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Extraction of chondroitin sulfate and type II collagen from sturgeon *(Acipenser gueldenstaedti)* notochord and characterization of their hybrid fibrils

Dawei Meng[a*], Wen Li[b], Xiaoqian Leng[c], Yasuaki Takagi[d], Zhiyuan Dai[a], Hao Du[c], Qiwei Wei[c]

a Collaborative Innovation Center of Seafood Deep Processing, Zhejiang Province Joint Key Laboratory of Aquatic Products Processing, Institute of Seafood, Zhejiang Gongshang University, Hangzhou 310012, China

b Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

c Key Laboratory of Freshwater Biodiversity Conservation, Ministry of Agriculture and Rural Affairs, Yangtze River Fisheries Research Institute, Chinese academy of Fishery Sciences, Wuhan 430223, China

d Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

Corresponding author at: Zhejiang Province Joint Key Laboratory of Aquatic Products Processing, Institute of Seafood, Zhejiang Gongshang University, Hangzhou 310012, China. E-mail addresses: dawei@mail.zjgsu.edu.cn (D. Meng)

1. Introduction

Chondroitin sulfate (CS) and type II collagen (Col II) are the two major biological macromolecules of the cartilage matrix [1, 2]. They form a stable fibrous structure, which contributes to the strength against abrasive force during joint movement *in vivo* [3]. Both CS and undenatured Col II, extracted from animal cartilaginous tissues, are widely applied in pharmaceutical and biomedical materials and nutraceuticals industries. The CSs extracted from animal tissues have various biological activities *in vitro*, including anti-viral, anticoagulant, anti-inflammatory, and antioxidant activities [4], [5]. Meanwhile, undenatured Col II, which maintained triple helical structure, could relieve osteoarthritis [6], [7]. In addition, due to their special structures and biological characteristics, CS and undenatured collagens are often used as the cell scaffold materials in tissue engineering [8].

Currently, commercially available CS and Col II are mainly derived from porcine throat, bovine trachea, chicken clavicle and shark cartilage [9], [10]. However, outbreaks of zoonoses, such as bovine spongiform encephalopathy, swine fever and avian influenza, have raised concerns about the products of terrestrial origin [11]. In addition, religious beliefs restrict the use of porcine sources, and shark catches are decreasing year by year [11], [12]. Therefore, alternative CS and Col II from fish tissues have attracted increasing attention. The current researches reported the cartilages of fishes such as skate fish, lumpsucker fish, rabbit fish, sturgeon are good sustainable sources of CS and Col II [13], [14], [15]. However, existing researches were all about the extraction of one certain substance from fish cartilage-related

tissues [16], [17]. Shen et al. reported the co-production of CS and low molecular weight peptides in one process using the hot-pressure extraction technique [18]. Many researchers have focused on collagen peptides because of their functional activities. However, as a biomaterial, the unique macromolecular and structural properties of undenatured collagen could not be replaced by collagen peptides. So far, there was still no method to simultaneously obtain both CS and undenatured Col II in one extraction process. It meant that one or more valuable macromolecular substances would be lost during the extraction of CS or undenatured Col II, resulting in incomplete utilization of fish body and serious waste of resources.

Protease digestion extraction is the most common procedure for extracting CS [14], [15]. However, it is difficult to extract with undenatured collagen because protease digestion destroys the secondary structure of collagen and hydrolyzes collagen into peptides. In terms of the traditional CS extraction procedures, alkaline treatment is a common method to breakdown the covalent connection between CS and core protein [19]. Meanwhile, low-temperature alkaline pretreatment is widely used to remove non-collagenous substances during collagen extraction [17]. Our recent study reported that, under certain condition, alkaline treatment increased the extraction efficiency of sturgeon notochord Col II and showed no effect on triple helical structure of Col II, but the extraction and structure properties of notochord CS were not addressed [19]. Based on previous studies, we hypothesis that it is possible to extract both CS and undenatured Col II using alkaline treatment. In addition, further exploration of the interaction between CS and Col II will facilitate the application of

sturgeon CS and Col II in biomedical materials. To our knowledge, no studies discuss the *in vitro* interaction of CS and Col II in the sturgeon notochord.

The objectives of this study were to develop a new method for the simultaneous extraction of CS and undenatured Col II from sturgeon notochord, furthermore, to clarify the interaction between CS and Col II fibrils. Firstly, we isolated CS and undenatured Col II from notochord tissue using low-temperature alkaline treatment and then extracted CS and undenatured Col II, respectively. Secondly, we analyzed their structural properties using spectroscopy methods. Finally, we discussed the effects of CS on Col II fibril, including the speed and degree of fibril formation, fibril morphology, and antioxidant activity of fibril membranes.

2. Materials and methods

2.1 Materials

Sturgeon (*Acipenser gueldenstaedtii*) notochord was obtained from the Sturgeon Biological Technology Co. Ltd. (Xinchang county, Zhejiang province, China). The notochord was kept in dry ice to be transported to the laboratory. The internal semisolid of notochord tube was removed and then the notochord sheath was lyophilized in a freeze dryer (FreeZone 2.5 L, American Labconco Co., Ltd., Kansas City, USA). The dry notochord sheath was stored at -30 °C until use.

2.2 Alkaline treatment of notochord

Notochord sheath was cut into small pieces (approximately 0.2 × 0.2 cm) for CS and undenatured Col II extraction. The tissues were continuously stirred in a solution of 0.1 M NaOH for 24 h at 4 °C, with a sample: solution ratio of 1: 50 (dry weight,

w/v, g/mL; two solution-changes). After alkaline treatment, the mixtures were centrifuged at 10,000 ×g for 30 min at 4 °C (Sorvall LYNX4000, Thermo fisher scientific, Waltham, USA). The supernatant and precipitate were used for CS and undenatured Col II extraction, respectively. To discuss the effects of alkaline treatment time and NaOH concentration on the yields of CS and Col II, the alkaline treatment time (12, 24, and 48 h) and NaOH concentration (0.01, and 0.1 M) were used to treat notochord tissue, respectively.

2.3 Isolation and purification of CS

The alkaline treated supernatant was neutralized to pH 8.0 with 3 M HCl. Enzymatic hydrolysis was achieved by the addition of papain (EC 3.4.23.1, 1:10,000, Solarbio Science&Technology Co, Ltd, BeiJing, China) with a ratio of papain : solution 1:5000 (w/v, g/mL). The reaction was kept at 50 °C for 4 h under continuous stirring. The reaction was then terminated by boiling for 10 min. The crude CS was precipitated by the addition of 3 volumes of ethanol at 21 °C for 12 h. The precipitate was collected by centrifugation at 10,000 ×g for 30 min and dried at 60 °C until constant weight.

The crude CS was dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl. The solution was applied to a column (1.5 cm × 7 cm) packed with UNOsphere Q strong anion-exchange media (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and equilibrated in 20 mM Tris-HCl and 50 mM NaCl. The crude CS was eluted with a linear gradient of NaCl (50 mM to 2 M in 90 min) in 20 mM Tris-HCl using Biologic LP system (Bio-Rad Laboratories, Inc, Hercules, CA, USA) at flow

rate of 1 mL/min. The eluate solution was collected in each 5 min. Three volumes of ethanol were added into collection tube to purify CS at 21 °C for 12 h. The precipitate was collected and re-dissolved in deionized water, and then dialyzed against 50 times volume of deionized water (two solution-changes) using a 200-Da dialysis membrane to remove NaCl at 21°C for 12 h. Finally, the dialysis fluid was lyophilized and stored at -30 °C until used. The percentage of dry weight of CS extracted in comparison with the dry weight of the initial tissues was calculated as the CS yield. Experiments were conducted for 3 times, and the data were expressed as means ± standard errors (SE).

2.4 Isolation and purification of Col II

The precipitate obtained from section 2.2 was used for undenatured Col II extraction. Extraction progress performed according to the method of Meng et al. [19]. The precipitate was washed to neutral with cold deionized water, and then continuously stirred in a solution of HCl (pH 2.0) containing 0.5% (w/v) porcine gastric mucosa pepsin (1:10,000., Solarbio Science & Technology Co, Ltd., BeiJing., China) at a sample (dry weight based) / solvent solution of 1:100 (w/v) for 24 h at 4 °C (two solution changes). The mixture was centrifuged at 10,000 g for 30 min (Sorvall LYNX4000., Thermo fisher scientific., Waltham, USA) to obtain the supernatants. The supernatant was precipitated by adding NaCl to a final concentration of 1 M. The precipitate was redissolved by a small amount of HCl (pH 2.0) at 4 °C. After redissolving, the purified collagen was dialyzed against 50 volumes of distilled water at 4 °C for 24 h with two changes of water. The dialysate was lyophilized using a freeze dryer (FreeZone 2.5 L, American Labconco Co., Ltd.,

Kansas City, USA). The percentage of dry weight of collagen in comparison with dry weight of the initial tissues was calculated as the collagen yield. Experiments were conducted for 3 times, and the data were expressed as means ± standard errors (SE).

2.5 Determination of purity and molecular weight of CS and undenatured Col II

2.5.1 Purity and molecular weight distribution of CS

The purity and average molecular weight of CS was analyzed by Gel permeation chromatography/size-exclusion chromatography (GPC/SEC). An Agilent 1260 Infinity II Multi-detector GPC/SEC system (Agilient Technologies., CA, USA) was coupled with a refractive index (RI) detector and a multi-angle laser light scattering (MALLS, Wyatt Dawn Heleos-II., USA). Separation was performed using a Agilent PL aquagel-OH Mixed-H column (8 μm, 7.5 mm × 300 mm) and was eluted with 0.1 M $NaNO_3$ at 1.0 mL/min (45 °C). The CS was prepared at a concentration of 1 mg/mL using deionized water and the injection volume was 50 μL. The average molecular weight calculations were performed by Agilent GPC/SEC software (Agilient Technologies, CA, USA).

2.5.2 SDS-PAGE of Col II

SDS-PAGE was performed according to the method of Laemmli. [20]. The lyophilized Col II was dissolved in diluted HCl (pH 2.0) at a concentration of 1 mg/mL. The Col II were mixed at a ratio of 1:1 (v/v) with buffer (0.5 M Tris-HCl buffer, pH 6.8, with 4% SDS and 20% glycerol) containing 10% β-mercaptoethanol. The sample solution was boiled for 3 min. Ten micrograms of sample solution were loaded onto each lane. Electrophoresis was performed at 15 mA for the stacking gel,

and 20 mA for the 8% running gel. After electrophoresis, the gel was stained for 20 min with a 0.1% Coomassie Brilliant Blue R250 solution. Then, the gel de-stained with a mixture of 20% methanol, 2.5% glycerin, and 5% acetic acid.

2.6 Structure characteristics of CS and Col II

2.6.1 UV spectra of CS and Col II

The CS and Col II samples were prepared as a 1 mg/mL solution with deionized water and dilute HCl (pH:2.0), respectively. The UV spectra were recorded in the wavelength range from 190 to 400 nm (Evolution 60 s, Thermo fisher scientific, Waltham, USA). Deionized water was used as blank control.

2.6.2 FTIR of CS and Col II

FTIR spectroscopy of CS and Col II samples were recorded with an FTIR spectrophotometer (Nicolet iS10, Thermo Scientific, Madison, USA). The CS and Col II powers were ground together with potassium bromide (w/w 1:200), respectively, and then pressed into a 1 mm pellet for measurement in the range of 500-4000 cm^{-1}. The potassium bromide powder was used as the background.

2.6.3 NMR of CS

^{1}H- and ^{13}C-NMR spectra of CS were recorded with a Bruker Avance 600 (Karl-scrube, Germany) operating at 600 MHz. The lyophilized sample was dissolved in D_2O at a 30 mg/mL concentration and the spectra were recorded at 298 K. The chemical shifts (d, ppm) were quoted with respect to external sodium 4, 4-dimethyl-4-silapentane-1-sulfonate (0.0 ppm).

2.6.4 Disaccharide composition of CS

CS was dissolved in Tris-acetate buffer (50 mM Tris and 60 mM of sodium acetate, pH 8.0) to 10 mg/mL. Eighty microliter chondroitinase ABC (6mU/μL, EC 4.2.2.4, Sigma-Aldrich., Saint louis., USA) was mixed into 400 μL CS solution. The reaction was performed for 24 h at 37 °C. Then, the mixture was boiled for 10 min to terminate reaction and filtered through 0.22 μm syringe filters. The solution was fractionated by ultrafiltration using molecular weight cutoff of 3 kDa (Merck Millipore Ltd., Darmstadt., Germany). The fraction of molecule weight below 3 kDa was collected for analysis of disaccharide composition. The disaccharide sample was detected by HPLC with Waters 2695 Pump system, Waters 2489 UV/Vis detector (Waters Co., USA) and a Spherisorb SAX column (4.6 mm × 150 mm, 5 μL, Waters Co., USA), with a linear gradient from 5 to 35 min using 0 to 1 M NaCl (pH 3.5) solution as mobile phase. The absorbance was monitored at 232 nm. Bovine and shark cartilage CSs (Sigma-Aldrich., Saint louis., USA) were used as standard and were analyzed by the same method. Each experiment was replicated three times.

2.6.5 Circular dichroism (CD) of Col II

CD spectra of Col II was measured on Brighttime Chirascan (Applied Photophysics Ltd, UK). The measurement was performed following our previous method [19]. Col II was dissolved in pH 2 HCl solution to 1 mg/mL, and placed into a quartz cell. The spectra were measured at wavelength range of 190 to 250 nm at 10 °C with an interval of 0.1 nm. The scan speed was 50 nm/min.

2.7 Fibril formation *in vitro*

The fibril-forming process of Col II was evaluated by the method of Meng et al.

[19]. Lyophilized Col II was dissolved in pH 2.0 HCl solution to 0.3% (w/v). CS was added to Na-phosphate buffer (45mM, pH 7.4) at concentrations of 0, 0.75, and 1.5 mg/mL, respectively. The Col II solution was mixed with a Na-phosphate buffer with a ratio of 1:2 (v/v). After mixing, the final concentrations of CS were 0, 0.5, and 1 mg/mL, respectively. The mixed solution was pipetted into a quartz cuvette. Fibril formation at 21°C was monitored by measuring turbidity change via optical absorbance at 340 nm, using a spectral monitor (Evolution 60 s., Thermo fisher scientific., Waltham, USA).

The fibril formation degree was determined followed by the method of Zhang et al. [21]. When the turbidity was constant, the mixture was centrifuged at 20,000 ×*g* for 20 min at 4 °C. Quantification of protein in supernatant was measured by Lowry method [22]. The bovine serum albumin was used as a standard. Fibril formation degree was defined as the percentage of the decrease of collagen concentration in the solution, which meant the percentage of collagen molecules that formed the fibrils.

2.8 Morphology and elemental analysis of fibrils

The microstructure of notochord Col II fibrils was observed using a scanning electron microscope (SEM; Sigma 500, Carl Zeiss Ltd, Germany). The Col II fibrils were formed and obtained by centrifugation as described in Section 2.7. The precipitated fibrils were fixed by 2.5% (v/v) glutaraldehyde in 30 mM Na-phosphate buffer (pH 7.4) for 3 h at room temperature, and then rinsed with Na-phosphate buffer to remove the fixative. The fixed fibril was dehydrated using a graded series of ethanol solutions, and soaked in a t-butyl alcohol solution for two 15-min intervals.

Finally, the sample was freeze-dried using a freeze-drying device, and then coated with gold-platinum, using an auto fine coater (JFC-1600; JEOL Ltd, Japan). SEM and energy dispersive X-ray spectroscopy (EDX; Link ISIS 300, Oxford Instrument, UK) were used to analyze the elemental content of the fibril surfaces.

2.9 Statistical analyses

The data of yields, fibril formation degree, and antioxidant activity were expressed as means ± standard errors. The data were analyzed using ANOVA and Tukey–Kramer post-hoc tests for multiple comparisons, which were performed using a statistical add-in for Microsoft Excel.

3. Results and discussion

3.1 Yields of CS and Col II

In actual production, shortening the extraction time will save processing cost and minimizing the NaOH concentration will reduce environmental pollution. In this study, we discussed the effects of alkaline treatment time and NaOH concentration on CS and Col II yields. The results were shown in Table. 1 and 2. As shown in Table. 1, long alkaline treatment time increased the yield of CS. When alkaline treatment time was 24 h, the yield of Col II was the highest. Our previous study showed that alkaline pretreatment could improve the efficiency of Col II extraction because it could remove non-collagenous proteins and polysaccharides bound to Col II in the tissue. Meanwhile, long alkaline treatment could also lead to collagen loss [19]. Thus, 24 h alkaline treatment was suitable to obtain high yields of CS and Col II. As shown in Table. 2, low NaOH concentration (0.01 M) obtained higher yield of CS. However,

the yield of Col II extracted at 0.01 M NaOH was much lower than that at 0.1 M NaOH. It suggested that CS was easier to dissolved into diluted NaOH solution, but was not conducive to the removal of non-collagenous protein. Residue of non-collagenous protein in the tissue decreased the Col II extraction efficiency, resulting in lower yield of Col II. Therefore, 24 h, 0.1 M NaOH treatment was used for CS and Col II extraction, and the products were used for further structural analysis.

The yields of notochord CS and Col II were 5.34 ± 0.74% and 45.25 ± 5.25% (dry weight basis), respectively. Notochord CS yield was lower than the CSs derived from sturgeon cranial and backbone cartilages (19.5% and 13.3%, respectively) [15], [23], but was higher than that of the CSs extracted from smooth hound cartilage (2.52%) and *Holothutis scabra* cartilage (2.89%) [24], [25]. It is difficult to compare the yield of sturgeon notochord CS with other notochordal tissues because there have been few other studies on notochord CS extraction. In this study, CS was purified by ethanol precipitation and ion-exchange chromatography, which might lead to loss of CS during purification process. For large scale production, more efficient and environmentally friendly purification methods, such as ultrafiltration or membrane filtration, should be used instead of ethanol precipitation and ion-exchange chromatography [26].

The yield of Col II (45.25 ± 5.25%) was higher than Bester sturgeon notochord Col II (37.5%) reported in our previous study [19]. Different ages and species of sturgeon thought to be responsible for different yields. Our previous study reported that alkaline pretreatment accelerated the extraction efficiency of undenatured Col II

[19]. Based on amino acid analysis results, we found non-collagenous proteins were removed by alkaline pretreatment [19]. From this research, we knew that the polysaccharides also could be efficiently removed by alkaline pretreatment. Comparing with previous method, alkaline pretreatment time was extended from 12 h to 24 h in this study. The longer alkaline pretreatment time might be the reason for the higher Col II yield, because the polysaccharide components in the notochord might be more fully removed after the prolonged alkaline pretreatment time. As known, in the cartilage-related tissues, CS and Col II interlace to form the stable fibril sheath that resists high pressure. After removal of CS and non-collagenous proteins, Col II is better to exposure to pepsin solution without steric hindrance, and the pepsin will more easily break their intermolecular cross-links, thereby increasing the solubility of collagen molecules. In this study, we proposed a preliminary process for extraction of CS and undenatured Col II, respectively. Although, according to this method, the utilization of sturgeon notochord was increased from 37.5% to 50.6% comparing to our previous study [19]. The remaining 49.4% of notochord body was still not efficiently utilized. In order to reduce aquatic resource waste and make full use of sturgeon notochord, more suitable extraction conditions were need to be discussed in further study.

The amount of farmed sturgeon in China exceeded 80,000 tons in 2017 [27]. The notochord accounts for approx. 1.7% of sturgeon body weight (wet weight basis) and the moisture content of notochord is about 83.2% (data of the present research material). It is predicted that ca. 10.7 t CS and 90.5 t Col II can be obtained annually

from sturgeon notochord. Especially, type II collagen is highly valuable in the market because of much less availability than type I collagen. Thus, we conclude that sturgeon notochord has huge potential for the large-scale industrial production of CS and Col II.

3.2 Purity and molecular weight of CS and Col II

The purity and molecular weight of CS was analyzed by SEC-RI-MALLS as shown in Fig 1-A. CS appeared a symmetric peak in the HPGPC chromatography. The average molecular weight of notochord CS was ca. 38.4 kDa, and the polydispersity indices of notochord CS was 1.198. It indicated the component of CS was relatively uniform. Purified CS did not contain keratan sulfate with molecular weight was lower than CS [28]. Zhu et al. reported that the molecular weights of bovine, shark, and Chinese giant salamander CSs were 34.4, 75.8, and 49.2 kDa [29]. Maccari et al. [30] reported that the average molecular weight of sturgeon (unknown species) cartilage CS (unknown location) was 39.8 kDa. Gui et al. [15] reported that the molecular weights of hybrid sturgeon *(Acipenser baerii × Acipenser schrenckii)* skull and backbone CSs were 38.5 and 49.2 kDa, respectively. The molecular weight of sturgeon notochord CS was different with other fish CS and bovine CS, but similar with sturgeon cartilage CS. These results suggested that the molecular weight of CS was closely related to species source. This study was the first to report the molecular weight of sturgeon notochord CS.

SDS-PAGE was performed to detect the purity of notochord Col II, as shown in Fig 1-B. Notochord Col II had only one α-chain at ca. 130 kDa. The result was

consistent with our previous study [19]. These results suggested that the pure CS and Col II were obtained simultaneously by the new extraction method.

3.3 Structure characteristics of CS

The ultraviolet absorption of CS was identified using ultraviolet-visible spectrophotometer in the wavelength range from 190 to 400 nm, as shown in Fig 2 (A). The CS had a strong absorption peak at 190 to 210 nm, which was the characteristic absorption peak of polysaccharides [24], [31]. There had no absorbance at 260 nm and 280 nm, indicating that the nucleic acids and other protein were not mixed in CS [31].

The molecular structural characteristics of CS were identified by FTIR spectroscopy (Fig 2-C). The presence of sulfate group in C-O-S was detected at 856.59 cm^{-1}. Garnjanagoonchorn et al. reported that the absorption of the C-O-S axial and equatorial orientations of chondroitin 4-sulfate (CS-A) and chondroitin 6-sulfate (CS-C) exhibited the special peaks at 854.5 cm^{-1} and 823.7 cm^{-1}, respectively [32]. It suggested that the main type of sturgeon notochord CS was CS-A. Furthermore, the peak observed at 1068.29 cm^{-1} was attributed to the C-C ring vibrations [33]. The peaks shown at 1415.31 cm^{-1} and 1233.37 cm^{-1} were characteristic of C-O stretching [18]. The peak detected at 1640.16 cm^{-1} was represented -CONH structure [33]. The peak observed around 2922.86 cm^{-1} was attributed to the stretching vibration of C-H [18]. The strong absorbance peak at 3415 cm^{-1} indicated the stretching of -OH [18], [33].

^{1}H and ^{13}C-NMR spectroscopy were performed to confirm the integrity and

characteristics of CS molecular structure (Fig 3). ^{1}H-NMR spectroscopy was shown in Fig 3-A. All proton signals of CS appeared in two spectral regions, one between 2.0 and 2.1 ppm, and the other between 3.0 and 5.0 ppm. It indicated that high purity CS was obtained. The characteristic signal at 4.80 ppm was assigned to H4 of GalNAc-4SO_4 [34]. It indicated that CS of sturgeon notochord was composed by CS-A. Furthermore, the peak between 2.0 to 2.1 ppm was the signal of acetyl methyl group proton [35]. The signals at 3.74 and 3.98 ppm were assigned to H6 and H2 of N-acetyl-galactosamine, while the signals at 3.32, 3.54 and 3.69 ppm were assigned to H2, H3 and H4/5 of glucuronic acid, respectively.

^{13}C-NMR spectroscopy was shown in Fig 3-B. All signals were found in the region 50-80 ppm and 100-110 ppm except for the acetamido methyl carbon at around 25.5 ppm and the carbonyl at around 174.0 ppm. It indicated CS had high sulfate content at the 4 and/or 6 positions of the GalNAc [30]. The signals at 102.3, 53.0, 81.4, 78.1, 62.5 ppm were assigned to the C1, C2, C3, C4 (C5) and C6 of GalNac-4SO_4. The signals at 105.0, 73.8, 75.1, 78.1 and 175.7 ppm were assigned to the C1, C2, C3, C5 and C6 of GlcA [36]. Therefore, the sturgeon notochord CS contained a high content of CS-A, which was consistent with FTIR spectroscopy results. The structure of the sturgeon notochord CS differed from that of sturgeon cartilage CS, which was composed of CS-C [15], [23]. Differences in the functional activities of sturgeon notochord and cartilage CSs required further discussion.

To further investigate the disaccharide composition of CS, we hydrolyzed the CS with chondroitinase ABC and analyzed the disaccharide structure using SAX-HPLC,

as shown in Fig. 4. The disaccharide composition of sturgeon CS was analyzed by comparison with shark cartilage CS and bovine cartilage CS. Bovine Cart-CS consisted of non-sulfated disaccharide CS-0 (5.70%), and mono-sulfated disaccharide CS-A (63.79%) and CS-C (30.51%). Shark Cart-CS consisted of non-sulfated disaccharide CS-0 (2.79%), and mono-sulfated disaccharide CS-A (28.91%) and CS-C (50.46%), and di-sulfated disaccharide CS-D (17.85%). This disaccharide composition was consistent with the previous report [28]. Sturgeon notochord CS was composed of CS-0 (3.06%), CS-A (86.59%), and CS-C (10.35%). The di-sulfated disaccharide was not detected in sturgeon notochord CS. CS-A was the major component of sturgeon notochord CS. This result was consistent with the FTIR and NMR results. The effects of alkaline treatment time and NaOH concentration on disaccharide composition of CS was shown in Supplementary Fig.1. The result indicated that alkaline treatment time and NaOH concentration had no effect on disaccharide composition of sturgeon notochord CS.

3.4 Structure characteristic of Col II

The ultraviolet absorption of Col II was shown in Fig 2-B. The Col II was emerged a strong absorption peak at 200 to 220 nm, and the maximum absorption peak was at 206.9 nm. It was the characteristic absorption peak of type II collagen [37]. The ultraviolet absorption peak of type I collagen was at approx. 232 nm [38], slightly higher than Col II. Low absorbance at 280 nm because there were few aromatic amino acids in collagen [19].

The FTIR spectra of Col II was presented in Fig 2-D. The main characteristic

absorption peaks contained amide A (3416.17 cm^{-1}), amide B (2922.11 cm^{-1}), amide I (1618.61 cm^{-1}), amide II (1554.92 cm^{-1}) and amide III (1239.06 cm^{-1}). The amide A band of Col II was observed at 3416.17 cm^{-1}. Doyle et al. reported that the absorption band of amide A, associated with N-H stretching vibration appeared in the range of 3400–3440 cm^{-1} [39]. The amide B band was observed at 2922.11 cm^{-1}, which was related to the asymmetrical stretching of CH_2 [40], [41]. The amide I band frequencies from 1600 to 1700 cm^{-1} were mainly related to carbonyl group stretching vibrations and were characteristic of the secondary coil structure [40]. The amide I bond of Col II was observed at 1637.00 cm^{-1}. This observation confirmed the formation of hydrogen bond between N-H stretch, where the C-O residues were responsible for stabilizing triple helical structure [41]. The amide II band corresponded to N-H bending vibrations, and the amide III band represented C-H stretching [40]. The amide II and III of Col II were observed at 1554.92 and 1239.66 cm^{-1}, respectively. According to Plepis et al, the ratio of absorbance of amide III and to the peak between 1400 to 1454 cm^{-1} was close to 1.0, indicating that the triple helical structure of collagen was intact [42]. In this result, the ratio of amide III and 1454.14 cm^{-1} was 1.01, which confirmed the intact triple helical structures in Col II.

The CD spectra of notochord Col II was shown in Fig 5. The Col II had a rotatory maximum at 221 nm and a crossover point at approximately at 212 nm, which were typical characteristics of collagen triple-helical conformation [43]. FTIR and CD spectra results demonstrated that the Col II maintained an intact triple helical structure after extraction.

3.5 Effects of CS on the Col II fibril formation

Collagen fibril formation *in vitro* was a self-assembly process from soluble collagen molecule to insoluble fibrils. The changing process of solution turbidity reflected the different stages of fibril formation, and the turbidity value reflected fibril diameter [22]. The effects of CS on Col II fibril formation process were shown in Fig 6-A. The turbidity curve of three samples exhibited a logarithmic growth trend, and no lag phase was detected in the three curves. It suggested that fibril formation was too fast to detect the fibril nucleation process in the lag phase. The initial turbidity values for the 0, 0.5, and 1 mg/mL CS mixed samples were 0.258, 0.359, and 0.388, respectively. All three turbidity curves increased significantly during the first 15 min, and then the turbidity growth slowed down over time. The turbidity value of the 0 mg/mL CS sample remained slowly increasing over 300 min. The turbidity value of the 0.5 mg/mL CS sample stopped increasing at 180 min, and the turbidity value of the 1 mg/mL CS mixed sample stopped increasing at 135 min. It meant that CS accelerated the completion of Col II self-assembly, but inhibited the lateral aggregation of fibrils. CS was a polyanionic chain structure composed of disaccharide units of glucuronic acid and sulfated N-acetyl galactosamine [44]. In section 3.3, we demonstrated that the notochord CS was mainly composed of CS-A with N-acetyl galactosamine sulfated at carbon 4. The negative carboxyl and sulfonic acid groups of the CS chain were both anions under neutral conditions. These anions interacted with the amino groups of basic amino acids such as lysine, hydroxylysine, histidine and arginine on the side chain of Col II through electrostatic attraction. Mathews (1968)

have reported that electrostatic interaction was important for polyanions on collagen aggregation [45]. Multiple anions on the CS chain combined with amino ions on different collagen molecules or fibrils to form steric hindrance, which was dispersed between Col II molecules or fibrils, thereby affecting the later aggregation of fibrils. It might be the reason why the growth phase was shortened and the turbidity curve rapidly reached a plateau phase after CS was added to the sample.

The results of the degree of fibril formation at different CS concentrations were shown in Fig 6-B. The degree of fibril formation was 97.8 ± 0.09%, 97.1 ± 0.13% and 95.9 ± 0.11% for the 0, 0.5, and 1 mg/m CS samples, respectively. The degree of fibril formation decreased with increasing CS concentration. It suggested that CS had an inhibitory effect on Col II fibril formation. Wood et al. (1960) found that CS-A retarded the rate of growth of type I collagen fibrils [46]. Keech (1961) reported CS produced individual fibrils of smaller diameter than those from control solutions of type I collagen [47]. Since CS inhibited the lateral aggregation of fibrils, more soluble collagen or fine fibrils remained in solution, resulting in a reduced degree of fibril formation.

3.6 Morphology observation of fibrils

The Col II fibrils formed under different CS concentration conditions were shown in Fig. 7. For no CS sample, the Col II formed a disordered fibril meshwork structure. The fibrils with different thickness were clearly visible. The long fusiform structure was interlaced on the surface and inside of the fine fibrils and the average diameter of Col II fibril was between 10 to 50 nm. The fibril morphology of the

samples with added 0.5 and 1 mg/mL CS was similar, but different from the sample without CS added. The structure of long fibrils was not clearly visible, and the fibrils were connected by a dense network structure. The formation of this dense network structure might be due to the interaction between CS and Col II fibrils. So far, most studies have discussed the effect of CS on type I collagen fibril formation, but there were few studies on the interaction between CS and Col II.

3.7 Binding of CS to Col II fibril

Sulfur elements were located on the side chains of methionine and cystine of Col II. However, our previous study showed that methionine and cystine were low in Col II of sturgeon notochord [19]. Meanwhile, sulfur was an abundant element on the CS chain [44]. Thus, the sulfur content on the fibril surface could reflect the interaction of CS with Col II fibrils. Sulfur mapping was conducted using SEM-EDX and the results were shown in Fig. 8. To our knowledge, it was the first time that elemental mapping has been used to analyze the interaction between CS and Col II fibrils. The sulfur ratios of 0, 0.5 and 1 mg/mL CS samples were 0.6, 0.8 and 0.8%, respectively. The sulfur elemental mapping shown that the 0.5 and 1 mg/mL CS samples had higher sulfur densities than the sample without CS. It suggested that CS directly bound to Col II fibrils. The interaction between CS and Col II fibrils was thought to be the binding of carboxyl and sulfonic groups on the CS chain to amino groups on the Col II fibrils [48].

4. Conclusion

In this study, a new extraction process was established basing on our previous

study. By this method, both CS and undenatured Col II were obtained from sturgeon notochord with the yield of 5.34 ± 0.74% and 45.25 ± 5.25%, respectively. The average molecular weight of notochord CS was 39.7 kDa, and the main structure of sturgeon notochord CS was CS-A. CS accelerated the completion of Col II self-assembly, but inhibited the lateral aggregation of fibrils. The new extraction process will improve the utilization rate and avoid the waste of sturgeon notochord. Furthermore, the studies on the interaction between CS and Col II fibrils will promote the application of sturgeon notochord extracts in the field of biomedical materials.

Funding

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Credit Authorship contribution statement

Dawei Meng: Conceptualization, Methodology, Data curation, Writing-original draft.

Wen Li: Methodology. Xiaoqian Leng: Methodology. Zhiyuan Dai: Resources. Qiwei Wei: Funding acquisition, Resources. Hao Du: Funding acquisition, Resources. Yasuaki Takagi: Methodology, Writing-review & Editing.

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Carbohyd. Polym. 233 (2020) 115831.

Fig. 1. A: SEC-RI-MALLS chromatogram of sturgeon notochord CS. B: SDS-PAGE of sturgeon notochord Col II. Lane 1: Molecular weight marker; Lane 2: Col II.

Fig. 2. UV spectra of the sturgeon notochord CS (A) and Col II (B); FTIR spectra of the sturgeon notochord CS (C) and Col II (D).

Fig. 3. ^{1}H-NMR (A) and ^{13}C-NMR (B) analysis of sturgeon notochord CS. NC: N-acetyl-galactosamine; UC: glucuronic acid; Number: Carbon location.

Fig. 4. Disaccharide composition of sturgeon notochord CS (A); Bovine cartilage CS (B); Shark cartilage CS (C).

Fig. 5. CD spectra of the sturgeon notochord Col II.

Fig. 6. A: Fibril formation *in vitro* by Col II measured by optical absorbance at 320 nm. B: The degree of fibril formation of notochord Col II at different reaction times. Columns and bars show mean and standard error of the means of three experiments. Differences between groups with different letters are significant (Tukey-Kramer post-hoc test, $p < 0.01$).

Fig. 7. Scanning electron micrographs of sturgeon notochord Col II fibrils formed at 21 °C for 10 h. A: 0 mg/mL CS; B: 0.5 mg/mL CS; C: 1 mg/mL CS. Scale bar: 2 μm (1); 1 μm (2); 300 nm (3).

Fig. 8. Elemental mapping of CS and Col II hybrid fibril surface. A: 0 mg/mL CS; B: 0.5 mg/mL CS; C: 1mg/mL CS; (1): Backscattered electron image of SEM; (2): Sulfur elemental distribution on the fibril surface; (3): EDX spectra of the Col II fibril

661 surface. Scale bar: 5 μm.

Highlights

・Chondroitin sulfate (CS) and collagen (Col II) extraction method was developed

・Yields of CS and Col II from sturgeon notochord were suitable for industrialization

・Sturgeon notochord CS was mainly composed by CS-A with molecular weight of 38.4 kDa

・The method had no effect on secondary structure and fibril-forming ability of Col II

・CS bound on Col II fibrils and inhibited the lateral aggregation of fibrils

Abstract

Chondroitin sulfate (CS) and undenatured type II collagen (Col II) are the two major biological macromolecules of cartilage-related tissues. In this study, a new extraction process was developed to obtain CS and Col II simultaneously. By this process, CS and undenatured Col II were extracted from sturgeon notochord with the yields of 5.34 ± 0.74% and 45.25 ± 5.25%, respectively. The SEC-RI-MALLS result showed that the average molecular weight of notochord CS was 38.4 kDa. FTIR, NMR, and SAX-HPLC results indicated the notochord CS was mainly composed of CS-A. The new extraction process had no effect on the triple helical structure of Col II. To analyze the interaction between the two macromolecules, the effect of CS on Col II fibril formation was examined using turbidity assay and SEM observation. CS accelerated the completion of Col II self-assembly and inhibited the lateral aggregation of fibrils. The results of this study suggested that the sturgeon notochord is a valuable source of CS and Col II. The new extraction method not only improves the utilization rate of sturgeon notochord, but also reduces the waste of aquatic resources. CS and Col II derived from sturgeon notochord have the potential for use in biomedical materials.

Keywords

Sturgeon; Notochord; Extraction; Chondroitin sulfate; Type II collagen; Fibril

One extraction-process, obtaining two products ; Hybrid fibril materials

★ Material utilization up ★ Production Waste down

Farmed sturgeon

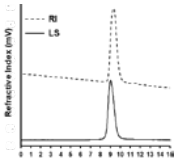
Notochord sheath

Co - extraction

Chondroitin-4-sulfate

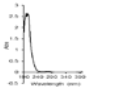
Undenatured type II collagen

Purity detection



SEC-RI-MALLS

Structural analysis



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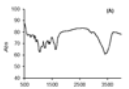

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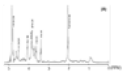

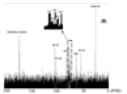

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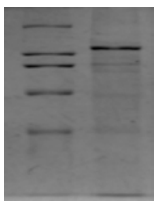
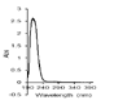
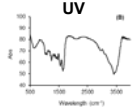


SDS-PAGE



UV



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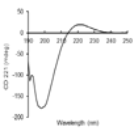
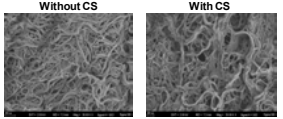
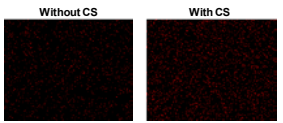


CD

Their interaction



SEM



Sulfur mapping

Table. 1. The effect of alkaline treatment time on CS and Col II yields (day weight base).

Time of alkaline treatment	Yield of CS	Yield of Col II
12 h	4.14±0.43%	42.53±7.97%
24 h	5.34±0.75%	45.25±5.25%
48 h	5.35±0.22%	40.83±3.17%

Table. 2. The effect of NaOH concentration on CS and Col II yields (dry weight base).

NaOH concentration	Yield of CS	Yield of Col II
0.01 M	6.80±0.25%	34.48±4.47%
0.1 M	5.34±0.75%	45.25±5.25%

Fig.1

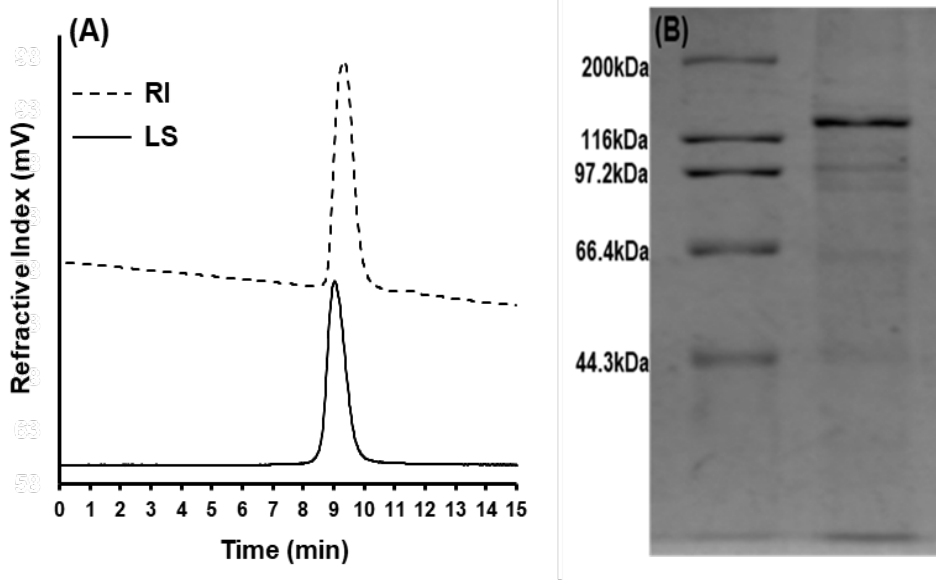

(A)
RI
LS
Refractive Index (mV)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (min)
(B)
200kDa
116kDa
97.2kDa
66.4kDa
44.3kDa

Fig.2

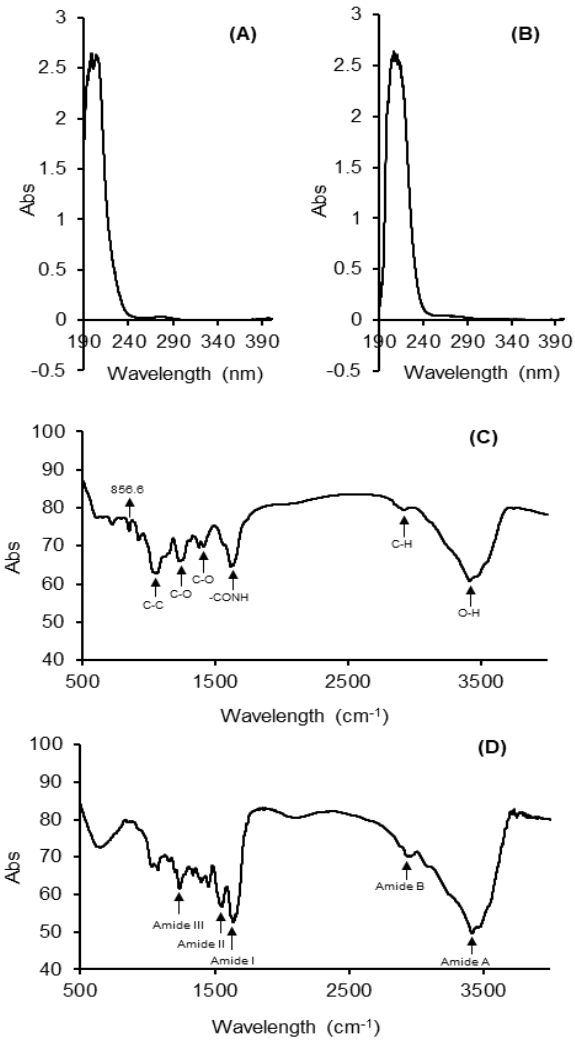

(A)
(B)
Abs
Wavelength (nm)
(C)
856.6
C-C
C-O
C-O
-CONH
C-H
O-H
Wavelength (cm-1)
(D)
Amide III
Amide II
Amide I
Amide B
Amide A
Wavelength (cm-1)

Fig.3

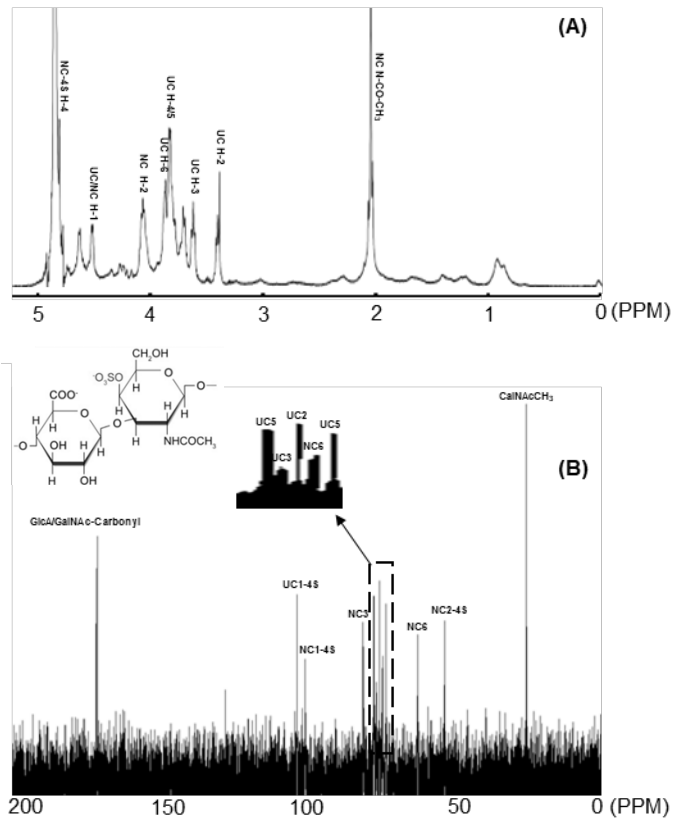

(A)
NC-4S H-4
UC/NC H-1
NC H-2
UC H-4S
UC H-5
UC H-3
UC H-2
NC N-CO-CH3
5
4
3
2
1
0 (PPM)
(B)
UC5
UC2
UC5
UC3
NC6
CalNAcCH3
GlcA/GalNAc-Carbonyl
UC1-4S
NC3
NC6
NC2-4S
NC1-4S
200
150
100
50
0 (PPM)

Fig.4

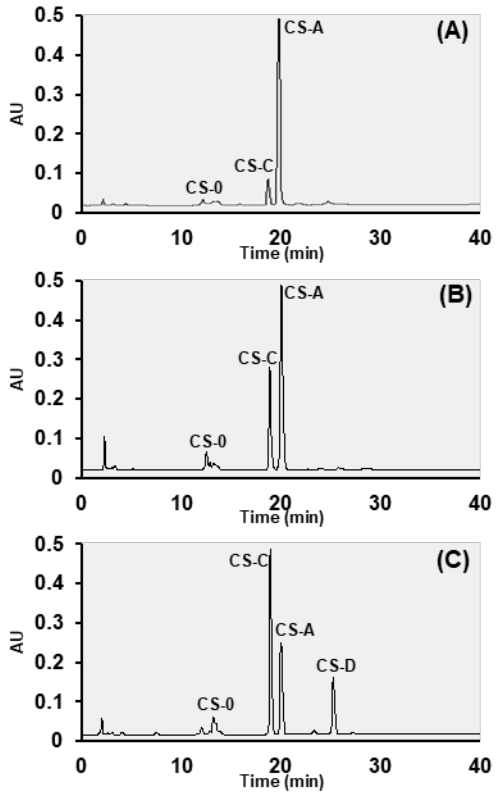

(A)
CS-A
CS-C
CS-0
AU
Time (min)
(B)
CS-A
CS-C
CS-0
AU
Time (min)
(C)
CS-C
CS-A
CS-D
CS-0
AU
Time (min)

Fig.5

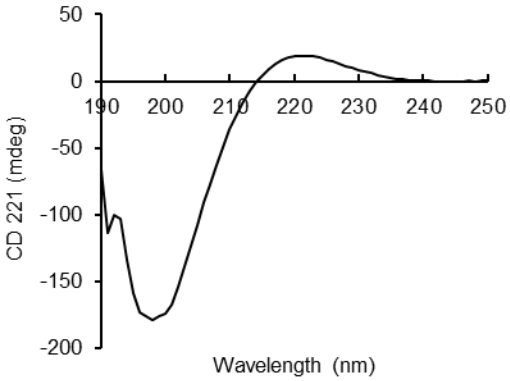

50
0
190
200
210
220
230
240
250
-50
-100
-150
-200
CD 221 (mdeg)
Wavelength (nm)

Fig.6

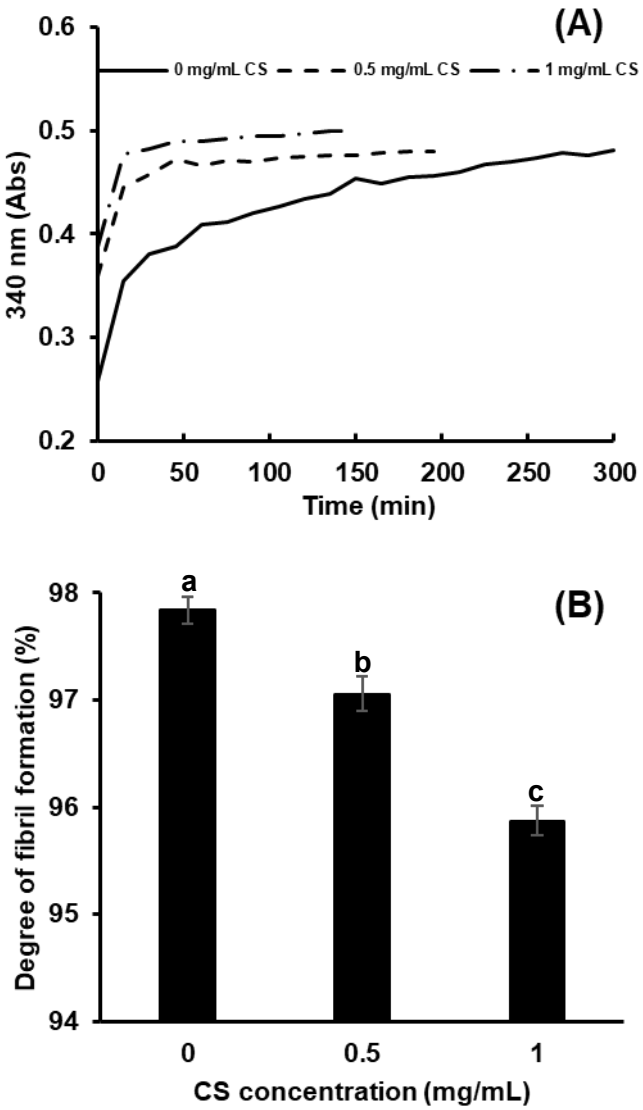

(A)
0 mg/mL CS
0.5 mg/mL CS
1 mg/mL CS
340 nm (Abs)
0.6
0.5
0.4
0.3
0.2
0
50
100
150
200
250
300
Time (min)
(B)
Degree of fibril formation (%)
98
97
96
95
94
a
b
c
0
0.5
1
CS concentration (mg/mL)

Fig.7

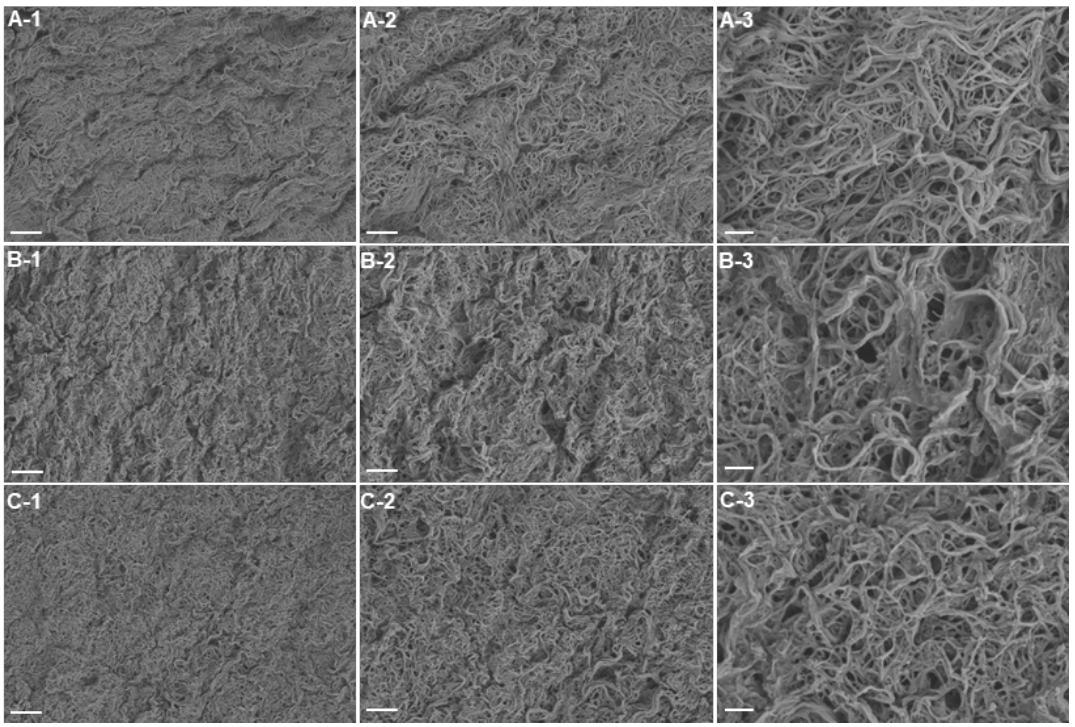
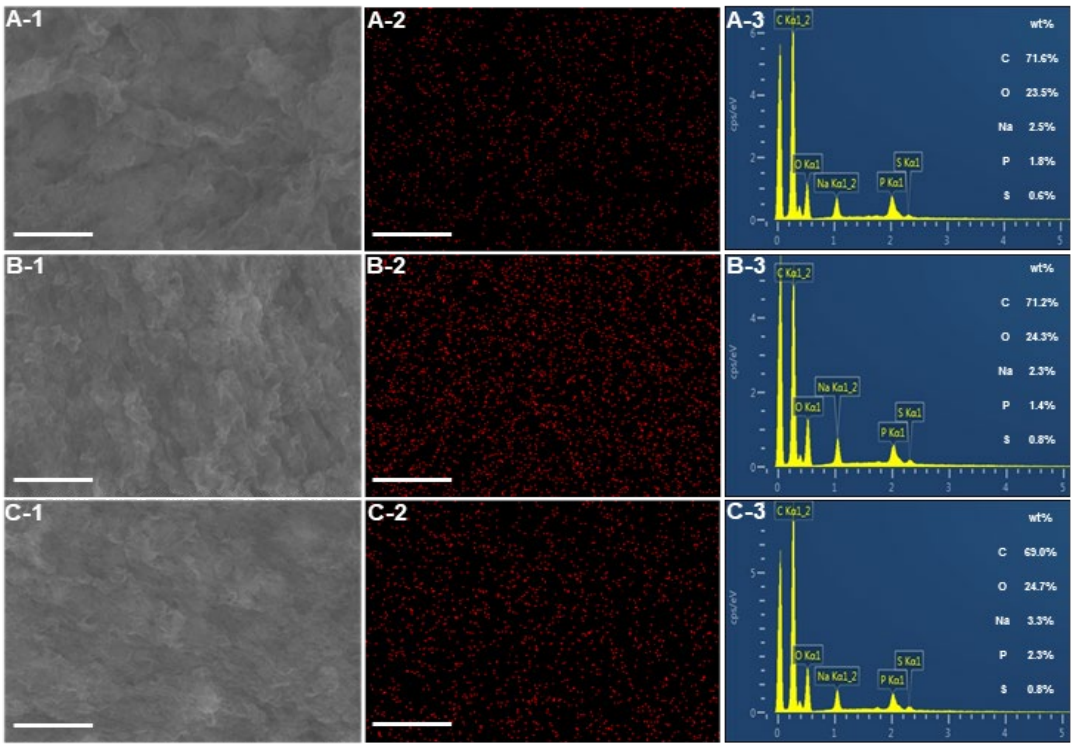
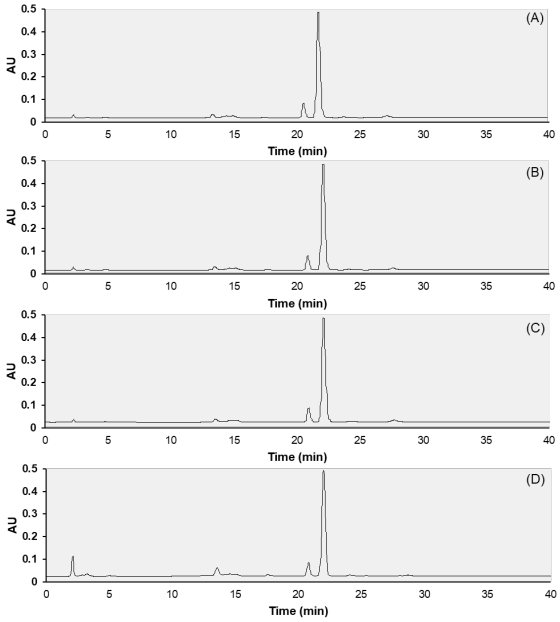


Fig.8


A-1
A-2
A-3
C Ka1_2
O Ka1
Na Ka1_2
P Ka1
S Ka1
cps/eV
wt%
C 71.6%
O 23.5%
Na 2.5%
P 1.8%
S 0.6%
B-1
B-2
B-3
C Ka1_2
Na Ka1_2
O Ka1
S Ka1
P Ka1
cps/eV
wt%
C 71.2%
O 24.3%
Na 2.3%
P 1.4%
S 0.8%
C-1
C-2
C-3
C Ka1_2
O Ka1
Na Ka1_2
S Ka1
P Ka1
cps/eV
wt%
C 69.0%
O 24.7%
Na 3.3%
P 2.3%
S 0.8%



Supplementary Fig.1. Disaccharide analysis of sturgeon notochord CS under different alkaline treatment time and NaOH concentration. (A): 12 h, 0.1 M NaOH; (B): 24 h, 0.1 M NaOH; (C): 48 h, 0.1 M NaOH; (D): 24h, 0.01 M NaOH.