [39].
3.1.2.3. Effect of soaking time on SPC of soybeans. The effect of soaking time on SPC of soybeans is studied, when soaking temperature and ultrasonic power are fixed at 30 °C and 60% (144 W). From Fig. 2d, SPC gradually increases as the soaking time increases from 120 to 240 min, and then significantly decreases. On one hand, it has been found that suitable ultrasonic treatment can dissociate proteins and increase solubility [40]; while regardless of the intensity of ultrasound, soluble proteins may partially degrade after a long time (20 min), and Khatkar et al. [41] also observed similar results. It is also possible that the soybean structure collapses due to too long soaking time, causing the loss of soluble protein to the soaking solution. On the other hand, in ultrasound itself, the number of cavitation bubble nuclei gradually decreases at extended time, thus there is not enough bubbles to participate in the oscillation process, resulting in the corresponding weakened cavitation effect.

Optimized parameters of SFU-assisted soaking parameters of soybeans are listed based on RSM (Fig. S1 in Supplementary): soaking temperature of 37 °C, ultrasonic power of 60% (144 W), and soaking time of 214 min. The experimental result of SPC is 16.836 ± 0.208 mg/g (n = 3). It is found that soaking time of soybeans without SFU is 390 min, and SPC is 14.734 mg/g. In contrast, soaking time of soybeans with SFU is shorten by 45.13% and SPC is improved by 14.27% in Fig. 3a. Based on the above-mentioned optimal parameters during SFU-assisted soaking process, the action process and mechanism of SFU, and its effect on physicochemical properties of soybeans are explained subsequently.

3.2. Analysis of action process and mechanism of SFU during soybean soaking process

3.2.1. Analysis of soybean soaking process

The M₀ of soybeans is 3.59% (d.b.%). Water absorption rate curves of soybeans in Control and SFU groups are shown in Fig. 3a during soaking process, namely the rehydration and the water absorption kinetic model is established. It can be seen that at the initial stage of soaking process, the water absorption rate shows an exponential trend; however, Mᵣ tends to stability as soaking time increases. Furthermore, the water absorption rate of soybeans in SFU group is faster compared with that in Control group. According to the research of Bello et al. [42], the increment of water absorption rate at the initial stage of rehydration was mainly due to the effect of natural capillary on the surface of the grain layer, as well as the gradient difference between grain tissue and external environment. A previous study [9] showed that the moisture content of soaked soybeans after ultrasonic pretreatment could reach 130 g/100 g at 180 min. Here, the moisture content of soybeans in SFU group can reach 130 g/100 g at 24 min, and the soaking time reduced by 86.7%, which shows that SFU-assisted technology obviously improves the soaking efficiency. In addition, the Peleg equation has been used to predict the water absorption of seeds in a variety of food applications and describe the water desorption of black beans and chickpeas [18,43]. By combining both test data and Peleg model (Eq. (2)), K₁ and K₂ of soybeans in Control and SFU groups are determined in Fig. 3a. Due to the coefficient R² from 0.96 to 0.97, the Peleg equation is suitable for describing the water absorption process of soaked soybeans. K₁ is inversely proportional to the rehydration rate, and smaller K₁ means faster rehydration process [44]. K₂ is inversely proportional to the maximum rehydration effect, and smaller K₂ means higher moisture content in equilibrium [43]. Hence, it indicates that SFU group has faster rehydration by K₁. It was reported that K₂ may also depend on the type of seeds and the loss of soluble solids during soaking process [45]. The soybean structure can be changed by SFU, resulting in a decrease in its water retention capacity. However, although K₂ is slightly smaller in SFU group, in fact, the maximum rehydration effect of soybeans will not be significantly changed [19].

In order to reveal water flow path during SFU-assisted soaking process of soybeans, magnetic resonance imaging (MRI) was used to visualize the soaking process. In Fig. 3b, MRI pseudo-color images at different soaking times shows the dynamic water flow during SFU-assisted soaking process. Water gradually diffuses from the inside of soybean cotyledons to outside. After soaking for 30 min, a large amount of soybean cell

![Image](image.png)

**Fig. 3.** Water absorption process of soybeans during soaking.
of water is still absorbed during soaking process (red part), although Mr of 3.59% (dbh%) is high. The internal moisture content of soybeans becomes higher; while the external tissue almost has no significant change after the soaking time of 90 min. The tissue with high moisture content gets thicker and the water has a tendency to fully diffuse outward after the soaking time of 150 min. Finally, the water almost completely penetrates into soybeans at 210 min. It was shown that the water first penetrated from the germ into the interior, and then expanded along the central axis [46], or the water gradually diffused from the inside of the soybean to the whole soybean cotyledon during the soaking process of soybeans [47].

LF-NMR can be further used to study water status and distribution in seeds [18], and the transverse relaxation time distribution curves during SPU-assisted soaking process of soybeans are determined. Fig. 3c shows T2 relaxation curves during SPU-assisted soaking process, the higher the molecular mobility, the longer the T2 relaxation time. There are four different proton groups: T2a, T21, T22 and T2b, as the relaxation signals. T21 is the first peak at a relaxation time of 0.1 ms. Researchers found that the relaxation time of starch, protein and other biomacromolecules was <1 ms through the neutron generation experiment, they believed that the protons in this part were mainly non-exchangeable C–H protons of large molecules such as protein and starch [48]. The main components of soybeans were protein and cellulose, so it was believed that these protons were mainly non-exchangeable protons of protein macromolecules [47]. The second component T21 has a relaxation time of about 3 ms and is classified as water that is tightly bound to macromolecules, which is defined as strongly bound water because of low mobility. The water of T2b is bound to the macromolecules and can also be considered as their parts. Although T2a has poor mobility, it still exchanges slowly with water molecules outside. During SPU-assisted soaking process, the peak of T21 shifts towards right, which indicates that more water penetrates into the macromolecules and rehydration occurs. T22 corresponds to the free water of soybeans. It is worth noting that when the soaking time reaches 10 ms, the peak area continues to expand with the right shift of T22 peak, and then this peak gradually becomes the main component. This is because T2a is mainly composed of free proton C–H and O–H in soybean cotyledons interstices, namely free water. Due to the low moisture content of dry soybeans, there is very little water in the intercellular space, and its signal cannot be displayed by T2 relaxation spectrum. During the soaking process, a large amount of water enters into interstitial spaces and then further into cells. As the soaking time extended, the peak generally shifts to the right, indicating that the degree of water dissociation in samples increases. Obviously, signals of each component increase after SPU-assisted soaking process, and thereafter, the increase of T22 is the most obvious, thus total relative water content increases mainly due to free water [18]. The relaxation time of T2b is the longest, which corresponds to the oil peak of dried soybeans. With the increment of soaking time, the oil peak shifts to the right and the peak area becomes larger. Since the oil is not affected by chemical exchange, it is believed that this phenomenon is caused by the overlap of water and oil signals. According to previous LF-NMR studies on plant tissue, this composition can be classified as water in the interstices of cytoplasm, oil bodies and protein bodies. The water relaxation time in these large spaces often exceeds 100 ms. The peak of T2b with the longest relaxation time is formed by these accumulated signals of water and oil [47]. Fig. 3d shows the model of T21 and T22 in the SPU-assisted soaking process. Soybean is affected by SPU from the outside to the inside, the closer to the ultrasonic transducer, the more significant effect of SPU, which can broaden the migration path of water and promote the mass transfer process.

3.2.2. Mechanism of SPU on soybeans during soaking process

3.2.2.1. Real-time online monitoring during SPU-assisted soaking process.

In order to realize the effect of SPU field itself on soaking process of soybeans, the maximum voltage amplitude of acoustic field and the oscillation period of cavitation bubbles are obtained by real-time online monitoring technology, as shown in Fig. 4a–d. The voltage amplitude is monitored by the PVDF sensor and is used to represent the intensity of SPU field in time domain. The stronger the intensity of ultrasonic field, the more obvious cavitation and mechanical effects. After the oscillation process, cavitation bubbles collapse with the greater pressure of micro-turbulence. The greater deformation of the PVDF sensor is induced, and more electrical energy is converted from mechanical energy in the form of the voltage amplitude [49]. Fig. 4a shows a typical ultrasonic voltage signals in one cycle of oscillation process, including multiple voltage amplitudes and a maximum voltage amplitude, which is similar to the waveform of damped oscillation. It indicates that the most obvious effect of SPU field is obtained as bubbles collapse, and then the intensity of ultrasonic field dissipates gradually over time. For the entire soybean soaking process (210 min), most cavitation nuclei have participated in the growth and collapse of cavitation bubbles, and only a few left nuclei correspond to weak cavitation effect; thereby the monitored voltage amplitude reduces. Since multiple amplitude voltages randomly distribute in one cycle of oscillation, and the maximum voltage amplitude is only one, it can be used as a characteristic parameter of ultrasonic field [50]. The maximum voltage amplitude of SPU field is detected in specific time periods (1, 30, 60, 90, 120, 150, 180, and 210 min) during soybean soaking process in Fig. 4b. The intensity of SPU field is weakened and becomes constant with the increment of soaking time. Further, the intensity of ultrasonic field is related to the oscillation of cavitation bubbles, which can be explained from cycles of the oscillation. Fig. 4c shows amplitude voltages of SPU fields in two oscillation periods. The period from maximum voltage amplitude 1 to 2 belongs to one oscillation period, that is, life cycle of cavitation bubbles. The oscillation period is the characteristic value of consumed time during the growth and collapse of cavitation bubbles in time domain. The maximum voltage amplitude 1 is generated by ultrasonic noise signals from expansion radiation of cavitation bubbles therein, and the maximum voltage amplitude 2 is induced by acoustic signals from collapse radiation [51]. The oscillation periods monitored during specific soaking time (1, 30, 60, 90, 120, 150, 180, and 210 min) is shown in Fig. 4d. At the initial stage of SPU-assisted soaking process, the number of bubble nuclei and the density of the cavitation clouds are large in the soybean soaking solution, and its life cycle is long, which is enough for the oscillation of cavitation bubbles [52]. Generally, the longer the life cycle, the larger radius of cavitation bubbles, and the greater intensity of SPU field is produced when bubbles collapse [53], thus the maximum voltage amplitude can be monitored (Fig. 4b). With the prolongation of soaking time during SPU-assisted soaking process, a large number of bubble nuclei have been contributed to the oscillation process in the soaking solution, so the number of bubble nuclei and the intensity of SPU field decrease. Correspondingly, the maximum voltage amplitude and life cycle monitored in SPU field decreases, and it is inferred that the number of cavitation bubbles greatly reduces when the soaking time reaches 120 min, and the effect of SPU field becomes equilibrium after 120 min [54]. In addition, it has shown that the intensity of ultrasonic field directly affects the structure of soybeans, which was the essential reason for improving the heat and mass transfer process, and SPC of soybeans increases [55].

3.2.2.2. Microstructure and cell membrane permeability of soybeans during SPU-assisted soaking process.

Soaking is to make soybeans absorb water and then expand, which is beneficial to fully extracting protein after crushing. SPU-assisted soaking technology promotes the heat and mass transfer process in soaked soybeans, and it is closely related to the change of the internal structure of soybeans. Fig. 5 provides microstructure morphologies of soybeans by OM (Fig. 5a-c) and SEM (Fig. 5a1-c2). As for dried soybeans before soaking (Fig. 5a), the space between cotyledon cells is small and cells are arranged tightly. The
network structure of soaked soybean cells without SFU (Fig. 5b) becomes a little more stretched, and the space between cells increases. In contrast, the structure of soaked soybeans with SFU gets looser and plumper further, and the intercellular space is significantly larger in Fig. 5c. SFU is conducive to mass transfer in soybeans, meanwhile, cells absorbing water and swelling accelerate significantly [56]. On this basis, the cross and longitudinal sections of soaked soybean cells is observed by SEM in Fig. 5c1-c2. On one hand, the microstructure of cross section is elliptical, while that of longitudinal section is round. On the other hand, compared with dried (Fig. 5a1-a2) and soaked soybeans without SFU (Fig. 5b1-b2), soaked soybeans with SFU absorb water and expand more obviously; this is because the alternating compression and extension of medium particles during the propagation of SFU and the pressure is changed. The repeated and alternating changes of pressure will promote mass transfer of water in soybean structure during soaking process, resulting in the looser cell structure of soybean after efficient water absorption. The shearing force and micro-turbulence from ultrasonic cavitation induces cell dispersion and promotes the formation of large deep pores (yellow dotted circles in Fig. 5c1-c2). The intercellular space is enlarged and the water diffusibility increases [56]. Moreover, it can be observed that the entry of water causes the gradual swelling of cell during SFU-assisted soaking process, and the cell shape in Fig. 5c becomes more round than that in Fig. 5a, b, thus the larger area of round cell structure is seen in Fig. c1. The more round the cell tissues, the looser the structure. Meanwhile, the separation between cells appears and the formation of channels is induced, but it does not cause large area of tissue rupture (Fig. 5). Therefore, SFU-assisted soaking process promotes the mass transfer of water and hydration process. Combined with the results of bar graph in Fig. 6a, it is found that SPC permeated into the soaking solution only slightly increases, and the SPC retained in soybeans still significantly is raised after SFU treatment. It indicates that although SFU makes the cell structure looser to a certain extent, but avoids large-scale destruction of cell structure and loss of nutrients. Hence, the SPC of soybeans have been greatly preserved.

Furthermore, the above-mentioned phenomenon in soybean cells is also related to the cell membrane permeability [57], which can cause cell swelling, leakage and contraction of cytoplasm. Cell damage analysis can be classified as ion leakage. Fig. 6a shows SPC retained in soybeans, and that permeated into the soaking solution as well as the corresponding electrical conductivity. It is observed that SPC in SFU group is the highest significantly, but that permeated into the soaking solution increases slightly. It indicates that the hydroxyl radical produced by cavitation can oxidize the cell membrane lipid and change the membrane permeability [58]. When the cell membrane loses semi-permeability, ions will leak from cells. However, SFU-assisted soaking process does not cause large area of tissue rupture (Fig. 5). Hence the soaking efficiency and quality is improved, which makes it an efficient assisted technology. At the same time, the electrical conductivity of soybean milk in SFU group increases significantly ($p < 0.05$), indicating that the permeability of cell membrane is enhanced. It is reported that the enhanced cell membrane permeability promotes the secretion and release of intracellular metabolites, which is beneficial to the dissolution of nutrients and the accumulation of nutrients in the subsequent production of tofu [59].

SFU produces ultrasonic cavitation and bubbles collapse in aqueous medium. Fig. 6b shows that microjets, shearing forces and local heating occur due to the collapse of cavitation bubbles. In the two-phase system of soybean-water during SFU-assisted soaking process, the formation of micropores, the change of cell membrane permeability and the relaxation of the matrix are induced in soybeans. The permeability of cell membrane directly affects the soaking process of soybeans.

3.3. Physicochemical properties and protein structure of soybeans

3.3.1. Volume arithmetic mean diameter, hardness and flavor of soybean

Fig. 7a shows the volume, arithmetic mean diameter and hardness of soybeans in Dried, Control and SFU groups. Compared with Dried group, the volume of soybeans in Control and SFU groups increases by 1.6 and 2
Fig. 5. OM (a-c) and SEM (a1-c2) images of soybean microstructure under different soaking conditions.
times, and the arithmetic average diameter increases by 35% and 46%, respectively. This indicates that SFU-assisted soaking technology can significantly improve the efficiency of soybean soaking, promote soybeans to absorb water and swell more quickly. Thus, the volume becomes larger and shape gets more round. The researcher also has found that ultrasonic pretreatment can increase the permeability of soybeans by interacting with plant cell walls, thereby promote water absorption [9]. The texture of soaked soybeans becomes soft, while the hardness of soybeans in SFU group decreases by about 30% compared with that in Control group. The hardness change reported by Pohl et al. [60] was due to the gelatinization of starch and pectin through heating, as some pectin substances became soluble, and the soybean was softened. Additionally, the disruption of the middle lamella and softening of the protein matrix of soybeans also reduced hardness.

The flavor of soybeans is analyzed by E-nose, as shown in Fig. 7b. Four flavor compounds (W2W, W2S, W1W and W1S) of soybeans in Dried group are not strong; while their proportional range in Control group covers a great extent from 81.86 to 108.23%, and unpleasant flavor such as beany significantly increases. However, it is worth noting that the four unpleasant flavors of soybeans are significantly reduced by 16.37%–47.6% in SFU group compared with that in Control group. The unpleasant beany flavors are mainly composed of volatile and non-volatile flavor, including various pungent ones such as grassy, fishy and ammonia. The difference between the flavor substances of soybeans in Control and SFU groups can be mainly reflected by organic sulfur and inorganic sulfur compounds, followed by short-chain alkanes, nitrogen oxides, alcohols, aldehydes and ketones [61]. It was reported that the beany flavor was mainly produced by some short-chain aliphatic alcohols, ketones and aldehydes formed by the oxidation and decomposition of fatty acids, and fatty acids were induced by lipoxygenase (LOX) and peroxidase (POD) [27]. Yu et al. (2014) proved that the enzyme activity was mainly affected by ultrasonic treatment, because its secondary and tertiary structures were affected. Thus, oxidase could be inactivated by SFU to eliminate unpleasant beany flavor to a certain extent. The main cause of ammonia flavor is related to high nitrogen content, which is mainly related to volatile nitrogen oxides and sulfur compounds.

![Fig. 6. Cell membrane permeability.](image-url)
generated by the reaction between amino acids and sugars in soybeans [9], which is significantly reduced through SFU.

### 3.3.2. Particle size, secondary and tertiary structure of protein in soybean milk

The particle size of soybean protein is shown in Table 1. It can be found that the hydrodynamic diameter of soybean protein in Control group increases compared with that in Dried group, but that is reduced by 3.83% in SFU group compared with Control group. The reduction of particle size is attributed to ultrasonic cavitation with high shear force and turbulence, thereby large protein molecules can be converted into smaller ones [40]. In addition, the hydrolyzed protein may have a higher degree of expansion and a relatively loose structure, so it is easier to decompose by ultrasonic treatment, and a smaller particle size is induced. Other studies have also reported the application of ultrasonic treatment to reduce the size. Zou et al. [62] reported that when the ultrasonic power of 150 W was used for 20 min, the average particle size of chicken actomyosin decreased from 670 nm to 282 nm. Similar conclusions could be found in studies of whey [63] and sunflower protein [64].

In Fig. 8a, the secondary structure of soybean protein is evaluated by CD spectrum in the far ultraviolet region (190–260 nm). The typical characteristic of \( \alpha \)-helix in the CD spectrum of a protein is demonstrated by a positive peak at 190 nm and two negative peaks at 208 and 220 nm. \( \beta \)-sheet has a positive peak at 195 nm and a negative peak at 215 nm. A protein with no dominant secondary structure (unordered) has a negative peak around 200 nm [65]. Compared with Control group, the peak intensity at 192 nm and 195 nm decreases and that at 208 and 222 nm is also changed. All these reveal that the secondary structure of the protein is changed by SFU. The content distribution of the secondary structure is further calculated in Fig. 8a, SFU treatment increases \( \beta \)-sheet and unordered, and decreases \( \alpha \)-helix and \( \beta \)-turn. Similarly, Li et al. [66] also found that SFU treatment resulted in a decrease of \( \alpha \)-helix and an increase of \( \beta \)-sheet of the protein, which is consistent with results of this study. Among protein molecules, \( \alpha \)-helix is the most tightly bound, and its decrease indicates protein molecules have a certain degree of

### Table 1

| Group | Hydrodynamic diameter (nm) |
|-------|---------------------------|
| Dried | 275.76 ± 23.10 \(^a\)     |
| Control | 399.98 ± 10.00 \(^b\)   |
| SFU | 384.65 ± 15.61 \(^c\)  |

![Fig. 7. Physicochemical properties of soybeans.](image)

![Fig. 8. Soybean protein spectrum.](image)
relaxation; the increase in unordered indicates the protein molecules become looser [67]. When soybean protein is treated by SFU, agglomeration are dissociated and particle size decreases, which is consistent with particle size results (Table 1). The process of aggregate unfolding may destroy α-helix to form a disordered structure [68]. SFU opens the disulfide bond between peptide bonds and disassembles α-helix, so hydrophobic groups of the protein are exposed to the outside. Thus, protein-water interactions and protein solubility is strengthened [69]. This is consistent with the increase of SPC in SFU group. The increase may be due to conformational changes during SFU-assisted soaking process, and the formation of soluble protein comes from insoluble protein aggregates. It was reported that ultrasonic treatment promoted the formation of soluble protein from insoluble precipitate of commercial soy protein isolate [70].

In order to obtain whole information about changes of soybean protein structure, the tertiary structure is described by intrinsic fluorescence spectrum in Fig. 8b. Amino acids such as tryptophan (Trp) and tyrosine (Tyr) residues in soybean protein have a strong fluorescence quantum yield, while the other amino acids either have weak fluorescence or no fluorescence at all. Therefore, the emitted inherent fluorescence mainly comes from Trp and Tyr residues [28]. In this way, the tertiary structural change of protein can be determined by the fluorescence intensity and the maximum wavelength (λ). Compared with Dried group, soaking process significantly increases the fluorescence intensity of the soybean protein in Control and SFU groups. Meanwhile, compared with Control group, the maximum emission wavelength of soybean protein detected in SFU group is red-shifted from 339.07 to 342.0 nm, the corresponding fluorescence intensity increases significantly from 687.36 to 842.77 au, and the fluorescence intensity increased by 22.6%. This is due to the unfolding of the protein, and Trp residues exposures and fluorescence intensity increases [28], Zou et al. [71] reported a similar trend about the effect of ultrasound on chicken plasma protein hydrolysates. SFU treatment shows an increase in fluorescence intensity of soybean protein, which is also in line with results of Huang et al. [40].

4. Conclusion

In this study, soybean soaking process assisted by SFU was used. Optimum parameters were obtained according to high SPC based on RSM. The temperature was 37 °C, ultrasonic power was 60% (144 W) and the soaking time was 214 min. Compared with Control group (390 min, SPC was 14.734 mg/mL), the soaking time can be shortened by 45.13% and SPC increased by 14.27% in SFU group. The curve of water absorption rate fitted well with Peleg model. Overall, SFU can change the cell membrane permeability and the protein structure, reduce the particle size of the protein, loose the structure and promote its unfolding, leading to the exposure of fluorescent groups. In addition, SFU group had lower hardness and better flavor. Results obtained have proved to be a promising way to provide information on physical processing and its use in further processing of soybean.

CRediT authorship contribution statement

Lei Zhang: Data curation, Writing – original draft. Yang Hu: Data curation, Writing – original draft. Xue Wang: Data curation, Writing – original draft. Olugbenga Abiola Fakayode: Writing – review & editing. Haile Ma: Supervision. Cunshan Zhou: Supervision. Aiming Xia: Supervision. Qun Li: Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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