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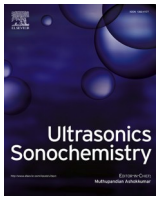

Ultrasound-assisted extraction of collagen from broiler chicken trachea and its biochemical characterization

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ABSTRACT

Broiler chicken tracheas are a co-product from chicken slaughterhouses which are normally turned into low value animal feed despite their high levels of collagen. Typical collagen extraction by acid and/or pepsin usually results in relatively low yield. Ultrasound-assisted extraction (UAE) could be a means to improve collagen yield. The objectives of this study were to investigate the effects of ultrasonic parameters on the yield and biochemical properties of trachea collagen (TC). Conventional extraction using acetic acid and pepsin for 48 h resulted in acid-soluble (AS) and pepsin-soluble (PS) collagen with a yield of 0.65% and 3.10%, respectively. When an ultrasound with an intensity of 17.46 $W{\cdot}cm^{-2}$ was applied for 20 min, followed by acid extraction for 42 h (U-AS), the collagen yield increased to 1.58%. A yield of 6.28% was obtained when the ultrasound treatment was followed by pepsin for 36 h (U-PS). PS and U-PS contained collagen of 82.84% and 85.70%, respectively. Scanning electron microscopy images revealed that the ultrasound did not affect the collagen microstructure. All collagen samples showed an obvious triple helix structure as measured by circular dichroism spectroscopy. Fourier transform infrared spectroscopy indicated that the ultrasound did not disturb the secondary structure of the protein in which approximately 30% of the α-helix content was a major structure for all collagen samples. Micro-differential scanning calorimetry demonstrated that the denaturation temperature of collagen in the presence of deionized water was higher than collagen solubilized in 0.5 M acetic acid, regardless of the extraction method. All collagen comprised of α_1 and α_2-units with molecular weights of approximately 135 and 116 kDa, respectively, corresponding to the type I characteristic. PS and U-PS collagen possessed higher imino acids than their AS and U-AS counterparts. Based on LC-MS/MS peptide mapping, PS and U-PS collagen showed a high similarity to type I collagen. These results suggest that chicken tracheas are an alternative source of type I collagen. UAE is a promising technique that could increase collagen yield without damaging its structure.

1. Introduction

Broiler chicken (*Gallus gallus domesticus*) is one of the most important global protein sources with an approximate annual production of 100 million MT of meat worldwide in 2022 (https://www.statista.com). Chicken meat processing generates abundant co-products, including viscera, feathers, tracheas, combs, frame bones, cartilage, etc. Cartilage has been classified as a special connective tissue which can be found in the trachea. It is constructed by C-shaped cartilaginous rings, a special structure designed to transport air with high flexibility [1]. Tracheas are generally turned into low value animal feed despite its high collagen content. Collagen is a substantial part of the extracellular matrix network accounting for about 60% of the cartilage on a dry basis [2]. Collagen extraction would lead to valorization of trachea co-products from the chicken meat processing.

Collagen is a natural biopolymer with a wide variety of applications in food, the cosmetic industry, as well as pharmaceuticals [3]. Co-products from various animals, such as fish, bovines, equines, porcine, among others have been sought as a source of collagen [4]. Tracheal co-products are also a good source of collagen in which bovine and porcine tracheal collagen have been studied [5]. Nevertheless, chicken trachea collagen has never been studied thus far. Generally, collagen extraction is carried out using acetic acid (Ac) and/or pepsin (Pep), by which the collagen yield is 0.1–6.4% and 0.3–36.2%, respectively, depending on the source of collagen [6–10]. The major disadvantage of these extraction methods is that they are time-consuming, requiring 2–4 days and they have a relatively low yield. Therefore, alternative approaches providing higher yields in shorter time spans should be sought.

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Recently, high-intensity ultrasound has gained enormous attention in the food industry because it provides several advantages including yield enhancement as well as being a non-toxic and eco-friendly process [11]. Ultrasonic waves creates an acoustic cavitation effect that generates high force-pressures and temperatures [12]. These lead to the disintegration of biological tissues. Ultrasound-assisted extraction (UAE) of collagen has been shown to increase yield from various co-products, such as featherback (*Chitala ornata*) skin, chicken sternal cartilage and lamb feet among others [13–15]. However, optimum conditions for ultrasound intensity, exposure time, and extraction time seem to vary between sources. An ultrasound power of 300 W with an exposure time of 25 min increased collagen yield from yellowfin tuna skin to 57.06% (dry weight basis) [16]. The maximum collagen yield from chicken sternal cartilage was 15.47% (dry weight basis). However, after applying UAE with an intensity of 11,350.32 $W{\cdot}cm^{-2}$ for 36 min, the secondary structure of the collagen was disrupted [17]. In addition, long-term ultrasonication reportedly led to damage in α-chain collagen from sea bass skin [18]. Although high intensity ultrasound seems to be promising for yield improvement, it might be destructive to the target protein. For this reason, the optimum conditions for UAE process should be taken into consideration. Furthermore, collagen structural changes induced by UAE should be explored in order to design a proper process to minimize its diverse effects, if any. Therefore, the objectives of this study were to extract chicken trachea collagen by UAE and to study the effect of ultrasound intensity, exposure time and extraction time on the structure and biochemical characteristics of the extracted collagen by conventional and UAE methods.

2. Materials and methods

2.1. Chemicals and samples

Type I collagen (bovine Achilles tendon), pepsin from porcine gastric mucosa (EC 3.4.23.1: activity of 250 units/mg solid), *trans*-4-hydroxy-L-proline (hydroxyproline or Hyp), chloramine-T hydrate, and Folin-ciocalteu's phenol reagent were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ninhydrin used for amino acid analysis was purchased from Biochrom (Cambridge, UK). Protein markers and chemicals used for gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals of analytical grade were purchased from Carlo Erba (Valde Reuil, France).

Chicken trachea samples were donated by Tanaosree Green Food Co., Ltd. (Nakhon Pathom, Thailand). Samples were vacuum-packed in polyethylene bags, packed with dry ice and transported to Suranaree University of Technology. Upon arrival, fat clots and meat residue were removed manually and washed with reverse osmosis (RO) water. Washed materials were stored at −20 °C throughout the study.

2.2. Chemical composition analyses

2.2.1. Proximate composition

Frozen samples were thawed at 4 °C overnight, cut and homogenized by a food blender (Tefal®, Rumilly, France) for 1 min. Moisture, ash and crude protein were analyzed according to AOAC [19] according to method No. 925.10, 923.03 and 990.03, respectively. Conversion factor of 6.25 was used for calculation of crude protein content.

2.2.2. Total lipids

Total lipids were analyzed according to Folch et al. [20] with slight modifications. Trachea samples (6 g) were added by chloroform: methanol (2:1) of 36 mL and homogenized using a homogenizer (Nissei AM-8, Nihonseiki Kaisha Ltd., Tokyo, Japan) at 10,000 rpm for 2 min. The homogenates were centrifuged (Sorvall ST16R, Thermo Fisher Scientific Inc., Langenselbold, Germany) at 10,000×g for 5 min. Subsequently, supernatants were collected and a mixture of chloroform, deionized (DI) water and 0.58% (w/v) NaCl solution (12, 12, and 2 mL) was added. The mixture was shaken and the bottom layer containing lipids was collected and added to 2 g of sodium sulfate anhydrous. The extracted lipids were filtered through a Whatman No. 4 and solvents were removed in a 100 °C sand bath for 15 min, and further dried in a hot air oven at 60 °C overnight. Total lipids were calculated based on gravity measurement.

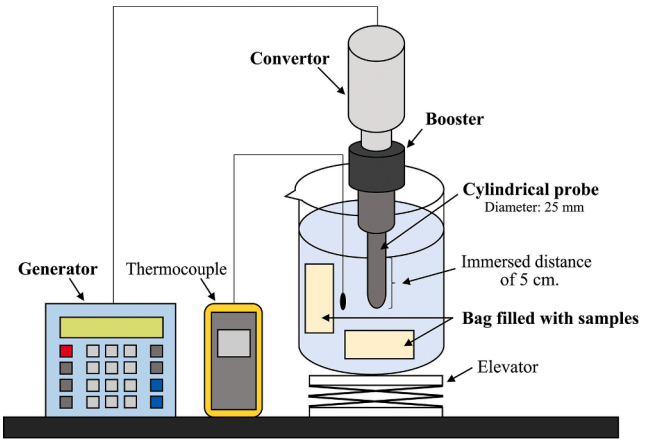

Fig. 1. Schematic diagram of ultrasound set up.

2.2.3. Hyp and collagen content

Hyp content was determined according to da-Silva et al. [21] with some modifications. Samples (10 mg) were hydrolyzed in 1 mL of 7.0 M NaOH in an autoclave at 121 °C for 40 min. Hydrolyzed samples were neutralized with 3.5 M sulfuric acid to pH 7 and filtered through Whatman no. 4. The neutralized mixture (50 μL) was added to 450 μL of 0.056 M oxidant reagent (1.38% of Chloramine-T hydrate in acetate-citrate buffer pH 6.5), and incubated in a dark at room temperature for 25 min. Subsequently, 500 μL of 1.0 M Ehrlich's aldehyde reagent (15.0% of *p*-dimethyl amino-benzaldehyde in 2:1 (v/v) isopropanol: perchloric acid) was added. The mixtures were then incubated in a 65 °C water bath for 25 min and cooled at room temperature for 3 min. Absorbance at 550 nm was measured (Genesys 10S UV–VIS, Thermo Fisher Scientific, Madison, WI, USA). Collagen content was calculated using conversion factors of 8.0 [22].

2.3. Collagen extraction

2.3.1. Conventional extraction

Non-collagenous proteins, fat and pigments were removed by soaking tracheas in 0.1 M NaOH at a ratio 1:10 (w/v) for 6 h. The fresh alkaline solution was replaced every 2 h. Subsequently, samples were washed with DI water until the pH of the wash water became neutral. The alkali-treated samples were added to a 0.5 M acetic acid at a ratio 1:15 (w/v) and extraction was carried out for 48 h at room temperature (25 ± 2 °C). Subsequently, the mixtures were centrifuged (CR22G-III, Hitachi Ltd., Tokyo, Japan) at 15,000×g for 20 min. Supernatants were added by 4.0 M NaCl to attain a final concentration of 2.6 M. Collagen pellets were collected by centrifugation at 17,000×g for 30 min. Then, the collagen was dialyzed against RO water using a 3.5-kDa molecular weight cut-off (MWCO) dialysis membrane (Thermo Fisher Scientific Inc., Rockford, IL, USA) for 48 h. Dialyzed collagen was lyophilized (GT2-S, GEA Lyophil GmbH, Hürth, Germany) and used for further analysis.

Precipitates remaining after acid extraction were extracted using 0.5 M acetic acid containing pepsin (50 units/g residue) at a ratio 1:15 (w/v) for 48 h at room temperature (25±2 °C). Soluble collagen was collected and precipitated as described above. Yield and collagen recovery were calculated, on a dry basis as follows:

$$\text{Yield}\,(\%) = \left(\frac{\text{weight of lyophilized sample}}{\text{weight of raw material, dry basis}}\right) \times 100$$

$$\text{Collagen recovery}\,(\%) = \left(\frac{\text{collagen content of lyophilized sample}}{\text{collagen content of raw material}}\right) \times 100$$

2.3.2. Ultrasound-assisted extraction

To avoid any metal contamination resulting from cavitation erosion of the titanium alloy probe, the alkali-pretreated trachea (35 g) was mixed with 100 mL DI water and packed in a polypropylene bag. Subsequently, the filled bag was attached at the bottom and on the wall of a glass beaker containing 300 mL DI water and 200 g ice as shown in Fig. 1. An ultrasonic generator (Q500, Qsonica LLC., Newtown, CT, USA) equipped with a 25-mm diameter cylindrical titanium alloy probe connected to a booster was used. The probe was immersed into the water at a depth of 5 cm, and a pulse mode of on-time 5 sec with off-time 5 sec was applied. Temperature of the system was monitored by a thermocouple type K (54IIB, Fluke Corporation, Everett, WA, USA) and was controlled to be lower than 25 °C throughout the ultrasound exposure.

Various ultrasound intensities (9.80, 17.46 and 27.56 W·cm^{-2}) and ultrasound exposure times (10, 20 and 30 min) were studied. The bag was flipped every 10 min within the specified exposure times to assure uniform ultrasonic treatment. When the exposure time was attained, samples were added by acetic acid to a final concentration of 0.5 M. Extraction by acid and pepsin was performed as described in 2.4.1, but with various extraction times (12, 18, 24, 30, 36, 42 and 48 h). Optimal conditions were determined based on yield and collagen content. Ultrasound power (W) was estimated from mechanical energy that is partially lost in the form of heat, which happens when ultrasound waves dissipate through a medium as described by Margulis and Margulis [23].

2.4. Scanning electron microscopy (SEM)

The microstructures of alkali-pretreated trachea and ultrasound-treated trachea were analyzed using SEM (JSM-6010LV, Japan Electron Optics Laboratory Technics Co. Ltd., Tokyo, Japan). All extracted collagen samples were observed using SEM (Quanta 450, Field Electron and Ion (FEI) Co., Hillsboro, OR). All samples were mounted on specimen stubs with double carbon tape and a gold coating was applied using an ion sputtered-coater (JFC-1100E, Japan Electron Optics Laboratory Technics Co. Ltd., Tokyo, Japan) for 1 min. The microstructures of the specimens were evaluated using an electron acceleration voltage of 15 kV at 500X magnification.

2.5. Characterization of collagen

2.5.1. Amino acid composition

Collagen samples (10 mg) were hydrolyzed in 1 mL of 6 M HCl at 115 °C for 24 h using a heat block (Boekel Scientific, Feasterville, PA, USA). Hydrolysates were neutralized with 3.5 M NaOH and filtered through Whatman No. 4. Norleucine was added as an internal standard. Amino acid profiles were analyzed using an amino acid analyzer (Biochrom 30 plus, Biochrom Ltd., Cambridge, UK) equipped with a cation exchange column (u-3183 High resolution, 200 mm of bed length and 4.6 mm of diameter, Biochrom Ltd., Cambridge, UK). Mobile phases included a lithium citrate buffer (pH 2.80–3.55) and a lithium hydroxide buffer (pH 14.0). Analysis of the system was performed using a post-column derivatization by ninhydrin. Mobile phases and ninhydrin were operated at 18 mL/h and the amino acid contents were expressed as amino acid residues/1,000 residues.

2.5.2. Micro-differential scanning calorimetry (μDSC)

Thermal denaturation of collagen samples were determined, according to Carsote and Badea [24] with slight modifications. Collagen samples (5 mg) were accurately weighed into a Hastelloy C crucible and added by a 500 mg of either DI water or 0.5 M acetic acid. The mixtures were allowed to rehydrate in a 4 °C -refrigerated incubator (KB240, Binder GmbH, Tuttlingen, Germany) for 24 h. Thermal analysis was performed using a μDSC (7 evo micro calorimetry, Setaram Instrumentation, Caluire-et-Cuire, France). The instrument was calibrated using a naphthalene standard. Samples were scanned from 20 to 70 °C at a heating rate of 1 °C/min. DI water and 0.5 M acetic acid were used as references for the respective solubilizing medium.

2.5.3. Spectral characterization

2.5.3.1. Ultraviolet (UV) spectroscopy. Collagen samples (5 mg) were dissolved in 5 mL 0.5 M acetic and shaken at 150 rpm at 4 °C for 12 h. Debris were removed by centrifugation at 10,000 × g for 10 min. Solubilized samples were diluted to obtain 0.5 mg protein/mL as quantified by the Biuret method [25]. Samples were placed in a 1-cm quartz cell and absorbance was measured using a UV spectrophotometer (Libra S22, Biochrom Ltd., Cambridge, UK). All spectra were scanned between 200 and 400 nm at a scan speed of 4 nm/s.

2.5.3.2. Circular dichroism (CD) spectroscopy. Collagen samples prepared as described in 2.5.3.1 were diluted to 0.1 mg/mL according to Anthis and Clore [26]. Samples were placed in a 1-mm quartz cell and analyzed using a CD spectrophotometer (Jasco J-815, Jasco International Co., Ltd., Tokyo, Japan). Spectra were recorded between 260 and 190 nm at a scan speed of 50 nm/ min. Measurements were performed at 25 °C using a peltier type cell holder.

2.5.3.3. Fourier transform infrared (FT-IR) spectroscopy. Collagen samples were placed on a crystal cell (Pike Technology Inc., Madison, WI, USA) and compressed on a FT-IR spectrometer (Tensor 27, Bruker Co., Ettlingen, Germany). Samples were measured on an attenuated total reflectance (ATR) mode. The IR spectra were recorded over the wavelength range of 4,000–900 cm^{-1}. Spectra were collected for 64 scans with a resolution of 4 cm^{-1} against background spectra which was measured from the clean empty cell at 25 °C. Spectra preprocessing was carried out by smoothing, baseline correction and normalization (X_{start} to $X_{end\ point}$: 1,700–1,600 cm^{-1}). Curve fitting of the amide I region to estimate protein secondary structure was analyzed, using a Lorentzian/Gaussian spectral line shape. Relative contents of β-sheets, random coils, α-helices, and β-turn structures were estimated at 1,626–1639, 1,640–1,649, 1,650–1,663, and 1,675–1,696 cm^{-1}, respectively [27]. Moreover, the absorption intensities of the 1,454 region (1,415–1,485 cm^{-1}) and the amide III region (1,200–1,300 cm^{-1}) were determined. All spectral data were collected and analyzed using OPUS software, version 7.2 (Bruker Co., Ettlingen, Germany).

2.5.4. Protein pattern

Collagen samples (5 mg) were dissolved in 1 mL of 10% sodium dodecyl sulfate (SDS), and shaken at 150 rpm at room temperature for 6 h. Solubilized samples were mixed with a treatment buffer (0.5 M Tris-HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercapthoethanol and 0.02% (w/v) bromophenol blue) to obtain 12 μg of protein as quantified by the Lowry method [28]. Samples were boiled at 90 °C for 3 min and separated on polyacrylamide gel electrophoresis made from 7.5% running gel, and 4% stacking gel. Electrophoresis was carried out at 80 V. Subsequently, gels were stained with Coomassie brilliant blue R-250 for 30 min, followed by de-staining with 10% (v/v) acetic acid in 25% (v/v) methanol for 2 h. The SDS-PAGE image was recorded (Fire reader V4, Uvitec Ltd., Cambridge, UK) and molecular weight (Mw) was estimated using UV1-ID software, version 16.0 (Uvitec Ltd., Cambridge, UK).

2.5.5. Protein identification

The type of collagen was identified according to the techniques of

Table 1
Chemical composition of broiler chicken trachea.

Composition	Content (%)	
	Raw material	Alkali-pretreated sample
Moisture	82.6^{a} ± 0.05	83.8^{a} ± 0.04
Protein	68.0^{b} ± 0.84	89.3^{a} ± 0.27
- Hyp	4.7^{b} ± 0.05	6.5^{a} ± 0.52
- Collagen	37.6^{b} ± 0.39	52.3^{a} ± 3.98
Ash	3.3^{a} ± 0.01	0.8^{b} ± 0.03
Total lipid	14.4^{a} ± 0.42	3.0^{b} ± 0.05
Carbohydrate	14.3^{a} ± 0.07	7.0^{b} ± 0.09

All are in dry basis except for moisture content; Carbohydrate content was calculated by 100 - (ash + total fat + protein); Different superscripts in row indicate significant difference ($p < 0.05$).

Sharma, Wongkham, Prasongwattana, Boonnate, Thanan, Reungjui and Cha'on [29] with some modifications. Protein bands on SDS-PAGE were excised from the gel using a scalpel. Gel pieces were washed with 20 mM ammonium bicarbonate (AmB), followed by a mixture of 20 mM AmB and acetonitrile (ACN) at a ratio of 1:1, and lastly by ACN for 10 min during each step. The gel pieces were then treated with 10 mM dithiothreitol (DDT) in 20 mM AmB at 56 °C for 45 min, followed by 55 mM iodoacetamide (IAA) in 20 mM AmB in the dark at room temperature for 30 min, and washed with 20 mM AmB: ACN (1:1) and ACN for 10 min during each step.

In-gel digestion was carried out in 20 mM AmB containing 20 ng/μL of sequencing-grade modified trypsin (V5111, Promega Corp., Madison, WI, USA) at 4 °C for 30 min. Gel pieces were incubated with 25 mM AmB at 37 °C overnight. Peptides were extracted with 1% formic acid (FA) in 50% ACN and followed by 1% FA in 85% ACN. Samples were dried using a refrigerated centrifugal vacuum concentrator (CentriVap, Labconco Corp., Kansas City, MO, USA) for 2 h, and reconstituted with 0.1% FA in 2% ACN. Undissolved debris were removed by centrifuging at 10,000 rpm for 10 min.

Digested samples were analyzed using a nano-liquid chromatography system (EASY-nLC II, Bruker Daltonik GmbH, Bremen, Germany) coupled with an ion trap mass spectrometer (Amazon Speed ETD, Bruker) equipped with an ESI nano-sprayer. The ESI-TRAP instrument was calibrated in the *m/z* range of 50–3,000 using an internal calibration standard. Digested samples (3 μL) were loaded by an autosampler onto an EASY-Column (10 cm, ID 75 μm, 3 μm, C18-A2, Thermo Fisher Scientific, Madison, WI, USA). Mobile phase A (0.1% FA) and B (0.1% FA in ACN) were used at a flow rate of 500 nL/min with a linear gradient from 5 to 35% B for 50 min, and 80% B for 10 min. Tryptic-digested bovine serum albumin (50 fmol) was used as a control. LC-MS/MS spectra were analyzed using Compass Data Analysis version 4.0.

Collagen identification was performed by searching against the NCBI's protein database on Metozoa (animals) using the Mascot MS/MS ion search program (https://www.matrixscience.com) with the initial searching parameters; Enzyme: Trypsin; carbamidomethylation (C) as fixed modification, and oxidation (HW) and oxidation (M) as variable modification; peptide mass tolerance of 0.5 Da and a fragment mass with

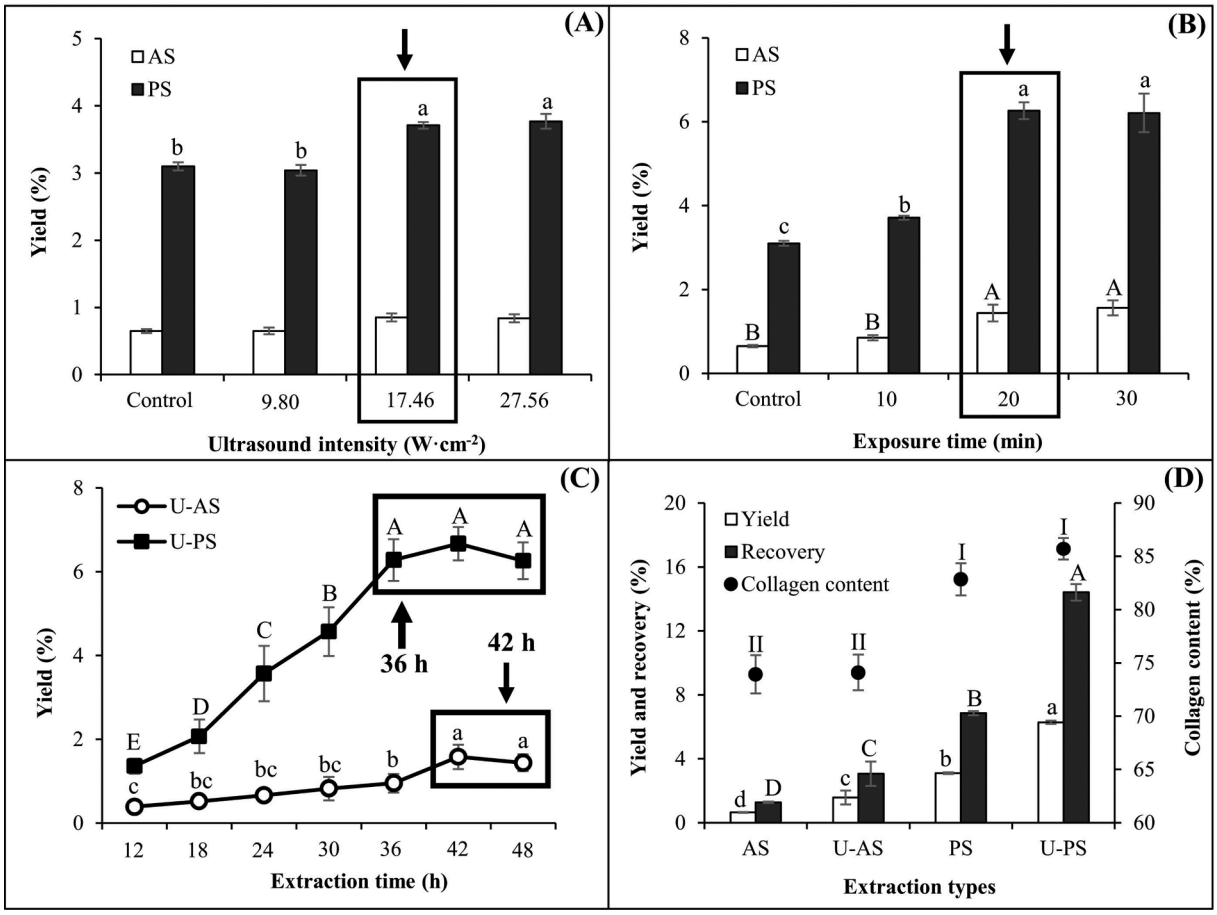

Fig. 2. Extraction yield of ultrasound-treated chicken trachea by various ultrasound intensities at 10-min exposure time (A), various exposure time at ultrasound intensity of 17.46 W·cm^{-2} (B), and various extraction times at ultrasound intensity of 17.46 W·cm^{-2} for exposure time of 20 min (C), extraction yield, recovery, and collagen content obtained from the conventional and ultrasound-assisted extraction method at the intensity of 17.46 W·cm^{-2} for 20 min (D). Different lowercase and uppercase letters indicate differences within each respective treatment ($p < 0.05$). AS: acid-soluble collagen, PS: pepsin-soluble collagen, U-AS: ultrasound-assisted extraction followed by acid extraction, U-PS: ultrasound-assisted extraction followed by pepsin extraction.

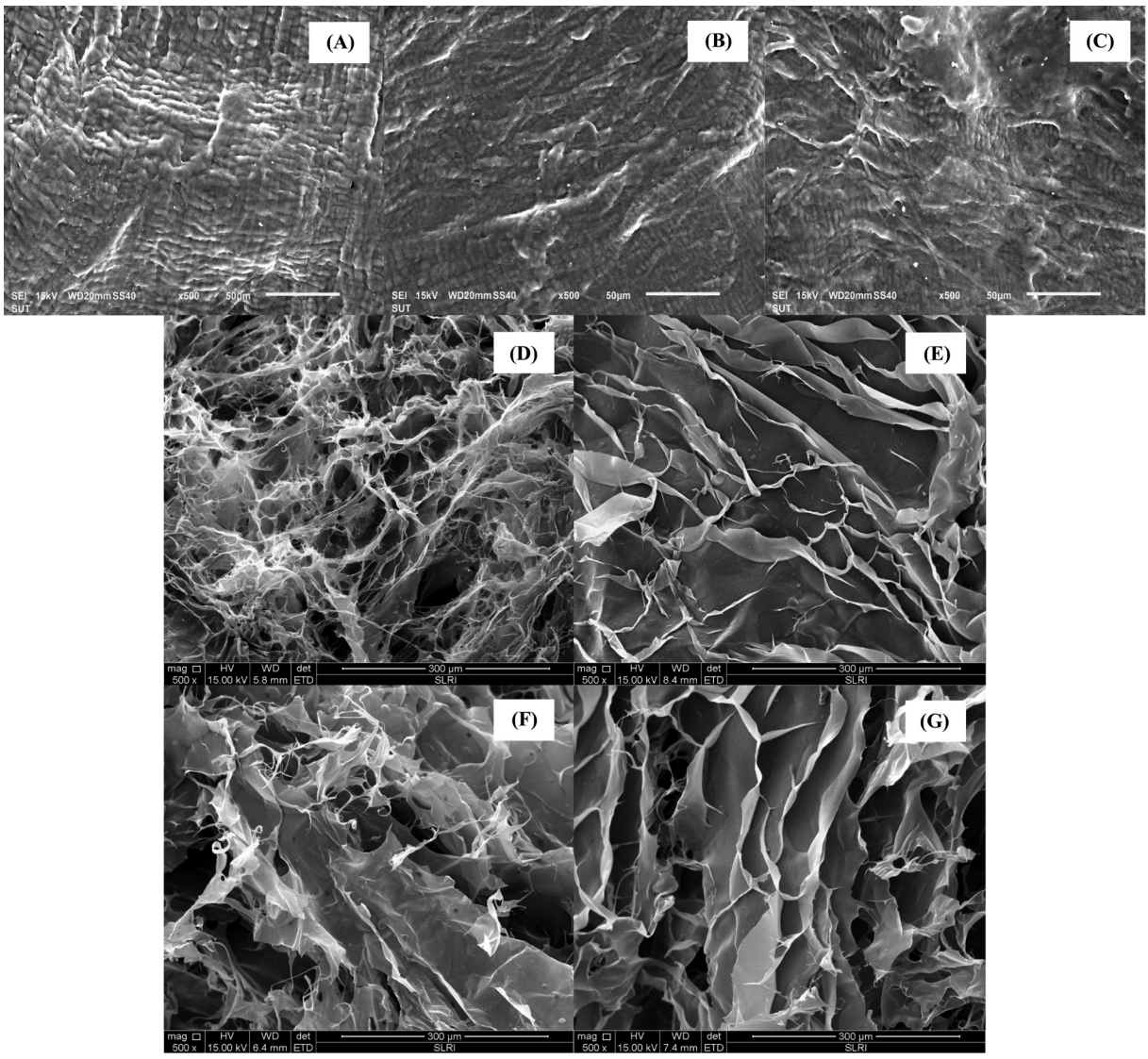

Fig. 3. SEM images of residues obtained after alkali-pretreatment (A), ultrasound treatment for 10 min (B) and 20 min (C), trachea collagen extracted by acid-soluble collagen (D), pepsin-soluble collagen (E), ultrasound assisted extraction followed acid extraction (F) and ultrasound assisted extraction followed pepsin extraction (G) at 500 magnifications.

a tolerance of 0.5 Da; a peptide charge state of +1, +2, +3; instrument type: ESI-TRAP; and report top (Auto).

2.6. Statistical analysis

All experiments were performed in triplicates. The mean values for the chemical composition of the raw material and alkali-treated samples were compared using the student's *t*-test for independent samples model with a 95% confidence level. In other experiments, an analysis of variance was performed and the mean comparisons were analyzed using Duncan's multiple range test with a 95% confidence level ($p < 0.05$). Statistical analyses were performed using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussions

3.1. Chemical composition

Major component of chicken trachea was protein, accounting for 68.0% (dry basis, Table 1). This was comparable with previously report on chicken (69.7%) and duck trachea (66.2%) and lower than ostrich trachea with the protein content of 84.4% [30]. Collagen content was estimated to be 37.6% based on Hyp content. This was higher than that reported in duck trachea (32.9%), sheep and lamb coproducts (including bone, cartilage, carcass trimmings and meat; 12.3% and 8.9%, respectively) [31,32]. However, chicken trachea contained relatively high total lipid (14.4%) and carbohydrate (14.3%). The composition of the trachea changed significantly after alkali pretreatment as its collagen content increased to 52.3% due to removal of non-collagenous proteins, fat, and other components.

3.2. Yield of collagen

The effects of ultrasound intensity, time of ultrasound exposure and extraction time on the yield are depicted in Fig. 2. In the acetic soluble process, UAE did not improve yield (Fig. 2A). UAE increased the yield of pepsin-soluble collagen extraction when the applied power ranged from 9.80 $W{\cdot}cm^{-2}$ to 17.46 $W{\cdot}cm^{-2}$ ($p < 0.05$), but further increases in intensity from 17.46 to 27.56 $W{\cdot}cm^{-2}$ did not increase yield ($p > 0.05$). The time of ultrasound exposure also significantly affected yield. Higher amounts of collagen were extracted with longer exposure times up to 20 min, but from 20 to 30 min the amount extracted did not change

significantly (Fig. 2B; $p > 0.05$). Hydrodynamic oscillation of 17.46 W·cm^{-2} could increase tissue disruption through cavitation phenomenon including a thermoacoustic effect and microjet formation via the implosion of cavitation bubbles. This tissue disruption exposes collagen molecules to acid and/or pepsin [13,33].

Ultrasound treatment could help in reaching a higher yield in a shorter amount of time compared to conventional methods. Collagen extraction of ultrasound-treated samples by pepsin (U-PS) reached a maximum level of 6.28% at 36 h, while it took 42 h for collagen extracted by acid (U-AS, Fig. 2C). Results of the present study revealed a reduction of 12.5% and 25% in extraction time for U-AS and U-PS, respectively (Fig. 2C). However, excessive intensity and/or exposure time could cause adverse effects on collagen solubility and structure [34]. Yield improvement was also reported in ultrasound treated chicken sternal cartilage, chicken lungs, jellyfish, bovine tendons among others [13,34–36]. Ultrasonic cavitation can open-up the structure of collagen fibrils, thus improving the acid extraction and pepsin solubilization of collagen [35]. In this study, an ultrasound treatment of chicken trachea at an intensity of 17.46 W·cm^{-2} and an exposure time of 20 min were found to be the optimum conditions.

Yield, recovery and purity of collagen obtained from trachea treated by ultrasound at the optimum conditions along with those prepared by conventional methods are given in Fig. 2D. The AS and PS conventional extraction yielded 0.7 and 3.1% collagen as well as only 1.3 and 6.9% collagen recovery, respectively, while these values increased by up to double through ultrasound treatment (Fig. 2D). A greater collagen yield was observed in pepsin extracted collagen (PS and U-PS), which might be correlated to higher efficacy of pepsin in cleaving specific peptide bonds in telopeptide regions. Besides, more individual collagen molecules from pepsin solubilization would lead to a higher amount of Hyp as well as higher collagen purity. PS and U-PS collagen exhibited a high purity of about 82–86%, whereas collagen content of AS and U-AS samples were 73–74%. However, all collagen in this study possessed higher purity than those prepared from soft-shelled turtle by acid extraction (43.6%) and ultrasound treatment at an intensity of 200 W for 4 min (50.7%) [37]. Therefore, an ultrasound intensity of 17.46 W·cm^{-2} with exposure time of 20 min in conjunction with pepsin provided the highest yield, recovery, and collagen purity.

3.3. SEM

SEM images of residues obtained after alkali-pretreatment and after ultrasound treatment are shown in Fig. 3A-C. It can be seen that ultrasound treatment disrupted the surface of the trachea tissues and a greater extent of tissue disintegration was observed with a longer exposure time. The implosion bubbles from acoustic cavitation resulted in high shear stress from compression and decompression pressure, leading to tissue rupture [38].

All extracted collagen samples exhibited fibrous structure (Fig. 3D-G). This was in agreement with previous studies [17,37]. AS collagen showed finer fibrous structures (Fig. 3D). Acetic acid and hydronium (H_3O^+) ions increased water accessibility to collagen fibers, rendering greater repulsive forces among polypeptide chains, thus increasing collagen extractability. However, PS and U-PS presented a dense multi-layered sheet-like structure (Fig. 3E, G). Non-helical regions were specifically cleaved by pepsin, causing aggregation of helical collagen molecules. The U-AS collagen exhibited less fibrous filament than AS collagen (Fig. 3F). Cavitation might induce assembly of extracted collagen molecules. These microscopic results suggested that the microstructures of acid-solubilized collagen and pepsin-solubilized collagen were different and ultrasonic processing did not affect the microstructure of the extracted collagen.

3.4. Amino acid composition

The distinct amino acids present in all collagen samples included glycine (Gly), proline (Pro), and hydroxyproline (Hyp); whereas histidine (His), hydroxylysine (Hyl), methionine (Met), phenylalanine (Phe), and tyrosine (Tyr) were present in low amounts (Table 2). These amino acid profiles were similar to those previously reported for type I collagen extracted from various sources, including Siberian sturgeon cartilage, chicken feet, and bigeye tuna bone [39–41]. Cysteine (Cys) and tryptophan (Trp) were not found in this study as they were destroyed during acid hydrolysis. Likewise, Trp and Cys were not found in collagen type I from yellowfin tuna co-product [16]. Cartilage sources normally composed of 80% type II collagen, contain relatively high amounts of Hyl (18 residues) and Glu/Gln (88 residues) [42]. However, chicken trachea exhibited amino acid profiles of type I collagen with relatively low Hyl and Glu/Gln, but high Ala. This profile was similar to that of silver carp scales, golden carp skin, and bovine tendons [10,43,44]. The whole trachea is composed of mucosa, submucosa, cartilage and adventitia (from innermost to the outermost order). Apart from cartilage, submucosa and adventitia contain connective tissues that might be a source of collagen type I [45]. A combination of collagen type I and II was also observed in porcine tracheas which reportedly comes from cartilage and the surrounding connective tissues of the perichondrium [5].

Hyp was derived from post-modification through hydroxylation of Pro by prolyl-hydroxylase. This amino acid is associated with the thermal stability of collagen triple helix. Collagen extracted by pepsin (PS and U-PS) exhibited higher imino acids (Pro + Hyp) than acid soluble collagen (AS and U-AS). High intermolecular cross-links at the telopeptide region via imino acids were cleaved by pepsin to a greater extent than through acetic acid alone. Moreover, proline hydroxylation (PH) for collagen extracted by pepsin was higher than for acid-soluble collagen. Hydroxylation is a critical process regulating collagen stability via imino acid rings [46]. A pyrolidine ring on Hyp and Pro led to a nucleation zone to generate a network structure, exerting high stability on the triple helix. Our results revealed that pepsin-soluble collagen exhibited higher stability than acid-soluble collagen. This was in agreement with those previously reported in collagen extracted from whale shark cartilage and golden carp skin [6,44]. A lower amount of

Table 2
Amino acid composition of collagen extracted by conventional and ultrasound-assisted extraction methods.

Amino acid	Content (residues/ 1,000 total residues)			
	AS	U-AS	PS	U-PS
Ala	116^{a} ± 1.96	112^{a} ± 2.70	97^{b} ± 3.47	101^{b} ± 0.88
Arg	58^{a} ± 3.16	57^{a} ± 2.83	52^{b} ± 2.57	51^{b} ± 4.05
Asp/Asn	48^{a} ± 2.67	47^{a} ± 1.20	42^{b} ± 3.46	46^{a} ± 1.62
Glu/Gln	72 ± 3.10	73 ± 3.11	74 ± 1.31	73 ± 3.25
Gly	308 ± 3.23	311 ± 4.89	310 ± 2.26	308 ± 2.30
His	6 ± 2.57	10 ± 1.20	11 ± 0.57	8 ± 0.52
Hyl	7 ± 1.02	7 ± 0.83	8 ± 2.81	9 ± 0.65
Hyp	76^{b} ± 1.03	77^{b} ± 1.31	105^{a} ± 0.88	104^{a} ± 3.81
Ile	20^{a} ± 2.60	17^{a} ± 1.00	15^{b} ± 0.19	14^{b} ± 1.11
Leu	47^{a} ± 2.18	45^{a} ± 3.69	42^{b} ± 1.61	42^{b} ± 1.19
Lys	28 ± 1.08	26 ± 3.12	25 ± 1.59	27 ± 0.51
Met	11 ± 1.35	9 ± 4.71	9 ± 0.63	8 ± 1.05
Phe	11 ± 1.49	10 ± 0.62	15 ± 0.85	13 ± 1.72
Pro	112^{ab} ± 3.75	108^{b} ± 0.93	115^{ab} ± 4.10	118^{a} ± 1.27
Ser	29^{a} ± 1.53	29^{a} ± 2.42	27^{b} ± 1.27	28^{ab} ± 1.82
Thr	26^{a} ± 0.92	25^{a} ± 2.52	20^{b} ± 2.25	19^{b} ± 1.56
Tyr	6 ± 0.37	7 ± 4.62	5 ± 0.57	4 ± 0.52
Val	32^{a} ± 1.92	30^{ab} ± 2.22	29^{b} ± 0.83	27^{b} ± 0.66
Imino acid	188^{b} ± 2.48	186^{b} ± 0.72	220^{a} ± 4.89	222^{a} ± 1.51
PH (%)	41.0^{b} ± 0.72	42.0^{b} ± 0.60	47.7^{a} ± 0.72	46.9^{a} ± 2.02

PH was degree of proline hydroxylation and calculated from [Hyp residue/ (Hyp residue + Pro residue)] × 100. Different superscripts in a row indicate significant difference ($p < 0.05$). AS: acid-soluble collagen, PS: pepsin-soluble collagen, U-AS: ultrasound assisted extraction followed acid extraction, U-PS: ultrasound assisted extraction followed pepsin extraction.

Table 3
Denaturation temperature (T_d), enthalpy (ΔH) values, absorption ratio obtained from FT-IR spectra and Rpn ratio calculated from CD spectra of collagen samples by various methods.

Sample	T_d (°C)		ΔH(J/g)		Absorption ratio (Amide III/ 1,454 cm^{-1})	Rpn*
	DI Water	0.5 M Acetic acid	DI Water	0.5 M Acetic acid		
AS	35.1^{b} ± 0.48	32.6^{b} ± 0.32	0.11^{b} ± 0.02	0.09^{b} ± 0.03	1.03 ± 0.12	0.11 ± 0.01
U-AS	34.3^{b} ± 0.34	31.9^{b} ± 0.41	0.13^{b} ± 0.01	0.09^{b} ± 0.01	1.07 ± 0.03	0.10 ± 0.20
PS	45.6^{a} ± 0.17	42.1^{a} ± 0.11	0.26^{a} ± 0.03	0.21^{a} ± 0.01	1.06 ± 0.16	0.11 ± 0.00
U-PS	45.0^{a} ± 0.22	41.8^{a} ± 0.28	0.23^{a} ± 0.03	0.18^{a} ± 0.06	1.04 ± 0.07	0.10 ± 0.43

Different superscripts in column indicate significant difference ($p < 0.05$). *Rpn was CD ellipticity ratio between maximum and minimum value. AS: acid-soluble collagen, PS: pepsin-soluble collagen, U-AS: ultrasound assisted extraction followed acid extraction, U-PS: ultrasound assisted extraction followed pepsin extraction.

Ala, Asp/Asn, Arg, Ile, Leu, Thr, Ser, and Val were found in PS collagen ($p < 0.05$). This might be due to the removal of the telopeptide region during pepsin hydrolysis. Similar findings were also reported in collagen from the skin of blue sharks and deer [47,48]. Additionally, collagen obtained from UAE showed similar amino acid profiles to those found using corresponding conventional processes. Pezeshk et al. [16] observed fluctuations in Pro, Gly and Hyp contents of acid soluble collagen from yellowfin tuna skin exposed to ultrasound at 300 W for 0–25 min, in which their contents were lowest at 15-min ultrasound exposure. Such a fluctuation was also observed in individual amino acids of pepsin soluble collagen type-II from chicken sternal cartilage treated by 10.7 $W{\cdot}cm^{-3}$ of ultrasound intensity for 0–36 min, and total amino acid contents were found to increase with ultrasound exposure time [13] Our results demonstrated that amino acid compositions of collagen were not affected by the ultrasonic process.

3.5. Micro-differential scanning calorimetry (μDSC)

T_d and ΔH values of collagen rehydrated in 0.5 M acetic acid were lower than those rehydrated in deionized (DI) water (Table 3). Lower T_d values in acetic acid were likely due to the electrostatic repulsion of helical structures and the destruction of hydrogen bonds. In addition, T_d and ΔH values of collagen extracted by pepsin were higher than those extracted by acid. Higher imino acid content in PS and U-PS (220 and 222 residues per 1000 residues, respectively) than AS and U-AS (188 and 186 residues per 1000 residues, respectively) might explain its higher thermal stability via pyrolidine rings [16]. In this study, the pepsin-extracted collagen showed a comparable T_d (45 °C) to pepsin-extracted chicken sternal cartilage collagen (44 °C) and was lower than pepsin-extracted collagen from porcine trachea (53 °C) [5,13]. In addition, the acid-extracted TC possessed higher T_d (35 °C) than acid-extracted collagen from a tuna coproduct (28 °C) [16]. The habitat and body temperature of animals greatly affected the thermal stability of collagen. The collagen extracted by UAE exhibited comparable T_d and ΔH values with those found using their corresponding conventional method, regardless of the rehydration solvent ($p > 0.05$). These results were in accordance with the imino acid content of samples and revealed that ultrasound treatment at the intensity of 17.46 $W{\cdot}cm^{-2}$ for 20 min did not damage the triple helix structures of the extracted collagen. This is the first study elucidating thermal behavior of TC.

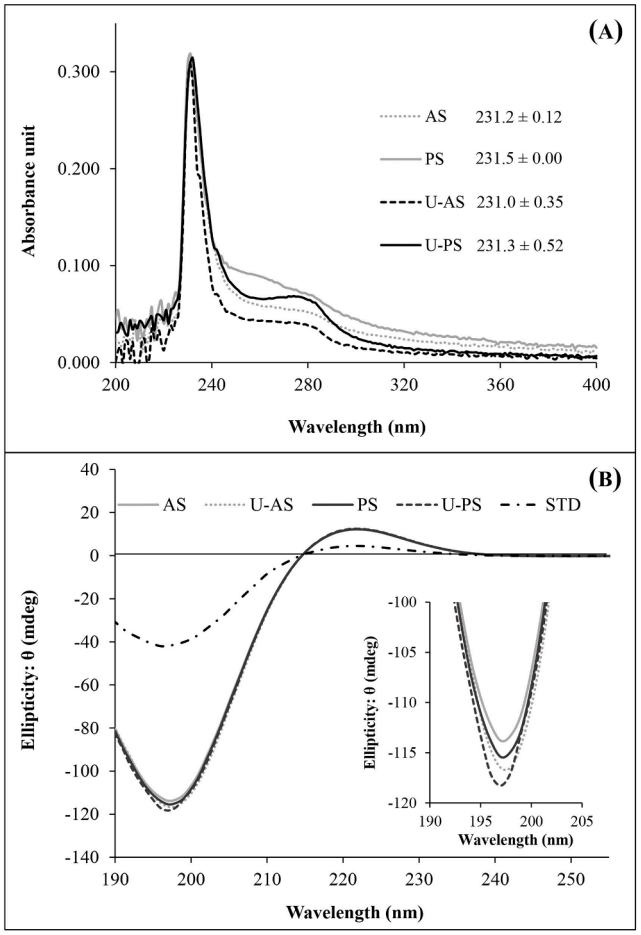

Fig. 4. UV (A) and CD spectra (B) of collagen samples extracted by different methods.

3.6. Ultraviolet (UV) spectroscopy

Collagen has a maximum absorbance at approximately 230 nm, at which point it can be distinguished from non-collagenous proteins with an absorbance at about 280 nm, corresponding to the aromatic amino acids presented in proteins. The UV-spectrum of all extracted collagen samples exhibited a maximum peak at approximately 231 nm (Fig. 4A). The UV-absorption was related to C = O, $CONH_2$, and $^{-}COOH$ in the polypeptide chains of collagen molecule [9]. Contamination of non-collagenous proteins would be minimal due to low absorbance of UV_{280}, a typical wavelength of protein absorption. The sensitive chromophores between $UV_{210\text{-}220}$ and $UV_{260\text{-}280}$ were minimal in all spectra, confirming low amounts of His, Tyr, and Phe residues, as well as a lack of Trp in all extracted collagen samples. It should be noted that AS and U-AS appeared to have less proteinaceous impurity than the pepsin-soluble collagen. In addition, collagen extracted by ultrasound showed comparable UV absorption with those extracted by the conventional method ($p > 0.05$). Hence, these results suggested that UAE treatment did not increase contamination of non-collagenous proteins when compared to the conventional extraction process.

3.7. Circular dichroism (CD) spectroscopy

Spectra of all samples showed a cross zero rotation at 215 nm, the maximum peak was observed at 222 nm, and the minimum peak was approximately 196–197 nm (Fig. 4B). These are characteristics of supercoil structure of collagen. Unfolding of a triple helix can be seen by a decrease in positive ellipticity in concomitant with an increase of

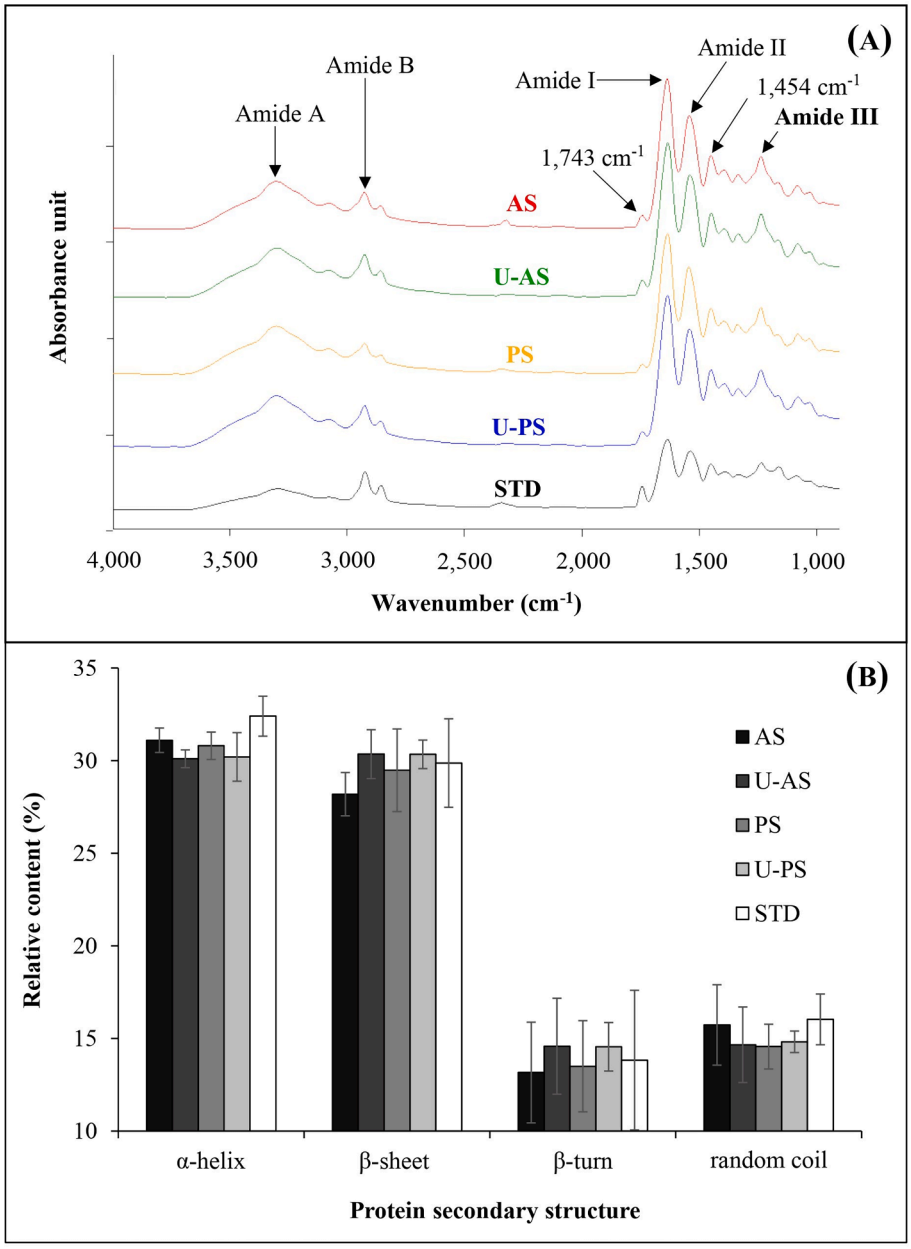

Fig. 5. FT-IR spectra (A) and protein secondary structure estimated from amide I region (B) of collagen samples extracted by different methods.

negative ellipticity as well as a red shift of negative bands to 203–210 nm [49]. Akram and Zhang [17] reported that chicken sternal collagen extracted by UAE at intensity of 11,350.32 W·cm^{-2} with an exposure time of 36 min exhibited a more negative ellipticity value, and the negative band shifted to 202.2 nm, indicating partial loss of the triple helix structure. Our study revealed that an ultrasound treatment of 17.46 W·cm^{-2} for 20 min did not significantly disturb the triple helical structure.

Most collagens showed distinct values of negative ellipticity with the AS being the highest value and the U-PS exhibited the lowest ($p < 0.05$, Fig. 4B inserted panel). Dynamic forces by acoustic pressure disrupted trachea cellular membranes and might simultaneously induce aggregation of triple-helix molecules by the pulse state of ultrasonication. Collagen extracted by pepsin followed by UAE led to high order collagen structure. The ratio of positive and negative ellipticity or Rpn value of all samples was about 0.1 (Table 3), indicating a triple helix for the native conformation of collagen. These results confirmed that TC retained a triple helix integrity even under high intensity ultrasound treatment.

3.8. Fourier transform infrared (FT-IR) spectroscopy

Spectra of all collagen samples exhibited a similar pattern with wavenumbers of amide I, II and III regions at 1,635–1,637, 1,539–1,547, and 1,236–1,239 cm^{-1}, respectively (Fig. 5A). Furthermore, most collagen contained weak vibrations at 1,743 cm^{-1}. This region, 1,737–1,744 cm^{-1}, represented C=O stretching bands which might be correlated to oxidative degradation products of proteins and/or lipids. [14]. The amide I peak is attributed to stretching vibrations of C=O coupled with N-H bond, which is associated with protein secondary structure. Based on the curve-fitting of the amide I band, the α-helix is a major structure in all collagen samples (Fig. 5B), estimating to be 30–32% ($p > 0.05$), whereas the β-turn and random coil were minor structures. Hong et al. [50] revealed that unwinding of triple helix collagen represented 15.44% α-helix content. This result demonstrated that an ultrasound intensity of 17.46 W·cm^{-2} for a 20 min exposure time did not disturb the secondary structure of TC. Akram and Zhang [17] stated that chicken sternal collagen extracted by an ultrasound intensity

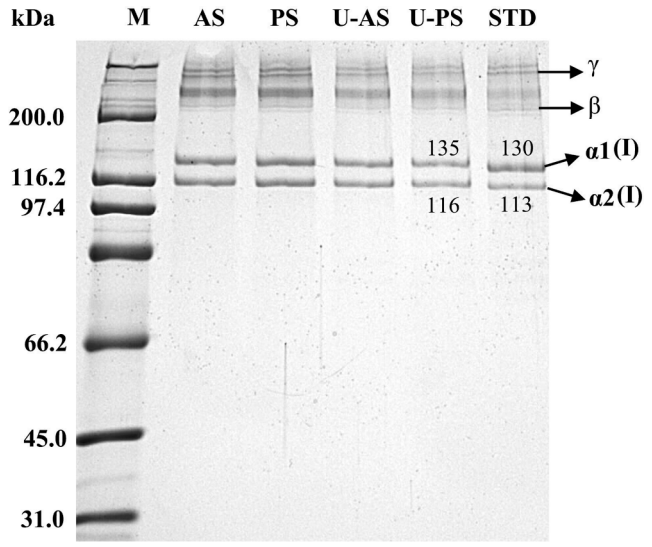

Fig. 6. Protein pattern of all collagen samples on 7.5% acrylamide gel. M: molecular weight marker, AS: acid-soluble collagen, PS: pepsin-soluble collagen, U-AS: ultrasound assisted extraction followed acid extraction, U-PS: ultrasound assisted extraction followed pepsin extraction, STD: type I collagen standard.

of 11,350.32 W·cm^{-2} for an exposure time of 36 min resulted in disruption of the secondary structure of collagen. Hence, extremely high intensity and long exposure time might induce structural changes of intact collagen structures.

Amide II bands are attributed to N-H bending and C-N stretching, while the amide III absorption peak reflects the N-H deformation and the C-N stretching [16]. The intensity ratio between amide III (1,237 cm^{-1}) and 1,454 cm^{-1}, indicates integrity of the triple helix structure. A ratio of about 1.0 typically indicates integrity of the triple helix. All collagen samples exhibited a ratio value of approximately 1.01–1.07 (Table 3, $p > 0.05$), confirming a triple helix structure. These results were similar with those previously reported from collagen extracted from golden carp and clown featherback co-products treated with 20–80% amplitude of ultrasound, which were estimated to be 1.00 [14,44]. Therefore, this study confirmed that UAE at intensity of 17.46 W·cm^{-2} for 20 min was not detrimental to the triple helix structure as well as the secondary structure of the extracted TC.

3.9. Protein patterns

All collagen comprised of α1(I)$_2$ and α2(I) chains as major components (Fig. 6). High molecular weight γ-chains (trimer) and β-chains (dimer) were evident, indicating that extracted collagen contained high amounts of covalent inter-molecular cross-linkages. The α1(I)$_2$ and α2(I) bands exhibited molecular weights (Mw) of 135 and 116 kDa, respectively, corresponding to Mw observed from the standard type I collagen of bovine Achilles tendons with α1(I) and α2(I) at 130 and 113 kDa, respectively. This is the first study classifying the collagen extracted from chicken trachea as a type I collagen. Collagen samples extracted from the conventional and UAE method showed comparable pattern. Low Mw proteins (<100 kDa) were not obviously noticed in the UAE sample. Therefore, the disruption of the trachea tissue membrane by UAE at an intensity of 17.46 W·cm^{-2} for 20 min did not cause severe hydrolysis of collagen. Collagen obtained from clown featherback fish skin subjected to 80% amplitude of ultrasound for 30 min showed degradation of α- and β-chains [14] Additionally, collagen extracted from chicken sternal cartilage by UAE at intensity of 11,350.32 W·cm^{-2} for an exposure time of 36 min exhibited partial degradation of the β- and α-chain structures [17]. Thus, extreme ultrasound intensity might cause detrimental effects on collagen structures. It should be noted that the disruption of polypeptide chains were not observed under the studied ultrasound treatment.

3.10. Protein identification

Gly and Pro were found in all matched peptides, except for GFSGLDGAK which complied with the general pattern of collagen, Gly-X-Y, with the majority of Pro in the X position (Table 4). Both α-subunits of PS and U-PS presented similar peptide sequences that showed high similarity with type I collagen. It was also correlated with amino acid composition of PS and U-PS, in which Gly, Pro, Ala, and Hyp were predominant (Table 2) as a typical characteristic of type I collagen [39–41].

Matched peptides, GFSGLDGAK and GQAGVMGFPGPK, corresponded with type I collagen of Nile tilapia (*Oreochromis niloticus*), donkey (*Equus asinus*), and sheep (*Ovis aries*) [51,52]. In addition, SAGVAVPGPMGPAGPR and DGEAGAQGPPGPTGPAGER matched with peptides of α$_1$ for type I collagen from broiler chickens. The α$_2$ (I) chain of PS and U-PS also exhibited 8 peptides, corresponding to α$_2$ of type I broiler collagen (Table 4). It has been reported that trachea cartilage was a rich source of type II collagen, however, TC revealed type I characteristics based on the amino acid profile, SDS-PAGE pattern, and LC-MS/MS, which might be attributed to the surrounding connective tissues as mentioned above [5]. Therefore, our study suggested that chicken trachea is an alternative source of type I collagen.

4. Conclusions

Chicken trachea was a rich source of protein for collagen extraction. Conventional extraction by pepsin resulted in 3.1% yield. Trachea collagen yield was increased to 6.28%, after ultrasound with an intensity of 17.46 W·cm^{-2} for an exposure time of 20 min, followed by pepsin

Table 4
Specific amino acid sequence of α$_1$ and α$_2$-chains obtained from collagen extracted by the conventional and ultrasound-assisted extraction methods.

Band	Peptide sequence hint	Coverage (%)	Protein identification	Taxonomy
α$_1$PS and α$_1$U-PS	SAGVAVP**GPMGPAGPR** GFSGLDGAK GQAGVMGFP**GPK** DGEAGAQ**GPPGPTGPA**GER	3.85	Collagen α1 (I) chain	*Gallus gallus*
α$_2$PS and α$_2$U-PS	AADFGP**GPM**GLM**GPR** GEI**GPA**GNY**GPTGPAGPR** V**GPIGPA**GNR GNVGLA**GPR** GEG**GPAGPAGPA**GAR GDP**GPVGPVGPA**GAF**GPR** GLA**GPQGPR** **GPPGPSGPP**GK	7.70	Collagen α2 (I) chain	*Gallus gallus*

Coverage values were calculated by comparing between numbers of amino acids obtained from LC-MS/MS and total amino acids of α$_1$ and α$_2$, which were 1,453 and 1,363 residues. PS: pepsin-soluble collagen, U-PS: ultrasound assisted extraction followed pepsin extraction.

extraction for 36 h was applied. Trachea collagen extracted by pepsin contained more than 80% collagen. UAE did not affect the collagen microstructure, and it did not damage the triple helix, nor the protein secondary structure. Collagen extracted by pepsin showed higher thermal denaturation than that extracted by acid in both DI water and 0.5 M acetic acid. Trachea collagen contained high imino acid content but low amounts of essential amino acids (His, Met, Phe and Trp). Trachea collagen was composed of α_1 and α_2 chains, indicating characteristics of type I collagen. Our study revealed that high-intensity ultrasound improves collagen extraction without disturbing the collagen structure. Valorization of chicken trachea can be achieved by collagen extraction.

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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