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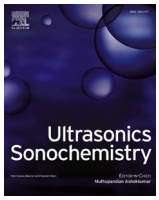

Effects of ultrasound on the structural and functional properties of sheep bone collagen

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ABSTRACT

The study evaluated the effect of an ultrasound-assisted treatment on the structural and functional properties of sheep bone collagen (SBC). The type and distribution of SBC were analyzed by proteome (shotgun) technology combined with liquid chromatography-tandem mass spectrometry. Compared with pepsin extraction, the ultrasound-assisted treatment significantly increased the collagen extraction rate by 17.4 pp ($P < 0.05$). The characteristic functional groups and structural integrity of collagen extracted by both methods were determined via Fourier transform infrared spectroscopy, ultraviolet absorption spectroscopy, and fluorescence spectroscopy. Circular dichroism spectra revealed that the ultrasound-assisted pretreatment reduced α-helix content by 1.6 pp, β-sheet content by 21.9 pp, and random coils content by 28.4 pp, whereas it increased β-turn content by 51.9 pp ($P < 0.05$), compared with pepsin extraction. Moreover, ultrasound-assisted treatment collagen had superior functional properties (e.g., solubility, water absorption, and oil absorption capacity) and foaming and emulsion properties, compared with pepsin extraction. Furthermore, the relative content of type I collagen in ultrasound-assisted extracted SBC was highest at 79.66%; only small proportions of type II, VI, X, and XI collagen were present. Peptide activity analysis showed that SBC had potential antioxidant activity, dipeptidyl peptidase 4 inhibitory activity, and angiotensin-converting enzyme inhibitory activity; it also had anticancer, antihypertensive, anti-inflammatory, and immunomodulatory effects.

1. Introduction

During slaughter, the meat industry produces large amounts of waste byproducts, such as bones, tendons, blood, internal organs, and skin [1]. These byproducts cannot be utilized effectively and are discarded, sometimes leading to environmental pollution. Challenges associated with population growth have prompted scientists to focus on the reprocessing of these commodities from a circular economy perspective [2].

In 2021, China's mutton meat output was 5.14 million tons, along with approximately 20 million tons of bones, which are rich in various nutrients (e.g., protein, minerals, vitamins, and mucopolysaccharides) [3]. Most sheep bones are processed into bone powderand bone mud, which are then added to animal feed or industrial raw materials. However, there have been few studies of the high-value processing and utilization of such products. >90% of the protein in sheep bone is collagen. With its excellent biocompatibility, biodegradability, low immunogenicity, biological activity and functional properties, collagen is widely used many industries [4].

Hot water extraction and chemical hydrolysis (by acids, bases, and/or salts) are conventional methods of collagen extraction [5]. Enzymatic hydrolysis has steadily taken the lead as the most popular extraction technique because of its mild reaction conditions, high extraction rate, limited side reaction products, and lack of effects on protein structure and activity [6]. Technologies such as high-voltage application, electrical pulses, microfiltration, and ultrasonication are used to assist conventional methods of collagen extraction [7]. The physicochemical properties of an ultrasound-assisted treatment and the cavitation force of ultrasonics presumably can improve the functional properties of protein [8,9], increase collagen yield, shorten extraction time, and alter

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its structural and functional properties [10,11]. Akram et al. [8] obtained chicken pectoral cartilage collagen by ultrasound-assisted enzymatic hydrolysis, and they discovered that the collagen yield, rheological characteristics, thermal stability, emulsification, and foaming properties had greatly improved. Ultrasonic technology combined with enzymatic hydrolysis has also been used to extract chicken bone protein [12], clown featherback (Chitala ornata) skin [13] and chicken lung collagen [14]. Here, sheep bone collagen (SBC) was prepared by ultrasound-assisted enzymatic hydrolysis, and its characterization, functional properties and collagen type were determined. This study will effectively improve the comprehensive utilization rate of sheep by-product resources and realize the high value of sheep by-product products.

2. Materials and methods

2.1. Materials and chemicals

Fresh sheep shoulder blades (5 Kg) were provided by Inner Mongolia Aofeili Food Co., Ltd. Pepsin (>1200 U/g) was obtained from Beijing Soleibo Technology Co., Ltd. All chemicals used for collagen extraction and analysis were analytical grade.

2.2. Preparation of defatted and decalcified sheep bone powder

Fresh sheep bones were washed under flowing water, and the meat and tendons were removed. After the bones had been softened by autoclaving for 40 min at 110 °C, they were repeatedly cleaned with hot water and dried at 80 °C for 12 h, then smashed into powder (80 mesh) [15]. This process yielded sheep bone powder.

Subsequently, the sheep bone powder was defatted and decalcified according to the method of Wu et al. [16]. Briefly, sheep bone powder was immersed in a solution of NaOH (0.1 M) at a ratio of 1:10 (w/v) and stirred for 24 h (the NaOH solution was replaced after 12 h of stirring); the residue was washed with distilled water, then drained. Then, ten volumes of 10% n-butanol solution were added to remove fat, and the mixture was stirred for 12 h (the n-butanol solution was replaced at 4 h intervals). The defatted sheep bone powder was repeatedly washed with distilled water, then decalcified with 0.25 M ethylenediamine tetraacetic acid disodium (pH 7.4) while stirring at 4 °C for 12 h. Then dried to obtain defatted and decalcified sheep bone powder.

2.3. Extraction of pepsin-soluble collagen from sheep bone powder (E-SBC)

According to the method of Akram & Zhang [17], collagen was extracted from sheep bone powder using a pepsin-based protocol, and this collagen was regarded as E-SBC. Briefly, defatted and decalcified sheep bone powder was immersed in 0.5 M acetic acid solution (1:10 powder: acetic acid, w/v), 4% (w/w) pepsin was added and the mixture was continuously stirred at 4 °C for 48 h (the acetic acid solution was replaced every 12 h). Subsequently, the solution was centrifuged at 10,000 r/min for 10 min at 4 °C. The pH of the supernatant was adjusted to 7 using a solution of 2 M NaOH, and NaCl was added to achieve a concentration of 0.9 M. The solution was left at room temperature for 8 h and followed by centrifugation at 8000 r/min for 15 min. The precipitate was dissolved in 0.5 M acetic acid, then placed into a dialysis bag (Mw 1200–1400, MD44-5 M, MYM, USA) and dialyzed with 20 volumes of 0.1 M acetic acid solution for 24 h (the acetic acid solution was replaced every 12 h). Then it was subsequently dialyzed with ultrapure water for 24 h. The samples were subjected to vacuum freeze-drying.

2.4. Ultrasound-treated pepsin-soluble collagen (UE-SBC)

Defatted and decalcified sheep bone powder (10 g) was soaked in an acetic acid solution and then placed in an ultrasonic bath for ultrasonication pretreatment. After ultrasonic pretreatment, the SBC was obtained using the enzymatic hydrolysis method described in Section 2.3, and this collagen was regarded as UE-SBC. The experiment was conducted with a fixed ultrasonication power of 480 W, ultrasonication time of 15 min, pepsin addition of 4%, acetic acid concentration of 0.5 M, and enzymatic hydrolysis time of 48 h. Ultrasonication powers of 320, 400, 480, 560, and 640 W; and ultrasonication times of 5, 10, 15, 20, and 25 min.

2.5. Determination of collagen extraction rate

2.5.1. Drawing the standard curve of hydroxyproline

A hydroxyproline standard was diluted to 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469, and 0.234 μg/ml with distilled water. A 60 μL standard solution was added to the same amount of chloramine T, thoroughly mixed, and incubated at 25±1 °C for 20 min. Then, the same amount of color-developing solution (60 μL) and 120 μL of water were added, mixed and submerged in a 60 °C water for 20 min. After removed and left for 15 min, and the light absorption value was detected at 560 nm. The standard curve was described by the equation $y = 0.0325x + 0.0077$ and had an R^2 value of 0.9985.

2.5.2. Determination of collagen extraction rate

In a digestion tube, 0.2 g of SBC powder and 2 mL of 6 M hydrochloric acid were mixed, and then heated at 100 °C for 3 h to facilitate hydrolysis. Next, the solution was adjusted to a pH of 6–8 with 10 M NaOH, and the supernatant's light absorption value was detected at 560 nm. The hydroxyproline concentration in the sample was calculated from the standard curve. The collagen content was calculated through multiplication of the hydroxyproline concentration a coefficient of 7.1. The collagen extraction rate was evaluated:

$$\textit{Collagen extraction rate}\ (\%) = \frac{X}{Y} \times 100\%$$

Where X is the quantity of collagen in the extracted solution after digestion, and Y is the quantity of collagen in the raw materials.

2.6. Characterization of collagen

2.6.1. UV absorption

The UV spectra of the sample was obtained using a TV-1810 UV–Vis spectrophotometer (Beijing Pu Analysis General Instrument Co., Ltd., Beijing, China). In accordance with the method of Caputo et al. [18], lyophilized collagen completely dissolved in 0.5 M glacial acetic acid (1:5, w/v). Spectrophotometry scanning was conducted at room temperature and at medium speed in the wavelength range of 190–400 nm.

2.6.2. Fluorescence spectrum

Fluorescence spectra were recorded using a FLS1000 fluorescence spectrophotometer (FLS, USA) at room temperature. SBC solution (0.6 mg/mL) was prepared with 0.5 M acetic acid. The solution was excited at 290 nm, emission was recorded over the range of 300–500 nm, and the constant slit for excitation and emission was 5 nm.

2.6.3. Fourier transform infrared spectroscopy

Lyophilized collagen powder and 200 mg of pure KBr were finely ground in a mortar. The FT-IR spectra were recorded using a FT-IR spectrometer Vertex 70 (Brook, Germany) at a scan range from 4000 to 500 cm^{-1}. Thirty-two background scans were conducted with a spectral resolution of 2 cm^{-1} at ambient temperature, using an attenuated total reflectance accessory[8].

2.6.4. CD spectra

The CD spectra of E-SBC and UE-SBC were recorded at 25 °C using a Chirascan V100 (UK Applied Photophysics Ltd., UK). All scans were performed from 190 to 400 nm with a scan rate of 100 nm/min and an

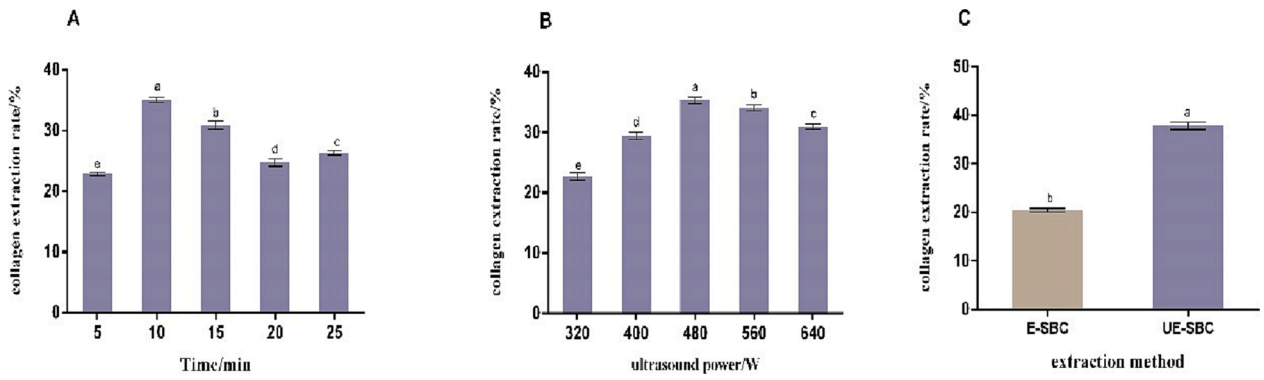

Fig. 1. Effect of ultrasonic-assist (UE) on pepsin-soluble (E) sheep bone collagen (SBC) extraction rate. (A) and (B) show the influences of ultrasonic power and time on collagen extraction rate of samples, respectively; (C) shows the difference between the E-SBC and UE-SBC. Different letters (a, b, c, …) indicate significant differences at $P < 0.05$.

interval of 0.5 nm [19].

2.7. Functional properties of collagen

2.7.1. Solubility

2.7.1.1. Influence of pH on collagen solubility. The solubility of collagen was executed on the base of the previous study [20]. SBC was dissolved in 0.5 M acetic acid solution to prepare a sheep bone collagen solution with a concentration of 3 mg/mL, and the solution was adjusted to 3–9 with 2 M NaOH and 2 M HCl; it was then diluted to 10 mL with distilled water and vortexed at room temperature for 5 min. Next, it was centrifuged at 8000 r/min for 15 min, and the supernatant was collected [21]. The soluble protein content in the supernatant was determined by the Lowry method.

$$Solubility\ (\%) = \frac{Supernatant\ protein\ content}{Total\ protein\ content\ in\ sample} \times 100\%$$

2.7.1.2. Salt solubility analysis. 10 mL of collagen solution (3 mg/mL) were mixed with NaCl to produce mass fractions of 1%, 2%, 3%, 4%, 5%, and 6%. The solution was vortexed at room temperature for 5 min, then centrifuged at 8000 r/min for 15 min. Measurement was conducted as described above.

2.7.2. Emulsifying and emulsifying stability

2.7.2.1. Effect of pH on emulsification and emulsion stability. A 5 mL solution of collagen (3 mg/mL) was prepared and its pH was adjusted to 3–9 with 1 M NaOH and 1 M HCl. The solution was mixed with soybean oil and homogenized at a speed of 8000 r/min for 2 min using an Ultra-fine homogenizer (HD-302, Taiwan, China). This mixture was then centrifuged at 1500 r/min for 5 min. The same homogenized 20 mL sample was heated in a 50 °C water bath for 1 h, and centrifuged at 1500 r/min for 15 min.

$$Emulsification\ (\%) = \frac{V_1}{V_0} \times 100\%$$

Where V_1 is the volume of the emulsified layer (mL), and V_0 is the total volume of the mixture (20 mL).

$$Emulsion\ stability\ (\%) = \frac{V_2}{V_1} \times 100\%$$

Where V_2 is the volume of the emulsified layer after heating (mL), and V_1 is the volume of the original emulsion layer (mL).

2.7.2.2. Effect of NaCl concentration on emulsification and emulsion stability. Sodium chloride was added to a collagen solution (3 mg/mL) to produce mass fractions of 1%, 2%, 3%, 4%, 5%, and 6%; then, it was evenly mixed with 10 mL of soybean oil. Subsequently, the solution was homogenized for 2 min with a super high-speed stirring homogenizer at 8000 r/min. The emulsion stabilities of the samples were determined as described above.

2.7.3. Oil absorption capacity (OAC)

OAC was determined in accordance with the method of Tomotake et al. [22] with some modifications. Briefly, SBC sample (0.1 g) was placed into a 15 mL centrifuge tube, combined with 5 mL of soybean oil, and vortexed for 2 min to ensure thorough mixing. The centrifuge tubes were individually placed in a water bath at 20–60 °C for 30 min, followed by centrifugation at 8000 r/min for 15 min.

$$OAC\ (g/g) = \frac{(m_2 - m_1)}{m}$$

Where m is the mass of collagen (g), m_1 is the total weight of collagen and centrifuge tube before oil absorption (g), and m_2 is the total weight of collagen and centrifuge tube after oil absorption (g).

2.7.4. Water absorption capacity (WAC)

A 100 mg sample of freeze-dried collagen was placed in a constant temperature and humidity cabinet. The temperature was adjusted to 20 °C, and the relative humidity was maintained at 80%. The sample was weighed at 0, 1, 2, 4, 6, 8, 10, 12, 24, and 48 h. The absorption of moisture by collagen was determined using the following equation:

$$WAC\ (\%) = \frac{m_t - m_0}{m_0} \times 100\%$$

Where m_t is the sample mass after t hours (g) and m_0 is the sample mass at the beginning of the test (g).

2.8. LC-MS/MS analysis

An aliquot was removed from each collagen sample (UE-SBC) for chromatographic separation using a nanoliter flow rate Easy nLC 1200 chromatography system (Easy-nLC1200, Thermo Fisher Scientific). The samples were then passed through a chromatographic analysis column (Thermo scientific EASY column (Reverse-phase), 75 μm × 150 mm (3 μm, C18), Dr. Maisch GmbH) for gradient separation. The peptides were separated and analyzed by data-dependent acquisition mass spectrometry using a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Q-Exactive Plus). Peptide secondary mass spectra was acquired under the following conditions: secondary mass spectra of the 20 highest intensity parent ions (MS2 scan) triggered after each full scan, secondary mass resolution: 17,500 @ *m/z* 200, AGC target: 2e5, secondary maximum IT: 60 ms, MS2 activation type: HCD, isolation window: 1.6 *m/z*, and normalized collision energy: 30. MS data were

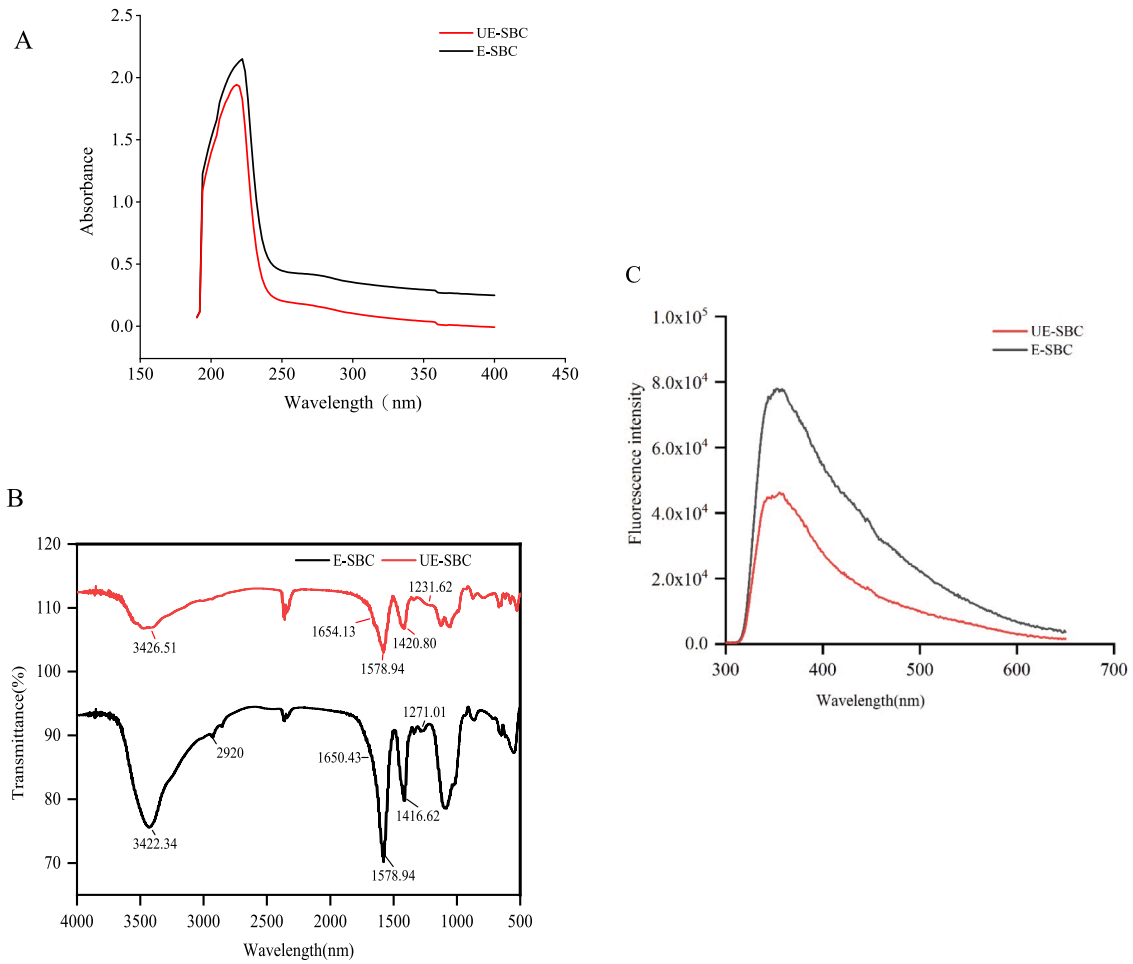

Fig. 2. UV–visible (A), FTIR (B) and Fluor spectra (C) of collagen samples extracted from sheep bone: collagen extracted with the pepsin method, E-SBC; collagen extracted by the ultrasonic-assist pepsin method, UE-SBC.

searched against the uniprot-Ovis aries (Sheep) [9940]-78314 (78314 total entries, downloaded 12/2021). Evaluation of bioactive peptides in protein sequences in the BIOPEPUWM (https://www.uwm.edu.pl/biochemia) database.

2.9. Statistical analysis

All experiments were repeated three times. The results are presented as means and standard deviations. Statistical analysis was conducted using the statistical package SPSS 20.0 (SPSS Inc., Chicago, IL, USA) for analyses of variance. Duncan's test was used to identify significant differences between means. *P*-values < 0.05 were considered statistically significant. Figures were constructed with Origin 8.0 software and GraphPad Prism 6.01 software.

3. Results and discussion

3.1. Extraction rate of collagen

With increasing ultrasonic treatment time, the rate of collagen extraction gradually increased, reaching the highest yield (35±0.44%) at 10 min, and then exhibited a decreasing trend (Fig. 1(B)). Ali et al. [23] found that the rate of protein extraction was directly affected by the duration of ultrasonication, and appropriate ultrasonication improved the protein extraction yield. An excessive duration of ultrasonication led to a significant decrease in collagen yield, presumably because increased ultrasonic cavitation caused exposure or destruction of active sites on the protein surface, thereby hindering further enzymatic hydrolysis [12]. Choi, et al. [24] also suggested that the duration of ultrasound-assisted treatment influenced the protein extraction yield of silkworm pupae, with the highest yields at 5 min. Additionally, the mechanical and thermal effects of ultrasonication can cause collagen denaturation and degradation, leading to reduced yield. These effects were also observed by Tu et al. [25]. Therefore, an ultrasonication time of 10 min was used in the present study.

According to Fig. 1(A), when the ultrasonication power in the pretreatment was 480 W, the rate of collagen extraction reached a maximum of 35.29±0.54%. The increase in ultrasonication power led to increased ultrasonic cavitation, which aided dissolution of collagen in the substrate; this dissolution facilitated interactions with pepsin in the solution, thereby improving the rate of collagen extraction [23]. Carcel et al. [2] reported that ultrasonication could release content by destroying cell walls and activating immobilized enzymes. When the ultrasonication power exceeded 480 W, the rate of collagen extraction decreased with additional ultrasonication power. This phenomenon presumably occurred because excessive ultrasonication power led to increased mechanical and thermal effects, which destroyed the molecular structure of collagen and reduced the extraction rate. Therefore, in this study, an ultrasonic power of 480 W was used to extract collagen from sheep bone.

Fig. 1(C) showed that the collagen extraction rate reached 37.9 ±0.78% after ultrasound-assisted treatment, an increase of 17.4 pp over pepsin extraction ($P < 0.05$). Ultrasound increases the kinetic energy of the particles through the impact effect, resulting in higher collagen

Table 1
Effect of ultrasonic pretreatment on the secondary structure of sheep bone collagen.

Samples	α-helix (%)	β-sheet (%)	β-turn (%)	Random (%)
UE-SBC	0.5±0.06^{b}	15.7±0.08^{b}	72.2±0.20^{a}	11.6±0.17^{b}
E-SBC	2.11±0.11^{a}	37.6±0.13^{a}	20.3±0.15^{b}	40±0.16^{a}

Different superscript letters within column are significantly different ($p < 0.05$).

yields.

3.2. Characterization of collagen

3.2.1. UV spectra

The peptide chains and side chains of collagen are the main components involved in UV absorption. The characteristic absorption peak of mammalian type I collagen is located at 218 nm [26]. As shown in Fig. 2(A), the maximum absorption peaks of E-SBC and UE-SBC at 222 and 218 nm were associated with the presence of C–O, –COOH, and CO–NH_2 in collagen polypeptide chains [27], these findings matched the UV absorption properties of the type I collagen triple helix structure [28]. There was no obvious absorption peak at 280 nm, implying that the concentrations of aromatic amino acids in SBC were extremely low. Similar phenomena have been identified in collagen from squid [29], loach [30], and Ujumuqin sheep [31].

3.2.2. FTIR spectra

FTIR analysis revealed some chemical characteristics of the collagen extracts. As shown in Fig. 2(B), the collagen absorption intensities differed between the two extraction methods, with a slight change in the position of the absorption peak. According to Andrews et al. [32], amide A consists of an N–H stretching vibration that usually occured in the range of 3440–3400 cm^{-1}, which is the characteristic absorption peak of a protein. The N–H stretching vibrations of E-SBC and UE-SBC occurred at 3422.34 and 3426.51 cm^{-1}, respectively; both exhibited a high peak intensity and were consistent with findings by Ju et al. [33]. The amide B band was mainly caused by the asymmetric stretching vibration of the –CH_2 group. The maximum absorption peaks of the amide B band appeared at 2920 cm^{-1}. The absorption peak of the amide I band, in the range of 1600–1700 cm^{-1}, was mainly related to the C = O stretching vibration. The amide I peak of E-SBC and UE-SBC were located at 1654.13 cm^{-1} and 1650 cm^{-1}, respectively. The amide II bands of E-SBC and UE-SBC exhibited an absorption peak of 1578.94 cm^{-1}, which was related to the C–N tensile vibration and N–H bending vibration (within the range of 1550–1600 cm^{-1}) [34]. Amide III signals indicative of N–H bending and C–N stretching were observed in the characteristic range of 1200–1350 cm^{-1}. The amide III bands were modified according to whether collagen maintained an intact triple helix structure; –CH_2 was the characteristic vibrational peak of glycine and proline residues [35]. The absorption peaks of E-SBC and UE-SBC were at 1271.01 cm^{-1} and 1231.62 cm^{-1}, respectively; accordingly, collagen extracted by each of the two methods generally had a complete triple helix structure.

3.2.3. Fluorescence spectrum

The fluorescence spectra of a protein are modified when the protein undergoes conformational changes. Compared with the results of enzymatic hydrolysis, the maximum fluorescence intensity wavelength of SBC extracted by ultrasound-assisted enzymatic hydrolysis increased from 353 to 355 nm (Fig. 2(C)). The fluorescence intensity of sheep bone collagen significantly decreased after ultrasound-assisted treatment because the cavitation and mechanical effects of ultrasonic waves unfolded the collagen structure and exposed additional color-emitting groups to the solvent, resulting in a fluorescence burst. Moreover, ultrasound treatment leads to changes in the state of protein aggregation and the local environment of these populations is affected, leading to a decrease in fluorescence intensity. Sonication also reportedly decreases the fluorescence emission intensities of soy proteins [36] and walnut proteins [37].

3.2.4. Secondary structure analysis

CD is an fantastic instrument to rapidly study the secondary protein structures. As shown in Table 1, compared with E-SBC, the ultrasound-assisted treatment significantly ($P < 0.05$) decreased the amounts of α-helices content by 1.6 pp, β-sheets content by 21.9 pp, and random coils content by 28.4 pp, while increasing the amount of β-turns by 51.9 pp. These findings suggested that ultrasonication caused a structural change in proteins where α-helices were stabilized by intrapeptide hydrogen bonds, whereas β-sheets were stabilized by interpeptide hydrogen bonds [38]. The decrease in α-helix content indicates a decrease in the number of hydrogen bonds between protein molecules [39]. This phenomenon presumably occurred because the ultrasound-assisted treatment unfolded the protein molecular chain by disrupting intermolecule bonds, disulfide bonds, and intramolecule hydrogen bonds; these changes led to the disruption of the ordered structures of α-helices and β-sheets, along with a conversion to β-turns. Downstream effects include altered protein conformation, enhanced protein flexibility, and looser protein packing [40,41]. Similar results were reported by Wang et al. [40]. However, Huang et al. [39] reported increases in the

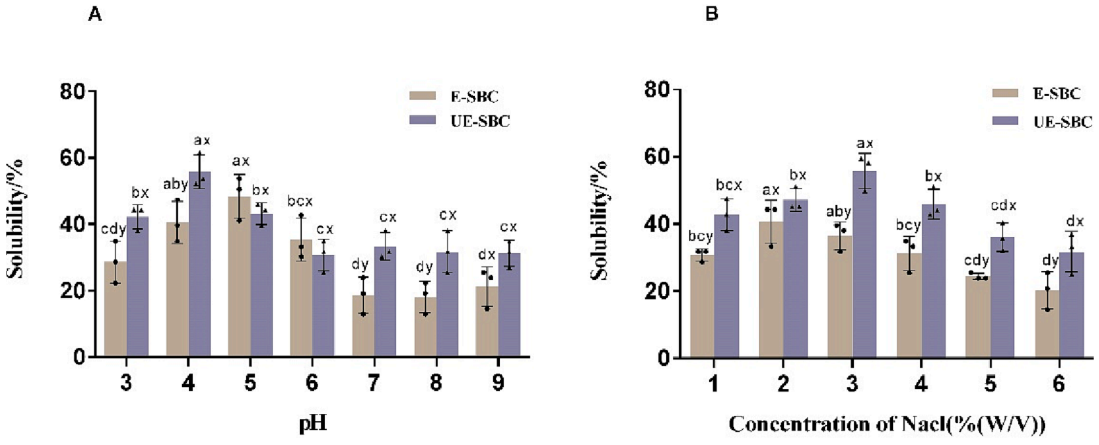

Fig. 3. pH (A) and nacl concentrations (B) dependent solubility of collagen samples extracted from sheep bone: collagen extracted with the pepsin method, e-sbc; collagen extra collagen extracted by the ultrasonic-assist pepsin method, UE-SBC. a-e indicates that the same extraction method has significant difference under different influence factors ($P < 0.05$), x-y indicates that the collagen extracted by different methods (E-SBC and UE-SBC) has significant difference under the same influence factors ($P < 0.05$).

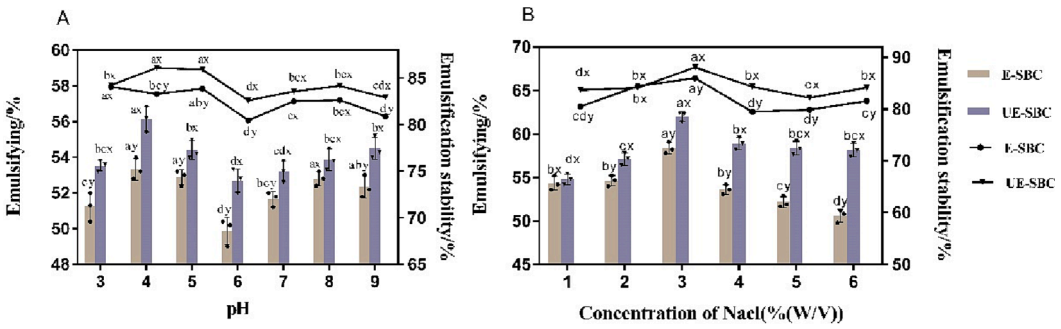

Fig. 4. Emulsifying and emulsifying stability of collagen extracted by pepsin (E-SBC) and collagen extracted by ultrasonic assisted pepsin (UE-SBC) at different pH (A) and NaCl concentrations (B). a-e indicates that the same extraction method has significant difference under different influence factors ($P < 0.05$), x-y indicates that the collagen extracted by different methods (E-SBC and UE-SBC) has significant difference under the same influence factors ($P < 0.05$).

amounts of α-helices and random coils, along with decreases in the amounts of β-sheets, in soybean protein aggregates after ultrasound-assisted treatment. These inconsistent findings suggested that, depending on the treatment variables, ultrasonication may have various effects on secondary structure.

3.3. The functional properties of SBC

3.3.1. Role of pH in solubility

Solubility is an important property of many industrial proteins [42]. The solubilities of E-SBC and UE-SBC in the pH range of 3–9 are shown in Fig. 3(A). Generally, collagen had the best solubility at a low pH [43]. Both extracted E-SBC and UE-SBC exhibited higher solubility under acidic conditions (i.e., pH of 3–5). The solubilities of UE-SBC and E-SBC were highest at pH 4 and 5, respectively. Because the solution pH tended to be neutral, the solubility of SBC began to decrease sharply as pH increased. The solubilities of UE-SBC and E-SBC were lowest at pH 6 and 7, respectively, and then remained stable with further increases in pH. These findings indicated that the isoelectric points of UE-SBC and E-SBC may be near pH 6 and 7, respectively. The mechanical and cavitation effects generated by ultrasonication presumably change the particle size and hydrogen bond composition of SBC, thereby affecting the size of the isoelectric point. Similar results were observed in a study of collagen from sea cucumber, which exhibited the lowest solubility at pH 6 [44]. When the pH of the solution was near the isoelectric point of collagen, the net charge of collagen was nearly zero, and electrostatic repulsion was minimal [21]. In those conditions, the collagen molecules aggregated and the amount of dissolved collagen decreased, leading to reduced solubility. Compared to E-SBC, the solubility of UE-SBC was generally greater in the pH range of 3 to 9, potentially because of decreases in crosslinking, bonding, and particle size in collagens extracted by ultrasound-assisted treatments [45]. Similarly, Zou et al. [46] reported that ultrasound-assisted treatment increased the relative solubility of acid-solubilized collagen from soft-shelled turtles.

3.3.2. Role of salt in solubility

As shown in Fig. 3(B), the solubility of SBC was significantly affected by the NaCl concentration. At NaCl concentrations of 3% and 2%, the solubility of UE-SBC and E-SBC were highest (55.79±5.15% and 40.62 ±6.35%, respectively). When the NaCl concentration was low, the salt ions could fully contact with collagen molecules; this led to an increased number of positive charges on the surface of collagen molecules and greater repulsive forces between molecules, which hindered aggregation and improved solubility. As the NaCl concentration continued to increase, the solubilities of SBC extracted by each of the two methods exhibited a decreasing trend. This trend reflected a “salting-out” phenomenon at higher NaCl concentrations, whereby hydrophobic sites were exposed to salt ions because the hydration shell was lost from the collagen surface and hydrophobic interactions among protein chains were increased, resulting in collagen precipitation [47]. Yang et al. [48] also reported that soft-shelled turtle collagen generally had higher solubilities under acidic conditions and lower solubilities at high NaCl concentrations. These results were related to differences in collagen characteristics and molecular properties [49].

On the whole, the ultrasound-assisted treatment improved the solubility of SBC, possibly because the conformation of UE-SBC changed to allow more hydrophilic amino acid residues to interact with the surrounding solution; thus, the initially insoluble protein aggregates became soluble [50].

3.3.3. Effect of pH on the emulsifying capacity

Emulsification is the ability of a protein to combine oil and water, creating a uniform dispersion system. Emulsion stability refers to the capacity of the emulsion to remain stable without separating into two phases. The effect of pH on collagen emulsification was investigated by modifying the pH of the solution. Fig. 4(A) shows that emulsification and emulsion stability were generally greater in UE-SBC than in E-SBC ($P < 0.05$). The emulsification and emulsion stability of UE-SBC were highest at pH 4. These findings may be the result of increased exposure of hydrophobic groups in collagen molecules after ultrasonication, which led to lose structures that more easily combined with lipids. Additionally, the sizes of dispersed protein particles were reduced by ultrasound-assisted treatment, producing greater protein adsorption capacity on oil droplets and improving emulsification performance. The emulsion stability and emulsification of UE-SBC and E-SBC were lowest at pH 6, consistent with the effect of pH on the solubility of SBC. pH 6 is presumably the isoelectric point of SBC. Near the isoelectric point, the net surface charge of collagen molecules at the oil–water interface is nearly zero, which does not promote emulsification; therefore, emulsification performance is lowest at this point. Additionally, the repulsive forces between collagen molecules are weak near the isoelectric point, thereby facilitating collagen aggregation and hindering emulsion stability. The SBC obtained by each of the two treatments had good emulsification under both acidic and alkaline conditions. Both acidic and alkaline environments can expose lipophilic functional groups in collagen, thereby improving its emulsification. Notably, Klompong et al. [51] reported that, under highly acidic or alkaline conditions, proteins had greater solubility and peptide chains easily moved to the oil–water interface, resulting in greater emulsification.

3.3.4. Effect of NaCl on the emulsifying capacity

Ultrasound affected emulsification performance by altering the surface hydrophobicity and flexibility of collagen molecules. Fig. 4(B) shows that as the NaCl concentration increased from 1% to 3%, the emulsification of UE-SBC and E-SBC continuously increased; they were highest at an NaCl concentration of 3%. Emulsification significantly decreased when the NaCl concentration was >3%, presumably because the increase in the NaCl concentration reduced the repulsive forces

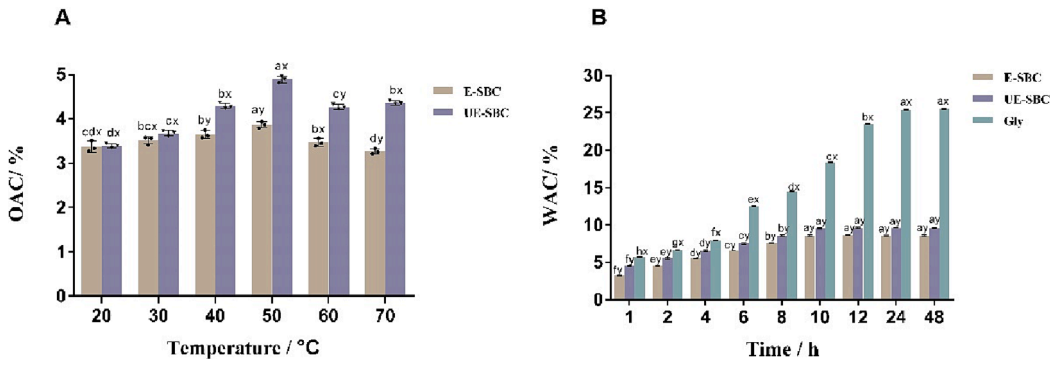

Fig. 5. Influences of temperature on oil absorption capacity (A) and water absorption capacity (B) of collagen extracted by pepsin (E-SBC) and collagen extracted by ultrasonic assisted pepsin (UE-SBC). a-e indicates that the same extraction method has significant difference under different influence factors ($P < 0.05$), x-y indicates that the collagen extracted by different methods (E-SBC and UE-SBC) or using glycerol has significant difference under the same influence factors ($P < 0.05$).

between collagen molecules. When a threshold concentration was reached, the diffuse double electrical layer was compressed by salt ions, which reduced the surface potential of emulsion droplets. Collagen aggregation and precipitation then occurred easily, reducing emulsification performance [52]. The emulsification performance of UE-SBC was higher than the emulsification performance of E-SBC at NaCl concentrations of 3–6%. Ultrasonic techniques have been effectively used in study concerning the emulsification and emulsion stability of lentil protein [53], tuna collagen [54], rainbow trout collagen [55]. Overall, ultrasound-assisted treatment improves protein emulsification.

3.3.5. Oil absorption capacity

OAC is an important property that influences the flavor and texture of the products [56]. Fig. 5(A) shows that over the temperature range of 20–50 °C, the OAC of collagen gradually increased. UE-SBC and E-SBC had the highest OAC values at 50 °C: 4.89±0.075 and 3.87±0.075 g/g, respectively. Further increases in temperature led to reductions in OAC, possibly because of protein denaturation. Initially, increases in temperature led to unfolding of protein structure and exposure of non-polar groups, thereby improving OAC. Further increases in temperature leads to increased protein denaturation and eventual precipitation, reducing OAC [12]. Overall, the OAC of UE-SBC was higher than that of E-SBC. The ultrasound-assisted treatment exposes a large number of non-polar groups on the molecular surface, creating a larger surface area for oil adsorption.

3.3.6. Water absorption capacity

WAC is a major functional property of collagens and their products. As shown in Fig. 5(B), with increasing time, the WAC of UE-SBC and E-SBC increased significantly at 1–10 h ($P < 0.05$) and then stabilized at 10–48 h ($P > 0.05$). At 48 h, the WAC of UE-SBC, E-SBC and glycerol were 9.58±0.08%, 8.57±0.08%, and 25.51±0.03%, respectively. Under the same conditions, the WAC of UE-SBC was numerically higher than that of E-SBC and statistically lower than that of glycerol. The glycerin molecule has three hydroxyl groups and contains more hydrophilic groups, leading to superior hygroscopicity. Collagen has a stable triple helix structure, with a small amount of exposed hydrophilic groups, so its has weaker moisture absorption.

3.4. Sheep bone collagen identification

SBC was identified by LC-MS/MS mass spectrometry; a protein false discovery rate ≤0.01 and peptide-to-spectrum match false discovery rate ≤0.01 were used as screening criteria for protein and peptide identification using the MaxQuant 1.6.1.0 mass spectrometry database. In total, 93 proteins were identified, among which there were six collagen subunits: collagen type I (alpha 1 and 2 chains), collagen type II (alpha 1 chain), collagen V type (alpha 2 chain), collagen type IX (alpha 3 chain), and collagen type XI (alpha 1 chain) (Table 2). The relative content of type I collagen was highest (79.66%), followed by the relative content of type V collagen (0.24%); thus, SBC was dominated by type I collagen. In total, 141 peptides were identified, including eight with antioxidant activity, 18 with dipeptidyl peptidase 4 inhibitory activity, 87 with angiotensin-converting enzyme inhibitory activity, 4 with anticancer activity, 1 with antihypertensive activity, 4 with anti-inflammatory activity, and 1 with immunomodulatory activity. These peptides yielded 21 peptide chains from the collagen type I α1 chain, 16 peptide chains from the collagen type I α2 chain, 4 peptide chains from the collagen type V α2 chain, 1 peptide chain from the collagen type XI α1 chain, 1 peptide chain from the collagen type II α1 chain, and 1peptide chain from the collagen type IX α3 chain. In summary, sheep bone collagen is mainly type I collagen, which has the potential for diverse biological activities and can be used for collagen peptide-focused research and development.

4. Conclusion

Ultrasound-assisted treatment improved the rate of SBC extraction. It also improved characteristics of SBC, resulting in better solubility, oil absorption, emulsification, and emulsion stability; however, it did not significantly improve moisture absorption. SBC extracted by each of the two methods maintained its structural integrity. The ultrasound-assisted

Table 2
Identification results of sheep bone collagen.

Protein names	Gene names	Number of proteins	Peptides	Mol. weight [kDa]	Sequence length	Score	Sequence coverage %	Relative content %
Collagen type I alpha 1 chain	COL1A1	6	21	139	1463	323.31	12.8	54.55%
Collagen type I alpha 2 chain	COL1A2	3	16	128.97	1364	299.12	10.4	25.11%
collagen alpha-2 chain	COL5A2	3	4	144.96	1499	66.396	2.6	0.24%
collagen alpha-1 chain isoform X2	COL11A1	4	1	176.57	1766	5.8188	0.7	0.03%
collagen alpha-3 chain	COL9A3	2	1	63.725	682	5.8149	0.9	0.00%
collagen alpha-1 chain	COL2A1	3	1	134.4	1418	41.102	0.6	0.05%

treatment resulted in decreased amounts of α-helices, β-sheets, and random coils, along with an increased amount of β-turns. LC-MS/MS identified different types of collagen; type I was the dominant type, followed by types II, VI, X, and XI. SBC has various potential biological activities, with broad application prospects and potentially high value. Therefore, ultrasound-assisted pretreatment is an effective method for extraction of sheep bone collagen, with good process efficiency and the potential for practical application.

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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