Carbohydrate Polymers 215 (2019) 73–81

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Liquefaction of chicken sternal cartilage by steam explosion to isolate chondroitin sulfate

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ARTICLE INFO

Keywords:
Steam explosion
Cartilage
Liquefaction
Chondroitin sulfate

ABSTRACT

Chondroitin sulfate (CS), together with peptide, was isolated from the liquid fraction of chicken sternal cartilage subjected to steam explosion (SE) by membrane separation. Cartilage was liquefied via the SE conditions, including various pressures (1.0–1.6 MPa) and times (60–140 s). The extraction procedure was optimized as follows: the amount of papain added, 0.11%; enzymolysis time, 10.5 h; and enzymolysis temperature, 56.5 °C, under which the highest recovery and total yield of CS were 92.15% and 18.55% at 1.4 MPa for120 s, and the counterparts of peptides were 87.35% (1.0 MPa, 140 s) and 63.07% (1.6 MPa, 140 s). The average molecular weight of CS samples ranged from 30 to 35 kDa. CS sample was confirmed using agarose-gel electrophoresis, and the structure was analysed Fourier transform infrared spectroscopy, chromatography and nuclear magnetic resonance. Taken together, SE can be an eco-friendly pretreatment method to liquefy cartilage for CS isolation.

1. Introduction

Chondroitin sulfate (CS) is a representative sulfated glycosaminoglycan, which is covalently attached to the core proteins, and exists the form of proteoglycans. The sizes of the core proteins ranged from 10 kDa to 500 kDa, and the overall sizes of the entire proteoglycans ranged from 80 kDa to 3500 kDa (Silbert & Sugumaran, 2002). CS consists of a repeating disaccharide unit that contains alternate sequences of D-glucuronic acid and N-acetyl-D-galactosamine (GalNAc) linked by β (1→3) bonds. It is recognized as the natural sulfated polysaccharide possessing structural microheterogeneity and various physiological functions (Lauder, 2009; Volpi, 2006). CS from different sources are usually a combination of different types due to the sulfate groups in varying amounts and different positions (Mikami & Kitagawa, 2013; Schiraldi, Cimini, & De Rosa, 2010), and the two most abundant CS types are chondroitin-4-sulfate and chondroitin-6-sulfate. CS has been associated with diverse physiological events such as organogenesis, cytokinesis, morphogenesis and central nervous system development (Volpi, 2014). Currently, CS has extensive applications in pharmaceutical, cosmetic and functional foods due to its special bioactivity and nutrient functions (Nunes et al., 2017; Shi et al., 2014). For instance, CS has served as a symptomatic slow acting drug or dietary supplement for osteoarthritis in Europe and some other countries (Mikami & Kitagawa, 2013; Volpi, 2014). Additionally, it has been reported that CS and its oligosaccharide can alleviate metabolic syndromes and gut microbiota dysbiosis (Li et al., 2019; Shang et al., 2016).

CS is mainly obtained from the cartilage of terrestrial animals such as bovine, porcine (Volpi, 2007, 2009), avian and marine organisms including bony fish (Maccari, Galeotti, & Volpi, 2015) by extraction and purification processes (Volpi, 2006). However, the safety should be considered because of its source of animal tissue materials, which could be contaminated by mad-cow disease, foot-and-mouth disease, or hog cholera. Moreover, there is limited raw available material from endangered marine organisms such as sharks. China is the biggest CS producer in the word, providing more than 80% of the CS. According to the United States Department of Agriculture, approximately 83 million tons of raw chicken was consumed worldwide in 2012, 28 million tons of which was from China (Wang et al., 2016), which means plenty of chicken sternal cartilage will be generated. Nevertheless, this cartilage is generally processed into some low-value-added snack foods in China. It would be advantageous if the chicken sternal cartilage is considered to be a raw material to isolate CS, which could not only relieve the pressure on CS material sources to some extent, but also increase the

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https://doi.org/10.1016/j.carbpol.2019.03.032
Received 24 January 2019; Received in revised form 11 March 2019; Accepted 12 March 2019
Available online 15 March 2019
0144-8617/

additional value of this cartilage.

Conventional CS isolation processes usually involve four steps that are the chemical hydrolysis of cartilage, breakdown of the proteoglycan core, elimination of proteins and CS recovery, and purification of CS (Shi et al., 2014). A high-concentration of NaOH, urea, guanidine HCl or similar chemicals is commonly used in the first two stages. However, the utilization of these reagents, especially high-concentration alkali, is not environmentally friendly. Therefore, various alternative extraction methods have been tried to replace the classical ones to pursue the sustainability, which include the digestion of cartilage and proteins with enzymes, selective precipitation with alcoholic solutions, and separation by molecular weight using ultrafiltration-diafiltration technologies. Compared with the traditional steps, the enzymolysis and membrane separation technologies seem to be more suitable for the large-scale industrial production of CS.

Recently, steam explosion (SE) has emerged as a pretreatment technology devoted for lignocellulosic biomass such as cellulose, hemicellulose and lignin (Carvalho et al., 2017; Liu et al., 2017; Rodriguez, Sanchez, & Parra, 2017; Zhao, Li, Zheng, Wang, & Yu, 2018). It is based on pressurization and forcing steam into fibrous tissues and cells of biomass, followed by instantly releasing the pressure within 0.01 s. The short time provides overwhelming explosion power to disrupt the compact structure and simultaneously avoid a long time of violent treatment under high temperature and pressure (Zhao, Yang, Zhang, & Wu, 2012). This process is identified as an adiabatic expansion process as well as a conversion process of thermal energy into mechanical energy (Yu, Zhang, Yu, Xu, & Song, 2012). After SE pretreatment, the constitutive components of biomass are released. For example, oligosaccharides can be produced from the sugarcane bagasse, and the enzyme and solvent accessibility of cellulose is increased. Another property of this technology is the water (steam) used as the medium is abundant, non-toxic and environmentally friendly. However, limited research is reported on the use of this technology to pre-treat animal material. Based on our previous study that CS could be obtained from the chicken leg bone by boiling at 120 °C for 120 min (Wang et al., 2019), SE is proposed to as a pretreatment method to liquefy the chicken sternal cartilage for CS isolation.

In the present study, chicken sternal cartilage was liquefied by SE, and CS was isolated from the liquid fraction in an environmentally friendly manner. The effects of SE parameters, including the pressure and time, on the liquefaction of cartilage were investigated. The microstructure changes of the solid residue from SE cartilage were verified by scanning electron microscopy (SEM), and CS was obtained from the liquid fraction by enzymolysis and membrane combination separation technologies. The chemical and structural properties of CS were confirmed by Fourier transform-infrared spectroscopy (FT-IR), agarose-gel electrophoresis, chromatography and nuclear magnetic resonance (NMR).

2. Materials and methods

Raw chicken sternal cartilage of adult white feather chicken used in the study was provided by the PROTIL Biotechnology Co. Ltd (Hebi, China). Before experiments, raw cartilage was washed with distilled water to remove the residuary meat, and then cut into pieces approximately 1 cm in length. The cartilage pieces were stored at −20 °C before use. 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. Papain was purchased from Solarbio (800 U/mg, Solarbio, Beijing, China). The standard unsaturated chondro/dermato disaccharides involving ΔDi0s (ΔUA-[1→3]-GalNAc), ΔDi4 s (ΔUA-[1→3]-GalNAc-4 s), ΔDi6 s (ΔUA-[1→3]-GalNAc-6 s), ΔDi2,4 dis (ΔDi-disB, ΔUA-2s-[1→3]-GalNAc-4 s), ΔDi2,6 dis (ΔDi-disD, ΔUA-2s-[1→3]-GalNAc-6 s), ΔDi4,6 dis (ΔDi-dis, ΔUA-4 s-[1→3]-GalNAc-6 s), and ΔDi2,4,6tris (ΔDitris, ΔUA-2s-[1→3]-GalNAc-4 s,6 s) were purchased from the Iduron Corporation (Alderley City, UK).

2.1. Proximate composition determination

The moisture in raw cartilage was determined through drying to a constant weight in an oven at 105 °C. After that, the dried cartilage was smashed into powder to estimate the contents of protein, fat and ash in the cartilage by AOAC methods (AOAC, 2000). The protein content was measured according to 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 fat content was obtained, according to AOAC method 960.39, with petroleum ether (40–60 °C) by a Soxhlet apparatus (VELP SER148, Italy). The ash content was estimated by weighing samples before and after heat treatment at 550 °C in a muffle furnace for 6 h (AOAC method 923.03). According to Khong et al. (Khong et al., 2018), the total carbohydrate content was calculated by Eq. (1):

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

where $P_{protein}$, P_{fat} and P_{ash} indicate the content (%) of protein, fat and ash in the dried cartilage, respectively. Six tests were used to calculate the mean as the result. The contents of protein, fat and ash in dry residual cartilage after SE treatment were determined as described above.

2.2. Steam explosion experiments

The SE procedure was carried out with a SE apparatus (QBS-80 SE, Hebi Gentle Bioenergy Co. Ltd., China). The SE process is shown in Fig. 1. Approximately 50 g of cartilage was loaded into a 400 mL chamber. The steam pressure was set at 1.0, 1.2, 1.4 and 1.6 MPa with a maintaining time of 60, 80, 100, 120 and 140 s, respectively, and then, the piston driving device was triggered to release the pressure instantly (within 0.01 s). After SE treatment, the liquid fraction and residual solid were separated by six-layer gauze. The liquid fraction was used to isolate the CS, and the residual solid was used to investigate the composition migration. The liquefaction rate of the sample was defined by Eq. (2):

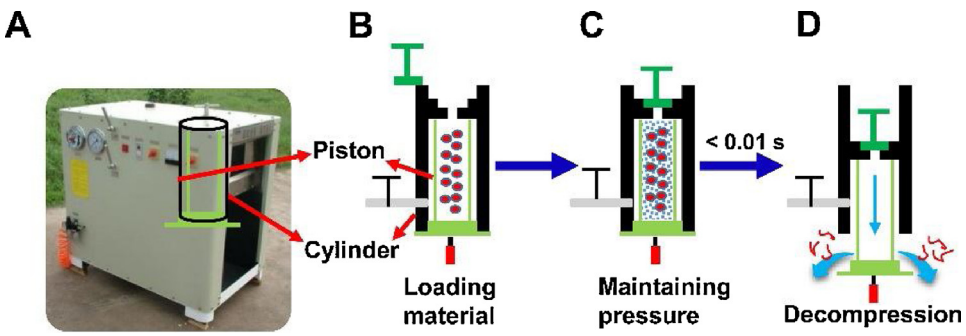

Fig. 1. Diagram of the SE process. (A) The SE apparatus was mainly composed of a cylinder and piston. (B) Raw material was loaded into the cylinder. (C) The steam pressurization phase, where the cylinder and piston were tightly coupled. (D) The explosion phase, where the piston was catapulted out of the cylinder (within 0.01 s), which was driven by the actuators and kinetic energy of the steam and material.

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

where m_0 and m_1 indicate the dry weight of the original sample and the residual sample after SE treatment, respectively. Three tests were performed for each set of SE conditions.

2.3. Scanning electron microscopy

Sample preparation was carried out as described previously with slight modifications (Reuter, Gilroyed, Xu, Mcallister, & Stanford, 2015). Briefly, samples were first fixed in 2.5% glutaraldehyde at room temperature for 4 h, and then washed 0.5 h with 0.1 M PBS (pH = 7.2) three times to remove the excess glutaraldehyde. Then, the samples were fixed again by 1% osmic acid for 2 h. Subsequently, samples were washed with distilled water to remove the osmic acid. Ultimately, dehydration with a graded ethanol series (from 30 to 100%) was performed, 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. CS isolation from the liquid sample

The liquid of SE cartilage liquefied at 1.0 MPa for 60 s was chosen to optimize the CS isolation conditions. The isolation procedures included three steps. In detail, the Brix of the liquid sample was determined with a portable Brix meter (EXTECH RF11, FLIR) at room temperature and then adjusted to 1% by adding distilled water. After that, the liquid sample was subjected to enzymolysis with varying amounts of papain added (0.02-0.12%, W/W), enzymolysis times (3–18 h), and enzymolysis temperatures (45–65 °C). Following enzymolysis, papain was inactivated by boiling at 100 °C for 5 min. Subsequently, the enzymatic solution was filtered successively through the 0.45 μm (MCE, JIN TENG, China) and 10 kD membranes (VF05P0, Sartorius Vivaflow 50) to separate the CS and peptides. During the separation procedures, the solution was first 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, WT600-2 J, Longer Pump®) equipped with a 10 kDa membrane. The cycling separation was performed four times by adding an equal volume of distilled water. The speed of the pump was set at 100 rpm according to the specification. Ultimately, the trapped fluid (CS solution) and filtrate (peptides solution) were lyophilized with a freeze drier (SR-A18N-80, Shanghai, China). The lyophilized powders were used for further analysis.

2.5. Optimization of the CS isolation

The effects of the amount of papain added, enzymolysis time, and enzymolysis temperature on the CS yield were determined by single factor tests. The CS yield was calculated by Eq. (3):

$$CS_0 \text{ yield (\%)} = m_{CS0}/m_{liquid} \times 100 \quad (3)$$

where m_{CS0} is the weight of the CS sample and m_{liquid} is the weight of the liquid fraction sample (Brix = 1%). The CS yield was chosen as the response value for response surface methodology (RSM). A Box-Behnken design (BBD) with three independent factors (X_1, the amount of papain added; X_2, enzymolysis time; and X_3, enzymolysis temperature) at three variation levels was performed.

Ranges of the amount of papain added (X_1), enzymolysis time (X_2), and enzymolysis temperature (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 CS isolation conditions (Zou, Chen, Yang, & Liu, 2011). The CS yield (Y) was the response value. 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. (4):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ii} X_i X_j \quad (4)$$

where Y is the response value, X_i, Xj is the independent variable, and β_0, β_i,β_{ii}, β_{ij} indicate the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively. Design-Expert software was applied to produce the response surfaces and contour plots while holding a variable constant in the second-order polynomial model. When the results showed a saddle point in the response surfaces, the estimated ridge of the optimum response was computed by the ridge analysis of the design expert procedure (Liyanapathirana & Shahidi, 2005). Based on the optimized conditions, CS samples were isolated from the other SE liquid samples.

2.6. FT-IR analysis

FT-IR spectra of CS samples and CS standard were recorded using an FT-IR spectrometer (TENSOR27, Bruker Company). The dried sample powder was ground with potassium bromide powder and pressed into a 1 mm pellet for FT-IR measurement in a frequency range of 4000 to 500 cm^{-1}.

2.7. Agarose-gel electrophoresis

Agarose-gel electrophoresis of the CS samples and CS standard were performed according to Dietrich et al. (Dietrich, Mcduffie, & Sampaio, 1977) and Volpi et al. (Volpi & Maccari, 2002) with slight modifications. CS samples and different glycosaminoglycans such as dermatan sulfate and heparan sulfate 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, and the solution was boiled at 100 °C for 5 min to inactivate the enzyme. Then, 20 μL of glycosaminoglycans with or without treatment of chondroitinase ABC were loaded for electrophoresis, respectively. Agarose-gel was prepared at a concentration of 1% in 0.04 M barium acetate buffer at a pH of 5.8. The run was conducted with an electrophoretic instrument (JY-SPCT) in 0.05 M 1, 3-diaminopropane buffer (pH = 9.0) at 100 mA for 4 h. After migration, the gel was soaked in 0.1% cetyltrimethylammonium bromide solution for 4 h and stained with 0.2% fresh toluidine blue for 6 h. The gel of the background was faded away with ethanol-water-acetic acid (50:49:1 v/v/v). The migrations of CS samples and glycosaminoglycans were recorded, respectively. The relative migration rate was calculated via the migration of glycosaminoglycan/migration of the CS standard.

2.8. Enzymatic treatment and constitutive disaccharide determination

CS samples were dissolved in chondroitinase ABC buffer with a 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 (Grondahl, Tveit, Akslenhoel, & Prydz, 2011). The unsaturated disaccharides in the solution were evaluated by strong anion exchange (SAX)-HPLC using HPLC equipment (Agilent 1260 Infinity II) equipped with a 150 mm × 4.6 mm stainless-steel Spherisorb 5-SAX column (5 μm, trimethylammoniopropyl groups $Si-CH_2-CH_2-CH_2-N^+(CH_3)_3$ in the Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK), and the signal was detected at 232 nm. Isocratic separation was performed using 50 mM sodium chloride at a pH of 4.0 for 5 min, followed by a linear gradient from 5 to 60 min of 50 mM to 1.0 M sodium chloride at a pH of 4.0 (Maccari et al., 2015). The flow rate was 1.0 mL/min. The injection volume was 10 μL, and standard disaccharides were used for qualitative and quantitative analysis.

2.9. Molecular weight determination

Based on the method of Roulard et al. (Roulard, Petit, Mesnard, & Rhazi, 2016) with some modifications, the molecular weights of CS samples were determined using gel permeation chromatography with a multi-angle laser light scattering system (GPC/MALLS). 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 used. Sodium chloride (0.1 M) was filtered through a 0.2 μm filter membrane, and degassed for 30 min as the mobile phase. CS samples were solubilized in the mobile phase at a final concentration of 1 mg/mL, and the dn/dc value (the refractive index increment) was 0.135 (Wang et al., 2019).The flow rate was 0.5 mL/min, and the injection volume was 200 μL.

2.10. Recovery, yield and uronic acid determination

The uronic acid content of CS samples and the CS standard were determined by the carbazole reaction as described by Kosakai et al. (Kosakai & Yosizawa, 1979). The protein contents of CS samples were evaluated by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The recovery and total yield of CS samples were calculated as follows by Eqs. (5) and (6):

$$CS_{recovery}\ (\%) = m_{CS}/m_{0carbohydrate}\text{-}m_{1carbohydrate} \times 100 \quad (5)$$

$$CS_{yield}\ (\%) = m_{CS}/m_{dry} \times 100 \quad (6)$$

where m_{CS} is the total weight of the CS sample while $m_{0carbohydrate}$ and $m_{1carbohydrate}$ are the total weight of the carbohydrates in the dry original cartilage and dry residual cartilage, respectively. Meanwhile, m_{dry} is the total dry weight of the raw cartilage.

2.11. Nuclear magnetic resonance spectroscopic analysis

The ^{1}H and ^{13}C NMR spectra of CS sample and CS standard were recorded by a Bruker AMX600 WB spectrometer equipped with a 5 mm diameter tunable probe, operating at 600 MHz. Thirty milligrams of sample was dissolved in 1.0 mL of D_2O at a high level of deuteration (99.997%) to avoid the presence of a relatively high percentage of water. The spectra were 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).

2.12. Statistical analyses

All experimental results were shown as mean (n = 3 or n = 6) ± standard deviation (SD). Data were analysed using 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. SE induced liquefaction of chicken sternal cartilage

The moisture content of fresh cartilage was 77.19 ± 1.01%. On a dry basis, cartilage powder was characterized as having a 73.98 ± 2.67% protein content, together with 4.34 ± 0.92% ash and 0.35 ± 0.04% fat as minor components. Thus, the carbohydrate content was 21.33 ± 1.81%, which meant that cartilage had potential to be a source of CS. Cartilage could be liquefied gradually under the given conditions. With the increase in pressure (from 1.0 to 1.6 MPa) and maintaining time (from 60 to 140 s), the liquefaction rate was improved from 41.65% to 75.72% (Fig. 2).

The conventional protocols separating CS required alkali such as NaOH to break the covalent bonds between CS and the core protein, which, especially for large-scale industrial production, aggravated environmental pollution and was against the sustainable strategy. Recently, a thermal liquefaction method (Kozliak et al., 2016; Posmanik et al., 2018) was proposed to manage food waste or lignin in an environmentally friendly manner. According to the report, the dissolution of keratin from feathers would be improved after SE treatment (Zhang, Zhao, & Yang, 2015). The chicken leg bone exposed to boiling could be used to extract CS (Wang et al., 2019). It was speculated that the liquid fraction from the SE cartilage sample had great potential for the isolation of CS.

3.2. SE induced microstructure changes and carbohydrate migration of the residual cartilage

To investigate the effect of SE on the cartilage structure, residual solid SE samples were chosen randomly to observe the microstructure by SEM. The results suggested the surfaces of cartilage changed significantly after SE treatment (Fig. 3A). Compared with the normal cartilage (raw material) characterized by a compact and smooth surface, SE samples showed plenty of cracks like ravines on the surface. To probe into the changes of SE cartilage constituents, the protein, fat, ash and carbohydrate contents of dry residual solid SE samples were measured. Compared with the original carbohydrate content of the dried sample, it was observably decreased in the residual solid with the increase of time at a certain pressure (Fig. 3B), which suggested that the carbohydrate migrated from the solid to liquid during the SE process. Additionally, the protein, fat and ash contents of the residual solid from SE cartilage changed as well (Supplementary Fig. 1).

SE is an innovative method for biomass pretreatment, which could be performed on a large-scale for industry. Based on pressurization, steam was forced into fibrous tissues and cells of biomass, followed by instant decompression. During this process, most of the steam in the biomass would quickly expand and break free of the structure, and ultimately the internal structure of the biomass was disrupted by a mechanical shearing force (Zhao et al., 2012). Interestingly, there were irregular holes in the normal cartilage. During explosion, the instant decompression of steam in holes might be the cause of inducing cracks, and it brought about the cartilage liquefaction.

3.3. Isolation optimization for CS from the SE cartilage liquid fraction using RSM

The effects of the extraction parameters, including the amount of papain added, enzymolysis time and enzymolysis temperature, on the CS yield were investigated. The results were shown in Supplementary Fig. 2. After performing the single-factor experiments, RSM was executed using a BBD with the design variables to investigate its effect on the responses. The design variables (papain, time, and temperature) with the response value were shown in Table 1.

Results obtained from single-factor experiments showed that CS yield increased with the increase of papain, time and temperature (Supplementary Fig. 2). Based on these results, the CS extraction conditions were further optimized by RSM, and the analysis of variance (ANOVA) of the BBD results was summarized in Table 2. The fitted quadratic model for the CS yield was estimated by RSM, which was shown in Eq. (7):

$$CS = 0.23 + 0.014X_1 + 9.550E\text{-}003X_2 + 8.673E\text{-}003X_3 + 2.154E\text{-}003X_1X_2 - 3.333E\text{-}004X_1X_3 + 5.596E\text{-}003X_2X_3 - 0.018X_1^2 - 0.011X_2^2 - 0.018X_3^2 \quad (7)$$

The quadratic regression model for the CS yield resulted in a determination coefficient ($R^2 = 0.9862$), representing that 98.62% of the variation could be explained excellently (Han et al., 2016). The lack of fit associated with P-values of 0.0968 (> 0.05), indicating a non-

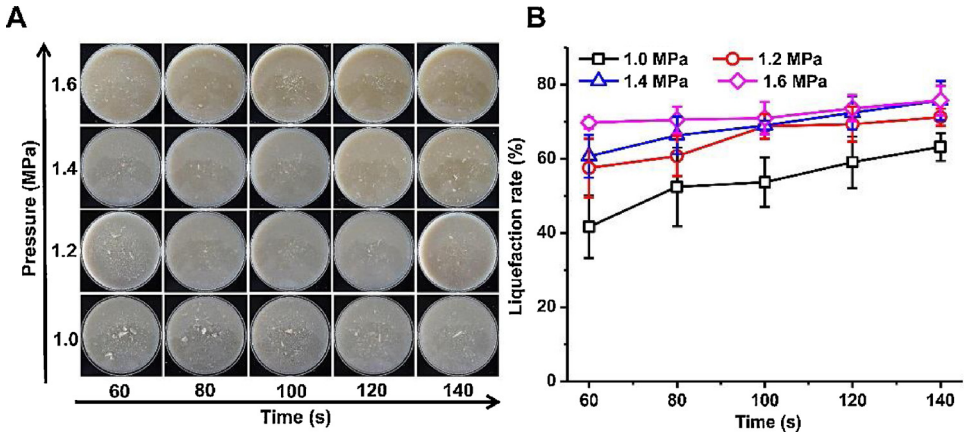

Fig. 2. Liquefaction of cartilage by SE. The mixture of the solid residue and liquid fraction (A), and liquefaction rate (B) of SE samples at different pressures and maintaining times. The error bars represent the standard deviation (SD) of the mean (n = 3).

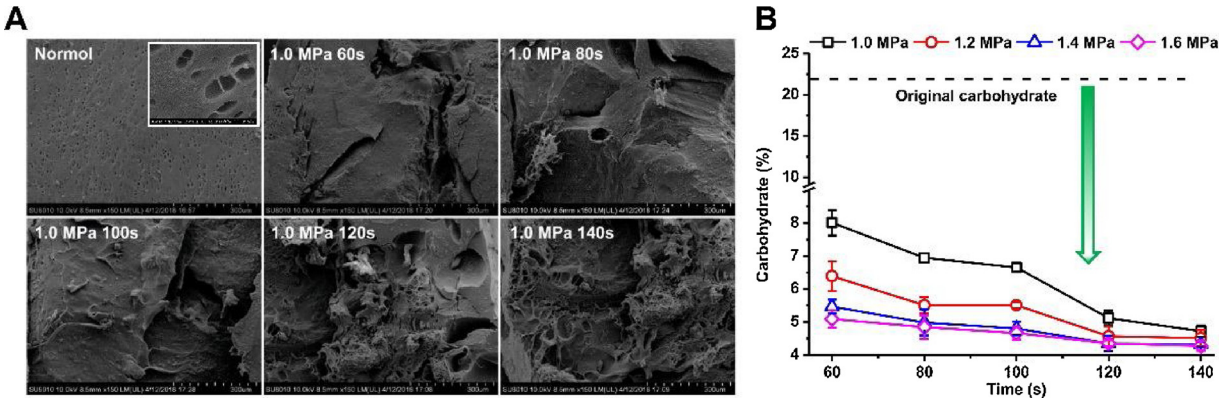

Fig. 3. Effects of SE on the microstructure and carbohydrate content of chicken sternal cartilage. (A) SEM photographs of normal (150× or 2000×) or SE cartilage samples (150×). (B) Carbohydrate content of dry residual solid SE samples. The error bars represent the SD of the mean (n = 3).

Table 1
Design approach and experimental results of RSM.

Run	Independent variables			CS yield (g/100 g)	
	X_1 (Papain, %)	X_2 (Time, h)	X_3 (Temperature, ˚C)	Measured	Predicted
1	0.12	9	50	0.20 ± 0.003	0.20
2	0.10	9	55	0.23 ± 0.009	0.23
3	0.08	9	50	0.18 ± 0.009	0.17
4	0.08	9	60	0.19 ± 0.006	0.19
5	0.10	9	55	0.23 ± 0.001	0.23
6	0.12	9	60	0.21 ± 0.007	0.22
7	0.10	6	60	0.20 ± 0.007	0.20
8	0.08	12	55	0.19 ± 0.011	0.19
9	0.10	9	55	0.23 ± 0.005	0.23
10	0.12	12	55	0.23 ± 0.001	0.23
11	0.10	9	55	0.23 ± 0.004	0.23
12	0.12	6	55	0.21 ± 0.007	0.20
13	0.10	6	50	0.19 ± 0.006	0.19
14	0.10	12	50	0.20 ± 0.006	0.20
15	0.10	12	60	0.23 ± 0.006	0.23
16	0.10	9	55	0.23 ± 0.006	0.23
17	0.08	6	55	0.18 ± 0.006	0.18

Note: The measured value of the CS yield is represented as the mean ± SD (n = 3).

Table 2
ANOVA for the response surface model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	6.61E-03	9	7.34E-04	55.78	< 0.0001	**
X_1	1.64E-03	1	1.64E-03	124.49	< 0.0001	**
X_2	7.30E-04	1	7.30E-04	55.41	0.0001	**
X_3	6.02E-04	1	6.02E-04	45.7	0.0003	**
X_1X_2	1.86E-05	1	1.86E-05	1.41	0.2738	
X_1X_3	4.44E-07	1	4.44E-07	0.034	0.8594	
X_2X_3	1.25E-04	1	1.25E-04	9.51	0.0177	*
X_1^2	1.35E-03	1	1.35E-03	102.76	< 0.0001	**
X_2^2	4.95E-04	1	4.95E-04	37.57	0.0005	**
X_3^2	1.30E-03	1	1.30E-03	98.7	< 0.0001	**
Residual	9.22E-05	7	1.32E-05			
Lack of Fit	7.03E-05	3	2.34E-05	4.29	0.0968	
Pure Error	2.19E-05	4	5.47E-06			
Cor Total	6.70E-03	16				
R-Squared	0.9862					

Note: $R^2 = 0.09862$, * $p < 0.05$, ** $p < 0.01$.

significance, supported that the model fits with the data. A P-value lower than 0.0001 was found, demonstrating again the high significance of the regression model and that it could be used to optimize the variables. More importantly, the amount of papain added (X_1), enzymolysis time (X_2) and enzymolysis temperature (X_3) significantly affected the CS yield. The quadratic term (X_1^2, X_2^2 and X_3^2) was highly significant ($p < 0.01$), and the X_2X_3 term was also significant ($p < 0.05$). The other terms were insignificant.

Three-dimensional response surfaces and two-dimensional contours were shown in Fig. 4. Response surface analysis was performed using Design-Expert software to determine the following optimal extraction conditions: the amount of papain added, 0.11%; enzymolysis time, 10.5 h; and enzymolysis temperature, 56.5 ˚C. The maximum predicted CS yield was 0.24%. To validate the model equations, a verification

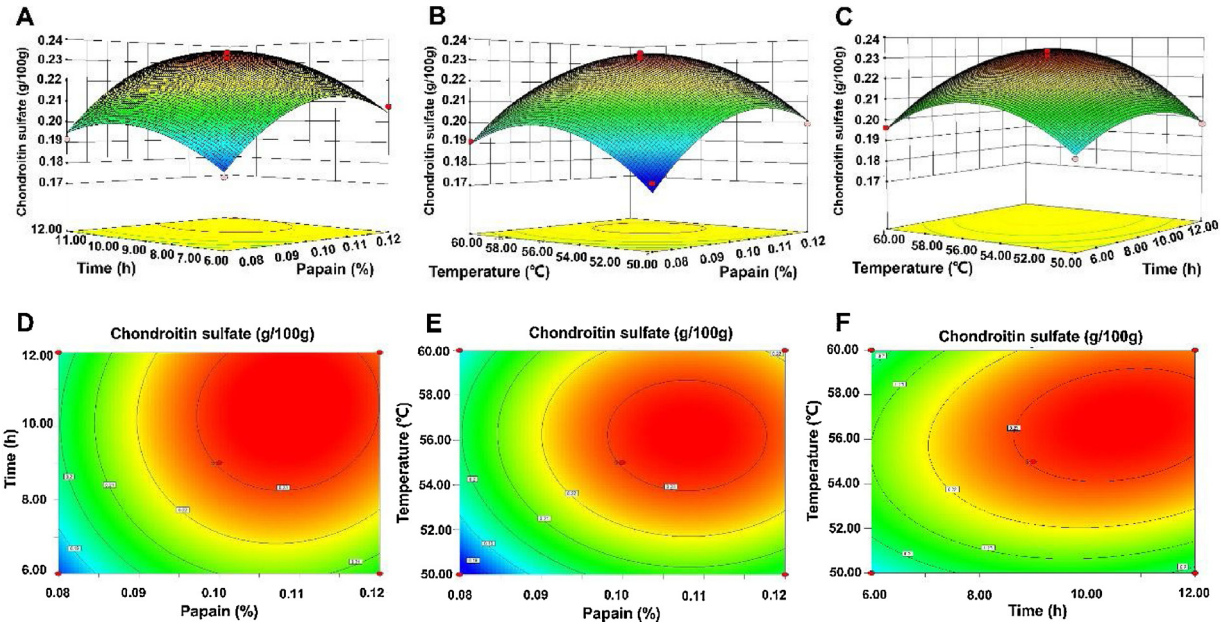

Fig. 4. Three-dimensional response surfaces (A, B and C) and contour graphs (D, E and F) for the interactive effects of the amount of papain added, enzymolysis time, and enzymolysis temperature on the CS yield.

experiment was conducted under these conditions, and the experimental CS yield was approximately 0.24 ± 0.002% (mean ± SD, n = 3), which was highly consistent with the predicted value. These optimal isolation conditions were employed to all of the other liquid fractions of SE samples to obtain CS. Papain could degrade intact proteoglycan completely (Karamanos, Aletras, Tsegenidis, Tsiganos, & Antonopoulos, 1992), and due to its low price, it might be suitable for large scale industrial production. Additionally, the CS isolation process, together with peptides, did not involve any other chemicals except for papain, which meant that it could reduce the pressure of environmental pollution.

3.4. Agarose-gel electrophoresis, FT-IR spectrum and NMR of CS

The results of the agarose-gel electrophoresis of glycosaminoglycans, the CS standard and CS samples with or without treatment of chondroitinase ABC were shown in Fig. 5A. The CS standard, dermatan sulfate, and CS samples were completely enzymatically hydrolysed by chondroitinase ABC, while heparan sulfate (Lane 3) was not, which was consistent with previous reports (Grondahl et al., 2011). The relative migration rates of glycosaminoglycans including heparan sulfate (0.73) and dermatan sulfate (0.84) were significantly less than those of the CS standard and CS samples (Fig. 5B). In 1, 3-diaminopropane buffer, the mobility rates of the CS samples were greater than other glyconsaminoglycans, and the migration of heparan sulfate was the slowest, which was in agreement with the research of Dietrich et al. and Maccari et al. (Dietrich et al., 1977; Maccari, Ferrarini, & Volpi, 2010).

The structures of CS samples, together with the CS standard, were further confirmed by FT-IR that was recorded in the range of 4000-500 cm^{-1} (Fig. 5C). The clear peak at 3435 cm^{-1} was observed in the standard CS, representing the hydroxyl structure of carbohydrate, which migrated to 3394–3359 cm^{-1} in CS samples. The characteristic peaks at 1620 cm^{-1} and 1560 cm^{-1} represented the carbonyl and N—H bands, indicating the presence of —COOH and —NH—C═O (Wang, Shen, & Lu, 2003). The peaks at 1420–1375 cm^{-1} indicated the coupling of C–O stretching vibrations and OH variable angle vibrations, suggesting the presence of free acid groups in the standard, and the peaks at 1257 cm^{-1} and 1057 cm^{-1} signified S—O and –C–O—S stretching vibrations, respectively, as previously reported by Khan et al. (Khan, Ashraf, Hashmi, Ahmad, & Anjum, 2013). The peak at approximately 850 cm^{-1} was used to identify chondroitin-4-sulfate, and the peak at 820 cm^{-1} was used to indicate chondroitin-6-sulfate (Brezinski, 1980). The peaks in spectra of the standard and samples were observed only at 877 cm^{-1}, indicating that samples mostly consisted of chondroitin-4-sulfate. Compared with the reports mentioned above, although both the CS standard and CS samples migrated slightly, the characteristic peaks were all shown in the spectra.

To further confirm the structure and the purity of CS sample (1.4 MPa 120 s), the spectra of ^{1}H and ^{13}C-NMR were recorded together with CS standard (Supplementary Fig. 3). Typical CS ^{1}H-NMR signals were concentrated in the region 1.5–5.0 ppm, and in this research, compared with the CS standard, there were some weak signals in the CS sample spectrum between 2.0 ppm and 3.0 ppm, which was most likely due to protein impurities (José et al., 2019). More importantly, the other stronger characteristic signals in the spectra of CS sample and CS standard were all shown almost at the same chemical shifts, which suggested that this CS sample had the same structure with the CS standard. The ^{13}C-NMR spectrum of the CS was found in the region 50–110 ppm, which indicated a high content of chondroitin sulfated in position 4 and/or 6 of the GalNAc (Fatma et al., 2018). During these regions, the signals that suggested the GalNAc-6SO_4 (at 103.5 ppm, 67.5 ppm, and 51.5 ppm) and GalNAc-4SO_4 (at 101 ppm, 61 ppm, and 50.9 ppm) were all found in the CS sample and standard spectra. Taken together, extremely few impurities were existed in this CS sample, and the ^{1}H and ^{13}C-NMR spectra of CS sample confirmed that this polymer contains high content of chondroitin sulfated in both positions 4 and 6 of the GalNAc. The results from agarose-gel electrophoresis, FT-IR and NMR suggested that the isolated samples from the liquid fraction of SE cartilage were confirmed as CS.

Migration of glyconsaminoglycans and CS samples with or without treatment of chondroitinase ABC. (B) Relative migration rates of glyconsaminoglycans and CS samples. (C) FT-IR spectra of the CS standard and CS samples. HS, DS and ST were heparan sulfate, dermatan sulfate and CS standard, respectively. Mix was a mixture of glyconsaminoglycans. "+" and "−" indicated glyconsaminoglycans and CS samples treated with or without chondroitinase ABC, respectively.

3.5. Disaccharide and molecular weight evaluation of CS

To further verify the CS sample structure, the unsaturated

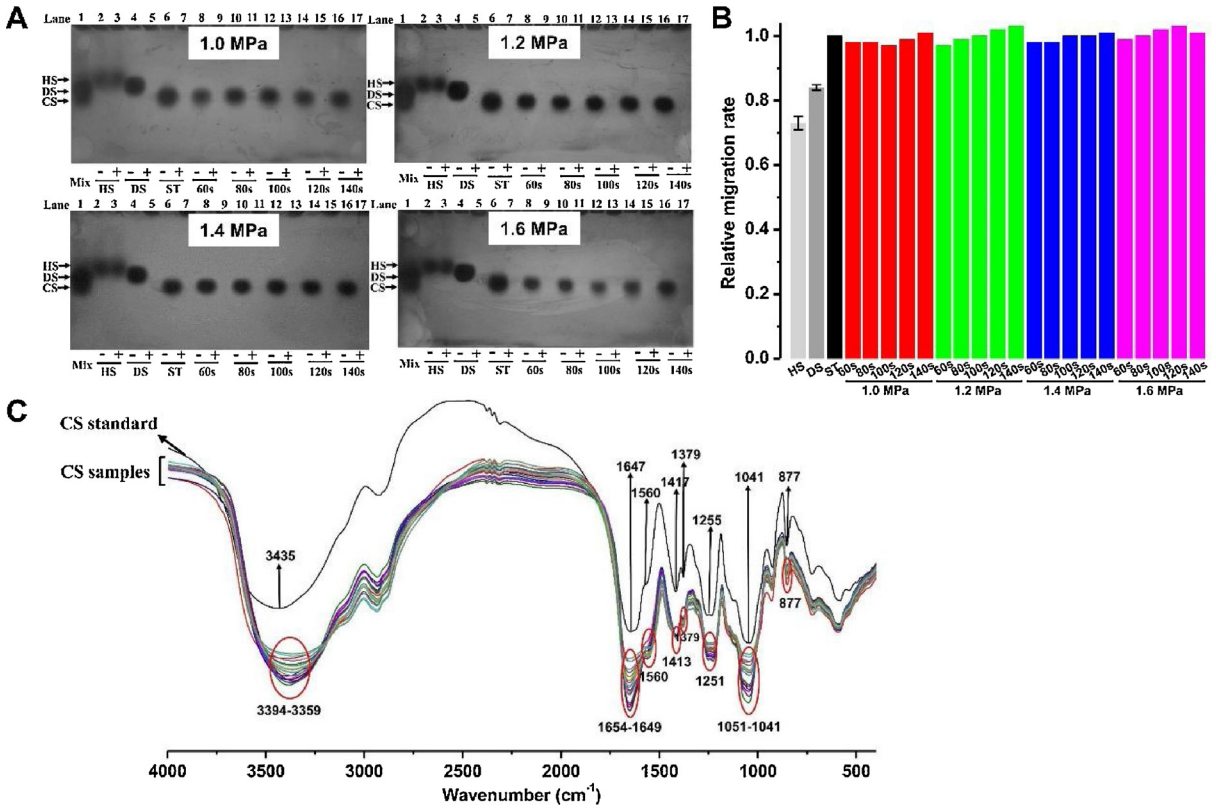

Fig. 5. Agarose-gel electrophoresis and FT-IR of CS samples. (A).

Table 3
Amount, disaccharide composition (%) and molecular weight (MW, kDa) values of CS samples.

Samples	Parameters								
	ΔDi0s	ΔDi6 s	ΔDi4 s	ΔDi4,6 dis	ΔDi2,6 dis	ΔDi2,4 dis	ΔDi2,4,6 tris	4 s/6 s	MW
1.0 MPa 60 s	6.21	21.08	72.23	0.48	trace	trace	trace	3.43	34.75
1.0 MPa 80 s	6.19	22.32	71.00	0.49	trace	trace	trace	3.18	33.71
1.0 MPa 100 s	6.34	22.27	70.89	0.49	trace	trace	trace	3.18	33.28
1.0 MPa 120 s	6.17	22.24	71.08	0.50	trace	trace	trace	3.20	34.98
1.0 MPa 140 s	6.19	22.26	71.04	0.51	trace	trace	trace	3.19	34.81
1.2 MPa 60 s	6.21	22.34	70.93	0.51	trace	trace	trace	3.18	34.35
1.2 MPa 80 s	6.16	22.20	71.12	0.52	trace	trace	trace	3.20	30.82
1.2 MPa 100 s	6.24	22.22	71.01	0.52	trace	trace	trace	3.20	33.24
1.2 MPa 120 s	6.10	22.06	71.32	0.52	trace	trace	trace	3.23	35.10
1.2 MPa 140 s	6.03	21.89	71.56	0.52	trace	trace	trace	3.27	34.57
1.4 MPa 60 s	6.11	22.38	71.01	0.50	trace	trace	trace	3.17	34.67
1.4 MPa 80 s	6.10	22.07	71.32	0.51	trace	trace	trace	3.23	35.38
1.4 MPa 100 s	6.06	22.03	71.36	0.51	trace	trace	trace	3.24	32.40
1.4 MPa 120 s	6.56	22.10	70.81	0.49	trace	trace	trace	3.20	32.30
1.4 MPa 140 s	6.43	22.00	70.94	0.49	0.14	trace	trace	3.22	28.72
1.6 MPa 60 s	6.29	22.17	70.90	0.48	0.16	trace	trace	3.20	29.29
1.6 MPa 80 s	6.46	22.18	70.71	0.48	0.18	trace	trace	3.19	28.58
1.6 MPa 100 s	6.54	22.11	70.69	0.47	0.19	trace	trace	3.20	31.86
1.6 MPa 120 s	6.90	22.04	70.42	0.47	0.17	trace	trace	3.20	29.92
1.6 MPa 140 s	7.50	22.31	69.52	0.49	0.18	trace	trace	3.12	34.70

Note: Scheme illustrated CS unsaturated disaccharides produced via chondroitinase ABC. ΔDi0s.

disaccharides generated by chondroitinase ABC were analysed by SAX-HPLC, and the chromatograms were provided in Supplementary Fig. 4. Various unsaturated disaccharides were obtained from the CS samples (Table 3). The nonsulfated disaccharide Di0S, monosulfated disaccharides Di6S and Di4S, and disulfated disaccharide Di4, 6 diS were all determined with different levels, while the disulfated disaccharide Di2, 6 diS was detected only in a portion of SE samples. Additionally, the disulfated disaccharide Di2, 4 diS and trisaccharide Di 2, 4, 6 were not determined. Compared with the previous research (Wang et al., 2019), the differences and similarities of CS samples in the quantity and composition of disaccharide were existed. Especially, the percentage of nonsulfated disaccharide Di0S in this research was more than 6% while it was trace (lower than 0.1%) in the previous report. Additionally, the disulfated disaccharide Di2, 6 diS was detected in CS from portion of SE samples but it was 0.29% CS from the ends of chicken leg bone by enzymatic method. Except the slight difference in quantity, the monosulfated disaccharides Di6S, Di4S and disulfated disaccharide Di4, 6 diS were all determined, and the disulfated disaccharide Di2, 4 diS and

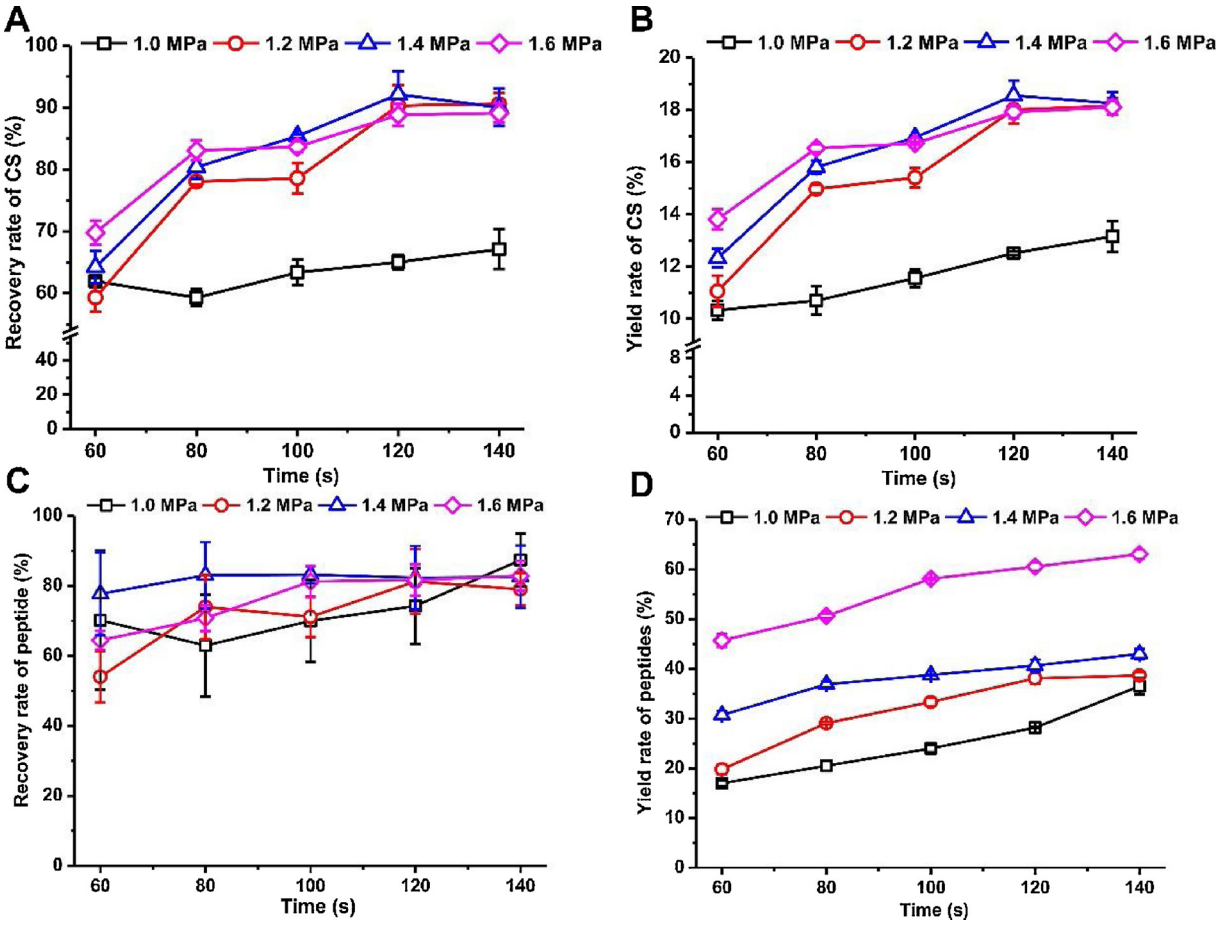

Fig. 6. The total recovery and total yield of CS (A, B) and peptides (C, D) from the liquid fraction of SE cartilage samples.

trisaccharide Di 2, 4, 6 were both trace, which was the consistent result from these two papers. The reason of the difference was probably caused by the temperature and the pressure during the SE treatment or the different material, and it remains to be investigated in future. The molecular weights of the CS samples were evaluated by GPC/MALLS, and the profiles were shown in Supplementary Fig. 5. The weight-average molecular weight of the CS samples ranged from 30 to 35 kDa (Table 3) that was a little smaller than the CS from the ends of chicken leg bone (37.18 kDa) (Wang et al., 2019). Overall, the molecular weight seemed to decrease slightly with the increase in pressure. It could be speculated that the slight difference in the CS structure was induced by the SE conditions of a higher pressure and longer maintaining time. Different sources of CS samples have varied structures and properties, especially related to the position and number of sulfate groups (Krichen et al., 2016). The 4-sulfated disaccharide content in the CS sample was significantly higher than the sulfated disaccharide in position 6, and the ratio of 4 s/6 s was approximately 3 (Table 3), suggesting that chondroitin sulfate A was the main component in the CS sample, which was consistent with the FT-IR results and recent report (Wang et al., 2019).

ΔUA-GalNAc; ΔDi6 s, ΔUA-GalNAc, 6 s; ΔDi4 s, ΔUA-GalNAc, 4 s; ΔDi4,6 dis, ΔUA-GalNAc4, 6 dis; ΔDi2,4 dis, ΔUA-GalNAc2, 4 dis; ΔDi2,4,6 tris, ΔUA2s-GalNAc4, 6 dis; ΔUA, 4, 5-unsaturated uronic acid; GalNAc, N-acetylgalactosamine; and s, sulfate group. The percentage of each identified disaccharide was determined using standard disaccharides. Trace indicates values lower than 0.1%. Quantitative data are the means of three repetitions.

3.6. Recovery and yield of CS and peptide

CS, together with peptides, was obtained from the liquid fraction of SE samples by membrane combination technology. As the pressure and maintaining time increased, the total recovery and total yield of CS and peptides increased (Fig. 6). The highest of recovery and yield of CS were 92.15% and 18.55%, respectively, at 1.4 MPa for 120 s, while the counterparts of peptide were 87.35% (1.0 MPa 140 s) and 63.07% (1.6 MPa 140 s). The uronic acid contents of CS samples ranged from 20.32% to 24.76%, which is slightly less than the CS standard, and the contents of protein ranged from 7.73% to 10.67% (Supplementary Fig. 6A). For the peptides from the liquid cartilage, the distribution of peptides was less than 2 kDa (Supplementary Fig. 6B). Compared with previous report (Luo, Fosmire, & Leach, 2002), even though CS was obtained with a higher yield using $MgCl_2$, guanidine HCl, papain and ethanol from the chicken keel cartilage, it took approximately 78 h for the entire extraction and digestion process. This process did not seem practical to produce CS in industry. Therefore, considering the CS recovery and yield, 1.4 MPa 120 s of SE could be chosen as the pretreatment condition to liquefy chicken sternal cartilage, and with the optimized isolation procedures, more than 90% CS could be obtained from the cartilage.

4. Conclusions

In this work, SE was firstly proposed as a pretreatment to liquefy chicken sternal cartilage, and based on this method, CS, together with peptide, could be obtained by environmentally friendly membrane combination technology. Further studies should be investigated to against the long enzymolysis time. Different enzymes combination may be considered. Additionally, pilot-scale production of this CS isolation method should be evaluated in the following work.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by “the Project Grant of National Key Research and Development Plan (2016YFD0400201)”, “Open project of Key Laboratory of Agro-Products Processing, Ministry of Agriculture and Rural Affairs, P. R. China”, and “Ten Thousand Experts Plan” Leading Talent.

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.2019.03.032.

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