



Title	Extraction of chondroitin sulfate and type II collagen from sturgeon (<i>Acipenser gueldenstaedti</i>) notochord and characterization of their hybrid fibrils
Author(s)	Meng, Dawei; Li, Wen; Leng, Xiaoqian; Takagi, Yasuaki; Dai, Zhiyuan; Du, Hao; Wei, Qiwei
Citation	Process Biochemistry, 124, 180-188 https://doi.org/10.1016/j.procbio.2022.11.026
Issue Date	2023-01
Doc URL	http://hdl.handle.net/2115/91040
Rights	© 2023. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	Process Biochemistry_Vol.124.pdf



[Instructions for use](#)

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

4 Dawei Meng^{a*}, Wen Li^b, Xiaoqian Leng^c, Yasuaki Takagi^d, Zhiyuan Dai^a, Hao Du^c,
5 Qiwei Wei^c

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

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

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

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

16

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

20

21

22

23 1. Introduction

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

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

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

54 Protease digestion extraction is the most common procedure for extracting CS
55 [14], [15]. However, it is difficult to extract with undenatured collagen because
56 protease digestion destroys the secondary structure of collagen and hydrolyzes
57 collagen into peptides. In terms of the traditional CS extraction procedures, alkaline
58 treatment is a common method to breakdown the covalent connection between CS and
59 core protein [19]. Meanwhile, low-temperature alkaline pretreatment is widely used to
60 remove non-collagenous substances during collagen extraction [17]. Our recent study
61 reported that, under certain condition, alkaline treatment increased the extraction
62 efficiency of sturgeon notochord Col II and showed no effect on triple helical
63 structure of Col II, but the extraction and structure properties of notochord CS were
64 not addressed [19]. Based on previous studies, we hypothesis that it is possible to
65 extract both CS and undenatured Col II using alkaline treatment. In addition, further
66 exploration of the interaction between CS and Col II will facilitate the application of

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

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

77 **2. Materials and methods**

78 2.1 Materials

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

85 2.2 Alkaline treatment of notochord

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

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

96 2.3 Isolation and purification of CS

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

105 The crude CS was dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 50
106 mM NaCl. The solution was applied to a column (1.5 cm × 7 cm) packed with
107 UNOsphere Q strong anion-exchange media (Bio-Rad Laboratories, Inc., Hercules,
108 CA, USA) and equilibrated in 20 mM Tris-HCl and 50 mM NaCl. The crude CS was
109 eluted with a linear gradient of NaCl (50 mM to 2 M in 90 min) in 20 mM Tris-HCl
110 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 \pm 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 (Agilent 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 μ m, 7.5 mm \times 300 mm) and was eluted with 0.1
144 M NaNO₃ 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 μ L. The average
146 molecular weight calculations were performed by Agilent GPC/SEC software
147 (Agilent 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 ^1H - 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/ μ L, EC
179 4.2.2.4, Sigma-Aldrich., Saint louis., USA) was mixed into 400 μ L 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 μ m 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 \times 150 mm, 5 μ L, 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.

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

225 2.9 Statistical analyses

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

230 **3. Results and discussion**

231 3.1 Yields of CS and Col II

232 In actual production, shortening the extraction time will save processing cost and
233 minimizing the NaOH concentration will reduce environmental pollution. In this
234 study, we discussed the effects of alkaline treatment time and NaOH concentration on
235 CS and Col II yields. The results were shown in Table. 1 and 2. As shown in Table. 1,
236 long alkaline treatment time increased the yield of CS. When alkaline treatment time
237 was 24 h, the yield of Col II was the highest. Our previous study showed that alkaline
238 pretreatment could improve the efficiency of Col II extraction because it could
239 remove non-collagenous proteins and polysaccharides bound to Col II in the tissue.
240 Meanwhile, long alkaline treatment could also lead to collagen loss [19]. Thus, 24 h
241 alkaline treatment was suitable to obtain high yields of CS and Col II. As shown in
242 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 $5.34 \pm 0.74\%$ and $45.25 \pm 5.25\%$
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 ($45.25 \pm 5.25\%$) 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 ^1H and ^{13}C -NMR spectroscopy were performed to confirm the integrity and

331 characteristics of CS molecular structure (Fig 3). ¹H-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 4SO₄ [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 ¹³C-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 4SO₄. 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 $3400\text{--}3440\text{ cm}^{-1}$ [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 band 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 $97.8 \pm 0.09\%$, $97.1 \pm 0.13\%$ and
427 $95.9 \pm 0.11\%$ 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

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

447 3.7 Binding of CS to Col II fibril

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

461 4. Conclusion

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

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

471 **Funding**

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

476 **Acknowledgments**

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

483 **Credit Authorship contribution statement**

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

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

488 **References**

- 489 [1] X. P. Zhou, J. K. Wang, W. J. Fang, Y. Q. Tao, T. F. Zhao, K. S. Xia, C. Z. Liang, J.
490 M. Hua, F. C. Li, Q. X. Chen, Genipin cross-linked type II collagen/chondroitin
491 sulfate composite hydrogen-like cell delivery system induces differentiation of
492 adipose-derived stem cells and regenerates degenerated nucleus pulposus, *Acta.*
493 *Biomater.* 71 (2018) 496-509.
- 494 [2] O. F. Restaino, C. Schiraldi, Chondroitin sulfate: are the purity and the structural
495 features well assessed? A review on the analytical challenges, *Carbohydr. Polym.*
496 292 (2022) 119690.
- 497 [3] D. R. Eyre, H. Muir, Type I and II collagens in intervertebral disc. Interchanging
498 radial distributions in annulus fibrosus, *J. Biochem.* 157 (1976) 267-270.
- 499 [4] J. J. Mou, Q. Li, X. H. Qi, J. Yang, Structural comparison, antioxidant and anti-
500 inflammatory properties of fucosylated chondroitin sulfate of three edible sea
501 cucumbers, *Carbohydr. Polym.* 185 (2018) 41-47.
- 502 [5] R. Lan, Y. Li, R. Shen, R. Yu, L. H. Jing, S. S. Guo, 2020. Preparation of low-
503 molecular-weight chondroitin sulfates by complex enzyme hydrolysis and their
504 antioxidant activities. *Carbohydr. Polym.* 241, 116302.
- 505 [6] D. E. Trentham, R. A. Dynesius-Trentham, E. J. Orav, D. Combitchi, C. Lorenzo,
506 K. L. Sewell, D. A. Hafler, H. L. Weiner, Effects of oral administration of Type II

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

508 [7] K. S. Park, M. J. Park, M. L. Cho, S. K. Kwok, J. H. Ju, H. J. Ko, S. H. Park, H. Y.
509 Kim, Type II collagen oral tolerance; mechanism and role in collagen-induced
510 arthritis and rheumatoid arthritis, *Modern Rheumatol.* 19 (2009) 581–589.

511 [8] F. X. Li, M. Ducker, B. Sun, F. G. Szele, J. T. Czernuszka, Czernuszk.
512 Interpenetrating polymer networks of collagen, hyaluronic acid, and chondroitin
513 sulfate as scaffolds for brain tissue engineering, *Acta. Biomater.* 112 (2020) 122-
514 135.

515 [9] R. N. Carballal, R. P. Martín, M. Blanco, C. G. Sotelo, D. Fassini, C. Nunes, M. A.
516 Coimbra, T. H. Silva, R. L. Reis, J. A. Vázquez, By-products of *Scyliorhinus*
517 *canicula*, *Prionace glauca* and *Raja clavata*: A valuable source of predominantly
518 6S sulfated chondroitin sulfate, *Carbohydr. Polym.* 157 (2017) 31-37.

519 [10] F. Maccari, F. Galeotti, N. Volpi, Isolation and structural characterization of
520 chondroitin sulfate from bony fishes, *Carbohydr Polym.* 129 (2015) 143–147.

521 [11] A. Jongjareonrak, S. Benjakul, W. Visessanguan, T. Nagai, M. Tanaka, Isolation
522 and characterisation of acid and pepsin-solubilised collagens from the skin of
523 brownstripe red snapper (*Lutjanus vitta*), *Food. Chem.* 93 (2005) 475–484.

524 [12] M. Braccini, A. Hesp, B. Molony, 2021. Risk-based weight of evidence
525 assessment of commercial sharks in western Australia. *Ocean. Coast. Manage.*
526 205, 105501.

527 [13] W. Li, T. Kobayashi, D.W. Meng, N. Miyamoto, N. Tsutsumi, K. Ura, Y. Takagi,
528 2021. Free radical scavenging activity of type II collagen peptides and

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

531 [14] J. A. Vázquez, J. Fraguas, R. N. Carballal, R. L. Reis, R. I. Pérez-Martín,
532 J. Valcarcel, Optimal isolation and characterisation of chondroitin sulfate from
533 rabbit fish (*Chimaera monstrosa*), Carbohydr. Polym. 210 (2019) 302-313.

534 [15] M. Gui, J. Y. Song, L. Zhang, S. Wang, R. Y. Wu, C. W. Ma, P. L. Li, Chemical
535 characteristics and antithrombotic effect of chondroitin sulfates from sturgeon
536 skull and sturgeon backbone, Carbohydr. Polym. 123 (2015) 454-460.

537 [16] H. C. Robinson, J. J. Hopwood, The alkaline cleavage and borohydride reduction
538 of cartilage proteoglycan, J. Biochem. 133 (1973) 457-470.

539 [17] E. Żelechowska, M. Sadowski, M. Turk, Isolation and some properties of collagen
540 from the backbone of Baltic cod (*Gadus morhua*), Food. Hydrocoll. 24 (2010)
541 325-329.

542 [18] Q. S. Shen, C. H. Zhang, W. Jia, X. J. Qin, Z. K. Cui, H. Z. Mo, A. Richel, 2019.
543 Co-production of chondroitin sulfate and peptide from liquefied chicken sternal
544 cartilage by hot-pressure. Carbohydr. Polym. 222, 115015.

545 [19] D. W. Meng, H. Tanaka, T. Kobayashi, H. Hatayama, X. Zhang, K. Ura, S.
546 Yunoki, Y. Takagi, The effect of alkaline pretreatment on the biochemical
547 characteristics and fibril-forming abilities of types I and II collagen extracted
548 from bester sturgeon by-products, Int. J. Biol. Macromol. 131 (2019) 572–580.

549 [20] U. K. Laemmli, Cleavage of structural proteins during assembly of head of
550 bacteriophage T4, Nature. 227 (1970) 680–685.

- 551 [21] X. Zhang, M. Ookawa, Y. K. Tan, K. Ura, S. Adachi, Y. Takagi, Biochemical
552 characterization and assessment of fibril-forming ability of collagens extracted
553 from bester sturgeon *Huso huso* × *Acipenser ruthenus*, Food. Chem. 160 (2014)
554 305–312.
- 555 [22] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, Protein measurement
556 with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
- 557 [23] R. IM, Y. Park, Y. Y. Shik Kim, Isolation and Characterization of Chondroitin
558 Sulfates from Sturgeon (*Acipenser sinensis*) and Their Effects on Growth of
559 Fibroblasts, Biolo. Pharm. Bul. 33 (2010) 1268–1273.
- 560 [24] F. Krichen, H. Bougatef, N. Sayari, F. Capitani, I. B. Amor, I. Koubaa, F. Maccari,
561 V. Mantovani, F. Galeotti, N. Volpi, A. Bougate, Isolation, Purification and
562 Structural Characterestics of Chondroitin Sulfate from Smooth hound Cartilage:
563 *In vitro* Anticoagulant and Antiproliferative Properties, Carbohyd. Polym. 197
564 (2018b) 451-459.
- 565 [25] L. Yang, Y. H. Wang, S. Yang, Z. H. Lv, Separation, purification, structures and
566 anticoagulant activities of fucosylated chondroitin sulfates from *Holothuria*
567 *scabra*, Int. J. Biol. Macromol. 108 (2018) 710-718.
- 568 [26] S. D’ambrosio, A. Alfano, E. Cassese, O. F. Restaino, S. B. Ferraiuolo, R.
569 Finamore, M. Cammarota, C. Schiraldi, D. Cimini, Production and purification
570 of higher molecular weight chondroitin by metabolically engineered *Escherichia*
571 *coli* K4 strains, Sci. Rep. 10 (2020) 13200.
- 572 [27] P. Bronzi, M. Chebanov, J. T. Michaels, Q. W. Wei, H. Rosenthal, J. Gessner,

573 Sturgeon meat and caviar production: Global update 2017, *J. App. Ichthyol.* 35
574 (2019) 257-266.

575 [28] O. F. Restaino, R. Finamore, P. Diana, M. Marseglia, M. Vitiello, A. Casillo, E.
576 Bedini, M. Parrilli, M. M. Corsaro, M. Trifuoggi, M. D. Rosa, C. Schiraldi, A
577 multi-analytical approach to better assess the keratan sulfate contamination in
578 animal origin chondroitin sulfate, *Anal. Chim. Acta*, 958 (2017) 59-70.

579 [29] W. M. Zhu, J. Yang, Y. Wang, D. He, Y. S. Yan, N. Su, C. Zhang, X. H. Xing,
580 Structural characterization and *in vitro* antioxidant activities of chondroitin
581 sulfate purified from *Andrias davidianus* cartilage, *Carbohydr. Polym.* 196 (2018)
582 398-404.

583 [30] F. Maccari, F. Ferrarini, N. Volpi, Structure characterization of chondroitin
584 sulfate from sturgeon bone, *Carbohydr. Res.* 345 (2010) 1575-1580.

585 [31] F. Krichen, H. Bougatef, F. Capitani, I. B. Amor, I. Koubaa, J. Gargouri, F.
586 Maccari, V. Mantovani, F. Galeotti, N. Volpi, A. Bougatef, Purification and
587 structural elucidation of chondroitin sulfate/dermatan sulfate from Atlantic
588 bluefin tuna (*Thunnus thynnus*) skins and their anticoagulant and ACE inhibitory
589 activities, *RSC. Advances.* 8 (2018a) 37965-37975.

590 [32] W. Garnjanagoonchorn, L. Wongekalak, A. Engkagul, Determination of
591 chondroitin sulfate from different sources of cartilage, *Chem. Eng. Process.* 46
592 (2007) 465-471.

593 [33] M. Foot, M. Mulholland, Classification of chondroitin sulfate A, chondroitin
594 sulfate C, glucosamine hydrochloride and glucosamine 6 sulfate using

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

596 [34] M. Lopez-Alvarez, P. Gonzalez, J. Serra, J. Fraguas, J. Valcarcel, J. A. Vazquez,
597 Chondroitin sulfate and hydroxyapatite from *Prionace glauca* shark jaw:
598 physicochemical and structural characterization, Int. J. Biol. Macromol. 156
599 (2020) 329-339.

600 [35] T. Toida, H. Toyoda, T. Imanari, High-resolution proton nuclear magnetic
601 resonance studies on chondroitin sulfates, Anal. Sci. 9 (1993) 53-58.

602 [36] A. Mucci, L. Schenetti, N. Volpi, ¹H and ¹³C nuclear magnetic resonance
603 identification and characterization of components of chondroitin sulfates of
604 various origin, Carbohydr. Polym. 41 (2000) 37-45.

605 [37] A. N. Akram, C. H. Zhang, 2020. Extraction of collagen-II with pepsin and
606 ultrasound treatment from chicken sternal cartilage; physicochemical and
607 functional properties. Ultrason. Sonochem. 64, 105053.

608 [38] P. G. Kumar, T. Nidheesh, P. V. Suresh, Comparative study on characteristics and
609 *in vitro* fibril formation ability of acid and pepsin soluble collagen from the skin
610 of catla (*Catla catla*) and rohu (*Labeo rohita*), Food. Res. Int. 76 (2015) 804-812.

611 [39] B. B. Doyle, E. G. Bendit, E. R. Blout, Infrared spectroscopy of collagen and
612 collagen-like polypeptides, Biopolym. 14 (1975) 937-957.

613 [40] J. H. Muyonga, C. G. B. Cole, K. G. Duodu, Characterisation of acid soluble
614 collagen from skins of young and adult Nile perch (*Lates niloticus*), Food. Chem.
615 85 (2004) 81-89.

616 [41] A. Veeruraj, M. Arumugam, T. Balasubramanian, Isolation and characterization

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

619 [42] A. M. D. G. Plepis, G. Goissis, D. D. Gupta, Dielectric and pyroelectric
620 characterization of anionic and native collagen, *Polym. Eng. Sci.* 36 (1996)
621 2932-2938.

622 [43] X. Zhang, S. Adachi, K. Ura, Y. Takagi, Properties of collagen extracted from
623 Amur sturgeon *Acipenser schrenckii* and assessment of collagen fibrils *in vitro*,
624 *Int. J. Bio. Macromol.* 137 (2019) 809-820.

625 [44] S. Yamada, K. Sugahara, Potential therapeutic application of chondroitin
626 sulfate/dermatan sulfate, *Curr. Drug. Discov. Technol.* 5 (2009) 289-301.

627 [45] M. B. Mathews, L. Decker, The effect of acid mucopolysaccharides and acid
628 mucopolysaccharide-proteins on fibril formation from collagen solutions,
629 *Biochem. J.* 109 (1968) 517-526.

630 [46] G. C. Wood, The formation of fibrils from collagen solutions. 3. Effect of
631 chondroitin sulphate and some other naturally occurring polyanions on the rate
632 of formation, *Biochem. J.* 75 (1960) 605-612.

633 [47] M. K. Keech, The formation of fibrils from collagen solutions: IV. effect of
634 mucopolysaccharides and nucleic acids: an electron microscope study, *J.*
635 *Biophys. Biochem. Cytol.* 9 (1961) 193-209.

636 [48] M. Y. Yan, X. J. Jiang, G. C. Wang, A. L. Wang, X. X. Wang, X. Y. Wang, X. C.
637 Zhao, H. Xu, X. S. An, Y. P. Li, Preparation of self-assembled collagen fibrillar
638 gel from tilapia skin and its formation in presence of acidic polysaccharides.

639 Carbohydr. Polym. 233 (2020) 115831.

640

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

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

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

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

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

650 Fig. 6. A: Fibril formation *in vitro* by Col II measured by optical absorbance at 320
651 nm. B: The degree of fibril formation of notochord Col II at different reaction times.
652 Columns and bars show mean and standard error of the means of three experiments.

653 Differences between groups with different letters are significant (Tukey-Kramer post-
654 hoc test, $p < 0.01$).

655 Fig. 7. Scanning electron micrographs of sturgeon notochord Col II fibrils formed at
656 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
657 (1); 1 μm (2); 300 nm (3).

658 Fig. 8. Elemental mapping of CS and Col II hybrid fibril surface. A: 0 mg/mL CS; B:
659 0.5 mg/mL CS; C: 1 mg/mL CS; (1): Backscattered electron image of SEM; (2): Sulfur
660 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 $5.34 \pm 0.74\%$ and $45.25 \pm 5.25\%$, 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

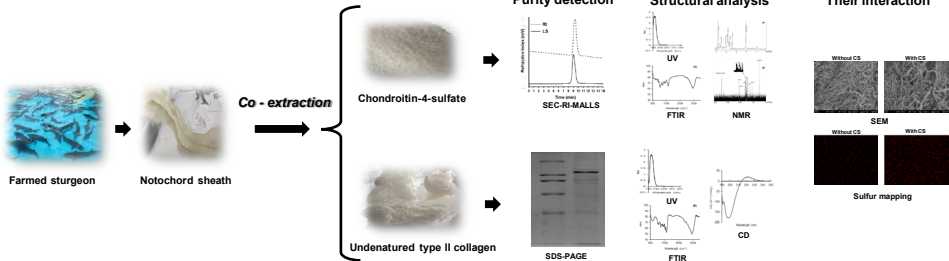


Table. 1. The effect of alkaline treatment time on CS and Col II yields (day weight base).

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

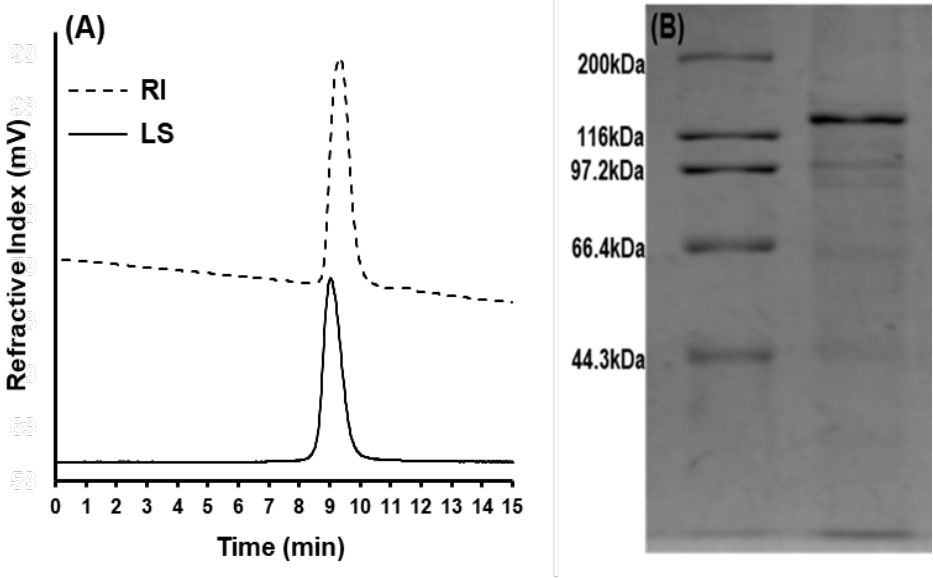


Fig.2

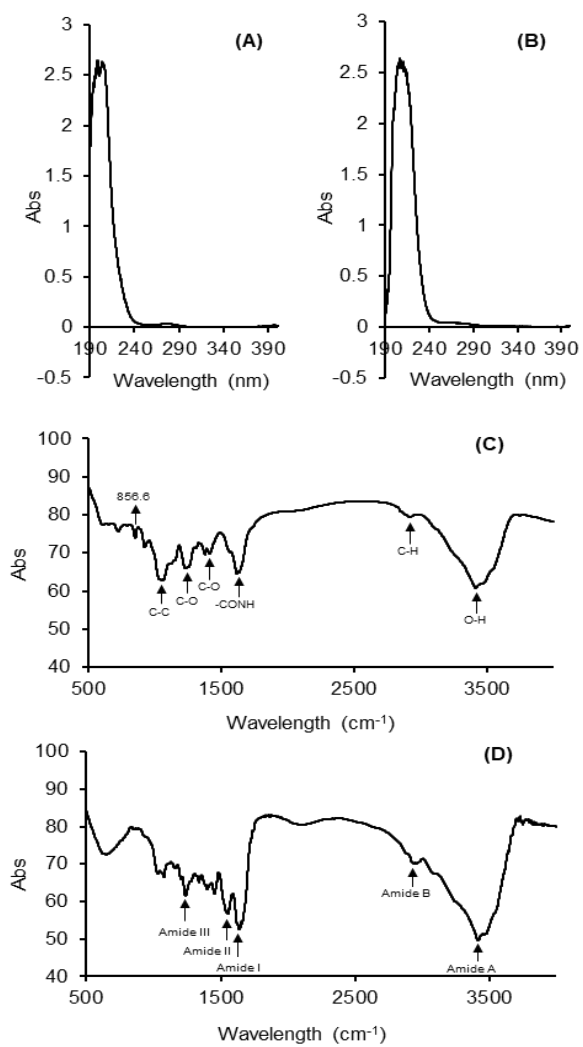


Fig.3

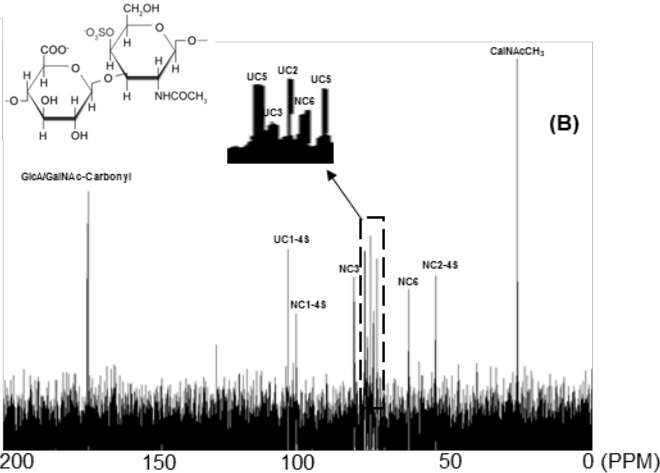
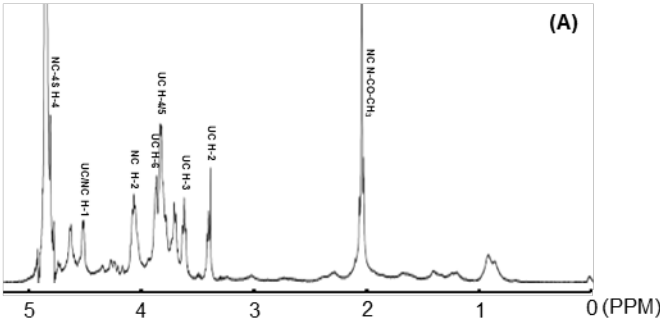


Fig.4

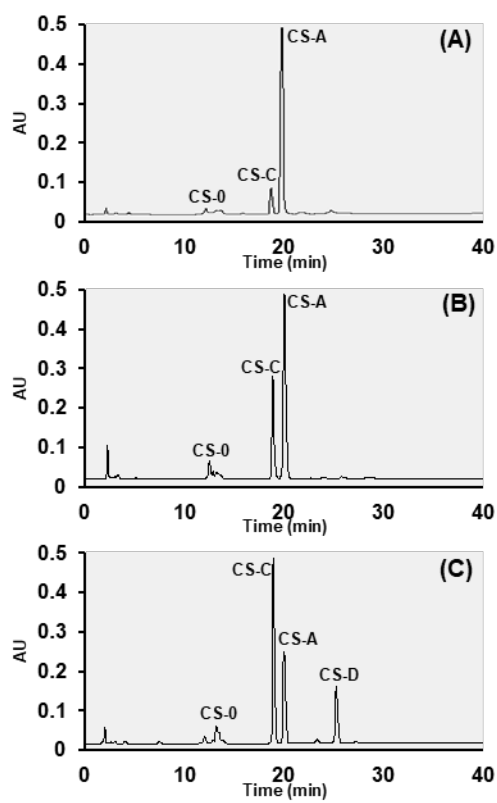


Fig.5

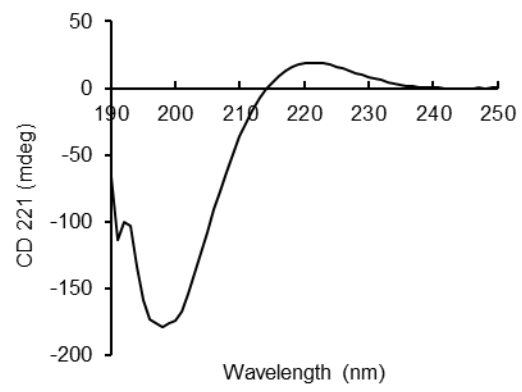


Fig.6

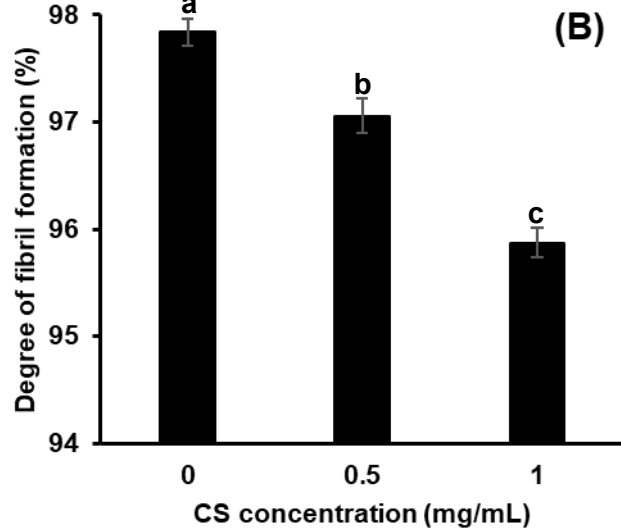
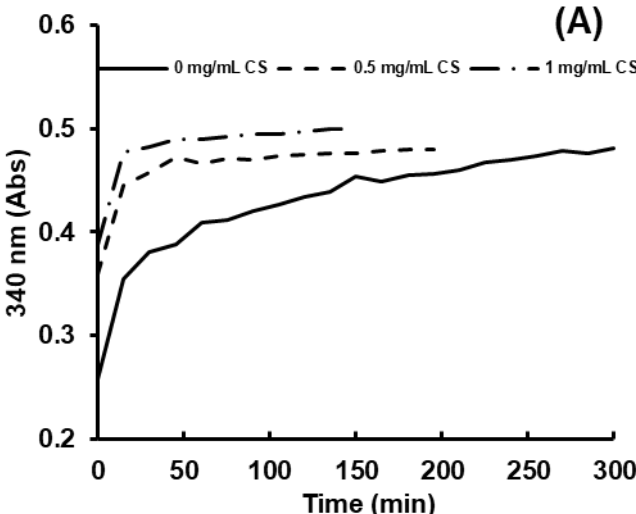


Fig.7

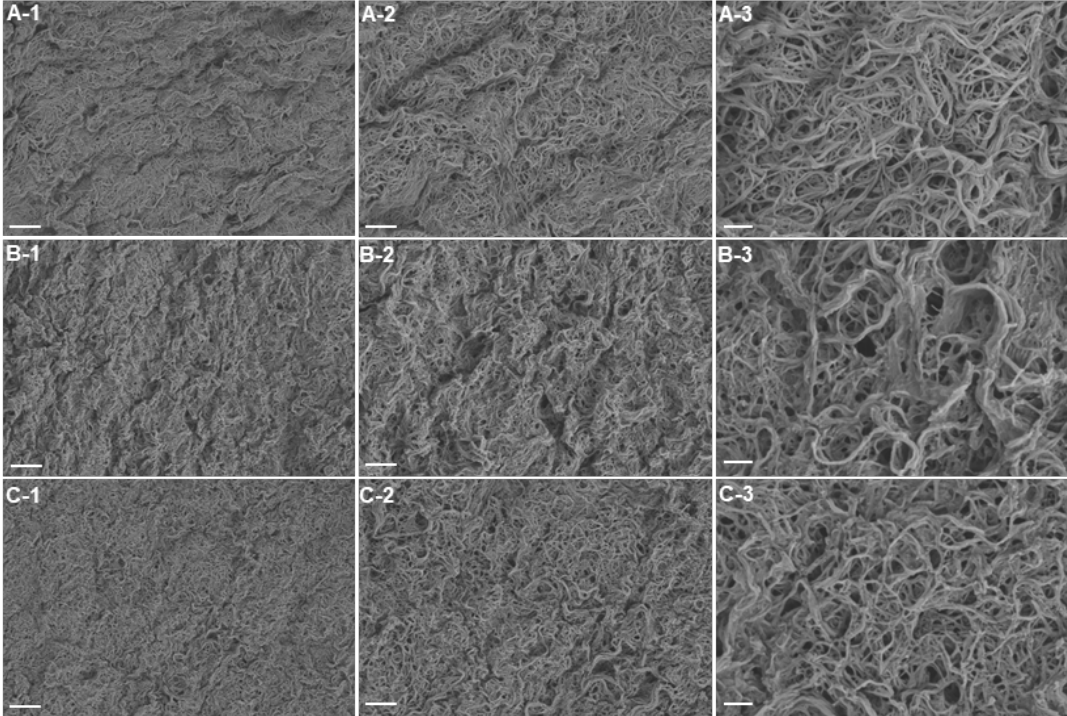
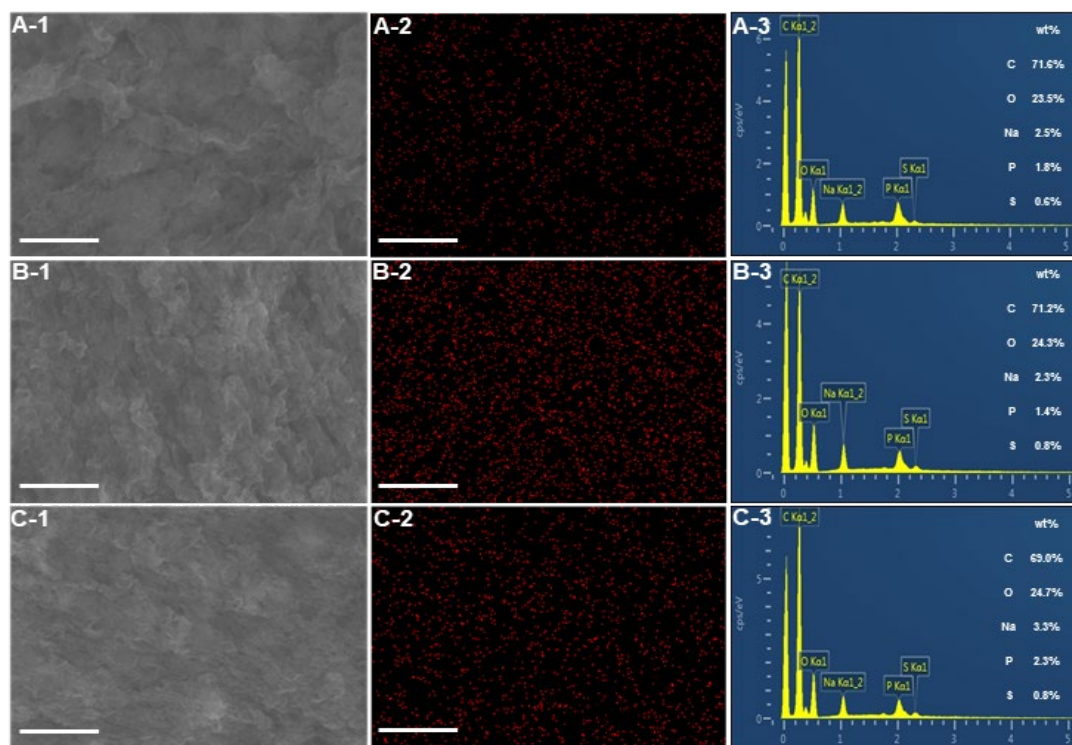
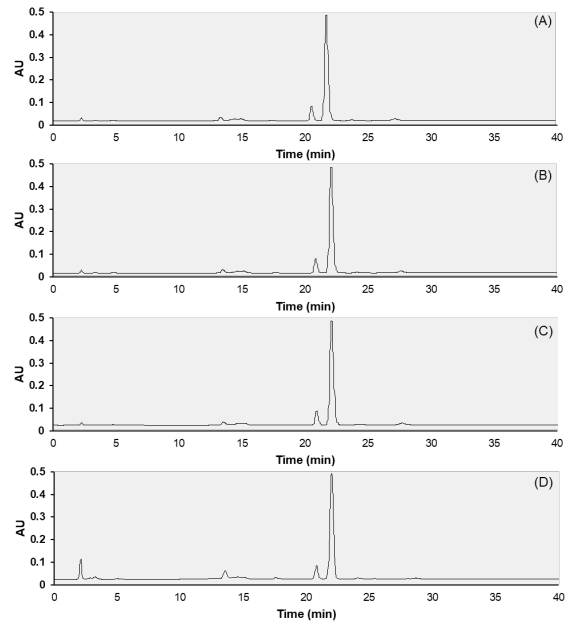


Fig.8





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.