Ultrasonics Sonochemistry 94 (2023) 106338

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Structural and physicochemical properties of the different ultrasound frequency modified Qingke protein

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ARTICLE INFO

Keywords:
Ultrasound
Modification
Qingke protein
Physicochemical properties
Structure

ABSTRACT

There is a burgeoning demand for modified plant-based proteins with desirable physicochemical and functional properties. The cereal Qingke is a promising alternative protein source, but its use has been limited by its imperfect functional characteristics. To investigate the effect of ultrasound treatment on Qingke protein, we applied single- (40 kHz), dual- (28/40 kHz), and tri- (28/40/50 kHz) frequency ultrasound on the isolated protein and measured subsequent physicochemical and structural changes. The results showed that the physicochemical properties of proteins were modified following ultrasound treatment, and many of these changes significantly increased with increasing frequency. Compared with the native Qingke protein (control), the solubility, foaming activity, stability, and water or oil holding capacity of tri-frequency ultrasound modified Qingke protein increased by 43.54%, 20.83%, 20.51%, 28.9%, and 45.2%, respectively. Furthermore, ultrasound treatment altered the secondary and tertiary structures of the protein resulting in more exposed chromophoric groups and inner hydrophobic groups, as well as reduced β-sheets and increased random coils, relative to the control. Rheological and texture characterization indicated that the values of G' and G'', hardness, gumminess, and chewiness decreased after ultrasound treatment. This study could provide a theoretical basis for the application of multi-frequency ultrasonic technology for modification of Qingke protein to expand its potential use as an alternative protein source.

1. Introduction

Protein is an abundant and sustainable component and additive often used in the food industry, and there is currently a growing demand for novel, nutritional plant-based proteins. Cereals are rich in protein and may serve as a suitable alternative source. Highland barley (*Hordeum vulgare* Linn. var. *nudum* Hook.f.), commonly known as Qingke, is a cereal mainly cultivated in the north- and southwest regions of China, particularly in plateau areas like Tibet and Qinghai[1,2]. Qingke possesses excellent nutritional qualities including high levels of protein, fiber, and vitamins, as well as low lipid and carbohydrate levels[3]. Due to its fortification with bioactive ingredients like β-glucan, polyphenol, and flavonoids, Qingke also exhibits unique biological benefits such as alleviation of oxidative stress and reduced risk of diseases including hypertension, diabetes mellitus, and atherosclerosis[4,5]. Thus, Qingke has been extensively used in noodles, wine making, functional food development, and animal fodder production[6].

Qingke protein is the second most abundant nutritional component of Qingke grains, accounting for up to 17.5% of its total content[7]. Moreover, Qingke protein is rich in amino acids, particularly lysine, and contains all the essential amino acids required by humans unlike wheat, corn and other grain crops. Proteins serve as high-function molecules with rich nutritional value in food ingredients; they can stabilize oils and bubbles, form gel structures, and improve system viscosity[8]. Its high

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https://doi.org/10.1016/j.ultsonch.2023.106338
Received 21 December 2022; Received in revised form 3 February 2023; Accepted 17 February 2023
Available online 20 February 2023

proportion of protein and potential nutritional applications make Qingke a promising alternative source of protein. However, not only the byproducts of Qingke processing, which include β-glucan and starch, have limited interest in and widespread use of Qingke, but the application of Qingke protein in food products has been impeded by its imperfect functional characteristics, including poor extensibility, solubility, foaming activity and stability, and water and oil holding capacity. Therefore, it is imperative to devise an effective and safe modification to both improve the utilization of Qingke protein and enhance its nutritional and economic value.

Ultrasonic technology, a mature and safe physical modification technology, has become an excellent alternative to traditional thermal processing. It is widely used in the food processing and quality control industries due to its high efficiency and stability which in turn save energy. When ultrasonic waves are propagated in a medium, they interact with liquid or gas to produce cavitation as well as mechanical, thermal, and chemical effects[9]. Hence, even without additives or excessive heat treatment, protein structures within the ultrasonic field can be remarkably modified, resulting in changes to functional characteristics that may improve food performance[10]. For example, Jin et al. [11] reported that ultrasonic wave treatment (20 kHz, 10 min) reduced both disulfide content and unstable secondary structures in buckwheat protein. O'Sullivan et al.[12] observed that ultrasonic treatment significantly improved hydrophobicity of potato protein isolates while also decreasing hydrodynamic volume, and this substantially increased emulsification ability and stability. It has been previously established that frequency combinations are important factors affecting the formation and intensity of liquid cavitation. Ample evidence indicates that compared with single-frequency ultrasound, multi-frequency ultrasound generates stronger cavitation activity, thus significantly enhancing the modifying effects[13]. For instance, the new ultrasound treatment mode, simultaneous multi-frequency ultrasound, has been successfully used in molecule-assisted extraction and starch modification[14]. According to previous research from our lab[15], the cavitation efficiency of tri-frequency ultrasound is markedly higher than that of dual- or single-frequency, and the solubility and water and oil holding capacities of gluten significantly increased with an increasing number of combinations.

Review of the literature has revealed that studies on Qingke have mostly focused on the functional characteristics of β-glucan and phenolic compounds [16–18], however there is little information available regarding the effects of different frequency ultrasound treatment on Qingke protein or its structural and functional characteristics. Therefore, the purposes of this study were to: (1) investigate the effects of single- and multi-frequency ultrasound on the functional and structural properties of Qingke protein, as well as explore gel property changes induced by ultrasonic treatment; (2) according the changed physicochemical and structural properties of the ultrasound modified Qingke protein, we expect to provide valuable insight in to the possible mechanisms of modifying cereal proteins and understand the advantages, limitations in the application of the cereal-derived protein in functional foods.

2. Materials and methods

2.1. Materials and chemicals

Whole grain Qingke (*Hordeum vulgare* Linn. var. *nudum* Hook. f. Himara 22[#]) was provided by Xigaze Agricultural Science Research Institute (Tibet, China). Rapeseed oil was purchased from Yonghui Supermarket (Beibei, Chongqing, China). BCA Total Protein Quantification Kit was obtained from Jiancheng Biotechnology Co., Ltd. (Nanjing, Jiangsu, China), and molecular weight protein markers from Yamei Biotechnology Co., Ltd. (Shanghai, China). 1-phenylamino-2-naphthalenesulfonic acid (ANS) and 5,5-Dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich., Ltd. (St. Louis, Missouri, USA). All chemicals were of analytical grade.

2.2. Pretreatment of raw material

2.2.1. Extraction of Qingke protein

Qingke protein was extracted using the method reported by Nieto-Nieto et al.[19] with minor modifications. Briefly, whole grain Qingke was crushed into flour and filtered through a sieve with aperture 0.60 mm. The powder suspension (50 g/L) was adjusted to pH 10.5 with 0.1 mol/L NaOH and stirred at 27 × *g* using an RW20 blender (IKA Company, Baden-Württemberg, German) for 2 h at 40 °C, then spun for 15 min at 1347 × *g* in a centrifuge (5810, Eppendorf Company, Hamburg, Germany). The pH of the supernatant was adjusted to 4.5 then centrifuged for 15 min at 1347 × *g*. The precipitate was washed three times with ultra-pure water and freeze-dried using a vacuum freeze drier (Alpha 2–4 LSC plus, Christ Company, Osterode, Germany) for 48 h to obtain Qingke protein (purity: 82.31 ± 1.47 g/100 g).

2.2.2. Ultrasonic treatment of Qingke protein

Qingke protein was processed using a lab-scale multi-frequency ultrasonic reactor (Jiangsu Jiangda Wukesong Biotechnology Co., Ltd., Jiangsu, China) consisting of an ultrasonic reaction tank, temperature control system (composed of circulating pump and water bath), and digital display control system. Protein suspensions (80 g/L) were treated using different frequencies for 10 min at 25 °C in the reaction tank. Based on preliminary experimental results, the processing conditions of single-frequency ultrasound were set at 40 kHz; the simultaneous dual-frequency conditions were set as 28/40 kHz; and the simultaneous tri-frequency conditions were set as 28/40/50 kHz. The control was shaken at 25 °C for 10 min without ultrasonic treatment. Samples were freeze-dried, crushed through a sieve with aperture 0.18 mm, and stored in the dryer.

2.3. Determination and analysis of functional properties of Qingke protein

2.3.1. Solubility

The BCA Total Protein Quantification Kit was used to determinate protein solubility. The absorbance of pretreated samples was measured at 562 nm using an ultraviolet spectrophotometer (UV-245, Shimadzu Corporation, Kyoto, Japan). Solubility was computed by the following equation:

$$\frac{Solubility}{(\mu g/mL)} = \frac{OD - 0.011}{0.117} \times 524 \times 100 \quad (1)$$

2.3.2. Foaming capacity and stability

Foaming capacity (FC) and stability (FS) of proteins were measured via the method described by Zhang et al.[20]. 100 mL of 20 g/L protein suspension samples were homogenized using a handheld high-speed homogenizer (IKA Co., Baden-Württemberg, Germany). Foam volume was measured immediately (V_0) and after standing for 30 min (V_{30}). Foaming capacity and stability were calculated using the following equations:

$$FC = \frac{V_0}{100} \times 100\% \quad (2)$$

$$FS = \frac{V_{30}}{100} \times 100\% \quad (3)$$

2.3.3. Emulsifying activity and emulsion stability

The emulsification properties were measured by a method reported by Meng et al.[21]. 2 mL of refined rapeseed oil and 6 mL of 10 g/L protein suspension were mixed and homogenized for 1 min. 50 μL of the bottom emulsion was then added to 5 mL of 1 g/L sodium dodecyl sulfate solution (SDS). The absorbance of the mixture was measured at 500 nm both immediately (A_0) and after standing for 10 min (A_{10}); the

absorbance of 1 g/L SDS was used as control. The emulsification activity index (EAI) and emulsification stability index (ESI) were calculated as follows:

$$\frac{EAI}{(m^2/g)} = \frac{2 \times T \times A_0 \times 100}{10 \times \varnothing \times 10^4} \quad (4)$$

$$\frac{ESI}{(min)} = \frac{\Delta t}{A_{10} - A_0} \times A_0 \quad (5)$$

Where T = 2.302; ∅ represents the volume fraction of oil in solution (0.25); Δt = 10 min.

2.3.4. Water holding capacity and oil holding capacity

Protein (1.00 g) was added to 10 mL distilled water or rapeseed oil in a centrifuge tube and mixed evenly. Samples were incubated at 25 °C for 0.5 h, then centrifuged at 440 × *g* for 30 min. The supernatant was removed to obtain the precipitate, which was then transferred onto filter paper and weighed after 10 min. Water holding capacity (WHC) and oil holding capacity (OHC) were expressed in terms of adsorbed water or rapeseed oil mass per gram of sample[22].

2.3.5. Surface hydrophobicity

The surface hydrophobicity of Qingke protein was determined by the ANS method using a spectrofluorometer. Briefly, 4 mL of 5 g/L protein solution and 20 μL of 8 mmol/L ANS solution were combined, and the mixture was incubated in the dark at 37 °C for 0.5 h. After then, fluorescence intensity was measured using a fluorescence spectrophotometer (F-2500, Hitachi LTD., Tokyo, Japan). The excitation wavelength was 390 nm, the emission wavelength was 400–600 nm, and the slit width of the excitation and emission wavelengths was 5 nm [23].

2.4. Determination and analysis of the structural properties of Qingke protein

2.4.1. Tertiary structure of Qingke protein

An ultraviolet spectrophotometer was used to measure the ultraviolet absorption spectrum from 190 to 600 nm of 5 g/L protein suspension at 25 °C. A fluorescence spectrophotometer was used to measure the ultraviolet absorption spectrum of 5 g/L protein suspension at 25 °C. The excitation wavelength was 280 nm, the emission wavelength was 220–500 nm, and the slit width of the excitation and emission wavelengths was 5 nm.

2.4.2. Fourier transform infrared spectroscopy (FT-IR)

Using the KBr pellet method, Qingke protein was measured using a Fourier transform infrared spectrometer (Spectrum100, PerkinElmer, Waltham, Massachusetts, USA) at 4000–500 cm^{-1}. Amide I was analyzed using Peak Fit Software (Systat Software, Inc., Richmond, Virginia, USA).

2.4.3. Sulfhydryl and disulfide bonds

To determine the free sulfhydryl groups (F-SH), 0.5 mL of 10 mg/mL protein solution, 2.5 mL of Tris-Gly-8M Urea solution, and 0.02 mL of 4 mg/mL DTNB solution were rapidly mixed and incubated at 25 °C for 25 min. Absorbance (A_{412}) was measured and the results were calculated according to the following formula:

$$\frac{SH}{(\mu mol/g)} = \frac{73.53 \times A_{412} \times D}{10} \quad (6)$$

Where D is the dilution factor, set as 6.04 (F-SH) and 15 (total sulfhydryl groups, T-SH).

To determine the disulfide bond (-S-S-) content, 0.2 mL of 10 mg/mL protein solution, 1.0 mL of Tris-Gly-10 M Urea solution, and 0.02 mL of 2-hydroxy-1-ethanethiol were rapidly mixed and incubated at 25 °C for 25 min. Then, 10 mL of 120 g/L trichloroacetic acid solution (TCA) was added to the mixture and the reaction was allowed to continue for 1 h. The reagent was centrifuged at 2750 × *g* for 10 min. After two washes with TCA, the precipitate was mixed with 3.0 mL of Tris-Gly-8 mol/L Urea solution and 0.04 mL of 4 mg/mL DTNB solution. Next, 0.04 mL of DTNB solution was added and the reaction was incubated at 25 °C for 25 min. Absorbance (A_{412}) was determined and the formula used to calculate T-SH content is shown in Formula (6). -S-S- content was calculated according to the following formula:

$$\frac{-S-S-}{(\mu mol/g)} = \frac{T - \mathrm{SH} - F - \mathrm{SH}}{2} \quad (7)$$

2.4.4. Scanning electron microscopy (SEM)

Dried Qingke protein was coated with gold membrane by ion sputtering, then observed and photographed under a scanning electron microscope (JSM-7800F, Electronics Co. LTD, Tokyo, Japan).

2.5. Determination and analysis of Qingke protein gel properties

2.5.1. Viscosity scanning of protein suspensions

Characterization of the rheological viscosity of protein dispersions (200 g/L) treated with 40, 28/40, or 28/40/50 kHz was performed at 25 °C using an MCR302 Modular rotary and an interface rheometer (Anton Paar GmbH, Graz, Austria) fitted with a parallel plate (40 mm diameter) for a shear rate range of 0.1–100 s^{-1}. The data were fitted according to the power-law model.

2.5.2. Preparation of thermally induced gel

Protein dispersions (200 g/L) treated with 40, 28/40 or 28/40/50 kHz were stirred at 10 × *g* for 10 min then cooled to 25 °C after 30 min in a boiling water bath. Gels were stored at 4 °C for 24 h.

2.5.3. Gel rheological frequency scanning

A parallel plate (25 mm diameter) was selected, strain was set at 1% according to the strain scanning results, and the angular frequency range was 0.1–100 rad/s. Data were fitted according to the power-law model.

2.5.4. Determination of gel texture

TPA texture properties of protein gel (200 g/L) were determined using an FA-XT Plus texture analyzer (Stable Mycro System, Britain) with a P/0.5 probe. The detection conditions were as follows: 1.0 mm/s speed, 5 g triggering force, 50% pressure, and the probe was pressed twice in the process of obtaining each measurement.

2.5.5. Determination of gel intermolecular forces

Gel samples were dissolved in four solutions, namely, (S1) 0.05 mol/L NaCl, (S2) 0.6 mol/L NaCl, (S3) 0.6 mol/L NaCl + 1.5 mol/L urea, and (S4) 0.6 mol/L NaCl + 8 mol/L urea. 1 g of gel was mixed with 5 mL of S1, S2, S3, and S4, respectively, and homogenized for 1 min. After standing at 25 °C for 1 h, the supernatant was centrifuged at 2750 × *g* for 10 min. Protein concentration of the supernatant was determined via BCA Total Protein Kit. Solubility of the sample protein in S1, S2-S1, S3-S2, and S4-S3 were interpreted as the contributions of non-specific binding, ionic bonding, hydrogen bonding, and hydrophobic interactions to gel formation, respectively.

2.6. Statistical analysis

All assays were performed in triplicate and the data are expressed as mean ± standard error. The results were analyzed by one-way ANOVA at a significance level of $P < 0.05$ using SPASS 17.0 software (SPSS Inc., Chicago, Illinois, America), and figures were drawn using Origin 8.0 (Origin Lab Corporation, Northampton, Massachusetts, America) and PowerPoint 2019 (Microsoft, Redmond, Washington, America).

3. Results and discussion

3.1. Solubility

Solubility is a functional indicator of protein denaturation and an important tool used to assess potential commercial applications of food and beverage products[24]. The solubility of Qingke protein significantly increased following ultrasound treatment (Fig. 1). Specifically, Qingke protein solubility increased by 12.24% following single-frequency ultrasound treatment relative to the control. This result may be due to cavitation and/or physical effects (e.g., turbulence, instantaneous high pressure, and high shear force) caused by ultrasonic-induced non-covalent force destruction (e.g., hydrogen bonds, hydrophobic interactions) and induction of protein conformational changes[25]. Protein solubility increased by 35.37% and 43.54% relative to the control following dual- and tri-frequency ultrasound treatments, respectively. Therefore, multi-frequency ultrasound caused more dramatic effects on proteins compared to treatment with the single-frequency mode. Additionally, dual- or tri-frequency ultrasound treatment caused more perturbation, turbulence, and increased formation of cavitation bubbles and nuclei in the liquid medium compared with single-frequency. Moreover, the superposition effect of tri-frequency treatment was greater than that of dual-frequency. More effective mass transfer improved both cavitation activity and treatment effectiveness[13].

3.2. Foaming and emulsifying properties

The foaming properties of a protein are important for maintaining stability of the gas–liquid interface and are known to affect the quality of sugary food products like baked goods and ice cream mixes[26]. As shown in Fig. 2(A), ultrasonic treatment significantly improved the protein's surface hydrophobicity which led to enhanced foaming activity and stability. Compared with single-frequency treatment, dual- and tri-frequency ultrasound improved the foaming capacity and foaming stability of Qingke protein by 10.42% and 15.38% (28/40 kHz), and 20.83% and 20.51% (28/40/50 kHz), respectively. These results are likely due to the transport, penetration, and recombination of molecules at the gas–liquid interface, as these are the main processes involved in foam formation that are affected by surface hydrophobicity and structural flexibility. Additionally, multiple-frequency combined ultrasound also reduces the cavitation dead angle thereby increasing the cavitation effect and modifying the structure of proteins. Similar results related to ultrasound-modified soy protein isolate from Morales et al.[27] revealed that the foaming properties of soybean isolate protein improved after ultrasonic treatment. Further, Zhang and colleagues reported that ultrasound treatment induced partial protein denaturation and exposed hydrophobic regions which consequently enhanced interactions at the interface and resulted in formation of more stable bubbles[28].

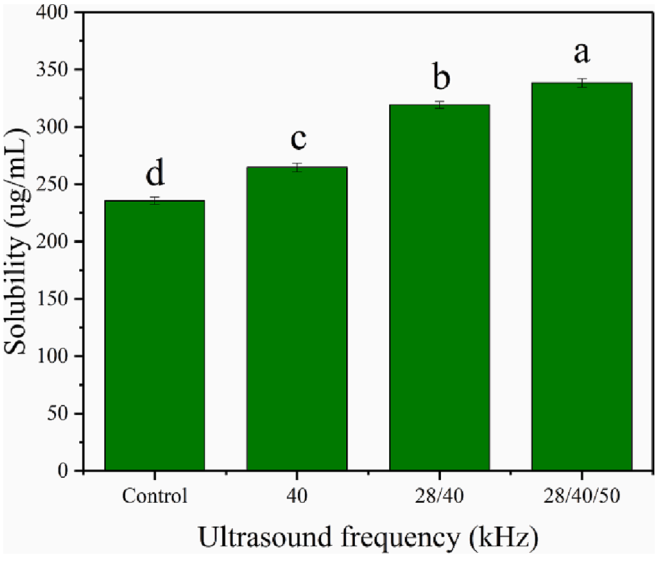

Fig. 1. Effect of ultrasound frequency on solubility of Qingke protein. (a-d represents a significant difference, $p < 0.05$).

EAI and ESI are critical values related to the utility and application of a specific protein as a food ingredient[29]. Fig. 2(B) conveys the influence of different ultrasound treatments on emulsifying activity and stability of Qingke protein. Notably, EAI increased by 5.42%, 12.82%, and 16.55%, and ESI increased by 17.43%, 27.09%, and 47.75% after treatment with single-, dual-, and tri-frequency ultrasound, respectively. The EAI and ESI of proteins may be highly susceptible to the resonance effect of cavitation bubbles, and the strong shearing forces may have given rise to partial high pressure and temperature which led to partial denaturation of tertiary and quaternary structures and the formation of a more disordered structure. In addition, exposed hydrophobic groups are more likely to adsorb lipids and interact with each other[30]. Ultimately, hydrophobicity and conformational stability are two major factors that impact emulsification[31].

3.3. Water/oil-holding capacity and surface hydrophobicity

Water- and oil-holding capacity (WHC and OHC, respectively) represent the ability of proteins to interact with water and oil, and they are closely related to the texture and flavor of products[32]. As shown in Fig. 3(A), the WHC and OHC of Qingke protein treated with single-frequency increased by 11.58% and 16.63%, respectively, as unconsolidated structures and exposed hydrophobic groups improved the ability of proteins to entrap water and oil[33]. Increased solubility could also explain the improved WHC and OHC of ultrasound-treated Qingke protein[34]. The results of synchronous dual- and tri-frequency treatments were similar to those of single-frequency albeit with stronger amplitudes of variation. Due to the superposition effect of synchronous ultrasound, tri-frequency treatment improved WHC and OHC the most, by 28.9% and 45.2%, respectively. The proportion of hydrophilicity and hydrophobicity was altered by physical modifications which increased protein activity and made the molecules more prone to expansion and dissociation. This in turn led to higher water and oil retention capabilities, which is consistent with results from a study on multi-frequency ultrasonic processing of gluten[15].

ANS binds to proteins in hydrophobic cavities formed by non-polar residues, therefore it is effectively used as a fluorescent probe to characterize a protein's surface hydrophobicity. Surface hydrophobicity of Qingke protein treated by single- or multi- frequency ultrasound was shown in Fig. 3(B). Compared with the control, Qingke protein treated with 28/40/50 kHz demonstrated the greatest enhancement. Additionally, the fluorescence intensity (FI) of samples treated with 28/40 kHz was significantly higher than that of samples treated with 40 kHz. In general, the physical and chemical effects induced by ultrasound play a key role in protein unfolding, resulting in the exposure of originally shielded hydrophobic groups to the external environment and an increase in the number and regions of surface hydrophobic groups. This increase in hydrophobicity contributes to the improvement of functional properties related to protein-solvent interactions[35], which was consistent with our solubility and water or oil retention results. Furthermore, surface hydrophobicity was reduced following ultrasound treatment primarily due to the extension of some denatured proteins.

3.4. Tertiary structure

3.4.1. UV–visible spectrum

UV–visible spectrum of the Qingke protein was shown in Fig. 4(A). Clear absorption peaks were observed in the near-ultraviolet region (250–300 nm) which were primarily attributed to the presence of chromophoric groups with aromatic side chains (e.g., phenylalanine

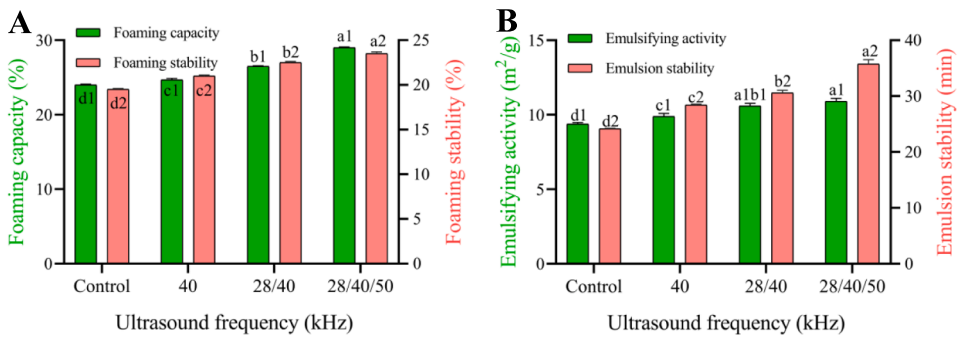

Fig. 2. Effect of ultrasound frequency on (A) foaming and (B) emulsifying properties of Qingke protein. (a-d represents a significant difference, $p < 0.05$).

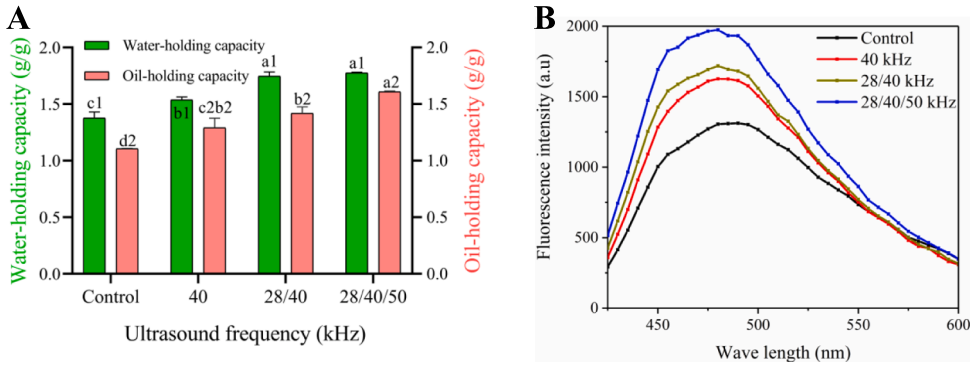

Fig. 3. Effect of ultrasound frequency on (A) water-holding capacity, oil-holding capacity and (B) surface hydrophobicity of Qingke protein. (a-d represents a significant difference, $p < 0.05$).

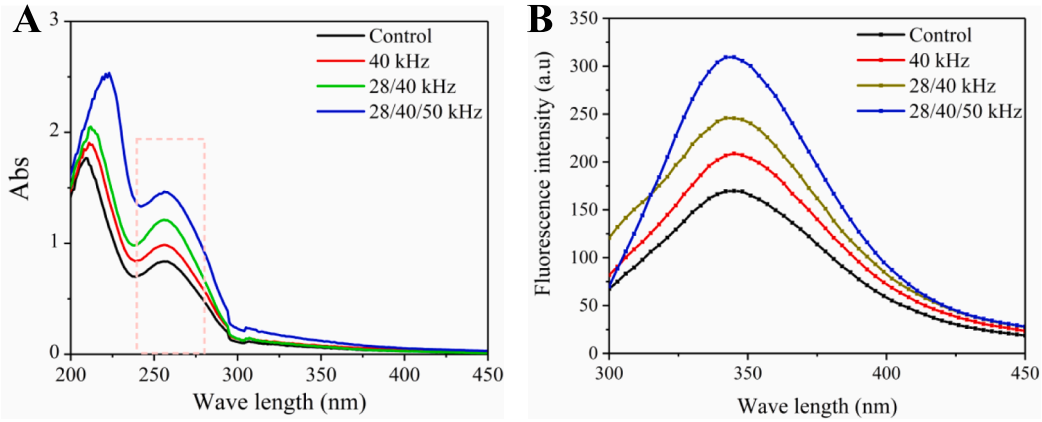

Fig. 4. Effect of ultrasound frequency on (A) UV–visible spectrum and (B) endogenous fluorescence spectrum of Qingke protein.

(257 nm), tyrosine (275 nm), and tryptophan (279 nm)). The increased intensity of this absorption peak in processed samples can be explained by ultrasonic treatment unfolding the protein and exposing chromophoric groups[36]. These results also revealed that the variation scopes for ultraviolet absorption peaks of samples treated with synchronous tri-frequency (28/40/50 kHz) were higher than those of samples treated with dual-frequency (28/40 kHz) or single frequency (40 kHz). This result indicated that coinstantaneous multi-frequency treatment can further influence the tertiary conformation of non-covalent bonds (e.g., hydrophobic and electrostatic interactions) and subsequently improve foaming and emulsification capabilities. The synergistic effect induced by amplitude superposition was primarily responsible for more intense cavitation and was more conducive to protein structure modification. Tri-frequency treatment resulted in alteration of the side-chain group distribution of aromatic residues[37]. This exacerbating transformation also caused larger changes in the functional features of samples treated with tri-frequency ultrasound. Spectra in the far-ultraviolet region (190–250 nm) can be used to characterize the peptide bond skeleton and electron transition frequency, as well as to detect the local secondary structure of proteins. Changes in the intensity and position of the absorption peak near 220 nm also reflect transformation of the secondary structure.

3.4.2. *Endogenous fluorescence spectrum*

Similar to the UV–visible spectrum, endogenous fluorescence spectra are mainly used to characterize the interactions of chromophoric groups in amino acid residues[38]. Among these residues, tryptophan is particularly sensitive to the environment and has the greatest influence

on the fluorescence spectrum. Therefore, an excitation wavelength of 280 nm was selected for our experiment, and changes to the tertiary structure were determined by detecting the fluorescence intensity of tryptophan. Fig. 4(B) illustrated that the peak locations did not move following ultrasonic treatment, as peaks were still present at 345 nm, whereas intensity of the treated samples was significantly higher than the control. Moreover, the tertiary conformation of modified protein molecules changed and more aromatic amino acids were exposed to the external environment, thus increasing the fluorescence intensity[39]. These changes were identical to previously observed changes in surface hydrophobicity, indicating that hydrophobic groups became more exposed due to cavitation and the mechanical effects generated by ultrasonic waves[40]. The fluorescence intensity of protein samples after tri-frequency ultrasonic treatment was higher than that of samples after dual- or single-frequency treatment, reaching a maximum value at 28/40/50 kHz. Afterall, the above results indicated that the conformational property of Qingke protein was changed to a certain extent after ultrasonic modification.

3.5. Secondary structure, Sulfhydryl, and disulfide bond analysis

Amide I peaks (1600–1700 cm^{-1}) were used to analyze the secondary structure transition of proteins, as the dipole coupling transition is sensitive to protein backbone structure[41]. By fitting the second derivative spectrum of amide I, peaks corresponding to β-sheets (1610–1640 cm^{-1}), random coils (1640–1650 cm^{-1}), α-helixes (1650–1660 cm^{-1}), and β-turns (1660–1670 cm^{-1}) were isolated; the area of each peak represented their content in the secondary structure (Fig. 5A)[42]. Among them, β-sheet content was the highest (45.21%), followed by β-turn (27.27%) and random coils (22.42%), while α -helix (5.09%) was the lowest for the control. After single-frequency (40 kHz) and dual-frequency (28/40 kHz) treatments, random coils increased by 3.88% and 8.57%, respectively, indicating that the ultrasound treatment reduced secondary structure order[43]. The secondary structure of protein treated with tri-frequency ultrasound (28/40/50 kHz) changed even more remarkably, with random coils increasing to 25.43 ± 0.43% and β-sheet decreasing to 42.04 ± 0.54%. In addition, the degradation of large aggregates, as well as the loosening and stretching of proteins, might underlie the increased incidence of random coils[11]. Therefore, the changed secondary structure of protein might improve the modified protein's properties such as emulsification and foaming ability.

Intermolecular and/or intramolecular SH changes are associated with foam formation. As shown in Fig. 5 (B), there were no significant-S-S- content differences between the control protein and proteins treated with ultrasound. However, there were increases in F-SH and T-SH among ultrasound-treated proteins relative to the control. After treatment with 40 kHz, 28/40 kHz, and 28/40/50 kHz, the F-SH content was 26.54 ± 0.22, 28.22 ± 0.28, 28.36 ± 0.03 μmol/g, respectively, which were 4.00%, 10.62%, and 11.13% higher than in the control. We speculate that tri-frequency ultrasound had a more intense effect due to enhanced cavitation, and that the increased F-SH might be related to surface sulfhydryl exposure. Additionally, the increase in F-SH and hydrophobicity indicates that sonication induced protein unfolding. Ultrasonic treatment often causes diameter reduction and due to the effects of turbulence, high pressure, and cavitation shear force, buried SHs may have been exposed which then contributed to the changes in solubility, WHC, and OHC[40]. Moreover, SH displayed an increasing trend, which could be accounted for by their gradual increased exposure with increasing frequency. This could be due to the fact that the stronger ultrasonic cavitation is associated with multiple frequency combinations, and tri-frequency ultrasound produced the greatest interference in a liquid medium and had a higher cavitation ratio, thereby damaging the surface of the liquid continuity and ultimately generating more cavitation bubbles.

3.6. SEM

The morphologies of native and modified Qingke protein were investigated by SEM which revealed that all of the proteins exhibited irregular shapes. As shown in Fig. 6(A), the structure of native Qingke protein particles was irregular with a rough surface, possibly due to erosion by chemical reagents like NaOH and HCl. We also found that the modified protein particles displayed a deformed structure. For instance, after 40 kHz ultrasound modification (Fig. 6B), a small number of irregular fragments were appeared on the surface of the proteins. Additionally, when the proteins were treated with dual-frequency, turbulence and microflow in fluid mass were heightened which led to the formation of a hole on the surface of these proteins (Fig. 6C). Due to a stronger cavitation effect, treated Qingke protein microstructures were loose, disorderly, and contained more holes and irregular fragments on the surface; this phenomenon was most prominent in tri-frequency treated protein (Fig. 6D). Evidently, turbulence and microflow in fluid mass increased at increasing frequencies and class numbers which in turn caused this phenomenon[44]. Moreover, bubble rupture caused by acoustic cavitation led to instantaneous high temperature and the formation of shear forces generated by microflow and shock wave, which

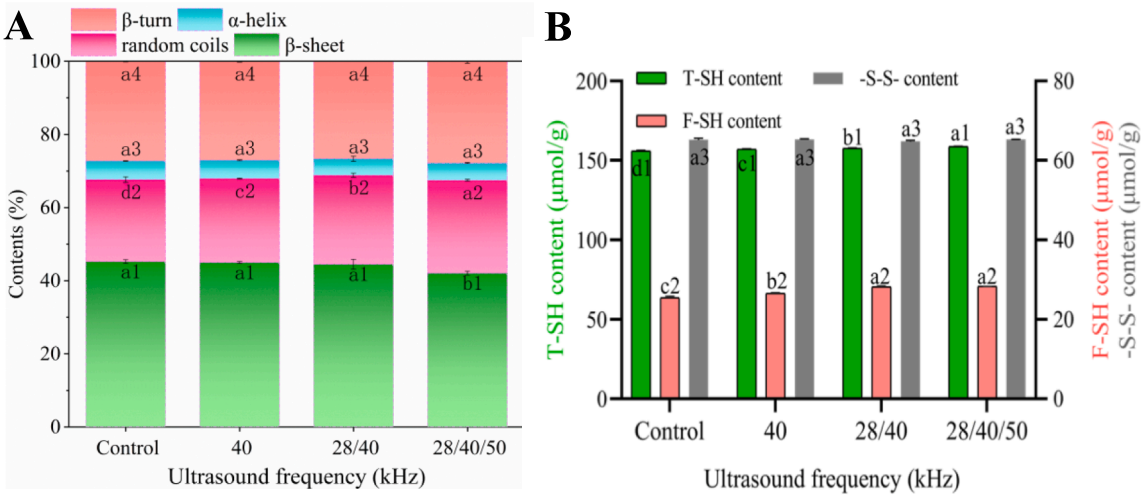

Fig. 5. Effect of ultrasound frequency on (A) secondary structure and (B) sulfhydryl and disulfide bonds of Qingke protein. (a-d represents a significant difference, $p < 0.05$).

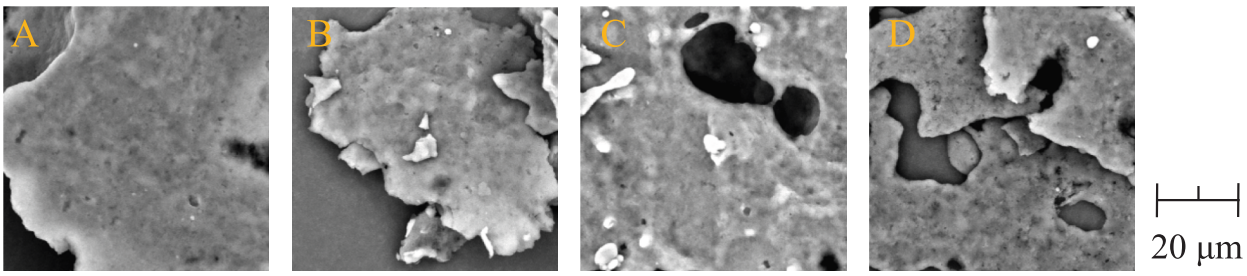

Fig. 6. SEM of Qingke protein, whereas A, B, C, D represented control, the modified Qingke protein treated by ultrasound frequency with 40 kHz, 28/40 kHz, 28/40/50 kHz, respectively.

destroyed the maintenance force between the peptide chains of Qingke protein[45].

3.7. Characteristics of ultrasound-modified thermal-induced Qingke protein gels

3.7.1. Viscosity scanning

The viscosity of pseudoplastic materials decreases with increasing shear rate, known as the shear thinning phenomenon. As shown in Fig. 7 (A), control and treated samples were all pseudoplastic fluids. As shear rate increased, samples became deformed and damaged resulting in decreased viscosity. After ultrasonic pretreatment, the apparent viscosity was markedly decreased, the consistency coefficient was reduced, and the flow characteristic index was significantly increased compared with the control. Specifically, the consistency coefficients after single-, dual-, and tri- frequency treatment decreased by 17.15%, 38.91%, and 48.70%, respectively. These results were attributed to the changed protein structure caused by acoustic cavitation which produces strong shear force and high-speed molecular motion[46]. In summary, ultrasound treatment reduced the expansion and interaction of some molecules leading to an increase in F-SH and surface hydrophobicity that, ultimately, improved solubility and fluid properties of Qingke protein.

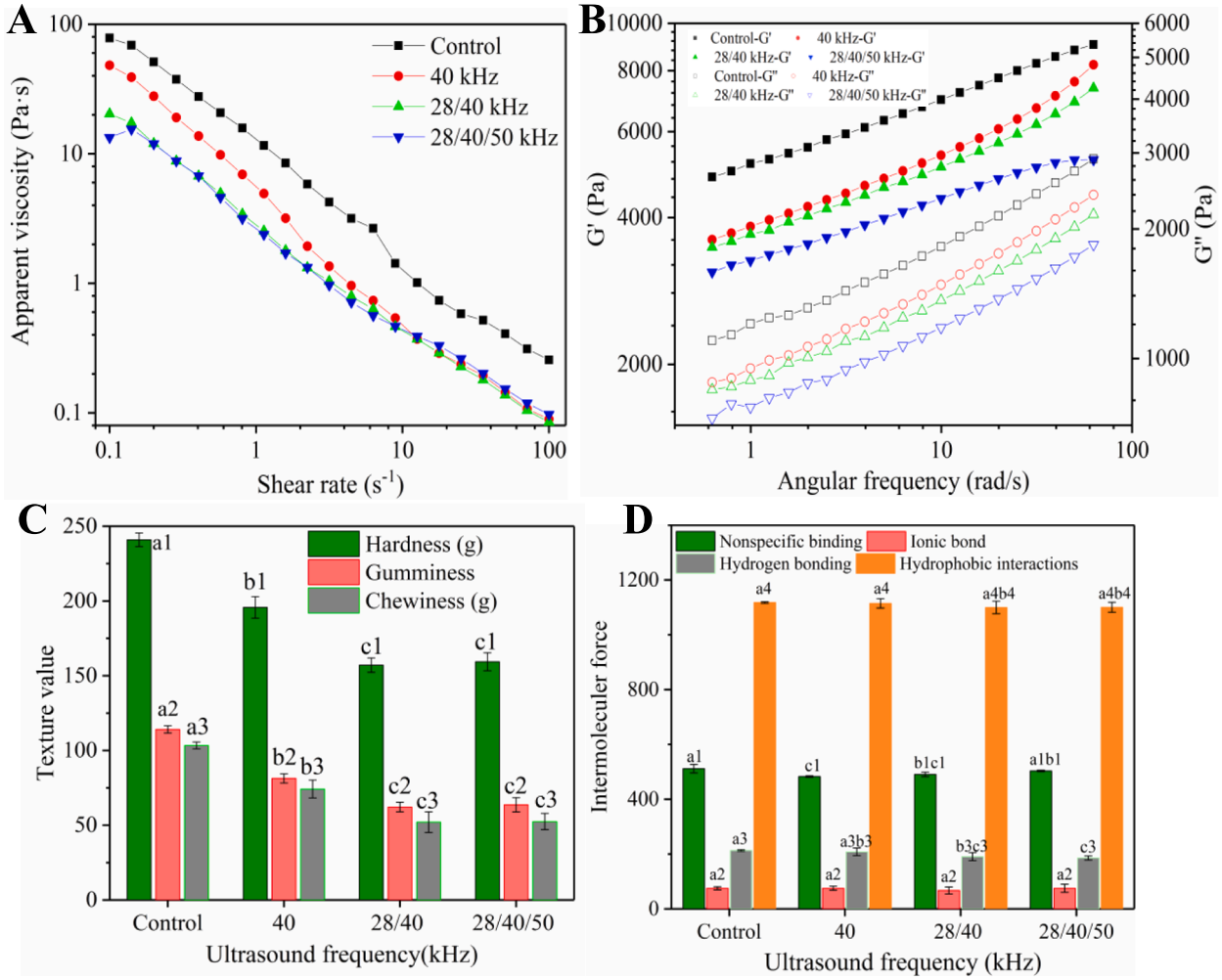

Fig. 7. Effect of ultrasound frequency on the rheological properties (A) viscosity scanning, (B) frequency scanning, and (C) texture property, (D) intermolecular forces of Qingke protein. (a-d represents a significant difference, $p < 0.05$).

3.7.2. Frequency scanning

Trends in G' and G'' values for all samples under strain were similar throughout testing, and specifically G' was higher than G'' indicating preferential elastic behavior. G' and G'' of protein samples all changed after ultrasonic treatment and only G' is discussed henceforth in the study. Following tri-frequency ultrasonic treatment, G' decreased significantly (Fig. 7B), indicating a weaker three-dimensional gel network. In addition, the overall trend of frequency increased suggesting enhanced flow performance due to decreased structural stability caused by denaturation. The physical force generated by high-intensity ultrasound destroys protein structures leading to protein denaturation. Due to cavitation, microfluidization, and protein chain expansion, molecules move more rapidly resulting in changes to flow behavior.

3.7.3. Texture properties

The hardness, gumminess, and chewiness of ultrasound-treated gels showed an overall decrease (Fig. 7C). In particular, hardness decreased by 18.76–34.79% in treated samples as compared to the control. Ultrasonic waves destroy non-covalent bonds, including hydrogen bonds, inhibiting formation of a robust three-dimensional network structures [28]. With an increase in the number of combined frequencies, gel texture properties changed more significantly, yet no significant differences were observed between dual- and tri-frequency treatments. Cao et al.[47] reported that, in myosin, hydrogen bonds and other non-covalent interactions were weakened by local overheating during microwave processing, which then led to partial protein denaturation and resulted in substandard gelling properties. Thus, protein denaturation and structural changes contribute to altered gel properties. In addition, correlational analyses of various structural and physicochemical properties of proteins were shown in Fig. 1S and indicated that F-SH content and water holding capability were the most significant factors affecting the properties of gelation.

3.7.4. Intermolecular forces

As shown in Fig. 7(D), hydrophobic interactions accounted for the largest proportion of intermolecular forces driving the formation of Qingke protein gel, followed by non-specific binding, hydrogen bonding, and ionic bonding. While we did not observe highly significant changes to hydrophobic interactions or ionic bonds following multiple-frequency ultrasound treatment, non-specific binding and hydrogen bonds decreased by 5.64% and 12.78%, respectively, compared with the untreated control. These results further suggest that sonication causes partial protein unfolding and weakens non-covalent interactions such as hydrogen bonding[27]. This weakening of hydrogen bonds inhibits protein aggregation, further explaining the poor gelation ability observed.

4. Conclusion

In this study, single-frequency, dual-frequency, and tri-frequency processing modes were carried out at 40 kHz, 28/40 kHz and 28/40/50 kHz for modifying the Qingke protein, respectively. We observed that multi-frequency ultrasonic treatment destroyed the higher-order structures and ultimately changed the functional properties of Qingke protein. Furthermore, the cavitation effect was ranked as follows: triple-frequency > dual-frequency > single-frequency. After treatment with multi-frequency ultrasound, proteins became unfolded, hydrophobic and chromophoric groups were exposed, hydrogen and non-covalent bonds were fractured, β-sheets decreased, and random coils increased. These internal variations led to improvements in solubility, water and oil holding, emulsification, and foaming properties. The characteristics of protein gels were also affected, indicated by decreases in G' and G'', hardness, gumminess, and chewiness. However, while the secondary and tertiary structures of the protein were destroyed. On the whole, ultrasound treatment is an effective method to modify Qingke protein, the changed physicochemical and structural properties could extend the future application of the protein in functional foods and nutraceuticals.

This manuscript has been thoroughly edited by a native English speaker from an editing company. An editing certificate will be provided upon request.

CRediT authorship contribution statement

Huijing Chen: Resources, Conceptualization, Investigation, Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. **Zehang Guo:** Conceptualization, Investigation, Data curation, Formal analysis, Methodology, Software, Writing – review & editing. **Zhirong Wang:** Investigation, Data curation, Formal analysis, Software, Writing – review & editing, Supervision. **Bing Yang:** Data curation, Investigation, Writing – review & editing. **Xuhui Chen:** Investigation, Data curation, Supervision, Writing – review & editing, Project administration. **Leyan Wen:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Qingqing Yang:** Investigation, Data curation, Methodology, Writing – review & editing. **Jianquan Kan:** Conceptualization, Resources, Methodology, Writing – review & editing, Funding acquisition, Project administration.

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.

Acknowledgements

This work was financially supported by the Fundamental Research Funds for the Chongqing Three Gorges follow-up research project—Yunyang noodle quality improvement and new product development (YYNY-2017-01).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2023.106338.

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