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Co-production of chondroitin sulfate and peptide from liquefied chicken sternal cartilage by hot-pressure

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ABSTRACT

Co-production of chondroitin sulfate (CS) and peptides was realized from the liquid fraction of chicken sternal cartilage subjected to hot-pressure (HP) by membrane combination separation technology. Cartilage was liquefied via the HP treatment at 110 °C (0.07 MPa) and 120 °C (0.1 MPa) for 0.5 − 2.5 h, respectively. The optimized co-production procedure was as follows: enzymolysis temperature, 61.2 °C; the enzyme ratio of trypsin and papain, 1.3:1 (W/W); enzymolysis time ratio, 2:2 (h/h), under which the highest yields of CS and peptides were 18.85% and 67.99%, and the recoveries were 93.63% and 92.69%. The average molecular weight of CS sample was 67.79 kDa. CS sample was confirmed using agarose-gel electrophoresis, and the structure was analyzed by Fourier transform infrared spectroscopy, chromatography and nuclear magnetic resonance. Taken together, HP can be as a pretreatment method to liquefy cartilage for the industrial co-production of CS and peptides with eco-friendly.

1. Introduction

In 2015, China has being the biggest chondroitin sulfate (CS) producer and exporter, which account for 80% of global CS output (ReportBazzar, 2015). The cartilages of terrestrial animals, avian and bony fish were the primary sources of CS (Maccari, Galeotti, & Volpi, 2015; Volpi, 2006). However, considering the raw material safety such as the contamination by some infectious diseases (the mad-cow disease, foot-and-mouth disease, or hog cholera) and limitation such as some endangered marine organisms, it will be a great challenge to supply the growing demand of CS in the future. China is one of the biggest chicken producer country. According to United States Department of Agriculture's report, China's production of chicken meat will increase to 12 million metric tons in 2019 (United States Department of Agriculture (USDA), 2019). It means that approximately 30,000 tons of chicken sternal cartilage will be produced, most of which, however, are usually processed into some low-value-added snack foods in China.

Except the moisture, the main constituents of the chicken sternal cartilage are protein and carbohydrate. The protein in this cartilage can be hydrolyzed into bioactive peptides against the osteoporosis (Lin et al., 2018), and the carbohydrate in the cartilage exposed to steam explosion can be isolated as the CS (Shen et al., 2019). But, few research of the co-production of CS and peptides is reported. As a typical sulfated glycosaminoglycan, CS is covalently attached to a core proteins, and the over size of which ranges from 80 kDa to 3500 kDa (Silbert & Sugumaran, 2002). In terms of the traditional CS isolation procedures, high-concentration alkali treatment is commonly thought to be required to breakdown of the core protein (Shi et al., 2014). However, it will produce a lot of effluent during the industrial production, which is not environmentally friendly. Additionally, the sewage treatment system will increase cost of production. Therefore, the digestion of proteins with enzymes, and separation by ultrafiltration-diafiltration technologies has being the alternative strategy to replace the conventional isolation method. Single enzyme of alcalase, trypsin or papain is chosen to hydrolyze the protein for CS isolation, but only one step of the enzyme hydrolysis will take approximately 24 h (Krichen et al., 2018; Maccari, Ferrarini, & Volpi, 2010, 2015), which is probably not practical during the industrial production. The previous study indicates that CS can be isolated from liquefaction of cartilage by steam explosion, and hydrolysis time reduces to 10.5 h (Shen et al., 2019). Even so, it is too long for CS production in industry. Besides, the highest liquefaction rate of chicken sternal cartilage treated steam explosion is 75.72%,

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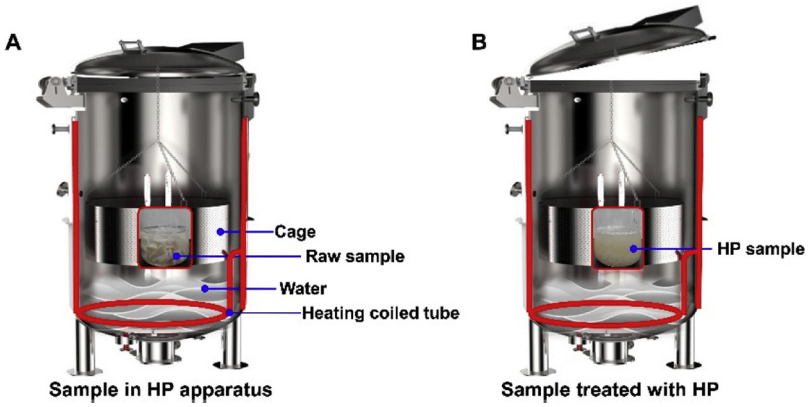

Fig. 1. Diagram of the HP process. The apparatus was mainly composed of a heating coiled tube and cage. (A) Sample was in the HP apparatus. Water heated by the heating coiled tube produces the steam, which provided the pressure and high temperature for the sample. (B) Sample has been treated with HP.

which means that more than 20% cartilage is not be used availably. The yield and the purity of CS obtained by this method were 18.55% and 91.53%.

Thermal liquefaction technology has been widely utilized in lignocellulosic biomass (Kozliak et al., 2016). The essence of thermal liquefaction can be illustrated that constituents of the material migrate to the media (such as the steam or water) during the treatment. In our lab, the hot-pressure (HP) has been developed for some constituent extraction. During the HP procedure, the materials such as the chicken bone or the residues, especially for the cartilage from the ends of the bone, can be liquefied partly. After the chicken bone treated with HP, the protein is isolated (Dong et al., 2014; Yue et al., 2017), and the substrate for flavorant can be prepared (Wang et al., 2016). To make the best of the cartilage and shorten the hydrolysis time, HP is proposed to liquefy the chicken sternal cartilage to realize the co-production of the CS and peptides.

In the present study, chicken sternal cartilage was almost liquefied by HP. Co-production of CS and peptides was realized environmentally friendly by double-enzyme hydrolyzing and membrane combination separation technologies from the HP cartilage liquid. The effects of HP parameters, including the temperature and time, on the liquefaction of cartilage were investigated. The microstructure changes and the migration of constituents of the HP cartilage solid residue were verified by scanning electron microscopy (SEM) and alcian blue staining. The chemical and structural properties of CS sample were confirmed by Fourier transform-infrared spectroscopy (FT-IR), agarose-gel electrophoresis, chromatography and nuclear magnetic resonance (NMR). The distribution of peptides molecular weight was determined by exclusion chromatography.

2. Materials and methods

Fresh chicken sternal cartilage from the adult White-feather chicken (42 d) was provided kindly by the PROTIL Biotechnology Co. Ltd (Hebi, Henan province, China). Before experiments, the residuary meat was removed from the cartilage, and the cartilage was stored at −20 °C until use (Avoid repeated freeze-thaw cycles). Chondroitin sulfate A standard and chondroitinase ABC (50 − 250 U/mg) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dermatan sulfate and heparan sulfate were bought from Medchem Express. Trypsin (1:250, Solarbio, Beijing, China) and papain (800 U/mg, Solarbio, Beijing, China) were purchased from Solarbio. The standard unsaturated chondro/dermato disaccharides involving ΔDi0 S (ΔUA-[1→3]-GalNAc), ΔDi4 S (ΔUA-[1→3]-GalNAc-4 S), ΔDi6 S (ΔUA-[1→3]-GalNAc-6 S), ΔDi2,4 diS (ΔDi-dis B, ΔUA-2 S-[1→3]-GalNAc-4 S), ΔDi2,6 diS (ΔDi-dis D, ΔUA-2 S-[1→3]-GalNAc-6 S), ΔDi4,6 diS (ΔDi-dis, ΔUA-4 S-[1→3]-GalNAc-6 S), and ΔDi2,4,6 triS (ΔDi tris, ΔUA-2 S-[1→3]-GalNAc-4 S,6 S) were bought from the Iduron Corporation (Alderley City, UK).

2.1. Proximate composition determination

The moisture in fresh cartilage was determined through drying to a constant weight in an oven at 105 °C. The dried cartilage was smashed into powder to estimate the contents of protein, fat and ash by AOAC methods (AOAC (Association of Analytical Chemists), 2000). The protein content was estimated through AOAC method 976.05, with a nitrogen to protein conversion factor of 6.25, by the Kjeldahl method using a Kjeltec 2300 Analyser (Foss Tecator, Hoganas, Sweden). The ash content was measured by weighing samples before and after heat treatment at 550 °C in a muffle furnace for 6 h (AOAC method 923.03). The fat content was assessed by AOAC method 960.39, with petroleum ether (40 − 60 °C) using a Soxhlet apparatus (VELP SER148, Italy). According to Vázquez et al. (Vázquez et al., 2019), the total carbohydrate content was calculated by Eq. (1):

$$\text{Carbohydrate (\%)} = 100\text{-}\ P_{protein}\text{-}P_{fat}\ \text{-}P_{ash} \quad (1)$$

Where, $P_{protein}$, P_{fat} and P_{ash} indicate the content (%) of protein, fat and ash in the dried cartilage, respectively.

2.2. Hot-pressure experiments

The HP procedures were executed with a HP apparatus. The HP process is shown in Fig. 1. The solid-liquid ratio of cartilage (approximately 50 g) and distilled water was 1: 2.5 (W/V), of which condition the cartilage was just barely submerged in the distilled water. The temperature was set at 110 °C (0.07 MPa) and 120 °C (0.1 MPa) with a maintaining time of 0.5, 1.0, 1.5, 2.0 and 2.5 h, respectively. After HP, a certain of distilled water was added to keep the original weight, and then the sample's Brix of the liquid was determined with a portable Brix meter (EXTECH RF11, FLIR) at room temperature. The liquid fraction together with the residue solid in the beaker were homogenized at 1500 rpm for 20 s by a homogenizer (IKA T18® digital ULTRA TURRAX®, Germany). Subsequently, the residue solid was separated by six-layer gauze. The liquid fraction was used to isolate the CS and peptides, and the residual solid was used to investigate the composition migration and SEM. The liquefaction rate of the sample was defined by Eq. (2):

$$\text{Liquefaction rate (\%)} = (1\text{-}m_1/m_0) \times 100 \quad (2)$$

Where, m_0 and m_1 indicate the dry weight of the original sample and the residual solid after HP and homogenizing, respectively.

2.3. Scanning electron microscopy

Sample preparation was performed as described previously (Shen et al., 2019). Briefly, samples were firstly fixed in 2.5% glutaraldehyde at room temperature for 4 h, and the excess glutaraldehyde was removed by 0.5 h with 0.1 M PBS (pH = 7.2). Then, the samples were fixed again by 1% osmic acid for 2 h. Subsequently, the osmic acid was removed with distilled water. Ultimately, dehydration with a graded ethanol series (from 30 to 100%) was carried out, and the samples were dried with critical point drying. All of the samples were fixed on aluminum sample stubs and coated with gold. The microstructure was observed by SEM (SU8010, Hitachi Ltd., Japan) at a 10 kV acceleration voltage with a magnification of 150× or 2000×.

2.4. Alcian blue staining

Based on the method of Dingerkus et al. (Dingerkus & Uhler, 1977), cartilages treated with or without HP at different conditions were fixed in 10% formalin-saline solution for 48 h, followed by decalcification with 0.1 M ethylene diamine tetraacetic acid for 7 d. Then the cartilages were embedded in paraffin, and prepared into 5 μm thick tissue sections to stain with alcian blue. The colour distribution was observed by a microscope (NIKON CI-S) with an imaging system (NIKON DS-U3).

2.5. CS and peptides isolation from the liquid sample

The liquid of HP cartilage liquefied at 120 °C for 1.5 h was chosen to optimize isolation conditions of the CS and peptides. The isolation procedures were performed according to method of Shen et al. (Shen et al., 2019). In detail, the Brix of the liquid sample was adjusted to 1% by adding distilled water. After that, the liquid sample was subjected to enzymolysis temperatures (45 − 65 °C), varying enzyme ratios (0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, W/W) of trypsin and papain (0.1%) (e.g. Enzyme ratio of trypsin and papain of 0.5: 1 meant that the amount of trypsin was 0.05% and the amount of papain was 0.1%), and enzymolysis time ratios (0.5:3.5, 1:3, 2:2, 3:1, 3.5:0.5, h/h) of trypsin and papain (e.g. Enzymolysis time ratio of 0.5: 3.5 meant that the solution was hydrolyzed by trypsin for 0.5 h, and then hydrolyzed by papain for 3.5 h). Following enzymolysis, enzymes were inactivated by boiling at 100 °C for 5 min. Subsequently, the enzymatic solution was filtered successively to separate the CS and peptides by the 0.45 μm (MCE, JIN TENG, China) and 10 kDa membranes (VF05P0, Sartorius Vivaflow 50). For the separation procedures, the solution was firstly filtered through a 0.45 μm membrane with a suction filter, and then, CS and peptides were separated from the filtrate by cycling with a peristaltic pump (YZ1515X, WT600-2J, and Longer Pump®) equipped with a 10 kDa membrane. The cycling separation was performed six times by adding an equal volume of distilled water. The speed of the pump was set at 100 rpm according to the specification. Ultimately, CS solution (the trapped fluid from the 10 kDa) and peptides solution (including the trapped fraction from the 0.45 μm membrane and the filtrate from the 10 kDa membrane) were lyophilized with a freeze drier (SR-A18N-80, Shanghai, and China). The lyophilized powders were used for further analysis.

2.6. Optimization of the CS and peptides isolation

The effects of enzymolysis temperatures, enzyme ratio of trypsin and papain, and enzymolysis time ratio on the yields of CS and peptides were determined by single factor tests. The yields of CS and peptides were calculated by Eqs. (3) and (4), respectively:

$$\text{CS yield (\%)} = m_{CS}/m_0 \times 100 \quad (3)$$

$$\text{Peptides yield (\%)} = m_{peptides}/m_0 \times 100 \quad (4)$$

Where, m_{CS} and $m_{peptides}$ are the weight of the CS sample and peptides sample and m_0 is the dry weight of the original sample. The yields of CS and peptides were chosen as the response value for response surface methodology (RSM). A Box-Behnken design (BBD) with three independent factors (X_1, enzymolysis temperature; X_2, the enzyme ratio of trypsin and papain; X_3, enzymolysis time ratio) at three variation levels was performed.

Ranges of enzymolysis temperature (X_1), the enzyme ratio of trypsin and papain (X_2) and enzymolysis time ratio (X_3) were employed to prepare seventeen experiments, which included twelve factorial points (levels ± 1) and five replicates of the central point, which were used to optimize the yield conditions of CS and peptides (Zou, Chen, Yang, & Liu, 2011). The CS yield (Y_1) and peptides yield (Y_2) were the response values, respectively. Design-Expert software (version 8.0.6, Stat-Ease Inc., Minneapolis, USA) was used to analyse the experimental data. Experimental data were fitted to a second-order polynomial model, and the regression coefficients were obtained (Liu et al., 2015). The generalized second-order polynomial model used in the response surface analysis was as follows in Eq. (5):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ii} X_i X_j \quad (5)$$

Where, Y is the response value; X_i and X_j are the independent variables; β_0, β_i, β_{ii} and β_{ij} indicate the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively. The response surfaces were obtained by Design-Expert software while holding a variable constant in the second-order polynomial model. According to the saddle point in the response surfaces, the estimated ridge of the optimum response was calculated by the ridge analysis of the design expert procedure (Liyanapathirana & Shahidi, 2005). Based on the optimized conditions, CS and peptides were obtained from the HP liquid sample. The protein contents of CS sample were evaluated by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). The recoveries of CS and peptides were calculated by Eqs. (6) and (7), respectively:

$$\text{CS recovery (\%)} = m_{CS}/m_{0carbohydrate}\text{-}m_{1carbohydrate} \quad (6)$$

$$\text{Peptides recovery (\%)} = m_{peptides}/m_{0protein}\text{-}m_{1protein} \times 100 \quad (7)$$

Where, m_{CS} and $m_{peptides}$ are the weight of the CS sample and peptides sample, and $m_{0carbohydrate}$ and $m_{1carbohydrate}$ are the total weight of the carbohydrates in the dry original cartilage and dry residual cartilage, respectively, and the $m_{0protein}$ and $m_{1protein}$ are the total weight of the protein in the dry original cartilage and dry residual cartilage.

2.7. Agarose-gel electrophoresis

Based on the methods of Dietrich et al. (Dietrich, Mcduffie, & Sampaio, 1977) and Volpi et al. (Volpi & Maccari, 2002) with slight modifications, agarose-gel electrophoresis of CS sample together with CS standard and other glycosaminoglycans including dermatan sulfate and heparan sulfate was carried out. CS sample and glycosaminoglycans were dissolved in chondroitinase ABC buffer (33 mM Tris − HCl, pH = 6.2, 33 mM sodium acetate, and 1 mU chondroitinase ABC) with a final concentration of 5 mg/mL at 37 °C for 8 h. Agarose-gel was prepared by a 1% agarose in barium acetate buffer (0.04 M, pH = 5.8). Twenty microliter of CS standard, the glycosaminoglycans and CS sample with or without treatment of chondroitinase ABC were loaded in the agarose-gel for electrophoresis. The run was conducted with an electrophoretic instrument (JY-SPCT) in 1, 3-diaminopropane buffer (0.05 M, pH = 9.0) at 100 mA for 4 h. After migration, the gel was soaked in cetyl pyridine chloride solution (0.2%) for 4 h and stained with fresh toluidine blue (0.2%) for 6 h. The gel of the background was faded away with distilled water. The relative migration rate was computed by the migration of glycosaminoglycan/migration of the CS standard.

2.8. FT-IR analysis

FT-IR spectra of CS sample, peptides sample and CS standard were recorded with an FT-IR spectrometer (Tensor-27, Bruker Company, Germany). The potassium bromide powder was as the background. The dried sample powder was ground together with potassium bromide powder (1:200) and pressed into a 1 mm pellet for measurement in a frequency range of 4000–500 cm^{-1} at a resolution of 4 cm^{-1} in the transmission mode.

2.9. Nuclear magnetic resonance spectroscopic analysis

The ^{13}C-NMR spectrum of CS sample was recorded by a Bruker AMX600 WB spectrometer equipped with a 5 mm diameter tunable probe, operating at 600 MHz. Fifty milligrams of sample was dissolved in 1.0 mL of D_2O at a high level of deuteration (99.997%). The spectrum was registered at 25 °C, and ^{13}C chemical shifts (δ, ppm) were quoted with respect to external sodium 4, 4-dimethyl-4-silapentane- 1-sulfonate (0.0 ppm). Spectra were processed with MestReNova 9.0.1 software (Mestrelab Research, Spain).

2.10. Enzymatic treatment and constitutive disaccharide determination

CS sample was dissolved in chondroitinase ABC buffer mentioned above with a final concentration of 5 mg/mL at 37 °C for 8 h, and then the solution was boiled at 100 °C for 5 min to inactivate the enzyme (Grøndahl, Tveit, Akslen-Hoel, & Prydz, 2011). The unsaturated disaccharides in the solution were detected by strong anion exchange (SAX)-HPLC apparatus (Agilent 1260 Infinity II) equipped with a 150 mm × 4.6 mm stainless-steel Spherisorb 5 − SAX column (5 μm, trimethylammoniopropyl groups $Si-(CH_2)_3-N^+(CH_3)_3$ in the Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK), and the signal was detected at 232 nm. Isocratic separation procedures were shown as follows: 50 mM sodium chloride (pH = 4.0) for 5 min, followed by a linear gradient from 5 to 35 min of 50 mM to 1.0 M sodium chloride (pH = 4.0) with a flow rate of 1.0 mL/min (Maccari et al., 2015). The injection volume was 10 μL, and standard disaccharides were used for qualitative and quantitative analysis.

2.11. Molecular weight determination of CS

The molecular weight of CS sample was evaluated by gel permeation chromatography with a multi-angle laser light scattering system (GPC/MALLS) following the method of Roulard et al. (Roulard, Petit, Mesnard, & Rhazi, 2016) with some modifications. HPLC (Hitachi High-Technologies Corporation, Tokyo Japan) equipped with DAWN HELEOS-II(Wyatt Technology Corporation, America) and Optilabr EX (Wyatt Technology Corporation, USA) detectors coupled with a TSK gel G4000PWxl column (7.8 × 300 mm) were employed. Column oven and multi-angle laser light scattering detector were kept at 30 °C, and the refractive index detector was maintained at 35 °C. Sodium chloride (0.1 M) was filtered through a 0.2 μm membrane, and degassed for 30 min as the mobile phase with a flow rate at 0.5 mL/min. CS sample was solubilized in the mobile phase at a final concentration of 1 mg/mL. According to Nordmeier (Nordmeier, 1993), the dn/dc value (the refractive index increment) was detected as 0.132. The injection volume of CS sample was 200 μL. Before the determining of the molecular weight of CS sample, the standard (SIGMA, 31389-25 G, Dextran from *Leuconostoc spp*. Mr~40,000) dissolved in the same mobile phase was assessed to calibrate the detector. And then the molecular weight of CS sample was evaluated under the same conditions.

2.12. Distribution of peptides molecular weight

The molecular weight distribution of peptide was determined via the Agilent liquid chromatograph (Agilent 1260 Infinity II) equipped with a TSK gel filtration column, G2000 SWXL 300 mm × 7.8 mm (Tosoh Co., Tokyo, Japan), and the signal was detected at 214 nm. The method was as previously described with modifications (Irvine & Shaw, 1986). The mobile phase composed of acetonitrile/water/trifluoroacetic acid (45/55/0.1, v/v/v) was delivered at a flow rate of 0.5 mL/min. The column was thermostated at 40 °C and 20 μL of sample was injected into the HPLC system. A molecular weight calibration curve ($Y = -3.8929X + 27.825$, $R^2 = 0.9806$) was obtained from the following standards from Sigma: Cytochrome C (12384 Da), aprotinin (6495 Da), bactracin (1421 Da), tetrapeptide (451 Da), and dipeptide (146 Da).

2.13. Ultraviolet absorption spectrum of peptides

The ultraviolet absorption spectrum (UV) of peptides was determined by a spectrophotometer (UV-1800, Mapada Instruments Co., Ltd., Shanghai, China) from 200 to 600 nm. The sample was prepared by dissolving in distilled water at the final concentration of 2 mg/mL.

2.14. Amino acid analysis of peptides and CS

Amino acids were determined with L8900 AA auto-analyzer (Hitachi Co., Tokyo, Japan) based on the previous report with some modifications (Sun et al., 2015), the dry basis of peptides sample and CS sample were hydrolyzed with 10 mL 6 M HCl at 110 °C for 24 h in evacuated sealed tubes, respectively. After hydrolysis, the samples were filtered and transferred into a 50 mL volumetric flask with shaking vigorously. One milliliter hydrolysate was dried with nitrogen and then dissolved in 5 mL of 0.02 M HCl. Twenty microliter of preparation sample was taken to analyse the content of amino acids.

2.15. Statistical analyses

All experiments have three replications and the results were shown as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) and Duncan's multiple range tests. The level of statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. HP induced liquefaction of chicken sternal cartilage

In addition to the minor components of fat (0.26 ± 0.04%) and ash (6.29 ± 0.43%), the main constituents of the dried cartilage were protein (73.35 ± 0.36%) and carbohydrate (20.10 ± 0.71%), indicating that chicken sternal cartilage can be used to isolate the CS and peptides. However, in terms of the traditional CS isolation, alkali such as NaOH, causing the effluent in industry, was commonly required to break the covalent bonds between CS and the core protein. Interestingly, CS can be obtained environmentally friendly (chemical free) from the liquid cartilage by steam explosion (Shen et al., 2019). Additionally, high temperature (120 °C) for a long time (2 h) seemed not influence the CS structure obviously (Wang et al., 2019). Therefore, HP was proposed to liquefy the chicken sternal cartilage to realize the co-production of the CS and peptides. With the increase in the maintaining time (from 0.5 to 2.5 h) at 110 °C and 120 °C, the liquefaction rate of the cartilage increased from 26.74% to 99.43% (Fig. 2), which suggested that the cartilage could be almost liquefied, and the liquefaction rate reached the peak at 120 °C for 1.5 h. Compared with the similar research to isolate the CS from the liquefied cartilage by steam explosion (Shen et al., 2019), this liquefaction rate reached to 99.36% (120 °C for 1.5 h) that was much higher than that (from 41.65% to 75.72%) of steam explosion treatment. And it means this HP treatment method improves the utilization of the cartilage.

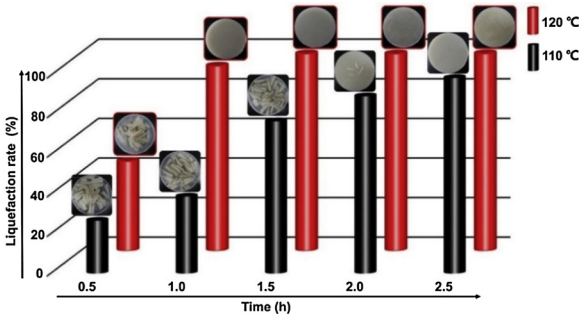

Fig. 2. Liquefaction of cartilage by HP. The liquefaction rate of HP samples at different temperatures and maintaining times. The insets indicate the mixture of the solid residue and liquid fraction at the corresponding HP conditions.

3.2. HP induced cartilage microstructure changes and carbohydrate migration

HP induced the microstructure of cartilage changes seriously, especially when the maintaining time was more than 1.5 h at 110 °C (Fig. 3A). Additionally, the Brix (soluble solid content) of the liquid from HP cartilage sample rose as the temperature and maintaining time increased (Fig. 3B), which means some component of the cartilage occurred migration. At 120 °C, when the maintaining time was more than 1.5 h, the Brix change became slowly. Compared with the normal cartilage, the smooth and compact surface of cartilage disappeared while amounts of irregular cracks were observed in the HP residual cartilage by SEM, which was probably caused by the migration of some components in the cartilage subjected to HP. To confirm this hypothesis, the residual cartilage was stained by alcian blue. Compared with the normal cartilage, the blue color became light gradually in the HP residual cartilage with the maintaining time increasing (Fig. 3C), which suggested that the content of mucopolysaccharide in the cartilage was decreased after HP treatment. Meanwhile, the contents of the constituents including the protein, fat, ash, and carbohydrate of the dried residual cartilage samples (portion) were investigated. Results indicated that the relative content of protein increased significantly (from 73.35% to 94.46%) while the carbohydrate content accordingly decreased (from 20.10% to 0.78%) (Fig. 3D). Taken together, HP could lead to the microstructure changes of the cartilage and cause the most of the carbohydrate to occur the migration from the solid to the liquid.

3.3. Isolation optimization for CS and peptides from the HP cartilage liquid fraction using RSM

Based on the results of liquefaction rate and Brix, the liquid sample from the cartilage subjected to 120 °C for 1.5 h was selected to isolate CS and peptides. The effects of enzymolysis temperature (X_1), the enzyme ratio of trypsin and papain (X_2) and enzymolysis time ratio (X_3) on the yields of CS and peptides were shown in Fig. 4. According to these single-factor experiments including the yields and purity of CS, RSM was performed by the BBD with the design variables to investigate the effect on the responses. The design variables including enzymolysis temperature (X_1), the enzyme ratio of trypsin and papain (X_2) and enzymolysis time ratio (X_3) with the response values were shown in Table 1. The co-production of CS and peptides conditions were further optimized by RSM, and the analysis of variance (ANOVA) of the BBD results were summarized in Table 2. The fitted quadratic models for the CS and peptides yields were shown in Eqs. (8) and (9), respectively.

$$Y_1\ (\text{CS yield, \%}) = 18.87 + 0.14X_1 - 0.22X_2 + 0.18X_3 - 0.23X_1X_2 + 0.066X_1X_3 + 0.12X_2X_3 - 0.64{X_1}^2 - 0.64{X_2}^2 - 0.47{X_3}^2 \quad (8)$$

$$Y_2\ (\text{Peptide yield, \%}) = 68.46 + 0.39X_1 - 0.27X_2 + 0.068X_3 - 0.26X_1X_2 - 0.39X_1X_3 + 0.095X_2X_3 - 0.58{X_1}^2 - 0.49{X_2}^2 - 0.73{X_3}^2 \quad (9)$$

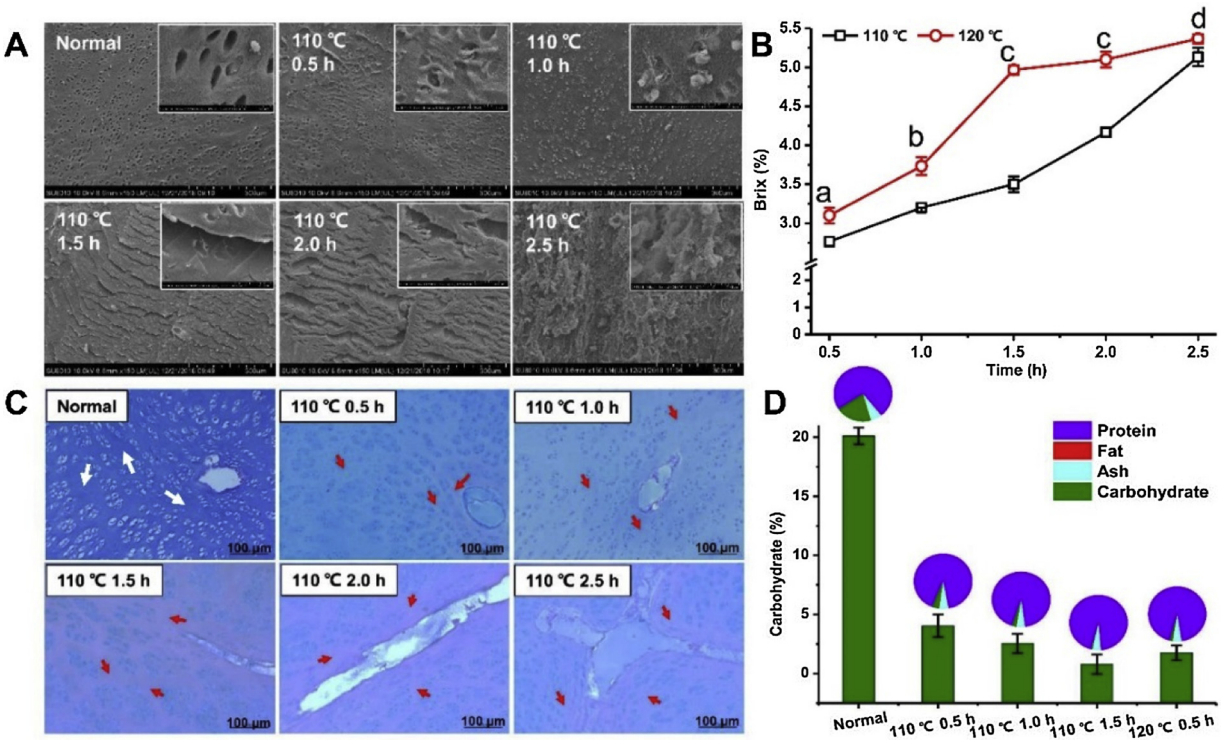

Fig. 3. Effects of the HP on the microstructure and constituent contents of chicken sternal cartilage. (A) SEM photographs (150× or 2000× (Inset)) of normal or HP residual cartilage samples. (B) The Brix of the liquid from the cartilage subjected to HP. (C) Distribution of polysaccharides in the normal and the HP cartilage. Polysaccharide can be stained blue (white arrows) by alcian blue. The red arrows indicate polysaccharide disappeared in the cartilage. (D) The contents of the constituents in the dried HP residual samples. The error bars represent the SD of the mean (n = 3), and different latters indicate the significant difference at 5% level (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

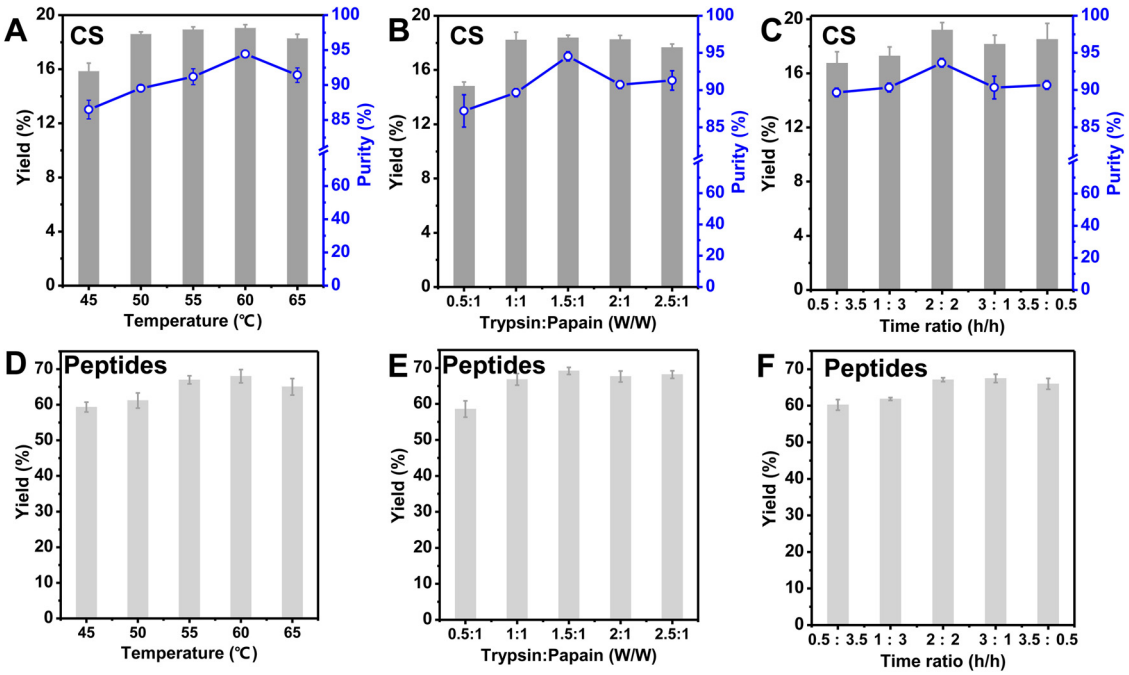

Fig. 4. Effects of enzymolysis temperature (A, D), the enzyme ratio of trypsin and papain (B, E) and enzymolysis time ratio (C, F) on the CS yield and peptides yield. The error bars represent the SD of the mean (n = 3). The purity of CS was calculated by subtracting protein from the CS sample.

The quadratic regression models for the CS and peptides yields resulted in a determination coefficients ($R_1^2 = 0.9746$ and $R_2^2 = 0.9452$), representing that 97.46% and 94.52% of the variation could be explained well (Han et al., 2016). The lack of fit associated with P-values of 0.1324 and 0.9043 ($P > 0.05$), indicating a non-significance, supported that the models fit with the data. P-values were < 0.0001 and 0.0012, suggesting the high significance of the regression models, both of which could be employed to optimize the variables. Additionally, for CS production, enzymolysis temperature (X_1), the enzyme ratio of trypsin and papain (X_2) and enzymolysis time ratio (X_3) significantly affected the yield. The quadratic terms (X_1^2, X_2^2 and X_3^2) were highly significant ($p < 0.05$), and the X_1X_2 term was also significant ($p < 0.05$). The X_1X_3 and X_2X_3 terms were insignificant. For the peptides production, enzymolysis temperature (X_1) and the enzyme ratio of trypsin and papain (X_2) could affected the yield dramatically ($p < 0.05$). The quadratic term (X_1^2, X_2^2 and X_3^2) was highly significant ($p < 0.01$) as well, and the X_1X_3 term was significant ($p < 0.05$). The other terms were insignificant.

Three-dimensional response surfaces were shown in Fig. 5. Response surface analysis was performed using Design-Expert software to acquire the following optimal extraction conditions: enzymolysis temperature, 61.2 ℃; the enzyme ratio of trypsin and papain (1.3:1, 0.13% of trypsin: 0.1% of papain) and enzymolysis time ratio (2:2, 2 h of trypsin: 2 h of papain). The maximum predicted CS yield and peptides yield were18.90% and 68.57%, respectively. To validate the models equations for the co-production of CS and peptides, a verification experiment was performed by the optimized conditions, and the experimental CS and peptides yields were 18.85 ± 0.09% and 67.99 ± 0.83% (mean ± SD, n = 3), respectively, which was highly consistent with the predicted value. These optimized conditions were

Table 1
Design approach and experimental results of RSM.

Run	Independent variables			CS yield (%)		Peptides yield (%)	
	X1	X2	X3	Measured	Predicted	Measured	Predicted
1	65	2:1	2:2	17.19 ± 0.15	17.29	67.13 ± 0.71	67.25
2	60	1.5:1	2:2	18.84 ± 0.45	18.87	68.60 ± 0.33	68.46
3	60	1:1	1:3	17.92 ± 0.15	17.92	67.49 ± 0.55	67.54
4	65	1.5:1	1:3	17.78 ± 0.59	17.65	67.91 ± 0.21	67.86
5	55	1.5:1	1:3	17.40 ± 0.46	17.50	66.24 ± 0.26	66.31
6	60	1.5:1	2:2	18.72 ± 0.41	18.87	68.10 ± 0.05	68.46
7	55	2:1	2:2	17.59 ± 0.32	17.46	66.99 ± 0.16	66.98
8	60	2:1	1:3	17.22 ± 0.44	17.24	66.87 ± 0.13	66.80
9	55	1.5:1	3:1	17.60 ± 0.32	17.73	67.17 ± 0.32	67.22
10	60	1.5:1	2:2	18.86 ± 0.09	18.87	68.94 ± 0.06	68.46
11	60	1.5:1	2:2	18.93 ± 0.45	18.87	68.36 ± 0.32	68.46
12	65	1.5:1	3:1	18.25 ± 0.21	18.15	67.29 ± 0.13	67.22
13	60	2:1	3:1	17.84 ± 0.24	17.84	67.18 ± 0.26	67.13
14	55	1:1	2:2	17.54 ± 0.51	17.44	67.13 ± 0.12	67.01
15	60	1.5:1	2:2	19.00 ± 0.12	18.87	68.31 ± 0.48	68.46
16	60	1:1	3:1	18.06 ± 0.40	18.03	67.42 ± 0.21	67.48
17	65	1:1	2:2	18.05 ± 0.47	18.18	68.30 ± 1.02	68.30

Note: X_1, X_2, and X_3 indicate enzymolysis temperature (℃), the enzyme ratio of trypsin and papain (W/W) and enzymolysis time ratio (h/h), respectively. The measured value of the CS yield and peptide yield is represented as the mean ± SD (n = 3).

Table 2
ANOVA for the response surface model.

Source	CS						Peptide					
	Sum of Squares	df	Mean Squares	F Value	p-value Prob > F	Significant	Sum of Squares	df	Mean Squares	F Value	p-value Prob > F	Significant
Model	5.970	9	0.660	29.87	< 0.0001	**	7.950	9	0.880	13.41	0.0012	**
X_1	0.160	1	0.160	7.30	0.0306	*	1.210	1	1.210	18.40	0.0036	**
X_2	0.370	1	0.370	16.88	0.0045	**	0.590	1	0.590	8.91	0.0204	*
X_3	0.260	1	0.260	11.62	0.0113	*	0.037	1	0.037	0.57	0.4759	
X_1X_2	0.210	1	0.210	9.50	0.0178	*	0.260	1	0.260	4.00	0.0856	
X_1X_3	0.017	1	0.017	0.78	0.4062		0.600	1	0.600	9.08	0.0196	*
X_2X_3	0.058	1	0.058	2.59	0.1513		0.036	1	0.036	0.55	0.4831	
X_1^2	1.720	1	1.720	77.61	< 0.0001	**	1.420	1	1.420	21.50	0.0024	**
X_2^2	1.720	1	1.720	77.41	< 0.0001	**	1.020	1	1.020	15.55	0.0056	**
$C^2X_3^2$	0.940	1	0.940	42.51	0.0003	**	2.240	1	2.240	34.06	0.0006	**
Residual	0.160	7	0.022				0.460	7	0.066			
Lack of Fit	0.110	3	0.037	3.43	0.1324		0.055	3	0.018	0.18	0.9043	
Pure Error	0.043	4	0.011				0.410	4	0.100			
Cor Total	6.120	16					8.410	16				
R-Squared	0.9746						0.9452					

Note: * $p < 0.05$, ** $p < 0.01$.

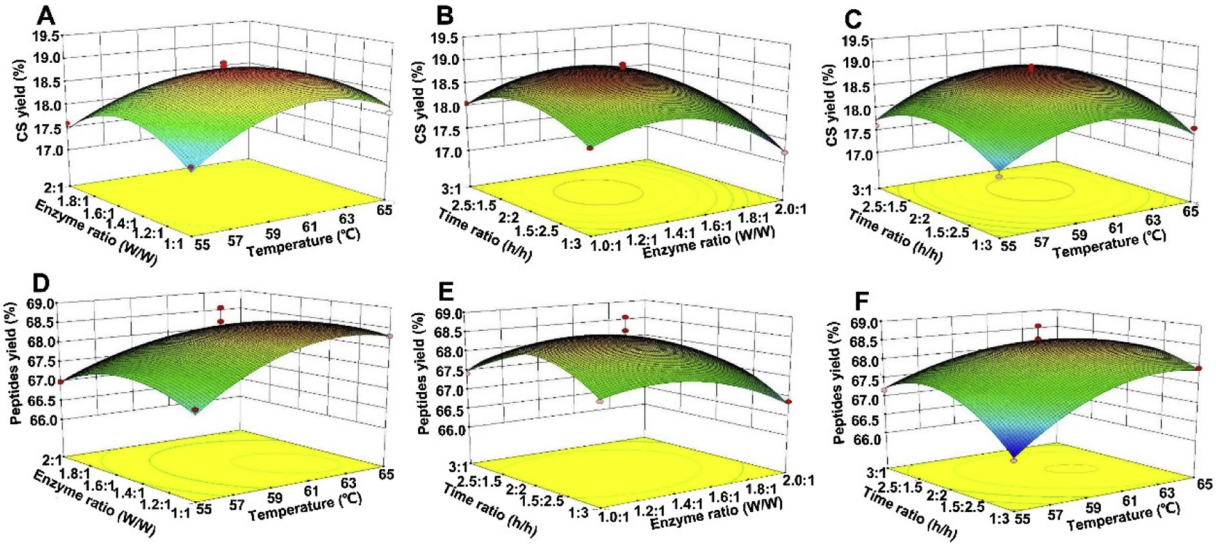

Fig. 5. Three-dimensional response surfaces for interactive effects of enzymolysis temperature, the enzyme ratio of trypsin and papain and enzymolysis time ratio on CS yield (A–C) and peptides yield (D–F).

used to realize the co-production of CS and peptides. This double-enzyme hydrolyzing system decreased the enzymolysis time to 4 h. Compared with the single enzyme such as the alcalase to hydrolyze the cartilage, it required 24 h (Krichen et al., 2018). The reason of shortening the time probably attributed to the pretreatment of HP for the cartilage and the double-enzyme hydrolyzing system. The pretreatment of HP for the cartilage (120 °C for1.5 h) may change the structure of the core protein, which made the enzymolysis easier. Although a report said that only papain could degrade the proteoglycan completely (Karamanos, Aletras, Tsegenidis, Tsiganos, & Antonopoulos, 1992), trypsin was able to hydrolyze the proteoglycan as well (Wang et al., 2019). It can be attributed to the different sources of the proteoglycan with different core protein characterized by the various amino acid sequences, which ultimately contributed to the synergistic effect of trypsin and papain.

3.4. CS confirmation by agarose-gel electrophoresis, FT-IR spectrum and NMR

Chondroitinase ABC is capable of hydrolyzing CS and dermatan sulfate instead of digesting heparan sulfate, and their mobility rates were totally different in 1, 3-diaminopropane buffer (Dietrich et al., 1977; Maccari et al., 2010). To preliminarily confirm the polysaccharide (carbohydrate) from the liquefied cartilage as CS, the relative migration rates of CS standard, CS sample, dermatan sulfate and heparan sulfate were analysed together. CS sample and CS standard were hydrolyzed by chondroitinase ABC (Fig. 6A), and the relative migration rates of CS standard and CS sample were almost the same and greater than the other two glycosaminoglycans' (Fig. 6B), which was agreement with the previous reports (Shen et al., 2019; Wang et al., 2019). It indicated preliminary that the polysaccharide (carbohydrate) from the liquefied cartilage was CS.

CS sample was identified further by FT-IR spectroscopy employing CS standard (chondroitin sulfate A) as the standard (Fig. 6C). The peaks of chondroitin-4-sulfate (chondroitin sulfate A) and chondroitin-6-sulfate were shown at 854.5 cm^{-1} and 823.7 cm^{-1}, respectively (Uchisawa, Okuzaki, Ichita, & Matsue, 2001). In this research, the obvious peak was detected at 852 cm^{-1} and a very weak absorption at 827 cm^{-1}, indicating the main type of CS in the sample was chondroitin sulfate A. The band observed at around 1051 cm^{-1} was attributed to the C—O—C ring vibrations (Krichen et al., 2018). The absorbance peaks shown at 1415 cm^{-1} and 1257 cm^{-1} (CS sample and CS standard) is characteristic of S═O stretching (Khan, Ashraf, Hashmi, Ahmad, & Anjum, 2013; Krichen et al., 2018). The peaks at 1560 cm^{-1}

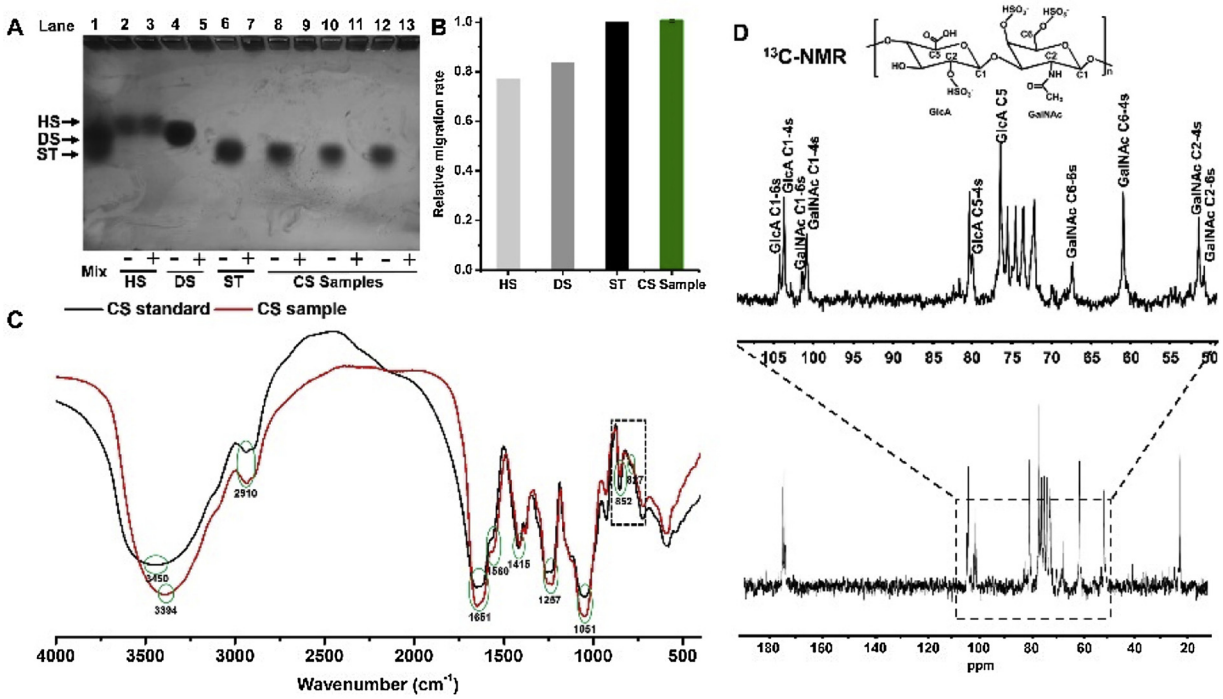

Fig. 6. Agarose-gel electrophoresis, FT-IR and NMR of CS sample. (A) Migration of glyconsaminoglycans and CS sample with or without treatment of chondroitinase ABC. (B) Relative migration rates of glyconsaminoglycans and CS sample. (C) FT-IR spectra of CS standard and CS sample. (D) The ^{13}C-NMR spectrum of CS sample. HS, DS and ST represent heparan sulfate, dermatan sulfate and CS standard, respectively. Mix is the mixture of glyconsaminoglycans. "+" and "−" indicate glyconsaminoglycans and CS sample treated with or without chondroitinase ABC, respectively.

represented the N−H band, indicating the presence of −NH−C═O (Wang, Shen, & Lu, 2003). A strong band detected at 1651 cm^{-1} for both CS sample and CS standard suggested the presence of uronic acid (Santhiya, Subramanian, & Natarajan, 2002). The peak observed around 2910 cm^{-1}was attributed to the stretching vibration of C−H (Li et al., 2019). The strong absorbance peaks at 3394 cm^{-1} (CS sample) and 3450 cm^{-1} (CS standard) indicated the stretching of the hydroxyl groups.

In addition, the structural characteristics of CS sample were demonstrated by ^{13}C-NMR spectral analysis (Fig. 6D). Chondroitin sulfated in position 4 and/or 6 of the GalNAc could be examined at the regions of 50–70 ppm and 100–110 ppm except for those of carbonyl (around 174 ppm) and acetamido methyl carbons (around at 22.5 ppm) (Mucci, Schenetti, & Volpi, 2000). The signals at 104.26 ppm and 101.36 ppm were attributed to the C1 of GlcA and to the C1 of the GalNAc-6SO$_4$, respectively, and the signals at 103.73 ppm and 100.91 ppm were attributed to the C1 of C1of GlcA and to the GalNAc-4SO$_4$, respectively, which was similar to results of Krichen et al. and Maccari et al. (Krichen et al., 2018; Maccari et al., 2010). The signals at 79.69 ppm and 76.50 ppm were attributed to uronic acid, which was consistent with the previous reports (Li et al., 2019; Mucci et al., 2000). The signal at 37.40 ppm was related to C6 of GalNAc-6SO$_4$ and the signal at 61.04 ppm was assigned to C6 of GalNAc-4SO$_4$. Therefore, the ^{13}C-NMR spectrum of CS indicated that the sample was consisted of chondroitin sulfated in both positions 4 and 6 of the GalNAc. Taken together, the polymer isolated from the HP cartilage was confirmed as the CS by agarose-gel electrophoresis, FT-IR and NMR.

3.5. Disaccharide and molecular weight evaluation of CS

To obtain more accurate structure information of the CS sample, the molecular weight, unsaturated disaccharides of the CS sample were analysed by GPC/MALLS (Fig. 7A and B) and SAX-HPLC, respectively (Fig. 7C). The specific results were shown in Table 3. Obviously, CS sample contained 5.99% of nonsulfated disaccharide ΔDi0 S. Abundant ΔDi4 S (72.94%) and ΔDi6 S (20.55%) constituted the CS sample. The disulfated disaccharide ΔDi4, 6 diS was presented in little percentage (0.52%) in CS sample. And the contents of other disulfated disaccharide ΔDi2, 6 diS, ΔDi2, 4 diS and trisaccharide were all trace (lower than 0.01%). The content of 4-sulfated disaccharide in CS sample was higher than sulfated disaccharide in position 6 producing a 4 S/6 S ratio of 3.58, which was the same with FT-IR result that chondroitin sulfate A was the major constituent in CS sample. The weight-average molecular weight of the CS sample was 67.79 kDa. The amount and disaccharide composition of CS sample from the HP cartilage liquid was almost consistent with the previous study (Shen et al., 2019). Interestingly, the percentage of ΔDi2, 6 diS was trace while it was 0.16 − 0.19% in CS obtained from the steam explosion (1.6 MPa, > 200 °C) cartilage liquid (Shen et al., 2019). Additionally, the molecular weight of CS sample was 67.79 kDa, and it was more than which of CS sample (28.58 − 35.38 kDa) from the steam explosion cartilage liquid. When the pressure was during 1.0 − 1.4 MPa (< 200 °C), the percentage of ΔDi2, 6 diS was trace as well. And the molecular weight decreased with the increase in pressure. These results suggested that the reason of the differences in this study were probably caused by the lower temperature and pressure.

3.6. Co-production of CS and peptides

The main constituents of the chicken sternal cartilage are protein (73.35%) and carbohydrate (20.10%). In addition, peptides can be obtained as well during the CS isolation. Therefore, it has important practical significance for the industrial production of the CS. The uronic acid in CS sample was determined as 27.48 ± 0.34%. The protein content of CS sample was 6.42 ± 0.42%, which suggested that the CS sample was relative purity (93.58 ± 0.42%). The amino acid composition of CS sample was determined to confirm the purity. The contents of the seventeen kinds of amino acids were shown and the total content reached to 8.75% lower than that (10.73%) of the CS sample from the liquefied cartilage by steam explosion (Shen et al., 2019)

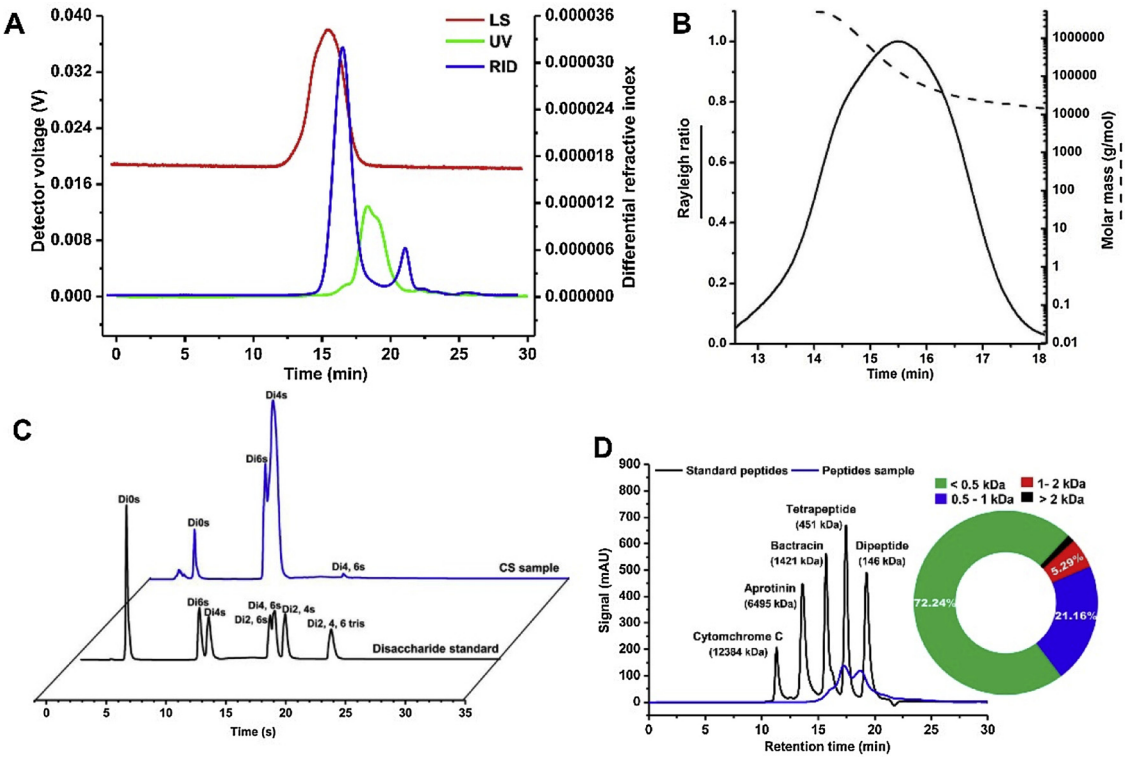

Fig. 7. Analysis of CS and peptides by chromatography. (A, B)The GPC/MALLS profiles of molecular weight about CS sample. LS, light scattering detection signal; UV, ultraviolet detection signal; RID, differential refraction detection signal. (C) The disaccharide chromatograms of CS sample and disaccharide standard. (D) The distribution of peptides obtained from the HP cartilage.

Table 3
Amount, disaccharide composition and molecular weight values of CS sample.

Parameters	CS sample
ΔDi0 S (ΔUA-GalNAc) (%)	5.99 ± 0.14
ΔDi4 S (ΔUA-GalNAc, 4 S) (%)	72.94 ± 1.91
ΔDi6 S (ΔUA-GalNAc, 6 S) (%)	20.55 ± 2.00
ΔDi4, 6 diS (ΔUA 4 S-GalNAc 6 S) (%)	0.52 ± 0.02
ΔDi2, 6 diS (ΔUA 2 S-GalNAc 6 S) (%)	trace
ΔDi2, 4 diS (ΔUA 2 S-GalNAc 4 S) (%)	trace
ΔDi2, 4, 6 triS (ΔUA 2 S-GalNAc 4 S, 6 S) (%)	trace
4 S/6 S	3.58 ± 0.47
Molecular weight (kDa)	67.79 ± 0.64

Note: Scheme illustrated CS unsaturated disaccharides produced via chondroitinase ABC. ΔUA, 4, 5-unsaturated uronic acid; GalNAc, N-acetylgalactosamine; S, sulfate group. Percentage of each identified disaccharide was determined using standard disaccharide. Trace indicates values lower than 0.01%. Data are means ± SD (n = 3).

(Supplementary Table 1). After optimal isolation, CS yield and recovery reached to 18.85 ± 0.09% and 93.63% ± 0.42%, respectively. Simultaneously, the yield and recovery of peptides were 67.99 ± 0.83% and 92.69% ± 1.13%, respectively. In terms of the yield and purity of CS, they were slightly higher than that from the liquefied cartilage by steam explosion technology (Shen et al., 2019), and the yield and recovery of peptides were improved as well.

The protein subjected to enzymes became short peptides (Fig. 7D). The percentage of small peptides (molecular weight < 1 kDa) was up to 93.40%, which suggested that this oligopeptides had a great potential to be used as the bioactive peptide (Lin et al., 2018). The physicochemical properties of the peptides were analysed by UV and FT-IR, and simultaneously the amino acid composition was evaluated (Supplementary Fig. 1). The maximum absorption peak of peptides from UV spectrum was 231 nm, which was associated with the chromophores of $CONH_2$, −COOH, and C═O in polypeptides chains (Luo, Chi, Yang, Zhao, & Wang, 2018). The FT-IR spectrum indicated that characteristic absorption bands of peptides including the amide A (3271 cm^{-1}), amide B (2835 cm^{-1}), amide I (1643 cm^{-1}), amide II (1537 cm^{-1}) and the amide III (1244 cm^{-1}) were all observed, and these bands were all existing in the college type II from the chicken sternal cartilage (Cao & Xu, 2008). The amino acids composition of the peptides suggested that the most abundant amino acid was Gly with the concentration of 11.25%, followed by Glu and Pro with the concentration of 8.80% and 7.37%, which, especially for Gly and Pro, were the most abundant amino acid in the college type II (Cao & Xu, 2008).Taken together, compared the steam explosion liquefaction (Shen et al., 2019), HP realized cartilage liquefaction completely and dramatically improved the chicken sternal cartilage utilization.

4. Conclusions

In this research, HP was proposed to liquefy chicken sternal cartilage completely. Co-production of CS and peptides was realized through the eco-friendly membrane combination technology from the HP cartilage liquid. Double-enzyme hydrolyzing system improved CS isolation efficiency. This co-production strategy should be suggested to employ the industrial production of CS.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2019.115015.

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