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Chicken leg bone as a source of chondroitin sulfate

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ABSTRACT

In this work, chondroitin sulfate (CS) was extracted from chicken leg bone soup using the heat-resin static adsorption extraction (HSAE) method. The HSAE method was optimized as follows: resin dosage, 10%; adsorption time, 4.3 h; eluent concentration, 2 M; eluent time, 1.3 h, under which the yield of CS1 from the bone soup reached 0.14% and the recovery rate was 67.35%. CS2, as reference, was obtained from the ends of chicken leg bone using enzymatic method. CS1 and CS2, together with other glycosaminoglycans, were confirmed using agarose-gel electrophoresis. The average molecular weight of CS1 and CS2 was 35.81 kDa and 37.18 kDa, respectively. The structures of CS1 and CS2 were compared using Fourier-transform infrared spectroscopy and high-performance liquid chromatography, and no significant difference was observed. Overall, the HSAE method was proposed to be a promising approach for the coproduction of CS and bone soup.

1. Introduction

Waste from food industry is an important factor causing environmental contamination (Martone, Pérez, & Sánchez, 2005). Significant research have been reported to develop methods to transform waste into valuable products (Coello, Montiel, Concepcion, & Christen, 2002; Larsen, Thilsted, Kongsbak, & Hansen, 2000; Laufenberg, Kunz, & Nystroem, 2003). The United States Department of Agriculture has reported that about 83 million tons of raw chicken was consumed worldwide in 2012 and 28 million tons of that was from China corresponding to producing approximately 16.6 to 41.5 million tons of chicken bones, and about 5.6 to 14 million tons in China (Wang, Dong et al., 2016). According to the report, chicken bones contain approximately 19% protein, 9% fat and 15% ash (Fonkwe & Singh, 1996), suggesting that it can be as a rich source of nutrients, as well as contaminants if not used properly. Unfortunately, these bones are usually recognized as by-products, and their value has not been appreciated in the poultry industry (Bhaskar, Modi, Govindaraju, Radha, & Lalitha, 2007). Although in China, a portion of chicken bones are used for bone soup as traditional food flavour enhancer in factories, most bones are discarded for aesthetic or microbial reasons, which will not only waste the nutrients in the bone but also contribute to environmental negative impacts.

In the previous research in our lab, chicken bone residue was used to prepare substrate for flavourants (Wang, Dong et al., 2016), chicken bone extract or protein (Dong et al., 2014) as well as peptone employed in the fermentation industry (Wang, Yue et al., 2016). Furthermore, the literature reported that chondroitin sulfate (CS) can be efficiently isolated from broiler chicken by-products from mechanical deboning of a mixture of crushed bone, cartilage, skin, adipose tissue and muscle (Nakano, Pietrasik, Ozimek, & Betti, 2012). However, it is unclear whether the chicken leg bone soup or the leg bone can be employed to extract polysaccharide such as CS. CS is a sulfated glycosaminoglycan comprised of repeating disaccharide units of N-acetyl-galactosamine and glucuronic acid linked by glycosidic bonds (Nakano, Betti, & Pietrasik, 2010). It has wide applications ranging from pharmaceutical, cosmetic and functional food uses due to its special bioactivity and nutrient functions (Nunes et al., 2017; Wu, Liu, & Chen, 2012). Currently, CS is mainly used as a food supplements, or as over the counter drugs sold in many countries (Henrotin, Marty, & Mobasheri, 2014). It is reported that a combination of CS and glucosamine can be used to partially treat osteoarthritis (Fox & Stephens, 2009). Recently, fucosylated CS, from *Holothuria Mexicana,* has shown potential for application in the field of anti-angiogenesis and anticoagulation (Li et al., 2018). More interesting results have shown that CS and disaccharide can ameliorate stress-induced intestinal inflammation and affect the structure of gut microbiota (Fang et al., 2017; Shang et al., 2016). Cartilage from animals, such as cow, pig or shark, are most commonly chosen as the raw materials for extracting CS (He, Yin, Yan, & Yu, 2014; Maccari, Ferrarini, & Volpi, 2010; Sugahara, Nadanaka, Takeda, & Kojima, 2010;

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Volpi, 2007, 2010). However, CS may be limited just from cartilage due to its prestige as a food supplement or drug for adjuvant therapy against osteoarthritis among the elderly. In addition to cartilage, non-cartilage, such as chicken leg bone, probably can be as the resources to obtain CS.

At present, CS isolated and purified from cartilage includes four main steps: hydrolysis cartilage by chemicals; break-down of the proteoglycan core; elimination of proteins and CS recovery; purification of CS (Shi et al., 2015). Alkali-enzymatic hydrolysis was considered the most common method to extract CS. Alkali was capable of destroying covalent bonds between CS and core protein, while enzymes, such as papain, alcalase, trypsin and pepsin, were usually chosen to hydrolyse the protein, which ultimately caused CS release from the cartilage. For these conducts, many reagents, such as sodium hydroxide, urea, or guanidinium chloride, can be utilized. Furthermore, to promote dissociation of proteoglycans during CS production, alkaline concentration may be increased, which aggravates environmental pollution. For the purification of CS, chromatography and membrane separation technologies were commonly employed. Davies et al. (Davies, Roubin, & Whitelock, 2008) used anion-exchange chromatography to purify coarse CS from bovine trachea. Lignot et al. (Lignot, Lahogue, & Bourseau, 2003) utilized enzymatic extraction followed by concentration-desalting by ultrafiltration–diafiltration technologies to purify CS from skate cartilage. Compared with an ion exchange resin, membrane separation technology appears to be more efficient. Moreover, the membrane separation technology does not involve phase transformation or secondary pollution, which should be more common employed in the scale production.

In the present study, we mainly investigated the heat-resin static adsorption extraction (HSAE) method to obtain CS from chicken leg bone soup, and the method was optimized using response surface methodology. Another conventional method of enzymatic extraction was also employed, as a reference, to extract CS from the end parts of chicken leg bone. The HSAE method and membrane purification process, based on the conventional cooking bone soup by the Chinese food industry, are convenient and suitable for application to industry. The properties of CS samples achieved using the two methods were analysed together with the standard. The molecular weight was almost the same, and the structure, except for the composition of disaccharides, showed no significant difference via Fourier transform-infrared spectroscopy (FT-IR) and high-performance liquid chromatography (HPLC), which indicates that CS can be obtained from chicken leg bone soup. This work provides a coproduction method for bone soup and CS in industry.

2. Materials and methods

2.1. Materials

Chicken leg bones were provided by Fengxiang Biotechnology Company (Liaocheng, Shandong Province, China) and stored at −20 °C. Both standard chondroitin sulfate A and chondroitinase ABC (CSase, from *Proteus vulgaris*, 50–250 units/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycosaminoglycans including dermatan sulfate, heparan sulfate and heparin were purchased from Medchem Express. Standard glucuronic acid was from Solarbio (Beijing,China). The standard unsaturated chondro/dermato disaccharides involving ΔDi0s (ΔUA-[1→3]-GalNAc), ΔDi4 s (ΔUA-[1→3]-GalNAc-4 s) were from Sigma-Aldrich, while ΔDi6 s (ΔUA-[1→3]-GalNAc-6 s), ΔDi2,4 dis (ΔDi-dis B, ΔUA-2s-[1→3]-GalNAc-4 s), ΔDi2,6 dis (ΔDi-dis D, ΔUA-2s-[1→3]-GalNAc-6 s), ΔDi4,6 dis (ΔDi-dis, ΔUA-4 s-[1→3]-GalNAc-6 s), ΔDi2,4,6tris (ΔDitris, ΔUA-2s-[1→3]-GalNAc-4 s,6 s) were purchased from the Iduron Corporation (Alderley City, UK). Amberlite™ FPA94CL anion-exchange resin (food grade) was obtained from the Dow Chemical Company. Other reagents were of analytical grade.

2.2. Alcian blue staining

According to the method of Dingerkus et al. (Dingerkus & Uhler, 1977), fresh chicken leg bones were fixed in 10% formalin-saline solution for 3 days, followed by decalcification with 0.1 M ethylene diamine tetraacetic acid. The leg bones were divided into three sections, including two ends and the middle section, for paraffin embedding, respectively. These parts were prepared into 5 μm thick tissue sections to stain with alcian blue. The colour distribution was observed with an Automatic digital slice scanning system (KF-PRO-005, Konfonng Biotech International Co., LTD.).

2.3. Extraction of CS with HSAE method and determination of the association yield

The residual meat, fat and connective tissue were removed from the fresh chicken leg bone, which were then soaked in water for 30 min to wash out the residual blood. The bones were placed in a crane cage mixed with a 1.5 fold weight of distilled water with respect to the bones, and boiled at 120 ± 0.5 °C, 0.1 MPa pressure for 120 min, mimicking the preparation of bone soup from Fengxiang Biotechnology Company (Liaocheng city, Shandong province, China). According to Dong et al. (Dong et al., 2014), the resulting soup was filtered through a 100-mesh sieve to remove the residues, and the filtrate was maintained at 85 ± 1.0 °C. Subsequently, the supernatant (oil) was removed using a tubular centrifuge at 12,000*g* for 10 min while the aqueous layer was collected as the bone soup and stored at −20 °C until further use.

HSAE method was performed as follows: various dosages of anion-exchange resin were added to the bone soup with stirring to absorb polysaccharides for various duration. The polysaccharides absorbed in the resin were eluted with various sodium chloride concentrations for various duration, and 0.1% trypsin was added to the eluent with incubating at 47 °C for 24 h. Trypsin was inactivated by boiling for 10 min. The protein in the mixture was removed by the follows (Nakano et al., 2012): pre-cooled trichloroacetic acid was added to the mixture with a final concentration of 7% (w/v) and left at 4 °C for 24 h. The precipitated protein was removed by centrifugation at 15,000*g* and 4 °C for 20 min. The supernatant obtained was quantitatively transferred to a beaker, and ethanol was added to a final concentration of 70% (v/v) to obtain the precipitate with standing at 4 °C for 24 h. The precipitate was collected by centrifuge at 5000*g* for 5 min and dried at 60 °C. The recovered precipitate was solubilized in distilled water to filter through the laboratory tangential ultrafiltration system (molecular-weight cut-off, 3 kDa; Minimate™, Pall Company) removing salt and other contaminants (Lignot et al., 2003; Murado, Fraguas, Montemayor, Vázquez, & González, 2010). The purified fractions were freeze-dried for further analysis.

The content of uronic acid was determined via the carbazole reaction as described by Kosakai et al. (Kosakai & Yosizawa, 1979). CS from chicken leg bone soup obtained using the HSAE method was designated CS1. The protein in the CS sample was determined by the method of Lowry et al. (Lowry, 1951) while the carbohydrate content (in terms of glucose) of CS sample was detected by phenol-sulfuric acid method (Taylor, 1995). The yield of CS from the bone soup was calculated as follows: CS yield (%) = the weight of CS / the weight of bone soup ×100 while the recovery rate of CS from the chicken leg bone soup was evaluated using the formula: recovery rate of CS = CS yield with HSAE / content of free CS in soup. The content of free CS in chicken leg bone soup was obtained as follows: 100 g chicken leg bone soup was treated with 7% (w/v) trichloroacetic acid to remove the protein as described above. Subsequently, 0.1% trypsin was added to the supernatant incubating in water at 47 °C for 24 h, followed by boiling at 100 °C for 10 min to inactivate enzyme. Afterwards, ethanol was added to the supernatant with a final concentration of 70% (v/v) and stored at 4 °C for 24 h to obtain the crude total free CS in 100 g bone soup. The purification was conducted as described above.

2.4. Extraction optimization using response surface methodology

The effects of resin dosage, adsorption time, eluent concentration and elution time on the yield of CS were analysed using response surface methodology. After four single-factor experiments, the preliminary ranges of the extraction variables were determined. The independent variables were set as follows: resin dosage (6, 8 and 10%), adsorption time (3, 4 and 5 h), eluent concentration (2, 3 and 4 M) and elution time (1, 2 and 3 h). Then, a Box-Behnken factorial design was adopted in which a 29-run with four variables and three levels, including five replicates at the centre point, was used to optimize the extraction conditions (Zou, Chen, Yang, & Liu, 2011). The symbols and coded factor levels are presented in the Supplementary File. The experimental design and regression analysis were conducted using Design-Expert software (version 8.0.6, Stat-Ease Inc., Minneapolis, USA). The interrelationships of the variables were based on a second-order polynomial model (Liu et al., 2015).

2.5. Enzymatic extraction of CS

According to Maccari et al. (Maccari, Galeotti, & Volpi, 2015), the ends of chicken leg bone (50 g) were crushed and defatted by grinding with 100 mL of acetone for three times, followed by filtration and drying at 60 °C for 24 h. Then, trypsin (1 g) was added and the solution was incubated at 47 °C for 6 h with stirring. After boiling for 10 min, the protein in the mixture was removed as described above. Ethanol was added to the supernatant with a final concentration of 70% (v/v), and stored at 4 °C for 24 h to obtain the precipitate. It was dried at 60 °C to obtain the rude CS by enzymatic method, and the CS from ends of leg bone with enzymatic method was named as CS2. The purification of CS was performed as described above. The uronic acid, protein and carbohydrate contents of CS sample was determined with the same methods as above.

2.6. Agarose-gel electrophoresis

Agarose-gel electrophoresis was performed according to Dietrich et al. (Dietrich, Mcduffie, & Sampaio, 1977) and Volpi et al. (Volpi & Maccari, 2002) with some modifications. CS samples and different standard glycosaminoglycans including dermatan sulfate, heparan sulfate and heparin were dissolved in CSase buffer (33 mM Tris-HCl, pH 6.2, 33 mM sodium acetate, 1 mU CSase) with a final concentration of 2 mg/mL at 37 °C for 8 h, and the solution was boiled at 100 °C for 3 min to inactivate the enzyme. Then, 20 μL of glycosaminoglycans with or without treatment of CSase were loaded for electrophoresis. An electrophoretic instrument JY-SPCT (JUNYI Electrophoresis Company, Beijing, China) was employed. The agarose-gel was prepared at a concentration of 1% in 0.04 M barium acetate buffer pH 5.8. The run was performed in 0.05 M 1, 3-diaminopropane (buffer at pH 9.0 with acetic acid) for 6 h at 50 mA. After migration, the plate was soaked in 0.1% cetyltrimethylammonium bromide solution for 3 h, and stained with 0.2% fresh toluidine blue for 8 h. The gel was destained with ethanol-water-acetic acid (50:49:1 v/v/v) until the background faded away. The migration of CS samples and other standard glycosaminoglycans was recorded using the camera, and their relative migration rates were calculated via the migration of glycosaminoglycan/migration of standard chondroitin sulfate.

2.7. FT-IR analysis

The FT-IR spectra of CS s amples and standard CS were determined using an FT-IR spectrometer (TENSOR27, Bruker Company). The dried powders samples were ground with potassium bromide powder and pressed into 1 mm pellets for FT-IR measurement frequencies ranging from 4000 to 500 cm^{-1}.

2.8. Enzymatic treatments and disaccharide evaluation determination

The enzymatic depolymerisation of CS was achieved using CSase. Different CS samples were dissolved in CSase buffer as described above with a concentration of 5.0 mg/mL. CS samples were digested at 37 °C for 12 h, and the solution was boiled at 100 °C for 2 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 using HPLC equipment from Agilent equipped with a 150 mm × 4.6 mm stainless-steel column spherisorb 5-SAX (5 μm, trimethylammoniopropyl groups $Si-CH_2-CH_2-CH_2-N^+(CH_3)_3$ in Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK) and detection at 232 nm. Isocratic separation was performed using 50 mM sodium chloride pH 4.0 for 5 min followed by a linear gradient from 5 to 60 min of 50 mM sodium chloride to 1.0 M sodium chloride pH 4.0, at 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 purposes.

2.9. CS molecular weight determination

The molecular weight of CS was determined using gel permeation chromatography with a multi-angle laser light scattering system (GPC/MALLS) according to Roulard et al. with some modifications (Roulard, Petit, Mesnard, & Rhazi, 2016). HPLC (HITACHI High-Technologies Corporation, Tokyo Japan) equipped with DAWN HELEOS-II (Wyatt Technology Corporation, America) and Optilabr EX (Wyatt technology corporation, America) detectors coupled with TSK gel G4000PWxl column (7.8 × 300 mm) were used. Before analysis, 0.1 M sodium nitrate as the mobile phase was prepared and filtered through a 0.2 μm filter membrane and degassed with an ultrasonic device for 30 min. 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 that was determined from Nordmeier (Nordmeier, 1993). The flow rate was 0.5 mL/min. Overall, these procedures successfully co-produced for chondroitin sulfate and bone soup as shown in the flow chart (Fig. 1)

3. Results and discussion

3.1. Distribution of polysaccharides in chicken leg bone

Alcian blue is specific for mucopolysaccharides, and can be

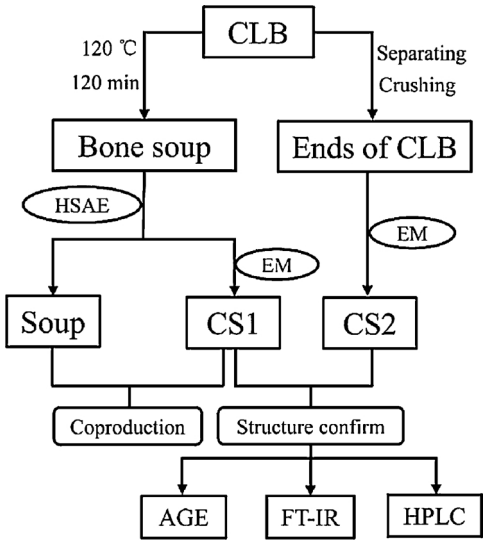

Fig. 1. Flow chart of coproduction for chondroitin sulfate and bone soup. CLB: chicken leg bone; EM: enzymatic method; AGE: agarose gel electrophoresis.

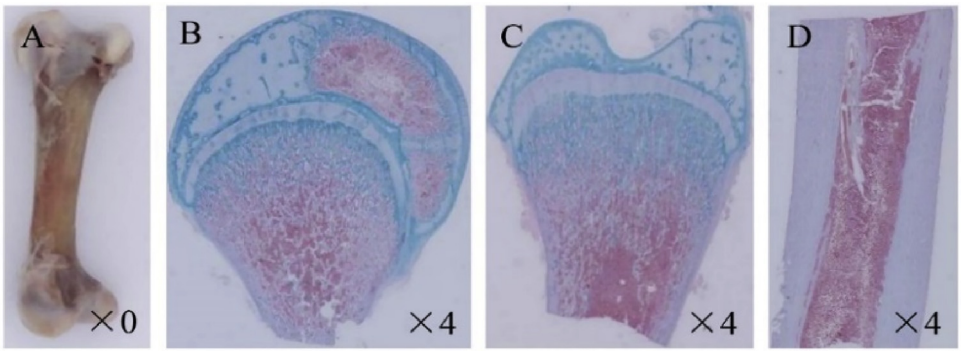

Fig. 2. Distribution of polysaccharides in chicken leg bone. A: chicken leg bone; B, C: ends of chicken leg bone; D: middle fraction of chicken leg bone. Acid polysaccharide was stained blue with alcian blue; ×4 indicated photograph was magnified 4 times, and ×0 indicates photograph was the original.

employed as the indicator of cartilage tissue (Dingerkus & Uhler, 1977). The ends of fresh chicken leg bone were stained blue, while the middle section was red (Fig. 2), indicating that the ends were comprised partly of cartilage-containing polysaccharides. Very little polysaccharide was found in the middle section of chicken leg bones. This was consistent with other results that CS, in animals, was mainly found in cartilage (Inerot, Heinegård, Audell, & Olsson, 1982; Nakano, Aherne, & Thompson, 1979). CS has been reported to be an important polysaccharide involved in endochondral ossification, a process of bone growth (Hunter, 1991). Nakano reported that the growth of animal bone involved the process of transformation from cartilage to bone tissue, and the concentration of CS, measured as uronic acid, was relatively constant (Nakano & Sim, 1995), suggesting that chicken leg bone has potential for use in extracting CS.

3.2. Optimization of extraction conditions of HSAE method by single-factor experiments

The effects of the extraction parameters on the yields of CS are shown in Fig. 3. As shown in Fig. 3A, when adsorption time, eluent concentration and elution time were fixed at 3 h, 3 M and 2 h, respectively, the yield of CS increased as resin dosage increased from 6% to 10%. However, when the amount of resin reached 8%, the upward trend was slow. Similarly, when resin dosage, eluent concentration and elution time was fixed at 8%, 3 M and 2 h, the yield of CS increased as adsorption time increased from 1 to 4 h (Fig. 3B). However, when increasing adsorption time to 5 h, the yield was not changed significantly. When resin dosage, adsorption time and elution time were fixed at 8%, 3 h and 2 h, respectively, the yield of CS increased as eluent concentration increased from 1 to 3 M, peaked at 3 M, and decreased when the concentration exceeded 3 M (Fig. 3C). Additionally, when resin dosage, adsorption time and eluent concentration were fixed at 8%, 3 h and 3 M, respectively, the yield increased as elution time increased from 1 to 2 h, and did not significantly increase further when extraction time exceeded 2 h (Fig. 3D).

3.3. Optimization of CS extraction yield with HSAE method by RSM

The appropriate ranges of resin dosage (6, 8 and 10%), adsorption time (3, 4 and 5 h), eluent concentration (2, 3 and 4 M) and elution time (1, 2 and 3 h) for CS extraction were determined in single-factor experiments. Based on these results, the extraction parameters were

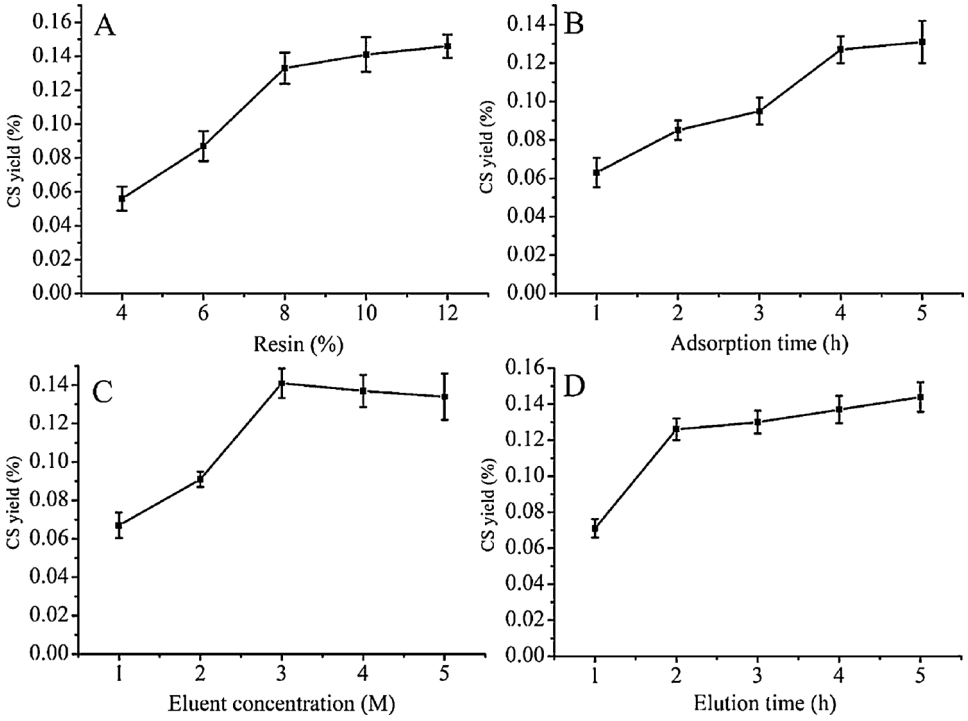

Fig. 3. Effects of resin dosage (A), adsorption time (B), eluent concentration (C), and elution time (D) on CS yield.

Table 1
Analysis of variance of response surface quadratic model for CS yield.

Source	Sum of square	Degree of freedom	Mean square	*F* value	*P* value	Significance
Model	0.015649	14	0.001118	10.42776	< 0.0001	**
A	0.010561	1	0.010561	98.52318	< 0.0001	**
B	0.001064	1	0.001064	9.926481	0.0071	**
C	0.001045	1	0.001045	9.751569	0.0075	**
D	1.01E-05	1	1.01E-05	0.094064	0.7636	
AB	6.4E-05	1	6.4E-05	0.597035	0.4526	
AC	1.6E-05	1	1.6E-05	0.149259	0.7051	
AD	0.000225	1	0.000225	2.098951	0.1694	
BC	2.25E-06	1	2.25E-06	0.02099	0.8869	
BD	1.6E-05	1	1.6E-05	0.149259	0.7051	
CD	0.000306	1	0.000306	2.856905	0.1131	
A^2	0.00226	1	0.00226	21.08447	0.0004	**
B^2	6E-05	1	6E-05	0.559825	0.4667	
C^2	8.72E-05	1	8.72E-05	0.813527	0.3823	
D^2	0.000299	1	0.000299	2.791142	0.1170	
Residual	0.001501	14	0.000107			
Lack of fit	0.000817	10	8.17E-05	0.477632	0.8435	
Pure error	0.000684	4	0.000171		< 0.0001	
Total	0.01715	28			< 0.0001	

Note: $R^2 = 0.9125$.
** Indicates significance ($p < 0.01$).

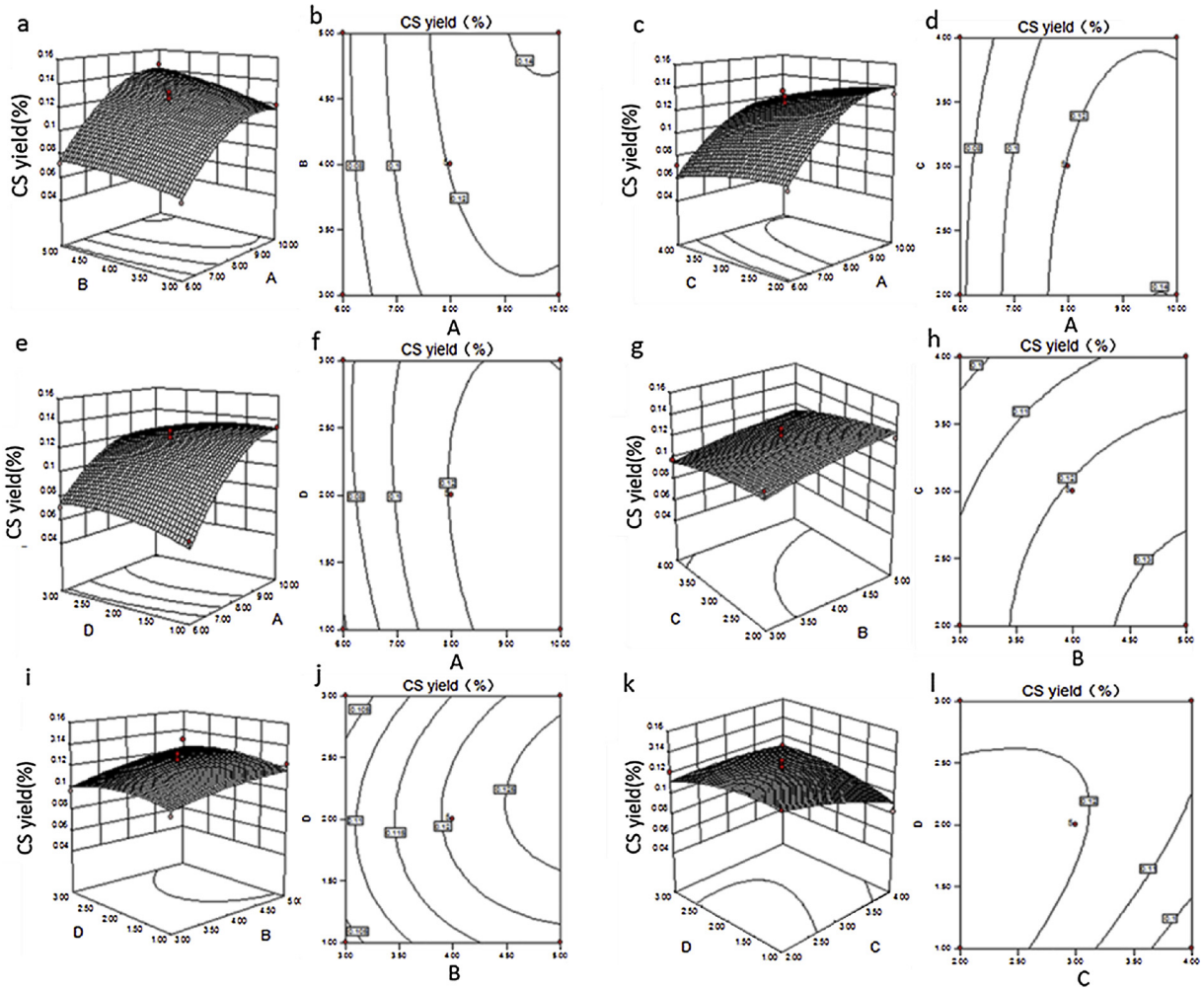

Fig. 4. Three-dimensional response surfaces (a, c, e, g, i and k) and contour graphs (b, d, f, h, j and l) for interactive effects of resin dosage, adsorption time, eluent concentration and elution time on CS yield.

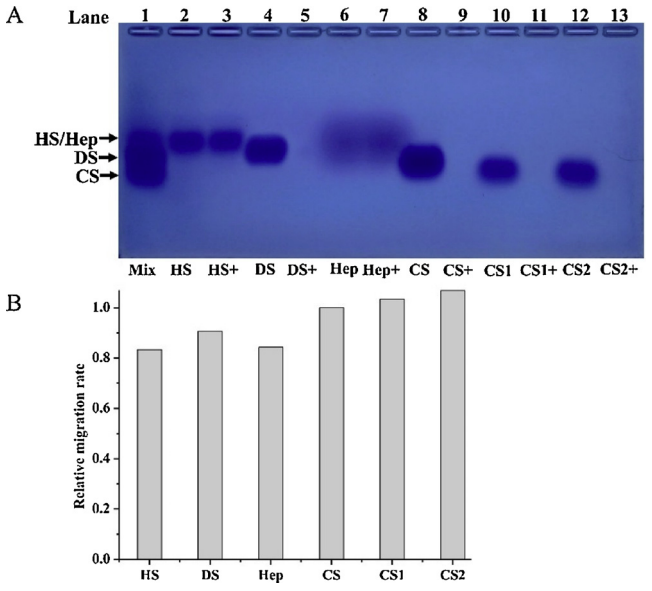

Fig. 5. Agarose-gel electrophoresis of glyconsaminoglycans and CS samples. (A) Migration of glyconsaminoglycans and CS samples with or without treatment of chondroitinase ABC. (B) Relative migration rates of glyconsaminoglycans and CS samples. HS, DS and Hep were heparan sulfate, dermatan sulfate and heparin, respectively. Mix was mixture of glyconsaminoglycans. CS1 and CS2 were chondroitin sulfates from bone soup and ends of leg bone, while CS was standard chondroitin sulfate A. "+" indicated glyconsaminoglycans and CS samples treated with chondroitinase ABC. Relative migration rates were calculated by migration of glycosaminoglycan/migration of standard chondroitin sulfate A.

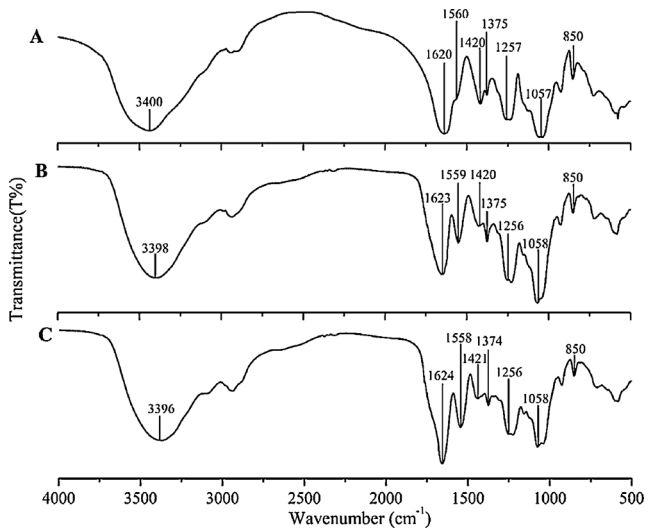

Fig. 6. FT-IR spectrum of CS. A: FT-IR spectrum of standard chondroitin sulfate A; B: FT-IR spectrum of CS1; C: FT-IR spectrum of CS2.

further investigated for optimum conditions using a Box-Behnken factorial design (Zhao, Zhang, Li, Dong, & Liu, 2015). Experimental conditions and the results of CS yields are listed in the Supplementary File.

Multiple regression analysis was applied to the experimental data, and the response variables and test variables were correlated according to the following second-order polynomial equation: $Y = 0.121 + 0.030A + 9.417 \times 10^{-3}B - 9.333 \times 10^{-3}C + 9.167 \times 10^{-4}D + 4.000 \times 10^{-3}AB - 2.000 \times 10^{-3}AC - 7.500 \times 10^{-3}AD - 7.500 \times 10^{-4}BC + 2.000 \times 10^{-3}BD + 8.750 \times 10^{-3}CD - 0.019A^2 - 3.042 \times 10^{-3}B^2 - 3.667 \times 10^{-3}C^2 - 6.792 \times 10^{-3}D^2$, where Y was the yield of CS calculated from the regression model, and A, B, C and D were the coded variables of resin dosage, adsorption time, eluent concentration and elution time, respectively.

Analysis of variance (ANOVA) of the response surface quadratic model was performed. For this model, the high F value was 10.42 while the low p value was < 0.0001 (Table 1), indicating that the polynomial model was highly statistically significant. The high determination coefficient ($R^2 = 0.9125$) indicated that 91.25% of the variability in the response could be explained by the model, demonstrating that the model equation had a high-quality fit and good precision and reliability (Han et al., 2016). Response surfaces were plotted using Design-Expert software to explain the interactions among variables and determine the optimal level of each variable for the maximum response. Three-dimensional response surfaces and two-dimensional contours are shown in Fig. 4. Response surface analysis was performed using Design-Expert software to determine the following optimal extraction conditions: resin dosage, 10%; adsorption time, 4.3 h; eluent concentration, 2 M; and elution time, 1.3 h. The maximum predicted yield of CS was 0.15%. To validate the model equations, a verification experiment was conducted under these conditions, and the experimental yield of CS was about 0.14 ± 0.01% (mean ± RSD, n = 3), highly consistent with the predicted value. The total free CS content in bone soup was 0.208 ± 0.006%, and the recovery rate of CS with the HASE method reached 67.35 ± 1.94% (mean ± RSD, n = 3). The above results demonstrated that the regression model was accurate and adequate for the prediction of CS extraction yield, and the HSAE method was appropriate to obtain CS from chicken leg bone soup.

3.4. Agarose gel electrophoresis of CS

In animal tissues, glycosaminoglycans are usually combined with proteins forming the corresponding proteoglycans (Schiraldi, Cimini, & De Rosa, 2010). In this experiment, we used HSAE methods and an enzymatic extraction method to extract CS from bone soup and the ends of bone. The yields were 0.14% and 4.25%, respectively. The uronic acid content of CS1 and CS2 was 40.92% and 41.15%, respectively, according to a carbazole assay. The protein content of CS1 and CS2 was 0.72% and 0.83%, respectively. The carbohydrate contents (in term of glucose) of CS1 and CS2 were 20.47% and 19.19%, respectively, while the content of that in standard chondroitin sulfate A was 20.85%. In this study, ultrafiltration technology, rather than conventional chromatography, was employed to purify CS, which was more suitable to the industry due to its low-cost, simplicity and efficiency (Lignot et al., 2003). In addition, the bone soup, after extraction, could still be used as original material for other applications. Simultaneously, the resin used in the experiment was recyclable. This method not only maximizes the value of chicken leg bone, but is also friendly to the environment.

The results agarose gel electrophoresis of glycosaminoglycans (Lane 1-7), standard CS (Lane 8-9), CS1 (Lane 10-11), and CS2 (Lane 12-13) samples with or without treatment of CSase are shown in Fig. 5A. Standard CS (Lane 9), dermatan sulfate (Lane 5), CS1 (Lane 11) and CS2 (Lane 13) samples were enzymatically hydrolysed completely by CSase, while heparan sulfate (Lane 3) and heparin (Lane 7) were not degraded, consistent with previous reports (Grøndahl et al., 2011). The relative migration rates of glycosaminoglycans including heparan sulfate (0.83), dermatan sulfate (0.91) and heparin (0.84) were significantly different from the standard CS (1), CS1 (1.03) and CS2 (1.07) samples (Fig. 5B). However, compared with the standard CS, the relative migration rates of CS1 and CS2 have a little increased, which may be caused by the difference of their molecular weight. Taken together, in 1, 3-diaminopropane buffer, the mobility rates of CS samples were greater than other glyconsaminoglycans and the migration of heparan sulfate was the slowest, which was agreement with the research of Dietrich et al. and Maccari et al. (Dietrich et al., 1977; Maccari et al., 2010). These results suggested that CS1 and CS2 samples from bone soup and the ends of bone were confirmed to be chondroitin sulfate, and the HSAE method can be applied to extract CS from the bone soup.

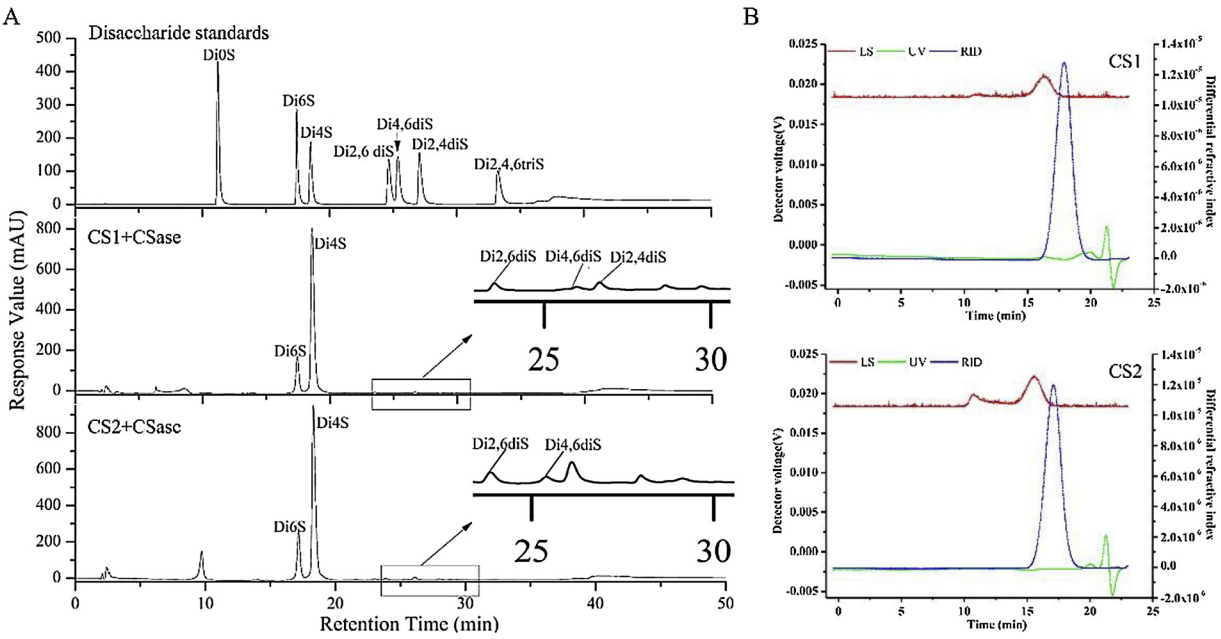

Fig. 7. SAX-HPLC separation of unsaturated disaccharides produced by CS samples treated with CSase (A). GPC/MALLS profiles of CS1 and CS2 for determination of molecular weight (B). CS1 + CSase and CS2 + CSase indicated CS samples were treated with chondroitinase ABC. ΔDi0s, UA-GalNAc; ΔDi6 s, UA-GalNAc6 s; ΔDi4 s, UA-GalNAc4 s; ΔDi2, 6 dis, UA2s-GalNAc6 s; ΔDi4, 6 dis, UA-GalNAc4, 6 dis; ΔDi2, 4 dis, UA2s-GalNAc4 s. LS, light scattering detection signal; UV, ultraviolet detection signal; RID, differential refraction detection signal. The identity of disaccharide species was assured by co-elution with purified standards.

Table 2
Amount, disaccharide composition and molecular weight values of CS purified from bone soup and bone.

Parameters	CS1	CS2
ΔDi0s (ΔUA-GalNAc) (%)	Trace	Trace
ΔDi6 s (ΔUA-GalNAc, 6 s) (%)	17.03	20.08
ΔDi4 s (ΔUA-GalNAc, 4 s) (%)	81.99	78.44
ΔDi2, 6 dis (ΔUA2s-GalNAc6 s) (%)	0.24	0.29
ΔDi4, 6 dis (ΔUA-GalNAc4, 6 dis) (%)	0.49	1.19
ΔDi2, 4 dis (ΔUA2s-GalNAc4 s) (%)	0.25	Trace
ΔDi2, 4, 6tris (ΔUA2s-GalNAc4, 6 dis) (%)	Trace	Trace
4 s/6 s	4.46	3.77
Molecular weight (kDa)	35.81	37.18

Note: Scheme illustrates CS unsaturated disaccharides produced via action of chondroitinase lyases. ΔUA, 4, 5-unsaturated uronic acid; GalNAc, N-acetyl-galactosamine; s, sulfate group. Percentage of each identified disaccharide was determined using purified standards and reported as weight percent. Trace indicates values lower than 0.1%. CS1: extracted from bone soup by HSAE method; CS2: extracted from end of bone by enzymatic method.

3.5. Identification of type of CS by FT-IR

FT-IR was used to identify the structure of CS1 (Fig. 6B) and CS2 (Fig. 6C) extracted by the HSAE method and enzymatic method, respectively. The FT-IR spectra was recorded in the range of 4000–500 cm^{-1}. Spectrograms of the two samples were substantially identical to that of standard chondroitin sulfate A (Fig. 6A). The characteristic peak at 3400 cm^{-1} was observed in the standard, representing the hydroxyl structure of carbohydrate, which was migrated to 3398 cm^{-1} and 3396 cm^{-1} in CS1 and CS2 samples, respectively. The characteristic peaks at 1620 cm^{-1} and 1560 cm^{-1} can be observed to represent the carbonyl and N—H band in standard, indicating the presence of —COOH and —NH—C═O (Wang, Shen, & Lu, 2003). The peaks at 1420-1375 cm^{-1} represented the coupling of the C—O stretching vibration and OH variable angle vibration, indicating 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 reported earlier by Khan et al. (Khan, Ashraf, Hashmi, Ahmad, & Anjum, 2013). Although these characteristic peaks were all existed, some migration was observed in the CS1 and CS2 samples (Fig. 6B and C), respectively. 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 peak spectra of the two samples and standard exhibited were observed only at 850 cm^{-1}, indicating that the samples mostly consisted of chondroitin-4-sulfate. The result was in agreement with the reports of Rani et al., in which the isolated chicken CS-keel spectrum displayed a distinct peak of sulfate group at 856.9 cm^{-1}, indicating the polysaccharide was chondroitin-4-sulfate (Rani, Baruah, & Goyal, 2017). However, the spectra of CS from bony fish always exhibited peaks at 820 cm^{-1}, suggesting that these cartilage samples mainly consisted of chondroitin-6-sulfate (Garnjanagoonchorn, Wongekalak, & Engkagul, 2007), suggesting that the type of CS may be species-related.

3.6. Disaccharide and molecular weight evaluation of CS

To further characterize the structure of CS, purified CS samples were subjected to treatment with chondroitin sulfate ABC, and the unsaturated disaccharides produced were analysed by SAX-HPLC (Fig. 7A). As shown in Table 2, various unsaturated disaccharides were generated from CS treated with chondroitin sulfate ABC. The non-sulfated disaccharide Di0S of CS1 and CS2 were both presented in trace amounts, and monosulfated disaccharides Di6S, Di4S of CS1 and CS2 were evaluated at 17.03%, 81.99% and 20.08%, 78.44%, respectively. Interestingly, the disulfated disaccharide Di2, 6 diS of CS1 and CS2 were 0.24% and 0.29%, while disaccharide Di2, 4 diS found only in CS1 was 0.25%. In addition, the disaccharide Di4, 6 diS (0.49% and 1.19%) differed. However, the trisaccharide was not determined. These results suggested that chondroitin sulfate A was the main component in the two CS samples, consistent with FT-IR results. The molecular weight of CS samples was evaluated by GPC/MALLS, and the profiles are shown in Fig. 7B. The average molecular weight of CS1 and CS2 was 35.81 kDa and 37.18 kDa, respectively (Table 2). These chromatographic results suggested that the structure and composition of CS obtained through the HSAE method and enzymatic extraction was similar, and the slight

differences in the content of disaccharide might be due to the difference in the extraction processes.

Various sources of CS samples have varied structures and properties. In particular, they can have repeating disaccharides with sulfate groups that vary in numbers and position, as well as their amount (Krichen et al., 2016). The degree and position of sulfate groups lead to different proprieties and biological capacities. SAX-HPLC analysis showed that the 4-sulfated disaccharide content in CS samples extracted by the two methods is higher than the sulfated disaccharide in position 6, producing a 4 s/6 s ratio of 3.77 and 4.46 (Table 2), respectively, quite similar to a previous report by Volpi (Volpi, 2010). Volpi studied the 4 s/6 s ratio of bovine, porcine, chicken and shark CS samples and found that 4-sulfated disaccharide accounted for the majority among terrestrial animals including avian CS samples, but Di6 s was the major disaccharide in fish. It is speculated that the composition of CS disaccharide has an important relationship with the raw materials from which it is derived.

4. Conclusions

In the present work, the HSAE method was proposed to extract chondroitin sulfate from chicken leg bone soup. After optimizing the extraction conditions, chondroitin sulfate was obtained successfully from the bone soup, and achieved the coproduction of chondroitin sulfate and bone soup. Further study should be investigated to against low yield of chondroitin sulfate from chicken leg bone soup.

Conflict of interest

The authors declare no conflict of interest.

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