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

111 rate of 1 mL/min. The eluate solution was collected in each 5 min. Three volumes of
112 ethanol were added into collection tube to purify CS at 21 °C for 12 h. The precipitate
113 was collected and re-dissolved in deionized water, and then dialyzed against 50 times
114 volume of deionized water (two solution-changes) using a 200-Da dialysis membrane
115 to remove NaCl at 21°C for 12 h. Finally, the dialysis fluid was lyophilized and stored
116 at -30 °C until used. The percentage of dry weight of CS extracted in comparison with
117 the dry weight of the initial tissues was calculated as the CS yield. Experiments were
118 conducted for 3 times, and the data were expressed as means ± standard errors (SE).
119 2.4 Isolation and purification of Col II
120 The precipitate obtained from section 2.2 was used for undenatured Col II
121 extraction. Extraction progress performed according to the method of Meng et al. [19].
122 The precipitate was washed to neutral with cold deionized water, and then
123 continuously stirred in a solution of HCl (pH 2.0) containing 0.5% (w/v) porcine
124 gastric mucosa pepsin (1:10,000., Solarbio Science & Technology Co, Ltd., BeiJing.,
125 China) at a sample (dry weight based) / solvent solution of 1:100 (w/v) for 24 h at
126 4 °C (two solution changes). The mixture was centrifuged at 10,000 g for 30 min
127 (Sorvall LYNX4000., Thermo fisher scientific., Waltham, USA) to obtain the
128 supernatants. The supernatant was precipitated by adding NaCl to a final
129 concentration of 1 M. The precipitate was redissolved by a small amount of HCl (pH
130 2.0) at 4 °C. After redissolving, the purified collagen was dialyzed against 50 volumes
131 of distilled water at 4 °C for 24 h with two changes of water. The dialysate was
132 lyophilized using a freeze dryer (FreeZone 2.5 L, American Labconco Co., Ltd.,

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

136 2.5 Determination of purity and molecular weight of CS and undenatured Col II

137 2.5.1 Purity and molecular weight distribution of CS

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

148 2.5.2 SDS-PAGE of Col II

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

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

158 2.6 Structure characteristics of CS and Col II

159 2.6.1 UV spectra of CS and Col II

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

164 2.6.2 FTIR of CS and Col II

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

170 2.6.3 NMR of CS

171 ^{1}H- and ^{13}C-NMR spectra of CS were recorded with a Bruker Avance 600 (Karl-
172 scrube, Germany) operating at 600 MHz. The lyophilized sample was dissolved in
173 D_2O at a 30 mg/mL concentration and the spectra were recorded at 298 K. The
174 chemical shifts (d, ppm) were quoted with respect to external sodium 4, 4-dimethyl-4-
175 silapentane-1-sulfonate (0.0 ppm).

176 2.6.4 Disaccharide composition of CS

177 CS was dissolved in Tris-acetate buffer (50 mM Tris and 60 mM of sodium
178 acetate, pH 8.0) to 10 mg/mL. Eighty microliter chondroitinase ABC (6mU/μL, EC
179 4.2.2.4, Sigma-Aldrich., Saint louis., USA) was mixed into 400 μL CS solution. The
180 reaction was performed for 24 h at 37 °C. Then, the mixture was boiled for 10 min to
181 terminate reaction and filtered through 0.22 μm syringe filters. The solution was
182 fractionated by ultrafiltration using molecular weight cutoff of 3 kDa (Merck
183 Millipore Ltd., Darmstadt., Germany). The fraction of molecule weight below 3 kDa
184 was collected for analysis of disaccharide composition. The disaccharide sample was
185 detected by HPLC with Waters 2695 Pump system, Waters 2489 UV/Vis detector
186 (Waters Co., USA) and a Spherisorb SAX column (4.6 mm × 150 mm, 5 μL, Waters
187 Co., USA), with a linear gradient from 5 to 35 min using 0 to 1 M NaCl (pH 3.5)
188 solution as mobile phase. The absorbance was monitored at 232 nm. Bovine and shark
189 cartilage CSs (Sigma-Aldrich., Saint louis., USA) were used as standard and were
190 analyzed by the same method. Each experiment was replicated three times.

191 2.6.5 Circular dichroism (CD) of Col II

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

197 2.7 Fibril formation *in vitro*

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

199 [19]. Lyophilized Col II was dissolved in pH 2.0 HCl solution to 0.3% (w/v). CS was
200 added to Na-phosphate buffer (45mM, pH 7.4) at concentrations of 0, 0.75, and 1.5
201 mg/mL, respectively. The Col II solution was mixed with a Na-phosphate buffer with
202 a ratio of 1:2 (v/v). After mixing, the final concentrations of CS were 0, 0.5, and 1
203 mg/mL, respectively. The mixed solution was pipetted into a quartz cuvette. Fibril
204 formation at 21°C was monitored by measuring turbidity change via optical
205 absorbance at 340 nm, using a spectral monitor (Evolution 60 s., Thermo fisher
206 scientific., Waltham, USA).

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

213 2.8 Morphology and elemental analysis of fibrils

214 The microstructure of notochord Col II fibrils was observed using a scanning
215 electron microscope (SEM; Sigma 500, Carl Zeiss Ltd, Germany). The Col II fibrils
216 were formed and obtained by centrifugation as described in Section 2.7. The
217 precipitated fibrils were fixed by 2.5% (v/v) glutaraldehyde in 30 mM Na-phosphate
218 buffer (pH 7.4) for 3 h at room temperature, and then rinsed with Na-phosphate buffer
219 to remove the fixative. The fixed fibril was dehydrated using a graded series of
220 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,

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

249 The yields of notochord CS and Col II were 5.34 ± 0.74% and 45.25 ± 5.25%
250 (dry weight basis), respectively. Notochord CS yield was lower than the CSs derived
251 from sturgeon cranial and backbone cartilages (19.5% and 13.3%, respectively) [15],
252 [23], but was higher than that of the CSs extracted from smooth hound cartilage
253 (2.52%) and *Holothutis scabra* cartilage (2.89%) [24], [25]. It is difficult to compare
254 the yield of sturgeon notochord CS with other notochordal tissues because there have
255 been few other studies on notochord CS extraction. In this study, CS was purified by
256 ethanol precipitation and ion-exchange chromatography, which might lead to loss of
257 CS during purification process. For large scale production, more efficient and
258 environmentally friendly purification methods, such as ultrafiltration or membrane
259 filtration, should be used instead of ethanol precipitation and ion-exchange
260 chromatography [26].

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

265 [19]. Based on amino acid analysis results, we found non-collagenous proteins were
266 removed by alkaline pretreatment [19]. From this research, we knew that the
267 polysaccharides also could be efficiently removed by alkaline pretreatment.
268 Comparing with previous method, alkaline pretreatment time was extended from 12 h
269 to 24 h in this study. The longer alkaline pretreatment time might be the reason for the
270 higher Col II yield, because the polysaccharide components in the notochord might be
271 more fully removed after the prolonged alkaline pretreatment time. As known, in the
272 cartilage-related tissues, CS and Col II interlace to form the stable fibril sheath that
273 resists high pressure. After removal of CS and non-collagenous proteins, Col II is
274 better to exposure to pepsin solution without steric hindrance, and the pepsin will
275 more easily break their intermolecular cross-links, thereby increasing the solubility of
276 collagen molecules. In this study, we proposed a preliminary process for extraction of
277 CS and undenatured Col II, respectively. Although, according to this method, the
278 utilization of sturgeon notochord was increased from 37.5% to 50.6% comparing to
279 our previous study [19]. The remaining 49.4% of notochord body was still not
280 efficiently utilized. In order to reduce aquatic resource waste and make full use of
281 sturgeon notochord, more suitable extraction conditions were need to be discussed in
282 further study.
283 The amount of farmed sturgeon in China exceeded 80,000 tons in 2017 [27]. The
284 notochord accounts for approx. 1.7% of sturgeon body weight (wet weight basis) and
285 the moisture content of notochord is about 83.2% (data of the present research
286 material). It is predicted that ca. 10.7 t CS and 90.5 t Col II can be obtained annually

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

291 3.2 Purity and molecular weight of CS and Col II

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

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

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

311 3.3 Structure characteristics of CS

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

318 The molecular structural characteristics of CS were identified by FTIR
319 spectroscopy (Fig 2-C). The presence of sulfate group in C-O-S was detected at
320 856.59 cm^{-1}. Garnjanagoonchorn et al. reported that the absorption of the C-O-S axial
321 and equatorial orientations of chondroitin 4-sulfate (CS-A) and chondroitin 6-sulfate
322 (CS-C) exhibited the special peaks at 854.5 cm^{-1} and 823.7 cm^{-1}, respectively [32]. It
323 suggested that the main type of sturgeon notochord CS was CS-A. Furthermore, the
324 peak observed at 1068.29 cm^{-1} was attributed to the C-C ring vibrations [33]. The
325 peaks shown at 1415.31 cm^{-1} and 1233.37 cm^{-1} were characteristic of C-O stretching
326 [18]. The peak detected at 1640.16 cm^{-1} was represented -CONH structure [33]. The
327 peak observed around 2922.86 cm^{-1} was attributed to the stretching vibration of C-H
328 [18]. The strong absorbance peak at 3415 cm^{-1} indicated the stretching of -OH [18],
329 [33].

330 ^{1}H and ^{13}C-NMR spectroscopy were performed to confirm the integrity and

331 characteristics of CS molecular structure (Fig 3). ^{1}H-NMR spectroscopy was shown in
332 Fig 3-A. All proton signals of CS appeared in two spectral regions, one between 2.0
333 and 2.1 ppm, and the other between 3.0 and 5.0 ppm. It indicated that high purity CS
334 was obtained. The characteristic signal at 4.80 ppm was assigned to H4 of GalNAc-
335 4SO_4 [34]. It indicated that CS of sturgeon notochord was composed by CS-A.
336 Furthermore, the peak between 2.0 to 2.1 ppm was the signal of acetyl methyl group
337 proton [35]. The signals at 3.74 and 3.98 ppm were assigned to H6 and H2 of N-
338 acetyl-galactosamine, while the signals at 3.32, 3.54 and 3.69 ppm were assigned to
339 H2, H3 and H4/5 of glucuronic acid, respectively.

340 ^{13}C-NMR spectroscopy was shown in Fig 3-B. All signals were found in the
341 region 50-80 ppm and 100-110 ppm except for the acetamido methyl carbon at around
342 25.5 ppm and the carbonyl at around 174.0 ppm. It indicated CS had high sulfate
343 content at the 4 and/or 6 positions of the GalNAc [30]. The signals at 102.3, 53.0,
344 81.4, 78.1, 62.5 ppm were assigned to the C1, C2, C3, C4 (C5) and C6 of GalNac-
345 4SO_4. The signals at 105.0, 73.8, 75.1, 78.1 and 175.7 ppm were assigned to the C1,
346 C2, C3, C5 and C6 of GlcA [36]. Therefore, the sturgeon notochord CS contained a
347 high content of CS-A, which was consistent with FTIR spectroscopy results. The
348 structure of the sturgeon notochord CS differed from that of sturgeon cartilage CS,
349 which was composed of CS-C [15], [23]. Differences in the functional activities of
350 sturgeon notochord and cartilage CSs required further discussion.

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

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

367 3.4 Structure characteristic of Col II

368 The ultraviolet absorption of Col II was shown in Fig 2-B. The Col II was
369 emerged a strong absorption peak at 200 to 220 nm, and the maximum absorption
370 peak was at 206.9 nm. It was the characteristic absorption peak of type II collagen
371 [37]. The ultraviolet absorption peak of type I collagen was at approx. 232 nm [38],
372 slightly higher than Col II. Low absorbance at 280 nm because there were few
373 aromatic amino acids in collagen [19].

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

375 absorption peaks contained amide A (3416.17 cm^{-1}), amide B (2922.11 cm^{-1}), amide I
376 (1618.61 cm^{-1}), amide II (1554.92 cm^{-1}) and amide III (1239.06 cm^{-1}). The amide A
377 band of Col II was observed at 3416.17 cm^{-1}. Doyle et al. reported that the absorption
378 band of amide A, associated with N-H stretching vibration appeared in the range of
379 3400–3440 cm^{-1} [39]. The amide B band was observed at 2922.11 cm^{-1}, which was
380 related to the asymmetrical stretching of CH_2 [40], [41]. The amide I band frequencies
381 from 1600 to 1700 cm^{-1} were mainly related to carbonyl group stretching vibrations
382 and were characteristic of the secondary coil structure [40]. The amide I bond of Col
383 II was observed at 1637.00 cm^{-1}. This observation confirmed the formation of
384 hydrogen bond between N-H stretch, where the C-O residues were responsible for
385 stabilizing triple helical structure [41]. The amide II band corresponded to N-H
386 bending vibrations, and the amide III band represented C-H stretching [40]. The
387 amide II and III of Col II were observed at 1554.92 and 1239.66 cm^{-1}, respectively.
388 According to Plepis et al, the ratio of absorbance of amide III and to the peak between
389 1400 to 1454 cm^{-1} was close to 1.0, indicating that the triple helical structure of
390 collagen was intact [42]. In this result, the ratio of amide III and 1454.14 cm^{-1} was
391 1.01, which confirmed the intact triple helical structures in Col II.
392 The CD spectra of notochord Col II was shown in Fig 5. The Col II had a
393 rotatory maximum at 221 nm and a crossover point at approximately at 212 nm,
394 which were typical characteristics of collagen triple-helical conformation [43]. FTIR
395 and CD spectra results demonstrated that the Col II maintained an intact triple helical
396 structure after extraction.

397 3.5 Effects of CS on the Col II fibril formation

398 Collagen fibril formation *in vitro* was a self-assembly process from soluble
399 collagen molecule to insoluble fibrils. The changing process of solution turbidity
400 reflected the different stages of fibril formation, and the turbidity value reflected fibril
401 diameter [22]. The effects of CS on Col II fibril formation process were shown in Fig
402 6-A. The turbidity curve of three samples exhibited a logarithmic growth trend, and
403 no lag phase was detected in the three curves. It suggested that fibril formation was
404 too fast to detect the fibril nucleation process in the lag phase. The initial turbidity
405 values for the 0, 0.5, and 1 mg/mL CS mixed samples were 0.258, 0.359, and 0.388,
406 respectively. All three turbidity curves increased significantly during the first 15 min,
407 and then the turbidity growth slowed down over time. The turbidity value of the 0
408 mg/mL CS sample remained slowly increasing over 300 min. The turbidity value of
409 the 0.5 mg/mL CS sample stopped increasing at 180 min, and the turbidity value of
410 the 1 mg/mL CS mixed sample stopped increasing at 135 min. It meant that CS
411 accelerated the completion of Col II self-assembly, but inhibited the lateral
412 aggregation of fibrils. CS was a polyanionic chain structure composed of disaccharide
413 units of glucuronic acid and sulfated N-acetyl galactosamine [44]. In section 3.3, we
414 demonstrated that the notochord CS was mainly composed of CS-A with N-acetyl
415 galactosamine sulfated at carbon 4. The negative carboxyl and sulfonic acid groups of
416 the CS chain were both anions under neutral conditions. These anions interacted with
417 the amino groups of basic amino acids such as lysine, hydroxylysine, histidine and
418 arginine on the side chain of Col II through electrostatic attraction. Mathews (1968)

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

425 The results of the degree of fibril formation at different CS concentrations were
426 shown in Fig 6-B. The degree of fibril formation was 97.8 ± 0.09%, 97.1 ± 0.13% and
427 95.9 ± 0.11% for the 0, 0.5, and 1 mg/m CS samples, respectively. The degree of fibril
428 formation decreased with increasing CS concentration. It suggested that CS had an
429 inhibitory effect on Col II fibril formation. Wood et al. (1960) found that CS-A
430 retarded the rate of growth of type I collagen fibrils [46]. Keech (1961) reported CS
431 produced individual fibrils of smaller diameter than those from control solutions of
432 type I collagen [47]. Since CS inhibited the lateral aggregation of fibrils, more soluble
433 collagen or fine fibrils remained in solution, resulting in a reduced degree of fibril
434 formation.

435 3.6 Morphology observation of fibrils

436 The Col II fibrils formed under different CS concentration conditions were
437 shown in Fig. 7. For no CS sample, the Col II formed a disordered fibril meshwork
438 structure. The fibrils with different thickness were clearly visible. The long fusiform
439 structure was interlaced on the surface and inside of the fine fibrils and the average
440 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

This work was partially supported by key laboratory research fund open topics (LFBC1002) from Key Lab of Freshwater Biodiversity Conservation, Ministry of Agriculture and Rural Affairs of China; The discipline innovation and talent introducing program for high education institutions, the 111 project (D21012).

Acknowledgments

We gratefully acknowledge the Sturgeon Biological Technology Co. Ltd., Xinchang county, Zhejiang province, China for providing the farmed sturgeon materials for this research. Thanks for Prof. Yasuaki Takagi of Faculty of Fisheries Sciences, Hokkaido University supply to technical support and article modification. Thanks to Prof. Qiwei Wei of Key Lab of Freshwater Biodiversity Conservation, Ministry of Agriculture and Rural Affairs of China provide financial support.

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.

References

[1] X. P. Zhou, J. K. Wang, W. J. Fang, Y. Q. Tao, T. F. Zhao, K. S. Xia, C. Z. Liang, J. M. Hua, F. C. Li, Q. X. Chen, Genipin cross-linked type II collagen/chondroitin sulfate composite hydrogen-like cell delivery system induces differentiation of adipose-derived stem cells and regenerates degenerated nucleus pulposus, Acta. Biomater. 71 (2018) 496-509.

[2] O. F. Restaino, C. Schiraldi, Chondroitin sulfate: are the purity and the structural features well assessed? A review on the analytical challenges, Carbohyd. Polym. 292 (2022) 119690.

[3] D. R. Eyre, H. Muir, Type I and II collagens in intervertebral disc. Interchanging radial distributions in annulus fibrosus, J. Biochem. 157 (1976) 267-270.

[4] J. J. Mou, Q. Li, X. H. Qi, J. Yang, Structural comparison, antioxidant and anti-inflammatory properties of fucosylated chondroitin sulfate of three edible sea cucumbers, Carbohyd. Polym. 185 (2018) 41-47.

[5] R. Lan, Y. Li, R. Shen, R. Yu, L. H. Jing, S. S. Guo, 2020. Preparation of low-molecular-weight chondroitin sulfates by complex enzyme hydrolysis and their antioxidant activities. Carbohyd. Polym. 241, 116302.

[6] D. E. Trentham, R. A. Dynesius-Trentham, E. J. Orav, D. Combitchi, C. Lorenzo, K. L. Sewell, D. A. Hafler, H. L. Weiner, Effects of oral administration of Type II

collagen on rheumatoid arthritis, *Science.* 261 (1993) 1727-1730.

[7] K. S. Park, M. J. Park, M. L. Cho, S. K. Kwok, J. H. Ju, H. J. Ko, S. H. Park, H. Y. Kim, Type II collagen oral tolerance; mechanism and role in collagen-induced arthritis and rheumatoid arthritis, Modern Rheumatol. 19 (2009) 581–589.

[8] F. X. Li, M. Ducker, B. Sun, F. G. Szele, J. T. Czernuszka, Czernuszk. Interpenetrating polymer networks of collagen, hyaluronic acid, and chondroitin sulfate as scaffolds for brain tissue engineering, Acta. Biomater. 112 (2020) 122-135.

[9] R. N. Carballal, R. P. Martín, M. Blanco, C. G. Sotelo, D. Fassini, C. Nunes, M. A. Coimbra, T. H. Silva, R. L. Reis, J. A. Vázquez, By-products of Scyliorhinus canicula, Prionace glauca and Raja clavata: A valuable source of predominantly 6S sulfated chondroitin sulfate, Carbohyd. Polym. 157 (2017) 31-37.

[10] F. Maccari, F. Galeotti, N. Volpi, Isolation and structural characterization of chondroitin sulfate from bony fishes, Carbohyd Polym. 129 (2015) 143–147.

[11] A. Jongjareonrak, S. Benjakul, W. Visessanguan, T. Nagai, M. Tanaka, Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (Lutjanus vitta), Food. Chem. 93 (2005) 475–484.

[12] M. Braccini, A. Hesp, B. Molony, 2021. Risk-based weight of evidence assessment of commercial sharks in western Australia. Ocean. Coast. Manage. 205, 105501.

[13] W. Li, T. Kobayashi, D.W. Meng, N. Miyamoto, N. Tsutsumi, K. Ura, Y. Takagi, 2021. Free radical scavenging activity of type II collagen peptides and

chondroitin sulfate oligosaccharides from by-products of mottled skate processing. Food. Biosci. 41, 100991.

[14] J. A. Vázquez, J. Fraguas, R. N. Carballal, R. L. Reis, R. I. Pérez-Martín, J.Valcarcel, Optimal isolation and characterisation of chondroitin sulfate from rabbit fish *(Chimaera monstrosa)*, Carbohyd. Polym. 210 (2019) 302-313.

[15] M. Gui, J. Y. Song, L. Zhang, S. Wang, R. Y. Wu, C. W. Ma, P. L. Li, Chemical characteristics and antithrombotic effect of chondroitin sulfates from sturgeon skull and sturgeon backbone, Carbohyd. Polym. 123 (2015) 454-460.

[16] H. C. Robinson, J. J. Hopwood, The alkaline cleavage and borohydride reduction of cartilage proteoglycan, J. Biochem. 133 (1973) 457-470.

[17] E. Żelechowska, M. Sadowsk, M. Turk, Isolation and some properties of collagen from the backbone of Baltic cod *(Gadus morhua)*, Food. Hydrocoll. 24 (2010) 325-329.

[18] Q. S. Shen, C. H. Zhang, W. Jia, X. J. Qin, Z. K. Cui, H. Z. Mo, A. Richel, 2019. Co-production of chondroitin sulfate and peptide from liquefied chicken sternal cartilage by hot-pressure. Carbohyd. Polym. 222, 115015.

[19] D. W. Meng, H. Tanaka, T. Kobayashi, H. Hatayama, X. Zhang, K. Ura, S. Yunoki, Y. Takagi, The effect of alkaline pretreatment on the biochemical characteristics and fibril-forming abilities of types I and II collagen extracted from bester sturgeon by-products, Int. J. Biol. Macromol. 131 (2019) 572–580.

[20] U. K. Laemmli, Cleavage of structural proteins during assembly of head of bacteriophage T4, Nature. 277 (1970) 680–685.

[21] X. Zhang, M. Ookawa, Y. K. Tan, K. Ura, S. Adachi, Y. Takagi, Biochemical characterization and assessment of fibril-forming ability of collagens extracted from bester sturgeon *Huso huso* × *Acipenser ruthenus*, Food. Chem. 160 (2014) 305–312.

[22] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[23] R. IM, Y. Park, Y. Y. Shik Kim, Isolation and Characterization of Chondroitin Sulfates from Sturgeon *(Acipenser sinensis)* and Their Effects on Growth of Fibroblasts, Biolo. Pharm. Bul. 33 (2010) 1268–1273.

[24] F. Krichen, H. Bougatef, N. Sayari, F. Capitani, I. B. Amor, I. Koubaa, F. Maccari, V. Mantovani, F. Galeotti, N. Volpi, A. Bougate, Isolation, Purification and Structural Characterestics of Chondroitin Sulfate from Smooth hound Cartilage: *In vitro* Anticoagulant and Antiproliferative Properties, Carbohyd. Polym. 197 (2018b) 451-459.

[25] L. Yang, Y. H. Wang, S. Yang, Z. H. Lv, Separation, purification, structures and anticoagulant activities of fucosylated chondroitin sulfates from Holothuria scabra, Int. J. Biol. Macromol. 108 (2018) 710-718.

[26] S. D'ambrosio, A. Alfano, E. Cassese, O. F. Restaino, S. B. Ferraiuolo, R. Finamore, M. Cammarota, C. Schiraldi1, D. Cimini, Production and purifcation of higher molecular weight chondroitin by metabolically engineered Escherichia coli K4 strains, Sci. Rep. 10 (2020) 13200.

[27] P. Bronzi, M. Chebanov, J. T. Michaels, Q. W. Wei, H. Rosenthal, J. Gessner,

Sturgeon meat and caviar production: Global update 2017, J. App. Ichthyol. 35 (2019) 257-266.

[28] O. F. Restaino, R. Finamore, P. Diana, M. Marseglia, M. Vitiello, A. Casillo, E. Bedini, M. Parrilli, M. M. Corsaro, M. Trifuoggi, M. D. Rosa, C. Schiraldi, A multi-analytical approach to better assess the keratan sulfate contamination in animal origin chondroitin sulfate, Anal. Chim. Acta, 958 (2017) 59-70.

[29] W. M. Zhu, J. Yang, Y. Wang, D. He, Y. S. Yan, N. Su, C. Zhang, X. H. Xing, Structural characterization and *in vitro* antioxidant activities of chondroitin sulfate purified from Andrias davidianus cartilage, Carbohyd. Polym. 196 (2018) 398-404.

[30] F. Maccari, F. Ferrarini, N. Volpi, Structure characterization of chondroitin sulfate from sturgeon bone, Carbohyd. Res. 345 (2010) 1575-1580.

[31] F. Krichen, H. Bougatef, F. Capitani, I. B. Amor, I. Koubaa, J. Gargouri, F. Maccari, V. Mantovani, F. Galeotti, N. Volpi, A. Bougatef, Purification and structural elucidation of chondroitin sulfate/dermatan sulfate from Atlantic bluefin tuna *(Thunnus thynnus)* skins and their anticoagulant and ACE inhibitory activities, RSC. Advances. 8 (2018a) 37965-37975.

[32] W. Garnjanagoonchorn, L. Wongekalak, A. Engkagul, Determination of chondroitin sulfate from different sources of cartilage, Chem. Eng. Process. 46 (2007) 465-471.

[33] M. Foot, M. Mulholland, Classification of chondroitin sulfate A, chondroitin sulfate C, glucosamine hydrochloride and glucosamine 6 sulfate using

chemometric techniques, J. Pharmaceut. Biomed. 8 (2005) 397-407.

[34] M. Lopez-Alvarez, P. Gonzalez, J. Serra, J. Fraguas, J. Valcarcel, J. A. Vazquez, Chondroitin sulfate and hydroxyapatite from Prionace glauca shark jaw: physicochemical and structural characterization, Int. J. Biol. Macromol. 156 (2020) 329-339.

[35] T. Toida, H. Toyoda, T. Imanari, High-resolution proton nuclear magnetic resonance studies on chondroitin sulfates, Anal. Sci. 9 (1993) 53-58.

[36] A. Mucci, L. Schenetti, N.Volpi, 1H and 13C nuclear magnetic resonance identification and characterizationof components of chondroitin sulfates of various origin, Carbohyd. Polym. 41 (2000) 37-45.

[37] A. N. Akram, C. H. Zhang, 2020. Extraction of collagen-II with pepsin and ultrasound treatment from chicken sternal cartilage; physicochemical and functional properties. Ultrason. Sonochem. 64, 105053.

[38] P. G. Kumar, T. Nidheesh, P. V. Suresh, Comparative study on characteristics and *in vitro* fibril formation ability of acid and pepsin soluble collagen from the skin of catla *(Catla catla)* and rohu *(Labeo rohita)*, Food. Res. Int. 76 (2015) 804-812.

[39] B. B. Doyle, E. G. Bendit, E. R. Blout, Infrared spectroscopy of collagen and collagen-like polypeptides, Biopolym. 14 (1975) 937-957.

[40] J. H. Muyonga, C. G. B. Cole, K. G. Duodu, Characterisation of acid soluble collagen from skins of young and adult Nile perch *(Lates niloticus)*, Food. Chem. 85 (2004) 81-89.

[41] A. Veeruraj, M. Arumugam, T. Balasubramanian, Isolation and characterization

of thermostable collagen from the marine eel-fish *(Evenchelys macrura)*, Process. Biochem. 48 (2013) 1592-1602.

[42] A. M. D. G. Plepis, G. Goissis, D. D. Gupta, Dielectric and pyroelectric characterization of anionic and native collagen, Polym. Eng. Sci. 36 (1996) 2932-2938.

[43] X. Zhang, S. Adachi, K. Ura, Y. Takagi, Properties of collagen extracted from Amur sturgeon *Acipenser schrenckii* and assessment of collagen fibrils *in vitro*, Int. J. Bio. Macromol. 137 (2019) 809-820.

[44] S. Yamada, K. Sugahara, Potential therapeutic application of chondroitin sulfate/dermatan sulfate, Curr. Drug. Discov. Technol. 5 (2009) 289-301.

[45] M. B. Mathews, L. Decker, The effect of acid mucopolysaccharides and acid mucopolysaccharide-proteins on fibril formation from collagen solutions, Biochem. J. 109 (1968) 517-526.

[46] G. C. Wood, The formation of fibrils from collagen solutions. 3. Effect of chondroitin sulphate and some other naturally occurring polyanions on the rate of formation, Biochem. J. 75 (1960) 605-612.

[47] M. K. Keech, The formation of fibrils from collagen solutions: IV. effect of mucopolysaccharides and nucleic acids: an electron microscope study, J. Biophys. Biochem. Cytol. 9 (1961) 193-209.

[48] M. Y. Yan, X. J. Jiang, G. C. Wang, A. L. Wang, X. X. Wang, X. Y. Wang, X. C. Zhao, H. Xu, X. S. An, Y. P. Li, Preparation of self-assembled collagen fibrillar gel from tilapia skin and its formation in presence of acidic polysaccharides.

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

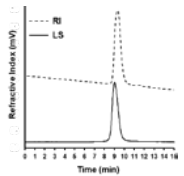
Notochord sheath

Co - extraction

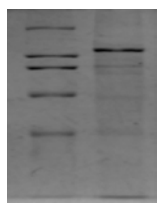
Chondroitin-4-sulfate

Undenatured type II collagen

Purity detection



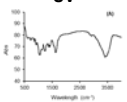
SEC-RI-MALLS



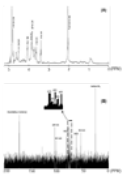
SDS-PAGE

Structural analysis

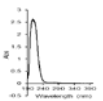
UV



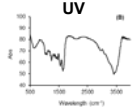
FTIR



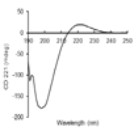
NMR



UV

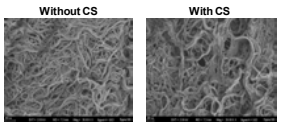


FTIR

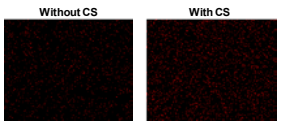


CD

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

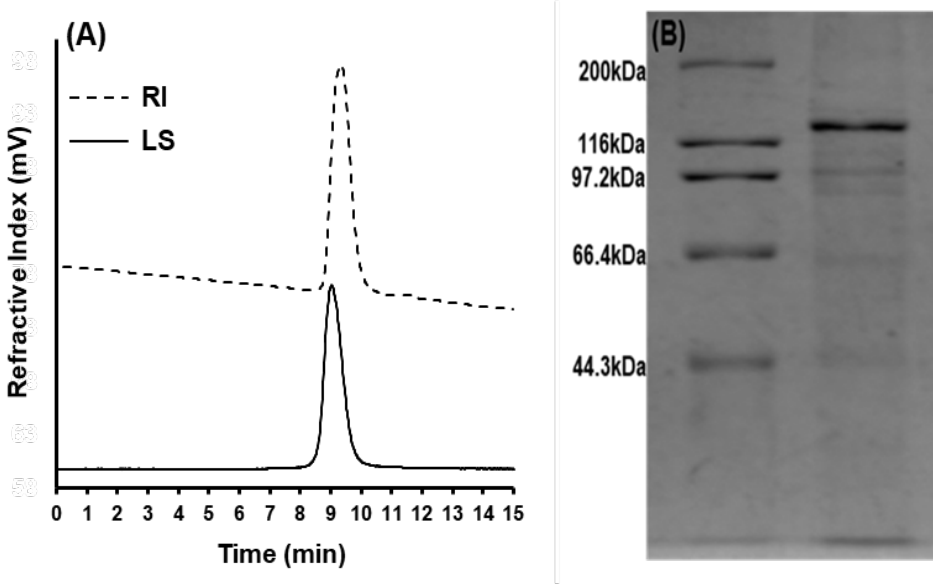

(A)
RI
LS
Refractive Index (mV)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (min)
(B)
200kDa
116kDa
97.2kDa
66.4kDa
44.3kDa

Fig.2

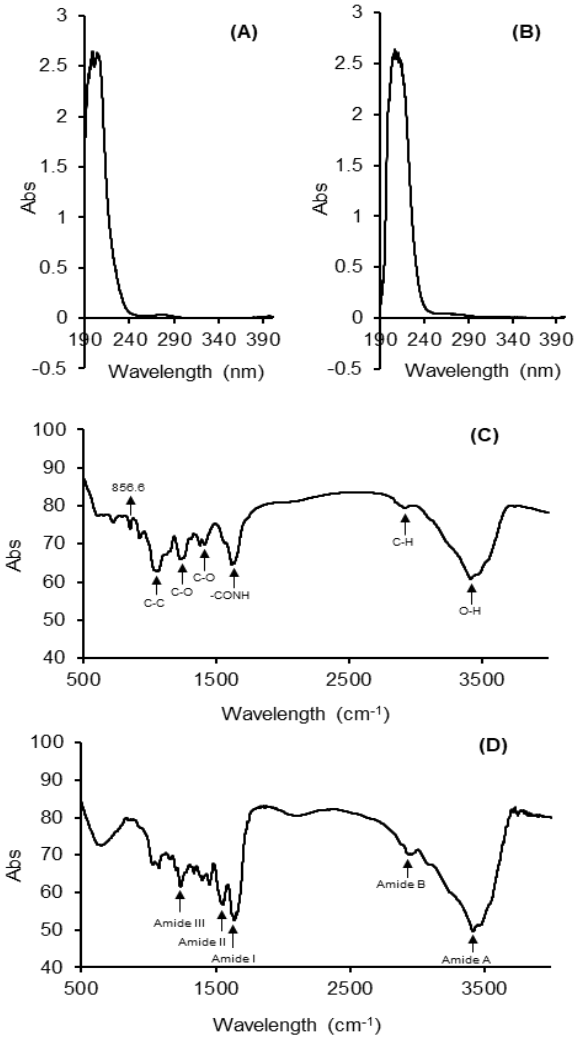

(A)
(B)
Abs
Wavelength (nm)
(C)
856.6
C-C
C-O
C-O
-CONH
C-H
O-H
Wavelength (cm-1)
(D)
Amide III
Amide II
Amide I
Amide B
Amide A
Wavelength (cm-1)

Fig.3

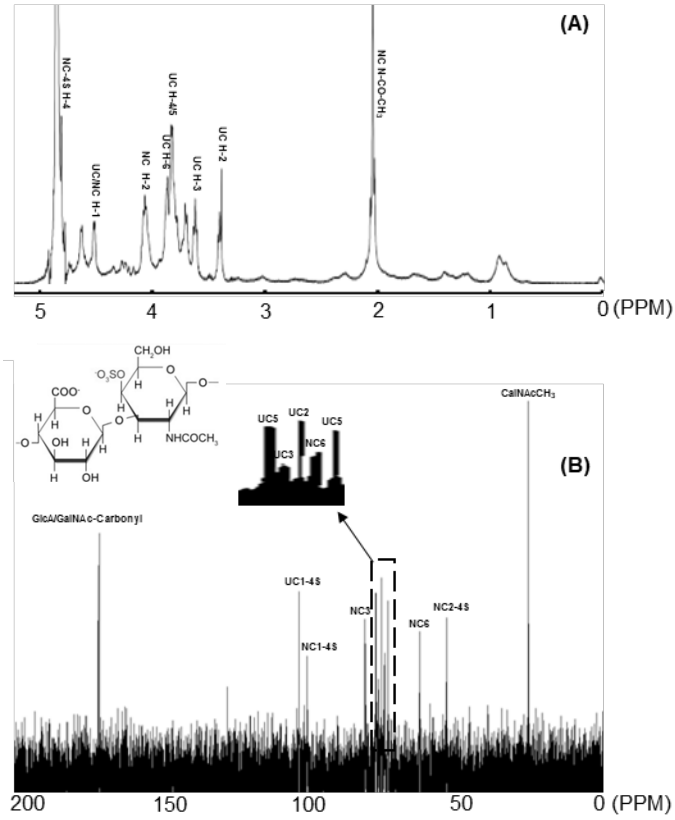

(A)
NC-4S H-4
UC/NC H-1
NC H-2
UC H-4/5
UC H-5
UC H-3
UC H-2
NC N-CO-CH3
5
4
3
2
1
0 (PPM)
(B)
CH2OH
COO-
NHCOCH3
OH
CalNAcCH3
UC5
UC2
UC5
UC3
NC6
GlcA/GalNAc-Carbonyl
UC1-4S
NC3
NC6
NC2-4S
NC1-4S
200
150
100
50
0 (PPM)

Fig.4

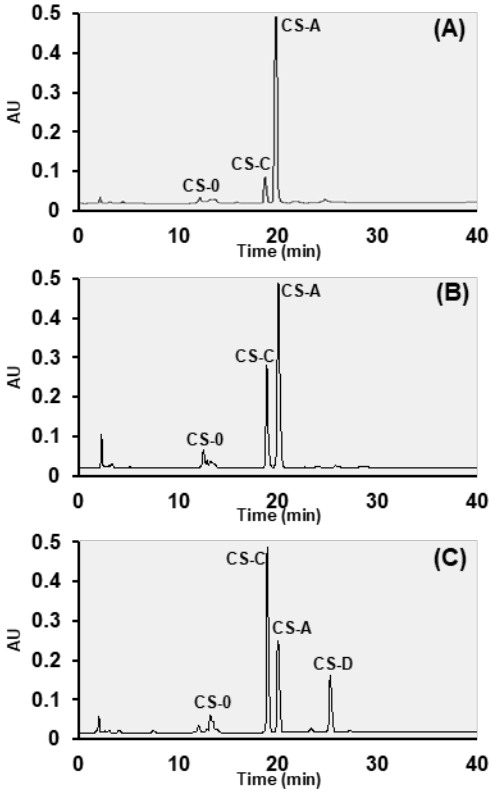

(A)
CS-A
CS-C
CS-0
AU
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Time (min)
(B)
CS-A
CS-C
CS-0
AU
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Time (min)
(C)
CS-C
CS-A
CS-D
CS-0
AU
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Time (min)

Fig.5

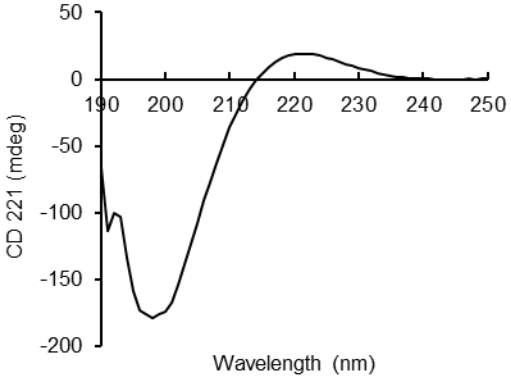

50
0
-50
-100
-150
-200
190
200
210
220
230
240
250
CD 221 (mdeg)
Wavelength (nm)

Fig.6

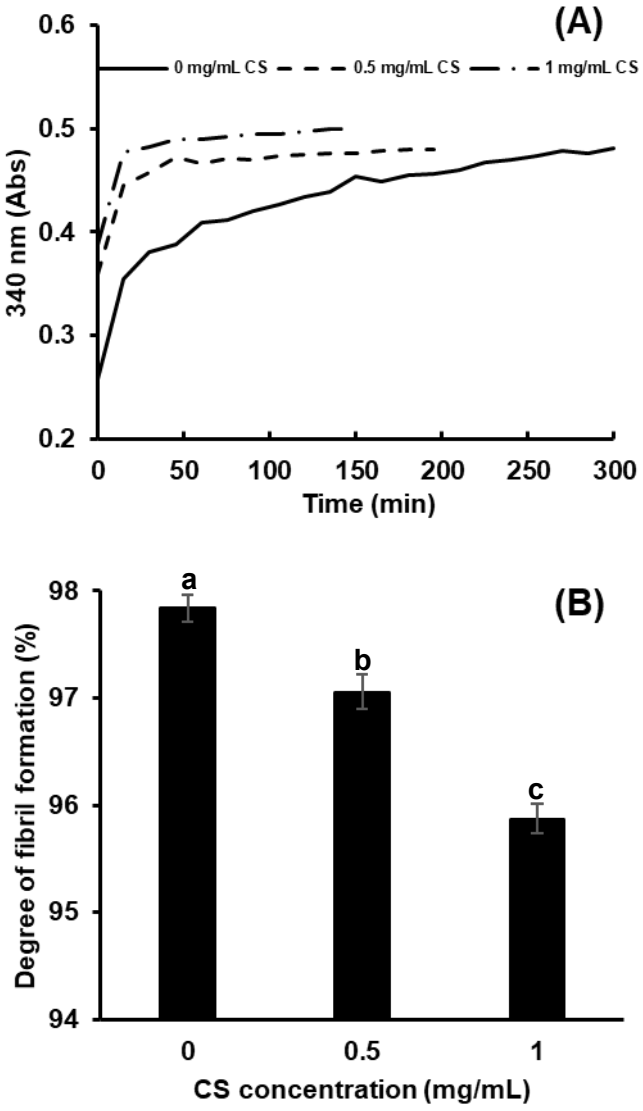

(A)
0 mg/mL CS
0.5 mg/mL CS
1 mg/mL CS
340 nm (Abs)
0.6
0.5
0.4
0.3
0.2
0
50
100
150
200
250
300
Time (min)
(B)
a
b
c
Degree of fibril formation (%)
98
97
96
95
94
0
0.5
1
CS concentration (mg/mL)

Fig.7

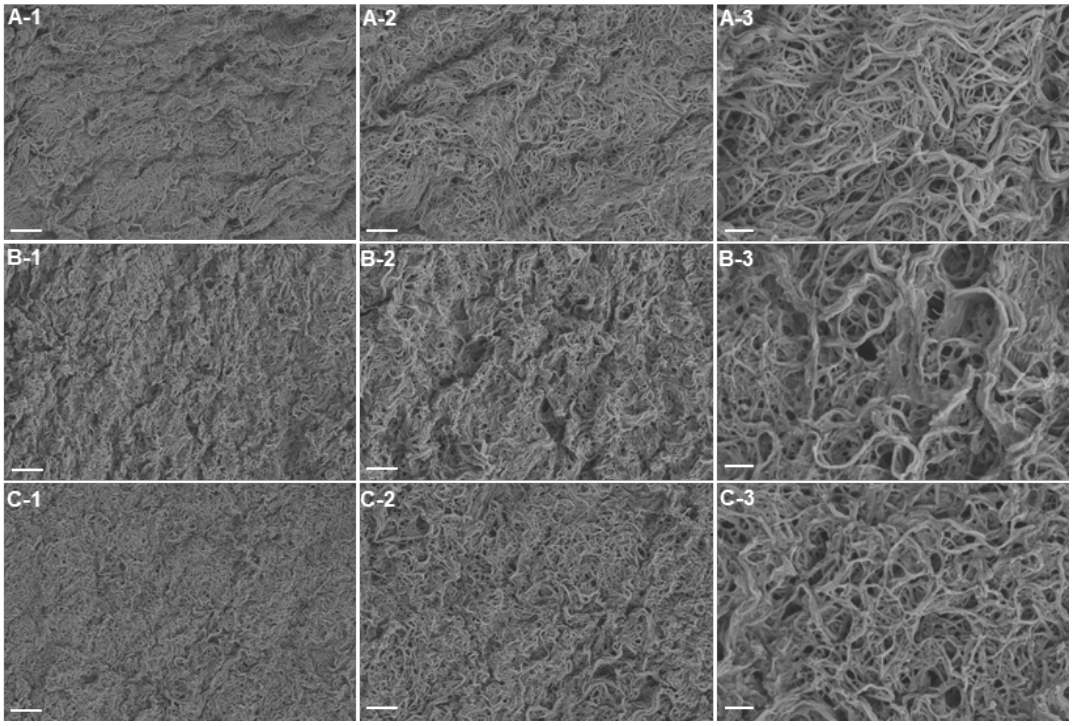
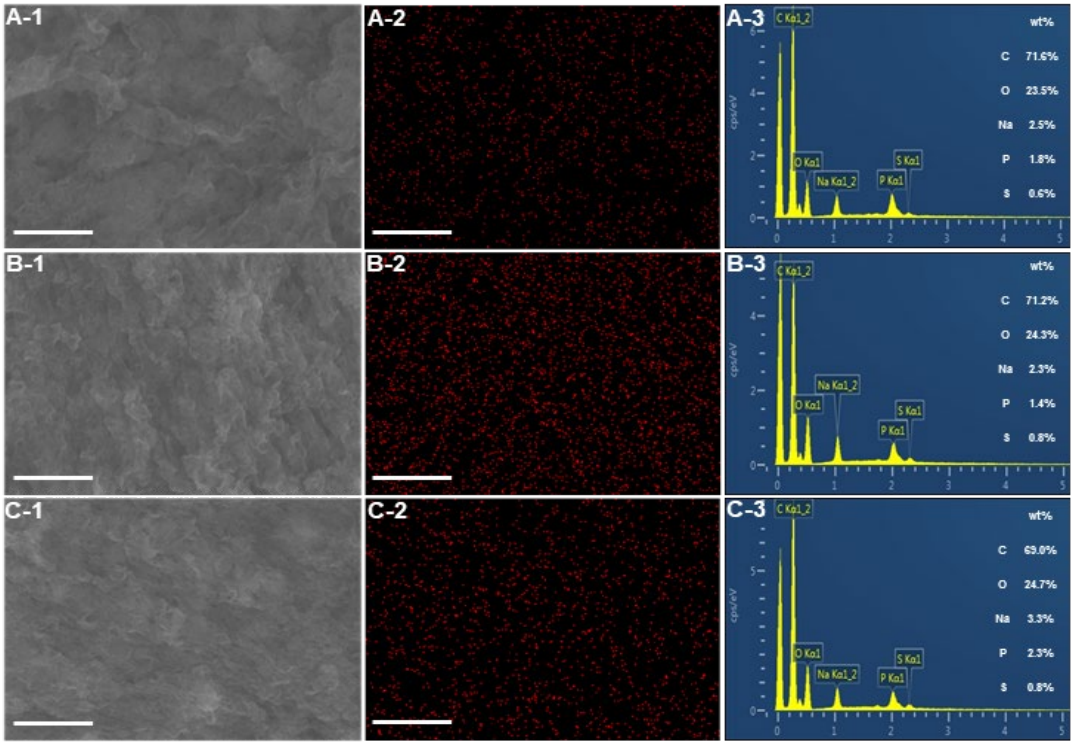
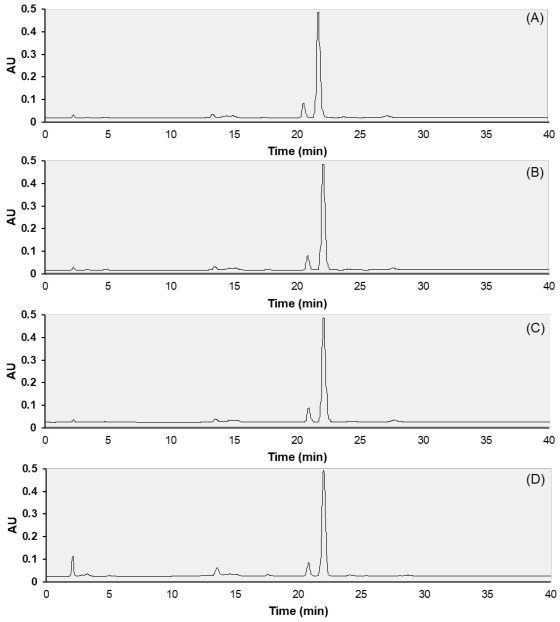


Fig.8


A-1
A-2
A-3
C Kα1_2
O Kα1
Na Kα1_2
P Kα1
S Kα1
wt%
C 71.6%
O 23.5%
Na 2.5%
P 1.8%
S 0.6%
B-1
B-2
B-3
C Kα1_2
Na Kα1_2
O Kα1
S Kα1
P Kα1
wt%
C 71.2%
O 24.3%
Na 2.3%
P 1.4%
S 0.8%
C-1
C-2
C-3
C Kα1_2
O Kα1
Na Kα1_2
S Kα1
P Kα1
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.