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Effect of ultrasound power on extraction kinetic model, and physicochemical and structural characteristics of collagen from chicken lung

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Abstract

The effects of ultrasound power on extraction kinetic model, and physicochemical and structural characteristics of collagen from chicken lung were studied. Ultrasound power caused a significant increase in extraction rate and equilibrium concentration, with the maximum extraction yield (31.25%) at 150 W. The experimental data were consistent with the predicted ones in this empirical equation, in which the percentage error differences was 0.026–4.159%. Besides, ultrasound treatment did not affect their triple-helical structure. The thermal stability of pepsin-soluble collagen by ultrasound pre-treatment (UPSC) was higher, due to the higher imino acid content (20.76%). UPSC also exhibited better solubility and fibril forming capacity. Overall, the kinetic model of UPSC from chicken lung could serve the purpose of obtaining collagen, which displayed a potential alternative source to mammal collagens for application in food, biomaterials and biomedical fields.

Keywords: Extraction kinetics, Chicken lung, Pepsin-soluble collagen, Ultrasound pretreatment

Introduction

According to Food and Agriculture Organization of the United Nations (FAO 2018) statistics, the world's chicken production in 2018 was about 97.8 million tons (of which China contributed ~ 11.7 million tons). Huge amounts of chicken by-products are produced due to rapid increase in the total production. The resultant by-products account for up to ~ 50% of the chicken weight and they are currently used partially as animal feed stuffs, pet attractant and crop fertilizer, resulting in serious environmental pollution and economic loss. Therefore, better and full utilization of these by-products is becoming urgent.

Collagen is an abundant component of extracellular matrix and its unique triple helix structure makes it stable in molecular structure. Collagen has low immunogenicity and excellent biocompatibility, hence it has been used in healthy food, packaging material, biomedical material, medical and cosmetic fields (Pal & Suresh 2016). More and more studies have focused on functional properties of collagen, especially those from the skin and bones of aquatic species compared to those from cow and pig (regional religious issues) (Bhagwat & Dandge 2016; Jana et al. 2016; Kobayashi et al. 2016), as they are important sources of easily soluble collagen. However, due to low thermal stability of aquatic collagen, it is urgent to find collagens with high thermal stability in the biomaterial application fields. Animal lungs are abundant in collagen and chicken lungs are basically donated to farmers as animal feed for foxes and minks or discarded, resulting in a huge waste of by-product resources. The results of our previous study showed that chicken lungs contain a high amount of collagen (~ 30%, dry weight). However, little is known about

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the extraction and physicochemical properties of collagen from chicken lung.

Extraction of collagen is solvent/raw material dependant process, known as leaching. Ultrasound pretreatment has emerged as a potential approach to extract substances from raw materials and has been certified to be an effective means to reduce processing time, energy, and chemical reagent consumption (Dahmoune et al. 2014). Furthermore, from an engineering view point, kinetic mathematical model is a meaningful tool, which greatly promotes process design, optimization, simulation, predetermination and manipulation (Bucić-Kojić et al. 2007; Saavedra et al. 2013). Therefore, in the process of collagen isolation, the extraction kinetic model of pepsin-soluble collagen from ultrasound pretreated (UPSC) chicken lung is essential and meaningful for reactor design. Additionally, the physicochemical and structural characteristics of UPSC were also investigated in this contribution.

Materials and methods

Materials and chemical reagents

The fat from chicken lungs was removed manually and the extracted lungs were then washed from the internal blood with tap water two times and then once with deionized water. The lungs were then cut into slices (~ 1.0 × 0.5 cm), stirred in a high-speed mixer until they were well homogenized. The mixture was then kept at − 20 °C according to the method described previously by Zou et al. (2017). Pepsin (4000 U mg^{-1}, dry matter), the standard $_{L}$-hydroxyproline ($_{L}$-$(OH)C_4H_7N(COOH)$), and dimethylaminobenzaldehyde ($((CH_3)_2NC_6H_4CHO)$) were bought from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and coomassie brilliant blue R-250 were purchased from Yuanye Laboratories Inc. (Shanghai, China). All the other reagents used in the experiment were of analytical grade.

Preparation of chicken lung

Chicken lungs were immersed in NaCl solution (20%, w v^{-1}) at 1:20 (w v^{-1}) and stirred for 8 h using a magnetic stirrer at 20 °C. The extraction mixture was subsequently centrifuged and the precipitate was immersed in 0.5 M Na_2CO_3 solution with 1:20 (w v^{-1}) for 24 h. The Na_2CO_3 solution was changed every 8 h. The minerals of chicken lung were removed by using Na_2-EDTA solution (0.3 M, pH 7.4) at a ratio of 1:20 (w v^{-1}) for 24 h with agitation. The solution of Na_2-EDTA (0.3 M, pH 7.4) was also renewed every 8 h. The sediment from centrifugation was immersed in isopropyl alcohol solution (10%, v v^{-1}) to fat removal then washed several times with distilled water until samples reached a pH of 7. Finally, the pretreated chicken lungs were kept at − 40 °C for further use.

Extraction and purification of collagen

Traditional extraction and purification of pepsin-soluble collagen (PSC)

Extraction and purification of PSC was performed according to the description of Chen et al. (2016) with slight modifications. PSC was extracted from the above operation steps with acetic acid solution (0.5 M, 1,20, w v^{-1}) containing pepsin (2000 U g^{-1} substrate) for 24 h. Subsequently, the supernatant of samples was collected by centrifugation. The residue of samples was extracted again using the same procedure. The obtained supernatant after centrifugation was added with NaCl to do a salting-out process (2.5 M and 1.0 M) for 12 h. The precipitate from salting-out process by centrifugation was re-dissolved in acetic acid solution with 1:10 (0.5 M, w v^{-1}) and then dialyzed in 0.1 M acetic acid solution (1,25, w v^{-1}), followed by double distilled water. PSC was lyophilized and then kept at − 20 °C for further use.

Extraction and purification of UPSC from chicken lung

The sample was extracted with acetic acid solution (0.5 M, 1:20, w v^{-1}) in an ultrasound processor (SCIENTZ-IID, Ningbo Xinzhi ultrasonic technology Co., Ltd., Zhejiang, China), where the flat tip probe immersion depth was around 1.0~2.0 cm. The operating mode was set as a pulsed on-time 2 s and off-time 3 s.The frequency and power of ultrasound were 24 kHz and 150 W, respectively. The extraction lasted 5 min. The temperature of cooling water passing steel jacket was set at 20 °C to avoid heating effects. Then pepsin (2000 U g^{-1} substrate) was added into the ultrasound pre-treatment samples. The next step was performed as given in the above section. UPSC was lyophilized and kept at − 20 °C for further determination.

Yield of collagen powder

The computational formula for the yield of PSC/UPSC was expressed as:

$$\%Yield = \frac{m_{PSC/UPSC}}{m} \times 100 \quad (1)$$

where $m_{PSC/UPSC}$ was the weight of collagen from chicken lung (dry weight after miscellaneous (heteroproteins, fats and mineral) removal) and m was the weight of chicken lung (dry weight after miscellaneous removal).

Kinetic model

A second-order model is usually employed to investigate kinetic model for solvent/raw material extraction. The second-order model could offer a representation of

extracting, as obvious from its important application in modeling extraction (Ho et al. 2005; Qu et al. 2010; Tao et al. 2014). The dynamic parameters in the second-order kinetic model could be illuminated. This model has also been derived to investigate the chicken lung collagen. The second-order kinetic model of extraction is as follows:

$$\frac{dCt}{dt} = k(C_e - C_t)^2 \quad (2)$$

where C_t is the collagen concentration (mg mL^{-1}) at time t, C_e is the equilibrium concentration of collagen (mg mL^{-1}) and k is the second order rate constant (mL mg^{-1} min^{-1}).

Solving Eq. (2) with the boundary conditions as $C_t|_{t=0} = 0$ and $C_t|_{t=t} = C_t$ gives

$$C_t = \frac{C_e^t kt}{1 + C_e kt} \quad (3)$$

Eq. (3) can be rewritten as Eq. (4) and subsequently reduced to Eq. (5) as follows

$$\frac{t}{C_t} = \frac{1}{kC_e^2} + \frac{t}{C_e} \quad (4)$$

when t approaches 0, the initial collagen extraction rate, h (mg mL^{-1} min^{-1}), can be written as:

$$h = kC_e^2 \quad (5)$$

$$\frac{t}{C_t} = \frac{1}{h} + \frac{t}{C_e} \quad (6)$$

A plot of t C_t^{-1} vs t can be drawn to determine C_e, k and h.

After rearranging Eq. (6), C_t can therefore be expressed as:

$$C_t = \frac{t}{\left(\frac{1}{h}\right) + \left(\frac{t}{C_e}\right)} \quad (7)$$

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyze the distribution of collagen subunits. The concentrations of polyacrylamide stacking gels and separating gels were 4 and 12%, respectively, and the sample wells were loaded with 25 μL. After dyeing and decolorizing, the electrophoretic bands were analyzed.

Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectrum of collagen was acquired in a FTIR spectrometer (Cary 600 Series, Agilent Technologies Inc., USA), with wavelength range from 4000 to 650 cm^{-1} and 32 scans. Two milligrams of the freeze-dried collagen powder were used and the measuring resolution was 4 cm^{-1}.

Amino acid composition

Five milligrams of sample power were hydrolyzed overnight in HCl solution (6 M) at 110–115 °C. The amino acid composition was measured by automatic amino acid analyzer (Hitachi L8800, Hitachi High-Technologies Co., Tokyo, Japan). The profile of amino acid was presented as the ratio of the individual amino acid to total amino acids. The results were reported as grams of amino acid per 100 g freeze-dried lyophilized sample, respectively. The percentage of tryptophan was not determined.

Determination of viscosity

Denaturation temperature (T_d) was determined by the method presented by Yang et al. (2016). Firstly, the Ostwald's viscometer was filled with 1.0 g L^{-1} collagen solution in acetic acid (0.1 M). The temperature increased from 10 to 50 °C and the interval was 5 °C. Every temperature was kept for 30 min and the viscosities were determined. The collagen T_d was considered as the mid-point of the linear portion, which was acquired by plotting fractional viscosity against temperatures. At least three measurements were carried on at each temperature.

Differential scanning calorimetry (DSC)

The sample melting temperature (T_m) was analyzed by DSC (Q20, instruments, New Castle, DE, USA). Samples of 8.0 mg were heated from 20 to 170 °C at a rate of 15 °C min^{-1}. T_m was defined as the temperature of endothermic peak. An empty pan was used as reference. The data of T_m for PSC and UPSC were obtained as the mean value of at least three determines.

Scanning electron microscopy (SEM)

The surface microstructure of the lyophilized PSC and UPSC powders were observed using a scanning electron microscope (EVO-LS10, ZEISSE, Baden Wurttemberg, Germany) with 10.0 kV of an accelerating voltage. Lyophilized samples were coated in an argon atmosphere using a gold/palladium alloy coater. The images of collagens were observed at 50 and 100 × magnification.

Solubility

The influences of pH and NaCl on the collagen solubility were studied based on the method of Yu et al. (2014). The collagen samples were dissolved in acetic acid solution (0.5 M) and mixed at 4 °C to get a 2.5 mg mL^{-1} solution. The pH of the sample solutions was adjusted to 2–10 with either HCl (1.0 M) or NaOH (1.0 M),

respectively. Distilled water was used to adjust the solution volume to 10 mL. The solutions were then centrifuged at 4 °C (10,000 *g*, 15 min). To study the effect of NaCl, 0, 2, 4, 6, 8, 10 and 12% of NaCl solutions were applied. The supernatants after centrifugation from the above solutions were used for determination of solubility of samples using the Kjeldahl method.

Protein analysis by NanoLC-ESI-MS/MS

The protein bands α_1 and α_2 on the gels were excised manually for NanoLC-ESI-MS/MS analysis following the method of Kang et al. (2017). In brief, each sample was firstly reduced by DTT and all cysteine residues alkylated by iodoacetamide and cleaned by desalting columns or ethanol precipitation. The sample was then digested with sequencing grade modified trypsin (Promega) in 100 mM of ammonium bicarbonate (pH 8.5). A dissolved peptide was determined by a NanoLC-ESI-MS/MS system.

The particle size of the C_{18} was 3 μM and the pore size was 300 Ä. Typical sample injection volume was 3 μL. All measured MS results were used to retrieve the most recent non-redundant protein database (NR database, NCBI) with ProtTech's ProtQuest software suite to obtain the information of collagen samples. The output from the database search was manually validated before reporting. The label-free quantitation method was used for the measurement of relative abundance of protein in each excised protein band.

Statistical analysis

Data were reported as mean ± SD. The results were analyzed with one-way analysis of variance (ANOVA) using SPSS 19.0. Significant differences were analyzed using the least significant difference (LSD) test. The significance was established at $P < 0.05$.

Results and discussion

Development of collagen extraction kinetic model

The appropriate ultrasonic power in collagen extraction from the chicken lung with ultrasound pretreatment can be identified through regression analysis. It was performed to establish empirical correlations for prediction of '*h*' and 'C_e', as well as the kinetic model. The results of C_t/t and t were obtained from the slope and intercept of Fig. 1 at a given liquid to material ratio of 20 mL g^{-1} and pepsin (2000 U g^{-1}). The data showed that the improvement in UPSC yield was obtained when higher ultrasonic power (P) was operated in the extraction process and the highest C_e was achieved at 150 W. However, a reverse trend was obtained at the treatment 200 W. This was due to the excessive ultrasonic power that might depress the solubility or destroy collagen structure in the extraction process. Meanwhile, the different

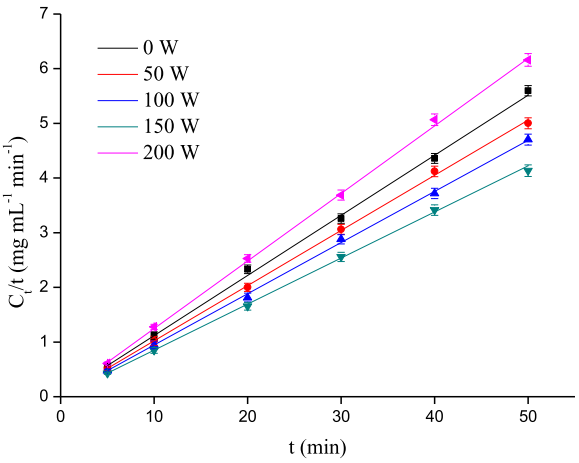

Fig. 1 Effect of extraction time on the concentration of collagen (mg mL^{-1}) at any time *t* during ultrasound power carried out at liquid to solid ratio of 20 mL g^{-1} and pepsin (2000 U g^{-1})

ultrasonic power of the extraction rate constant, *k*, initial extraction rate, *h*, and equilibrium concentration, C_e, are presented in Table 1. Therefore, the changes of kinetic parameters with ultrasonic power were represented by polynomial order polynomial functions as:

$$C_{e(P)} = 9.07 + 0.0486P - 0.00116P^2 + 1.215E^{-5}P^3 - 3.853E^{-8}P^4 \quad (9)$$

$$h_{(P)} = 54.3 + 1.570P - 0.0366P^2 + 3.858E^{-4}P^3 - 1.186E^{-6}P^4 \quad (10)$$

$$k_{(P)} = 0.668 + 0.00281P + 5.143E^{-6}P^2 \quad (11)$$

Therefore, C_t based upon ultrasonic power is obtained by substituting the above equations in Eq. (7) as:

$$C_{t,P} = \frac{t}{\frac{1}{54.3 + 1.570P - 0.0366P^2 + 3.858E^{-4}P^3 - 1.186E^{-6}P^4} + \frac{t}{9.07 + 0.0486P - 0.00116P^2 + 1.215E^{-5}P^3 - 3.853E^{-8}P^4}} \quad (12)$$

The above equation could be applied to predict a collagen yield from chicken lung under various ultrasonic powers. The obtained low errors ranges were 0.026–4.159% from the satisfactorily fitted experimental data. Therefore, the developed models could be applied to predict the extraction performances.

SDS-page

SDS-PAGE patterns of collagens from two extractions are shown in Fig. 2. Both PSC and UPSC were composed of α_1 chain and α_2 chain with approximate molecular weights below 130 kDa. The band intensities of α_1-chain are twice higher than that of α_2-chain in this pattern. The higher molecular weight components, particularly

Table 1 Extraction rate constant, initial extraction rate and equilibrium concentration for different process conditions of ultrasonic extraction

Ultrasonic processing (W)	Initial extraction rate, h (mg mL^{-1} min^{-1})	Equilibrium concentration of collagen, C_e (mg mL)$^{-1}$	Extraction rate constant, k (mL mg^{-1} min^{-1})
0	29.77	4.97	0.66
50	45.04	5.42	0.84
100	61.79	5.85	0.99
150	92.38	6.49	1.20
200	51.67	4.43	1.44

β-chains (dimmers of the α-chains), with a molecular weight of 200 kDa, were also present in our study. These SDS-PAGE patterns were similar to type I collagen triple helix from chicken bone (Oechsle et al. 2016). However, there were no γ-chains (trimers of the α-chains) in UPSC compared with PSC, implying that ultrasound could promote protein degradation in the extraction process. Therefore, SDS-PAGE patterns clearly demonstrated that the collagen acquired from the chicken lung was pure.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectrum provides special information on molecular structure, which allows investigation of the physicochemical property of proteins and collagen (Petibois & Déléris 2006). Amide A band observed at ~3410–3490 cm^{-1} is generally caused by NH stretching vibration. When the NH stretching of a protein or collagen contains a hydrogen bond, the absorption peak of amide A is shifted to lower frequencies; usually around 3300 cm^{-1} (Wang et al. 2014). The amide A band of PSC was found at 3319 cm-^{1} and had resemblance to that of UPSC from chicken lung in Fig. 3 (3316 cm^{-1}). Amide B is related to the asymmetric stretching vibration of CH alkyl chain, as well as NH_3^+ and has an absorption peak around 2850–2950 cm^{-1} (Peticolas 1979). In this study, as shown in Fig. 3, the amide B bands of PSC and UPSC occurred at 2891 and 2889 cm^{-1}, respectively.

The vibrational frequencies of amides I, II, and III bands are well-known to be directly linked to the shape of a side group polypeptide. Amide I, characterized in the range of 1600–1700 cm^{-1}, is the most important element to determine the secondary structure of a collagen (Chuaychan et al. 2015; Huang et al. 2016). The amide I band of PSC and UPSC appeared at 1673 and 1675 cm^{-1}, respectively, similar to the results of skin collagen of catla (*Catla catla*) and rohu (*Labeo rohita*) (Pal, Nidheesh & Suresh 2015). The amide II is generally associated with N-H in-plane bend as well as C-N stretching vibrations. The amide II of PSC and UPSC were present at 1582 and 1579 cm^{-1}, respectively. The amide III is responsible for C-N stretching and N-H from amide linkages, and is located in the collagen structure (Alfaro et al. 2014). Amide III bands of PSC and UPSC were located at the same

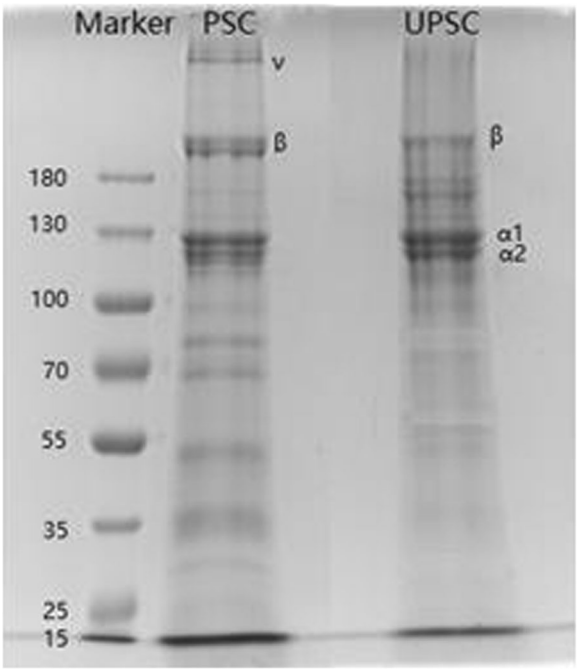

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of PSC and UPSC from chicken lung

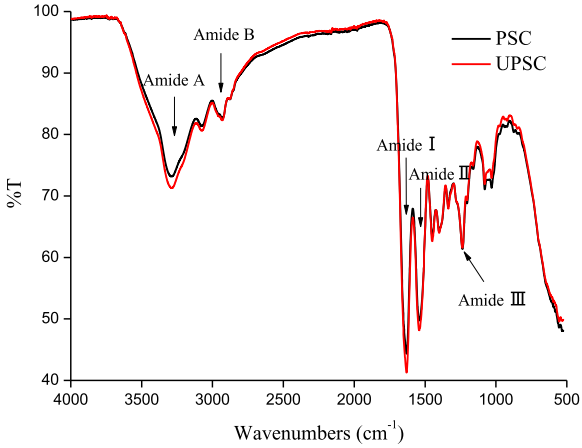

Fig. 3 Fourier transforms infrared spectra (FTIR) of PSC and UPSC from chicken lung

wave numbers (1237 cm^{-1}), and wave numbers were slightly lower than the collagen from *Loligo vulgaris* squid mantle (1246 cm^{-1}) (Cozza et al. 2016). Therefore, partial telopeptides were eliminated by pepsin during collagen preparation, probably resulting in the remove of active amino acids in the telopeptide area of the PSC and UPSC molecules (Dalla Valle et al. 2013). Additionally, strong C-H stretching at wave numbers of 1454 and 1452 cm^{-1} were observed for PSC and UPSC, respectively. This suggested that some differences existed between the secondary structural components between PSC and UPSC from chicken lung, but ultrasound pre-treatment had little effect on the triple-helical structure of collagen. In conclusion, the FTIR peak locations indicated that the inherent characteristics of PSC and UPSC were conserved.

Amino acid composition

The amino acid composition of PSC and UPSC are presented in Table 2. The compositions were similar to other collagens, in which glycine (Gly, 22.6%) was a major component, followed by alanine (Ala) and proline (Pro). The results in this study were also in accordance with those of Zhang et al. (2007) and Suleria et al. (2016), who also found that Gly was the most abundant amino acid in collagen. Ala was found as the major amino acid in the fins and scales of *C. catla* and *C. mrigala*. (Kittiphattanabawon et al. 2010). The contents of imino acids (proline and hydroxyproline, Pro and Hyp) of PSC and UPSC were 19.35 and 20.76%, respectively, which were greater than those of collagen from grass carp skin (18.6%) (Zhang et al. 2007). The imino acid contents in PSC and UPSC were also higher than those of skin collagens from cold-water fish, such as cod (15.4%) (Giraud-Guille et al. 2000) and warm-water fish bighead carp (*Hypophthalmichthys nobilis*) and grass carp (*Ctenopharyngodon idella*) (17.0–18.0%) (Hu et al. 2016). Regions of collagen containing Hyp and Pro participate in the production of connections stabilized by a hydrogen bond (Kaewdang et al. 2014). Therefore, imino acid contents are very significant for the collagen structural integrity. Thr, Met, Ile, Tyr, Phe, and His, however, showed significant lower concentrations, and Cys and Trp were not detected at all because HCl destroys them and their quantification acquires other procedures. The differences between PSC and UPSC for amino acid composition were statistically significant ($P < 0.05$), thereby, indicating a qualitative difference in these collagen (Mahboob 2015). Helices of PSC might be less stable with a lower imino acid content compared with that of UPSC. Therefore, thermal properties of PSC and UPSC were subsequently determined.

Table 2 Amino acid composition of PSC and UPSC from chicken lung (%, w/w)

Amino acid	PSC	UPSC
Asp	5.71 ± 0.13^{b}	4.87 ± 0.11^{a}
Glu	10.92 ± 0.21^{b}	10.35 ± 0.19^{a}
Ser	3.98 ± 0.09^{a}	3.74 ± 0.12^{a}
His	0.90 ± 0.04^{a}	0.86 ± 0.03^{a}
Gly	22.27 ± 0.25^{a}	22.58 ± 0.24^{a}
Thr	2.61 ± 0.07^{a}	3.07 ± 0.12^{b}
Arg	7.39 ± 0.14^{b}	6.47 ± 0.16^{a}
Ala	11.87 ± 0.20^{a}	12.75 ± 0.21^{b}
Pro	10.72 ± 0.16^{a}	11.31 ± 0.12^{b}
Hyp	8.63 ± 0.15^{a}	9.45 ± 0.13^{b}
Tyr	0.011 ± 0.0	–
Val	2.52 ± 0.05^{a}	2.96 ± 0.10^{b}
Met	0.12 ± 0.0^{a}	0.15 ± 0.01^{b}
Cys	–	–
Ile	1.35 ± 0.05^{a}	1.41 ± 0.07^{a}
Leu	3.20 ± 0.06^{a}	3.42 ± 0.09^{b}
Phe	0.67 ± 0.02^{a}	0.86 ± 0.02^{b}
Lys	3.74 ± 0.04^{a}	4.22 ± 0.05^{b}
Hydrophobic amino acids[#]	33.06 ± 0.22^{a}	35.93 ± 0.19^{b}
Imino acid[*]	19.35 ± 0.16^{a}	20.76 ± 0.15^{b}

[#] Hydrophobic amino acids: Ala + Thr + Val + Pro + Ile + Leu + Met + Phe
[*] Imino acid: Pro + Hyp
Different letters within the same line indicate significant difference; $P < 0.05$

Viscosity and the denaturation temperature (T_d)

The triple helix structure of collagen molecule can be transformed into an unordered coil construction by external cause, accompanied by reduction in viscosity values and solubility (Usha & Ramasami 2004). Therefore, viscosity determinations are usually used during the thermo-stability study of macromolecules. As shown in Fig. 4, the fractional change of PSC and UPSC from chicken lung was lessened continually when the temperature increased in the range of 10–60 °C. Rising temperature could break hydrogen bonds of collagen, and transform trimers into individual chains or dimmers. Finally, this treatment results in a change in the collagen denaturation (Kiew & Mashitah 2013). The T_d of UPSC and PSC was 38.5 and 35.3 °C, respectively. These results might be due to the Hyp ratio while it was highly correlated with thermal stability of UPSC and PSC. Additionally, the present T_d was obviously lower than that of mammalian collagen (T_d of ~ 40 °C) (Yousefi et al. 2017). The variation in T_d values might be due to the differences in species, body temperatures, living

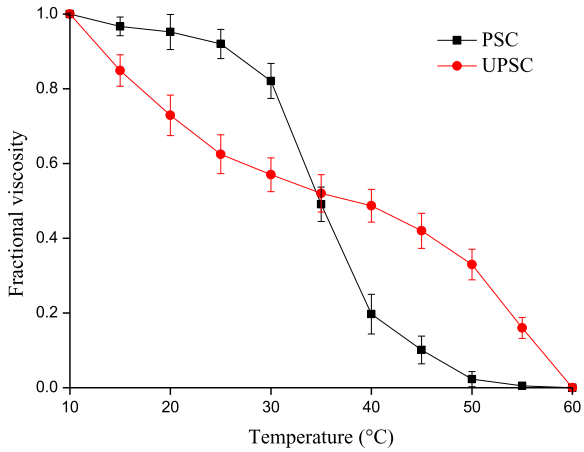

Fig. 4 Thermal denaturation curves of PSC and UPSC from chicken lung

conditions, and some differences in determination methods.

Differential scanning calorimetry (DSC)

The DSC pattern of PSC and UPSC is depicted in Fig. 5. The peak was related to continued conformational transformations of super-helix as a result of the destruction of materials. The T_m of PSC and UPSC was 90.16 and 94.16 °C, respectively, and the T_d (the above section) of UPSC was higher than that of PSC, consistent with the higher Hyp content of the UPSC (9.45%) than PSC (8.36%). The results indicated that thermal properties of collagens were related to their physicochemical changes caused by ultrasound treatment. They also demonstrated that ultrasound treatment partially alters the degrees of hydration, and the property of covalent cross-links. Hence, UPSC could have greater advantage in thermal stability and is promising in food processing, cosmetics and other industries.

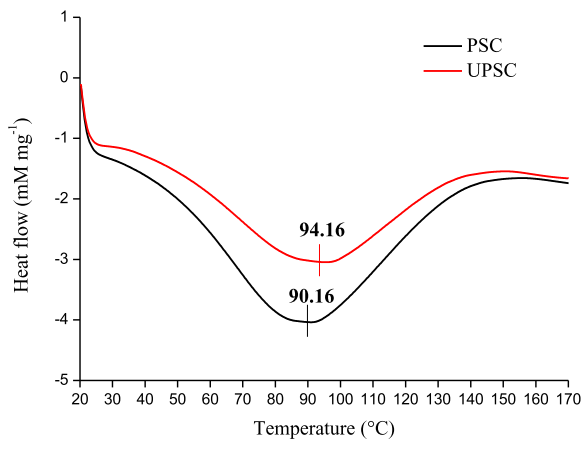

Fig. 5 Differential scanning calorimetry (DSC) thermograms of PSC and UPSC from chicken lung

Scanning electron microscopy (SEM)

Lyophilized PSC and UPSC were in loose, fibrous, porous and multi-layered aggregated structures observed by SEM (Fig. 6), similar to collagens from skeletal bone collagen (SCII) and head bone collagen (HCII) (Jeevithan et al. 2014). However, UPSC exhibited a looser and larger aperture structure than PSC. Moderate and uniformly distributed pore size of collagen was suitable for in vivo studies in various applications (Caliari et al. 2011; Cheng et al. 2017). For the difference in pore diameter of the two collagens, the different appearance might be due to the mechanical action and cavitation effect by ultrasound treatment, and different collagen concentrations before lyophilization. The average pore diameter and porosity of collagen are extensively considered as critical factors for biomaterials (Song et al. 2006). Some researchers have also found that the surface microstructure can be altered on the basis of the collagen content during sample preparation (Ikoma et al. 2003; Tang et al. 2015). Thus, UPSC may serve as an alternative source of collagens for application in food packaging, processing, and biomedical industries.

Solubility

Effect of pH

As shown in Fig. 7a, PSC and UPSC had a greater solubility in the acidic range of pH 1–4, and maximums solubility at pH 3–4. Denaturation of PSC and UPSC could occur to some extent under pH 1.0, resulting in lower solubility. Sharp decrease in solubility was then observed by increasing the pH and a minimum was reached at pH 8. Additionally, an increase in sample solubility was also presented in an alkali pH range. The reason for the higher relative solubility might be due to the greater net residue charges of collagen molecules, which improves inter-chain repulsion forces between chains, when the pH is higher or lower than the isoelectric point (pI) of collagen (Liu et al. 2012; Zhang et al. 2014). These results were similar to the study of Woo et al. (2008). In addition, UPSC exhibited higher solubilities than PSC in all tested pH range with the exception of pH 1–2, which implied UPSC could reduce the degree of cross-linking or weaken bonds due to ultrasound treatment in comparison with PSC from chicken lung (Jongjareonrak et al. 2005; Li et al. 2013; Yu et al. 2014).

Effect of NaCl

Both UPSC and PSC from chicken lung had similar solubility patterns in different NaCl concentrations (Fig. 7b). UPSC and PSC possessed better solubilities

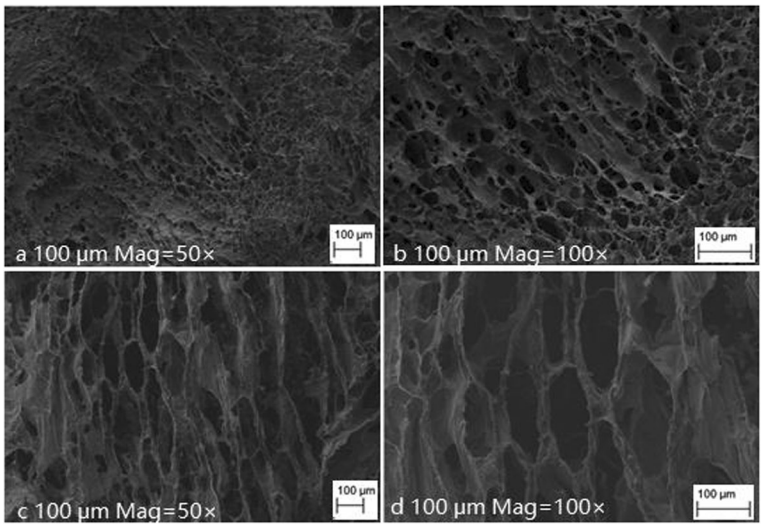

Fig. 6 Scanning electron microscopy (SEM) of collagen from chicken lung (**a**) PSC, Mag = 50 ×; (**b**) PSC, Mag = 100 ×; (**c**) UPSC, Mag = 50 ×; (**d**) UPSC, Mag = 100 ×

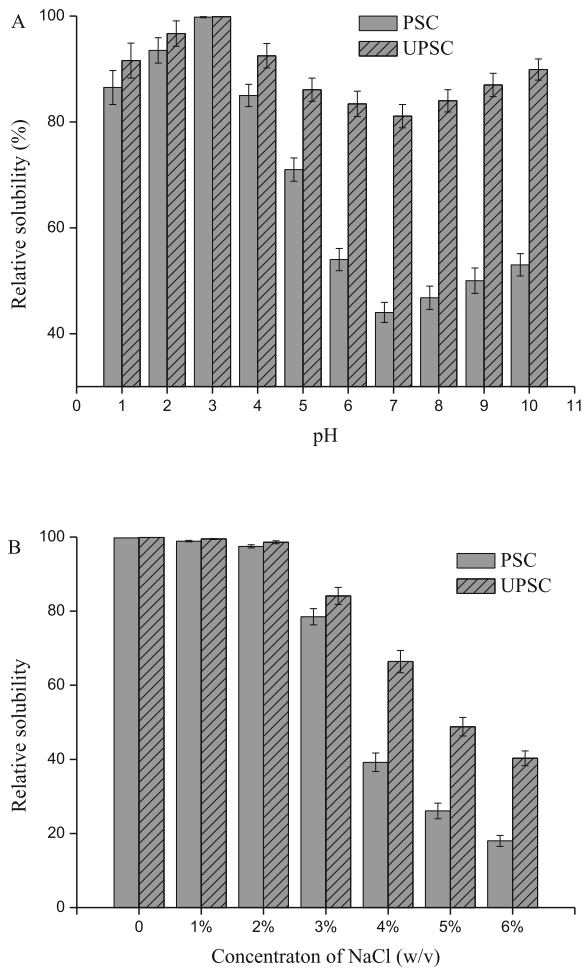

Fig. 7 Relative solubility (%) of PSC and UPSC from chicken lung as affected by different environmental factors. **a** pH; **b** concentration of NaCl

at NaCl concentrations below 2%, which was then significantly dropped when NaCl concentration was in the range of 3–6%. The solubility trend was consistent with collagens from the skin of trout, brown stripe red snapper and Spanish mackerel (Jongjareonrak et al. 2005; Li et al. 2013). The increase in the competition with water for NaCl is known to contribute to enhancing hydrophobic interactions between protein chains and lead to more collagen precipitation, as the ionic strength increased (Minh Thuy et al. 2014). Moreover, UPSC presented higher solubilities than PSC at NaCl concentration above 2%. This result showed that ultrasound treatment induced a partial hydrolysis of high molecular weight cross-linked collagen from pepsin extraction, leading to a higher solubility of UPSC from chicken lung.

Protein profiles of collagen after ultrasound pretreatment

NanoLC-ESI MS/MS is a sensitive technique to identify the sequencing peptides, so it was used in our study. The spectra resulting from data-dependent acquisitions in the NCBI database by using Mascot scores of peptide identification were for the α-1 and α-2 bands in UPSC gel. Among the peaks, a charged peak with m/z 835.4, 1505.8 and 811.4 was the main component in α-1 and α-2 band as determined by nanoLC-ESI MS/MS, and the sequencing peptide was identified as GPAGPQGPR, QLEEAEEESQR and GVAGPQGAR, respectively (Fig. 8). When this sequence was searched online in NCBI, we found a record of peptide from chick collagen alpha-2(I) chain [*Gallus gallus*] with 100% identification. The stabilization of this conformation requires

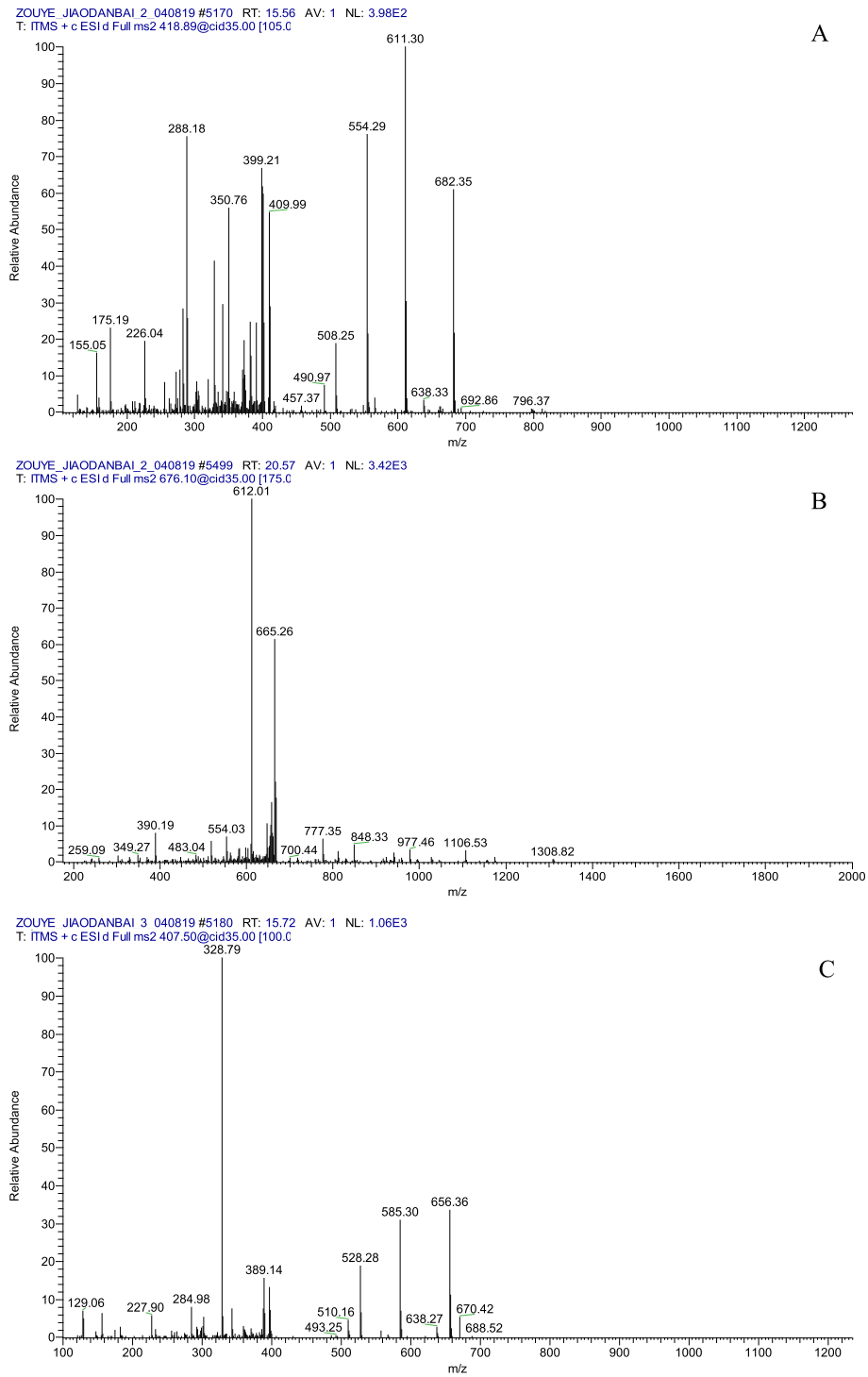

Fig. 8 MS fingerprints of main collagen from chicken lung. Sequencing peptide: **a** GPAGPQGPR, **b** QLEEAEEESQR and (**c**) GVAGPQGAR, respectively

the presence of Gly residues at every third position in the sequence and high contents of imino acids. The collagens in proteomic analysis shown in Table 3 could be classified on the basis of their biological function. Briefly, UPSC from chicken lung is classified into 10 main groups, such as collagen alpha-2(I) chain (Fragments), chick actin, cytoplasmic 1 OS, etc. Based on the SDS-PAGE subunit, amino acid composition and peptide profiles, it might be concluded that collagen from chicken lung, is type II collagen. Therefore, the results

Table 3 Identified UPSC from chicken lung sample by LC-ESI-MS/MS sequencing and analysis

Collagen α-1	Sequence Header	Protein mass	Protein count	Percentage (%)
	>sp.|P02457|CO1A1_CHICK Collagen alpha-1(I) chain OS = *Gallus gallus* GN=COL1A1 PE = 1 SV = 3	138,696.42	4	45.3%
	>sp.|P10587|MYH11_CHICK Myosin-11 OS = Gallus gallus GN = MYH11 PE = 1 SV = 4	229,509.19	14	40.7%
	>tr|A0A1D5PGZ0|A0A1D5PGZ0_CHICK Uncharacterized protein OS = Gallus gallus PE = 4 SV = 1	48,263.92	2	6.8%
	>sp.|P02467|CO1A2_CHICK Collagen alpha-2(I) chain (Fragments) OS = Gallus gallus GN=COL1A2 PE = 1 SV = 2	180,555.8	12	2.1%
	>tr|A0A1D5PX03|A0A1D5PX03_CHICK Uncharacterized protein OS = Gallus gallus PE = 4 SV = 1	95,230.95	2	3.0%
	>sp.|P60706|ACTB_CHICK Actin, cytoplasmic 1 OS = Gallus gallus GN = ACTB PE = 1 SV = 1	42,051.85	1	2.1%
Collagen α-2	>sp.|P02467|CO1A2_CHICK Collagen alpha-2(I) chain (Fragments) OS = Gallus gallus GN=COL1A2 PE = 1 SV = 2	180,555.8	41	83.5%
	>sp.|P02457|CO1A1_CHICK Collagen alpha-1(I) chain OS = Gallus gallus GN=COL1A1 PE = 1 SV = 3	138,696.42	17	12.8%
	>sp.|P10587|MYH11_CHICK Myosin-11 OS = Gallus gallus GN = MYH11 PE = 1 SV = 4	229,509.19	7	3.5%
	>sp.|P60706|ACTB_CHICK Actin, cytoplasmic 1 OS = Gallus gallus GN = ACTB PE = 1 SV = 1	42,051.85	1	0.1%

of protein profiles of UPSC could offer useful knowledge to better understand the collagen profile from chicken lung.

Conclusion

The chicken lung serves as an alternative source of collagen with a maximum collagen yield of 31.25% upon ultrasound pre-treatment at 150 W through the extraction kinetics. UPSC from chicken lung peptide was mainly identified as GPAGPQGPR, QLEEAEEESQR and GVAGPQGAR with a higher thermal stability, a better fibril forming capacity as well as better solubility in different pH and NaCl solution. Thus, UPSC from chicken lung serves as a potential alternative source of mammal collagens for applications in food processing, packaging and biomedical fields. The biological activity of peptides from chicken lung needs to be further studied.

Abbreviations

DSC: Differential scanning calorimetry; FT-IR: Fourier transform infrared; PSC: Pepsin-soluble collagen; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM: Scanning electron microscopy; T_d: Denaturation temperature; UPSC: Pepsin-soluble collagen from ultrasound pretreated chicken lung

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Authors' contributions

YZ designed this research, performed these experiments and wrote this manuscript; HY, XZ and MZ performed research; PX and DJ analyzed data; DW and WX conceived the study and exited the manuscript; and all authors read and approved the final article.

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Availability of data and materials

This is a research manuscript and all datasets on which the conclusions of the manuscript rely are included in the tables of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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