Research paper

Free radical scavenging activity of type II collagen peptides and chondroitin sulfate oligosaccharides from by-products of mottled skate processing

Running title: Antioxidant compounds from by-products of skate processing

Wen Li[a]*, Taishi Kobayashi[a], DaWei Meng[a], Nobuyuki Miyamoto[b], Naonobu Tsutsumi[c], Kazuhiro Ura[d], Yasuaki Takagi[d]

[a] Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[b] Marukyo Bio Foods, Wakkanai, Hokkaido 097-0022, Japan

[c] Sapporo Fine Chemical Research Center, Marukyo Bio Foods, Teine, Sapporo, Hokkaido 006-0004, Japan

[d] Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

E-mail addresses: fenzhongbahe@outlook.com (W Li), t-hand-895ce@docomo.ne.jp (T Kobayashi), mdw000@163.com (DW Meng), miyamoto@mbf-net.com (N Miyamoto), naonobu_tsutsumi@mbf-net.com (N. Tsutsumi), kazu@fish.hokudai.ac.jp (K Ura), and takagi@fish.hokudai.ac.jp (Y Takagi)

23 *Corresponding author: fenzhongbahe@outlook.com (W Li)

24 Tel/Fax: +81 138 40 5551

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Abstract

The by-products of the skate processing industry have bioactive properties but are mostly discarded. In the present study, the properties of skate cartilage, a major by-product of skate processing that is rich in chondroitin sulfate (CS) and type II collagen were investigated. Enzymatically-hydrolyzed type II collagen peptides (CP) and thermally hydrolyzed CS oligosaccharides (CSo) from skate cartilage were prepared and their antioxidant activities were studied. High free radical scavenging activity was observed in the <3 kDa MW fraction of the papain hydrolyzed CP (Pa-CP). CSo also had high free radical scavenging activity, but the activity was mainly due to the intermediate and final products of the non-enzymatic browning reaction resulting from high-temperature hydrolysis. These products may have included flavonoid-like compounds. Furthermore, Pa-CP and CSo were not cytotoxic. Thus, Pa-CP and CSo derived from skate by-products may be beneficial antioxidant ingredients. The present study may contribute to the development of functional health foods and achieving zero-waste skate processing.

Keywords: skate by-product, *Raja pulchra*, type II collagen peptides, chondroitin sulfate oligosaccharides, antioxidant activity, non-enzymatic browning products

Abbreviations: ABTS, 2,2 -Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; Ch-CP, Chymotrypsin hydrolyzed CP; CP, Collagen peptide; CS, Chondroitin sulfate; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; Pa-CP, Papain hydrolyzed CP; CSo, CS

48 oligosaccharides; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel

49 electrophoresis; and Tr-CP, Trypsin hydrolyzed CP

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1. Introduction

Capture fisheries and aquaculture products are important sources of food worldwide. The total fish production is increasing and was 171 tonnes in 2016 (FAO, 2018). However, in industrial fish processing, up to 70% of fish weight ends up as by-products (FAO, 2018) and is eliminated as solid waste or into the sea (Lopes et al., 2015). Thus, the by-products of fish processing cause serious environmental problems and represent a huge waste of resources, resulting in financial loss. The methods of disposal are also expensive (Pal & Suresh, 2016). The FAO (2018) report claimed that fish by-products represent a significant additional source of nutrition, and that currently available advanced processing technologies can be used to efficiently harness these nutrients. Fish by-products have good bioavailability and do not cause any common human diseases. Thus, fish by-products should be developed as value-added products to boost the efficiency of the fish processing industry and improve general health (Pal & Suresh, 2016).

Up to 50% of the carcasses of the mottled skate, *Raja pulchra*, which is an important marine commercial fish in northern Japan, end up as by-products during industrial skate processing (unpublished observation). These by-products include the head and axial cartilage, which are rich in type II collagen associated with proteoglycans bound to chondroitin sulfate (CS) chains (Murado et al., 2010). Recently, isolation and purification of CS polysaccharides (CSp) from mottled skate cartilage using enzymatic hydrolysis were done, and CS oligosaccharides (CSo) were produced using thermal hydrolysis of CSp using a subcritical water microreaction

system to improve the intestinal absorption and water solubility of CSp (Yamada et al., 2013). The preliminary study suggested that the bioavailability of CSo is 40-fold higher than that of CSp (unpublished observation). The anti-obesity effects of skate CSp and CSo had also been reported (Li et al., 2019). The molecular weight (MW) of CS affects the mode of action of skate CS: CSp shows stronger lipase inhibitory activity, whereas CSo suppresses adipocyte lipid accumulation at lower doses (Li et al., 2019). These results suggested that a formulation containing both CSp and CSo may be effective as an anti-obesity functional food. However, the functionalities of CSp and CSo other than the anti-obesity effect have had limited study, although CS is a multifunctional molecule (Volpi, 2019). Industrial usage of type II collagen, another major constituent of the mottled skate cartilage, has not yet been investigated either, although it accounts for ~60% of the cartilage dry weight (dw).

To maximize the use of existing resources, this study focused on the radical scavenging activities of type II collagen, CSp, and CSo. In general, cellular metabolism results in the production of reactive oxygen species (ROS) (Canas et al., 2007). Factors such as UV exposure and stress increase ROS *in vivo*. Overproduction of ROS leads to cell damage and is related to many diseases and aging, including skin aging, rheumatoid arthritis, and Alzheimer s disease (Canas et al., 2007). Dietary supplementation of antioxidants can reduce ROS in the human body; however, the application of artificial antioxidants is restricted because of their potential adverse effects on human health (Pan et al., 2016). Therefore, the pharmaceutical and food industries have been using natural antioxidants as a dietary supplement to reduce ROS.

98 Several studies have shown that hydrolysates/peptides from marine by-products have
99 antioxidant activity (Pal & Suresh, 2016; Sila & Bougatef, 2016). Thus, if type II
100 collagen peptides (CP), CSp, and CSo of the mottled skate cartilage have radical
101 scavenging activity, their industrial usage might be possible.

102 Data on the radical scavenging activity of type II CP in fish species are limited. Li
103 et al. (2013) reported the antioxidant activity of cartilage collagen hydrolysates from
104 cartilaginous fish (*Sphyrna lewini*, *Dasyatis akjei*, and *Raja porosa*), and confirmed
105 that DPPH-radical scavenging activity was higher in peptides with lower MW.
106 However, their studies have some limitations: their SDS-PAGE analysis showed
107 several protein bands other than the α, β, and γ bands of type II collagen, suggesting
108 relatively lower purity or hydrolysis of the collagen. They only used trypsin with a
109 high enzyme: collagen ratio (33%, w/w). Three antioxidant peptides were isolated
110 from the protein hydrolysate of the cartilage of the skate, *Raja porosa* (Pan et al.,
111 2016), but only non-collagenous peptides were identified. Meng (2019) investigated
112 the DPPH, ABTS, and OH- radical scavenging activities of type II CP derived from
113 sturgeon notochord, a special tissue industrially available only in sturgeon species.
114 However, the effects of enzymatic hydrolysis on the antioxidant activities of type II
115 CP from fish cartilage need more study.

116 Data on the radical scavenging ability of fish CS are also limited and have
117 generally been negative (Ajisaka et al., 2016). The CS from shark cartilage and
118 porcine intestinal mucosa showed low antioxidant activity (Ajisaka et al., 2016);
119 however, the high-temperature hydrolysis of CSp into CSo might affect the nature of

120 the product since the color of CSo is slightly brownish compared with the white color
121 of CSp. Using the high-temperature hydrolysis, CS may produce non-enzymatic
122 browning products, including caramelization and Maillard-reaction products, which
123 have higher antioxidant activity.

124 The present study aimed to evaluate the potential of the cartilage of mottled skate
125 as a source of antioxidants in functional foods. Type II CP using three enzymes were
126 prepared and their antioxidant activities were compared. Next, the relationship
127 between the MW distribution of type II CP and their antioxidant activity were
128 determined. And, the antioxidant activity of CSp and CSo was measured and the
129 compounds that possibly contribute to this activity were identified.

130

131 **2. Materials and methods**

132 *2.1. Materials*

133 Mottled skate cartilage, CSp (purity ~70%), and CSo with 2–14 sugar units
134 (purity ~80%) from mottled skate cartilage were obtained from Marukyo Bio Foods
135 Co., Ltd. (Wakkanai, Hokkaido, Japan). The purification method of CSp from skate
136 cartilage was reported by Hashiguchi et al. (2011). Briefly, the skate cartilage was
137 digested with actinase E and the CSp in the hydrolysates were precipitated using 80%
138 ethanol. The CSo was prepared using thermal hydrolysis of the CSp using the method
139 reported by Yamada et al. (2013). Briefly, CSp was hydrolyzed using a subcritical
140 water microreaction system at a pressure of 25 MPa. High-purity CSp (H-CSp) and
141 CSo (H-CSo, 6–14 sugar units) were purified from CSp and CSo, respectively, using

142 ethanol and activated charcoal by Marukyo Bio Foods Co. (purity ~83.1 and ~86.3%,
143 respectively). Briefly, samples were dissolved in water and stirred with activated
144 charcoal powder (10% w/v) for 24 h at 4°C. The charcoal was then removed by
145 filtration, and the CS was precipitated using ethanol (90% v/v). The precipitate was
146 separated using a glass filter and dried at room temperature (21–23°C). The other
147 components of purified CSp and CSo were minerals and water. Triglycine was
148 purchased from Sigma-Aldrich (Saint Louis, MO, USA). Precision Plus Protein^{TM} All
149 Blue Standard (protein markers) was obtained from Bio-Rad Laboratories (Hercules,
150 CA, USA). Other chemicals were purchased from Wako Pure Chemical Co. (Osaka,
151 Japan) unless otherwise specified.
152
153 *2.2 Enzymatic hydrolysis of skate type II collagen*
154 Skate type II collagen was extracted according to Zhang et al. (2014) with minor
155 modifications. Briefly, the cartilage powder (5 g) was stirred continuously in
156 deionized water (RFD270NC, Advantec, Tokyo, Japan; pH 2.0 adjusted using
157 0.01 mol/L HCl solution) containing 0.1% (w/v) pepsin (derived from porcine) for
158 48 h at 4°C. The mixture was centrifuged at 10,000 × *g* (12,000 rpm in a T15A42
159 rotor, high-speed micro centrifuge, CF15RXII, Hitachi, Ltd., Ibaraki, Japan) for 30
160 min at 4°C to obtain the supernatants, and the residue was re-extracted with the same
161 conditions. The supernatants were precipitated using NaCl at a final concentration of
162 1 M. The resulting precipitate was dissolved in water (pH 2.0). This process was
163 repeated three times to obtain purified collagen. The purified collagen was dialyzed

using a 100 kDa nominal MW dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against 50 volumes of water for 24 h with two changes of water. The dialysate was sequentially filtered with 3.0, 0.8, and 0.45 μm membrane filters (Advantec). All steps of the extraction and purification were done at 4°C or on an ice-bath, and the filtrate was lyophilized using a freeze-dryer (FDU-830, Tokyo Rikakikai Co., Tokyo, Japan). Samples were subjected to electrophoresis using a sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE). The lyophilized samples were dissolved in water (pH 2.0, 3 mg/mL) and then mixed at a ratio of 1:2 (v/v) with a sample buffer (0.5 M Tris–HCl buffer, pH 6.8, with 4% SDS, 10% β-mercaptoethanol, and 20% glycerol). The mixed solutions were boiled for 5 min, and 10 μL solutions were loaded in each lane. Electrophoresis was done at 15 mA for the stacking gel and 20 mA for the 7.5% running gel using the electrophoresis chamber (AE-6500, ATTO, Tokyo, Japan). After the electrophoresis, the gels were stained for 30 min using 0.1% Coomassie Brilliant Blue (CBB) R250 solution in 40% ethanol containing 10% acetic acid and destained with a mixture of 20% ethanol and 5% acetic acid until the band became visible.

The purified collagen was dissolved in water and denatured at 60°C for 1 h to make gelatin. The gelatin was digested with trypsin or chymotrypsin (2.5% w/w, derived from bovine pancreas) at 37°C for 4 h, or with papain (2.5% w/w, derived from the unripe fruit of the papaya tree) at 50°C for 4 h. After the digestion, the samples were boiled for 10 min to inactivate the enzymes. After filtration with a 0.22 μm filter membrane (Advantec), the samples were lyophilized. The lyophilized

samples were prepared as previously, and then subjected to electrophoresis on 15.5% Tricine-SDS-polyacrylamide gel (Schägger & Von Jagow, 1987). After electrophoresis, the gel was stained as previously. Subsequently, the gel was stained for 45 min using 0.25% Alcian blue in 2% acetic acid, then destained for 15 min in 2% acetic acid (Cowman et al., 1984). The percentage of the dw of peptides obtained compared to the dw of the initial collagen was calculated as the peptide yield.

2.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH (PubChem CID 2735032; Tokyo Chemical Industry, Tokyo, Japan) was dissolved in methanol to a final concentration of 115 μM. Each sample was dissolved in water to 0.625–5 mg/mL; L(+)-ascorbic acid (VC) was used as the positive control. The sample solution (500 μL) was reacted with DPPH methanol solution (500 μL) in the dark at room temperature for 30 min and the absorbance was measured at 517 nm using a microplate reader (Infinite F50R, Tecan Japan Co., Kawasaki, Japan). Each measurement was done in triplicate and the percentage scavenging effect was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = (A_b - A_s)/A_b \times 100,$$

where A_b and A_s indicate the absorbance of water and the sample solution, respectively.

2.4 2,2 -Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay

ABTS (PubChem CID 16240279; Tokyo Chemical Industry, 7 mM) and potassium persulfate ($K_2S_2O_8$, 2.45 mM) solutions (2:1 v/v) were reacted in the dark at room temperature for 12–16 h to generate the ABTS radical solution. The solution was diluted with phosphate-buffered saline (PBS, 0.01 M, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm and 500 μL was reacted with 500 μL of the sample solution for 10 min at room temperature, followed by measurement of the absorbance at 734 nm using the microplate reader. Each sample was dissolved in water to 0.04–5 mg/mL. VC was used as the positive control. Each measurement was done in triplicate, and the percentage scavenging effect was calculated as follows:

$$\text{ABTS radical scavenging activity (\%)} = (A_b - A_s)/A_b \times 100,$$

where A_b and A_s indicate the absorbance of water and the sample solution, respectively.

2.5 MW distribution

The MW distribution of the CP was determined using size exclusion chromatography on a TSKgel G2500PW column (7.5 mm × 30 cm, Tosoh Co., Tokyo, Japan) with UV detection at 214 nm. The mobile phase consisted of 35% acetonitrile in 0.05% trifluoroacetic acid (pH 2.1) with a flow rate of 0.6 mL/min. A MW calibration curve was obtained using the following standards: insulin (MW: 5,700, derived from bovine), vitamin B_{12} (MW: 1,355), and triglycine (MW: 189). A 30 μL sample or standards was injected into the high performance liquid chromatography (HPLC) system (SCL-10AVP, Shimadzu Corp., Tokyo, Japan). The percentage of

each MW fraction was calculated as follows:

$$\text{MW fraction (\%)} = (S_x/S_{total}) \times 100\%,$$

where S_x and S_{total} indicate the area of each fraction (>5 kDa, 3–5 kDa, 1–3 kDa, and <1 kDa nominal MW) and the total area of the chromatogram, respectively, assuming total equality of each fraction's responses.

2.6 Preparation of fractions with different MW

Papain hydrolyzed CP (Pa-CP) was separated using ultrafiltration membranes with a nominal MW cut-off (MWCO) of 3 and 10 kDa (Merck Millipore Ltd., Darmstadt, Germany). A 5 mL Pa-CP solution was centrifuged at 4,000 × *g* for 20 min and then eluted with 5 mL water at 4,000 × *g* for 20 min at room temperature. This process was repeated several times to obtain the <3 kDa MW peptides. Then, the >3 kDa MW peptides were added to the 10 kDa MWCO tube and eluted several times with water to separate the 3–10 kDa and >10 kDa MW peptides. The peptides were freeze-dried.

2.7. Non-enzymatic browning products and total flavonoid content (TFC)

The intermediate and final products of the non-enzymatic browning reaction (the Maillard and caramelization reactions) were estimated using the UV-absorbance and browning intensity of the sample solutions at 294 and 420 nm, respectively (Ajandouz et al., 2001). The TFC was determined using $AlCl_3$, according to Pontis et al. (2014), with minor modifications. A 0.5 mL sample solution was mixed with 0.75 mL 5%

252 (w/v) $AlCl_3$ in methanol. After 30 min of incubation, the absorbance was measured at
253 437 nm against methanol (blank). Each experiment was done in triplicate and the
254 absorbance (ΔA) was calculated using the following equation:

255 $$\Delta A = A_t - A_b,$$

256 where A_t is the absorbance of the sample after reacting with $AlCl_3$ in methanol, and A_b
257 is the absorbance of the sample alone reacted with methanol.

258

259 *2.8 Cytotoxicity*

260 L929 fibroblast cells were obtained from the Riken Cell Bank (Tsukuba,
261 Japan). Cells were cultured in 96-well plates in an atmosphere of 5% CO_2 at 37°C.
262 The culture medium was minimum essential medium (MEM, Gibco, Thermo Fisher
263 Scientific, Grand Island, NY, USA) containing 5% fetal bovine serum (FBS, Lot. No
264 451456, Gibco) and 1% penicillin and 1% streptomycin (both Gibco). Cells were
265 suspended at 2×10^5 cells/mL in the culture medium and seeded at 2×10^4 cells/well
266 (100 μL/well). A cell-counting chamber (Hirschmann, Eberstadt, Germany) was used
267 for cell counting. After 24 h of incubation, the culture medium was changed to include
268 CSo and Pa-CP (endotoxin <1 ng/mg) dissolved in endotoxin-free water (EFW,
269 GBiosciences, Saint Louis, MO, USA) at 1 mg/mL. EFW was used as the negative
270 control. The endotoxin was from lipopolysaccharides from the outer membrane of
271 certain Gram-negative bacteria that might contaminate the samples and evokes the
272 inflammatory response of cells (Li et al., 2019). Therefore, the endotoxin levels of
273 samples were measured according to Li et al. (2019) using a *Limulus amebocyte*

lysate (LAL) assay (ES-24S® Kit, lot No. 24S16004; Seikagaku Corp., Tokyo, Japan), using the manufacturer's instructions. Control Standard Endotoxin (CSE, lot No. 249025; Associates of Cape Cod Incorporated, East Falmouth, MA, USA) was used as a standard. Low endotoxin samples, which did not cause an inflammatory response, were used in this study.

Following a 48 h incubation with the samples, the cell number/metabolism was measured using a cell counting kit 8 (CCK8, Dojindo Ltd., Kumamoto, Japan). The kit measures the cellular activity of the dehydrogenase that converts NAD^+ and $NADP^+$ to NADH and NADPH and provides an estimate of total cell metabolic activity in each cell-culture well. Each wells total cell metabolic activity will change when cell number and/or metabolic activity of the cells change. After discarding the culture medium, 10% (v/v) CCK8 solution in culture medium was added to each well and incubated at 37°C for 30 min; and the absorbance was measured at 450 nm using the microplate reader.

2.9 *Statistical analysis*

Data were expressed as mean ± standard error. Statistical analysis was done using a Tukey-Kramer test after a two-way analysis of variance (ANOVA) in Microsoft Excel (Microsoft, Redmond, WA, USA) with the statistical software add-in (Ver. 2.12, Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical significance was accepted at $p<0.01$. Experiments were repeated twice to confirm the reproducibility of the results, and representative results are shown.

296

297 **3. Results and discussions**

298 *3.1 Yields and tricine SDS-PAGE*

299 The SDS-PAGE pattern of purified C-II sample is shown in Supplementary Fig. 1.
300 The sample showed only one α-chain band (~130 kDa MW), suggesting the relatively
301 higher purity of the sample.

302 The dw yields of trypsin-CP (Tr-CP), chymotrypsin-CP (Ch-CP), and papain-CP
303 (Pa-CP) were 83, 85, and 91%, respectively.

304 On the tricine-SDS-PAGE gel, CBB and Alcian blue stained peptides and acidic
305 polysaccharides with >6 sugar units can be observed (Cowman et al., 1984). The CP
306 showed no positive reaction with the Alcian blue stain, suggesting that the CP did not
307 contain reactive CS (Fig. 1A). Pa-CP was smeared after CBB staining with no clear
308 bands, whereas Tr- and Ch-CP showed several bands at MW >20 kDa (Fig. 1B).
309 These data suggested that Pa-CP had more peptides of lower MW than Tr- and Ch-CP.
310 The reason why Pa-CP had smaller peptides relates to the cleavage specificity of the
311 enzyme. Papain is a cysteine endoprotease and its cleavage specificity is broader than
312 that of trypsin and chymotrypsin (Slizyte et al., 2016).

313 Alcian blue staining showed that CSp and CSo had sugars of different MW (Fig.
314 1A). Smearing was observed for CBB in CSp and CSo (Fig. 1B), showing that these
315 samples had peptides, probably of a range of MW. These CS were obtained as CS
316 sodium salts. Thus, CSp and CSo contained CS, minerals, and peptides. The
317 preliminary measurements showed that the peptides accounted for <10% of the dw of

318 these samples. On the other hand, H-CSp and H-CSo had only CS and minerals, and
319 no peptides were observed (Supplementary Fig. 2).
320
321 *3.2 Antioxidant activity of CP*
322 *3.2.1 Free radical scavenging activity of CP*
323 The three enzymatically hydrolyzed CP samples showed free radical scavenging
324 activity in a dose-dependent manner (Fig. 2). The DPPH radical scavenging assay
325 (Fig. 2A) showed that the activity of Pa-CP was the highest among the three samples.
326 Ch-CP also showed DPPH radical scavenging activity. On the other hand, Tr-CP
327 showed almost no activity. The reason why Tr-CP has almost no DPPH radical
328 scavenging activity is not known. Meng (2019) also showed trypsin-hydrolyzed
329 collagen peptides from sturgeon notochord (type II collagen) and skin (type I collagen)
330 had low DPPH radical scavenging activity.
331 All CP samples showed strong ABTS radical scavenging activity and scavenged
332 almost all ABTS radicals at concentrations >1 mg/mL (Fig. 2B). Moreover, the ABTS
333 radical scavenging activities had minimum variability among the three CP samples. In
334 general, the ABTS method had higher values than the DPPH method probably due to
335 the lower stability of the ABTS radical. The advantage of the ABTS radical is its high
336 reactivity, but its lower stability may bias the results (Mareček et al., 2017).
337
338 *3.2.2 Relationship between antioxidant activity and MW distribution of CP*
339 The dominant MW of Tr-CP and Ch-CP were 3–5 kDa and >5 kDa, respectively

340 (Fig. 3). Pa-CP had a bigger proportion of smaller peptides: the proportion of the
341 <1 kDa and 1–3 kDa MW peptides of Pa-CP (48%) was higher than that of Tr-CP
342 (36%) and Ch-CP (12%). These results were consistent with those of the
343 tricine-SDS-PAGE analysis (Fig. 1). As discussed in 3.2.1, the broad cleavage
344 specificity may be responsible for the bigger proportion of smaller peptides in Pa-CP.
345 The higher antioxidant activity of Pa-CP can be attributed in part to its smaller
346 MW, as lower MW peptides generally had significantly higher antioxidant activities in
347 previous studies (Sila & Bougatef, 2016; Zou et al., 2016). For example, the tilapia
348 skin hydrolysate fractions with a MW <1 kDa had the highest free radical scavenging
349 capacity (Zheng et al., 2018). Moreover, small MW peptides may contain more
350 exposed bioactive groups than high MW peptides, which may facilitate contact with
351 free radicals.
352 To further confirm the relationship between the MW of peptides and the
353 antioxidant activity of CP, Pa-CP was divided into the three fractions with >10 kDa,
354 3–10 kDa, and <3 kDa MW. According to an ABTS assay (Fig. 4), Pa-CP with a MW
355 <3 kDa had stronger free radical scavenging activities than high MW Pa-CP.
356 The radical scavenging activity of type II CP from fish cartilage has only been
357 reported by Li et al. (2013). They studied cartilage collagen hydrolysates from
358 cartilaginous fish (*Sphyrna lewini*, *Dasyatis akjei*, and *Raja porosa*), and confirmed
359 that DPPH-radical scavenging activity was higher in peptides with lower MW.
360 However, their study differed in several ways from this study. They hydrolyzed
361 relatively low-purity collagen using only one enzyme (trypsin) with a much higher

362 enzyme:collagen ratio. Therefore, their trypsin-hydrolysates had peptides with
363 MW >60 kDa, whereas the hydrolysate in this study had mainly peptides with MW
364 <50 kDa.

365 Market available enzymes, i.e., trypsin, chymotrypsin, and papain, were used.
366 These were the enzymes commonly used for hydrolysis of fish by-products in
367 previous reports. Papain is comparatively less expensive than the other proteolytic
368 enzymes. Considering the cost of production, Pa-CP is, therefore, suitable for
369 large-scale production of CP. Furthermore, the relatively smaller MW of Pa-CP
370 suggested higher bioavailability, because of their digestive tract stability and intestinal
371 absorbability. For example, water-soluble oligopeptides can passively diffuse through
372 intercellular junctions, and di-/tri-peptides can be absorbed by the PepT1 transporter
373 (Wang et al., 2019). Also, peptides of <3 kDa MW are more stable during
374 gastrointestinal digestion than those with higher MW (Zou et al., 2016); therefore,
375 Pa-CP with MW <3 kDa derived from skate cartilage are potentially inexpensive
376 functional antioxidants to support human health.

377

378 *3.3 Antioxidant activities of CS*

379 *3.3.1 Free radical scavenging activity of CSp, H-CSp, CSo, and H-CSo*

380 Figure 5 shows the DPPH and ABTS radical scavenging activities of CSp, H-CSp,
381 CSo, and H-CSo. H-CSp was high-purity CS polysaccharides that were presumably in
382 the CS native form, and H-CSo was the high-purity CS oligosaccharides.

383 H-CSp showed almost no DPPH and ABTS radical scavenging activities, but CSp

384 had radical scavenging activities (Fig. 5). H-CSo had low but significant antioxidant
385 activity, and CSo had stronger radical scavenging activity than H-CSo (Fig. 5).
386 These data suggested that CS in its native form had no free radical scavenging
387 activity. The results were consistent with that of Ajisaka et al. (2016), who showed
388 that CS from shark cartilage and porcine intestinal mucosa had low DPPH radical
389 scavenging activity. These results strongly support the previous observations that
390 antioxidant activity of CS does not arise due to its direct action on free radicals, but
391 indirectly with its chelating activity (Campo et al., 2006). Low reactive radicals were
392 transferred to highly toxic hydroxyl radical using Fenton s reaction which is mediated
393 by metal cations (Campo et al., 2006). The carboxylate and sulfate groups of CS can
394 chelate metal ions, such as Fe^{2+} and Cu^{2+}, and may halt the Fenton s reaction, thereby
395 having antioxidant activity (cf. Campo et al., 2006).
396 The present study also showed that H-CSo, the highly purified CS
397 oligosaccharides, had low antioxidant activity. The precise mechanism underlying the
398 increase in antioxidant activity observed with the breakdown of CS polysaccharides
399 into oligosaccharides is not known. Arai et al. (1999) reported the production of
400 neutralizing molecules with the decomposition of glycosaminoglycans by ROS, which
401 may subsequently act as radical scavengers. Therefore, it is speculated that CS
402 oligosaccharides with low MW and simple structures may be easily decomposed by
403 ROS to produce more radical scavengers.
404 CSp had higher DPPH radical scavenging activity than H-CSp. The activity does
405 not relate to metal cations; thus, it may result from the peptides that were included in

406 CSp. Similarly, CSo had higher DPPH radical scavenging activity than H-CSo;
407 however, the difference between the activities of CSo and H-CSo was larger than that
408 between the CSp and H-CSp (Fig. 5A). This suggested that the high activity of H-CSo
409 may not be due to the peptides.
410
411 *3.3.2 Non-enzymatic browning products in CSo*
412 When CSo were produced in acidic and high-temperature environments, the
413 materials contained both sugar and peptides, and the product was brown in color. The
414 production of oligosaccharides possibly accompanies the non-enzymatic browning
415 reactions (Kroh, 1994), which include the Maillard and caramelization reactions.
416 During the reaction, high UV-absorbing but colorless compounds such as aldehydes
417 and ketones are formed in the intermediate stages, whereas brown polymers, e.g.,
418 melanoidins are produced in the final stages (Ajandouz et al., 2001). As these
419 compounds have free radical scavenging activity (Benjakul et al., 2005; Liu et al.,
420 2012), the products of the non-enzymatic browning reaction in CSo are candidate
421 antioxidants. The intermediate products absorb UV at 294 nm, whereas the final
422 products absorb at 420 nm. At both wavelengths, CSo showed higher absorbance in a
423 dose-dependent manner than Pa-CP and H-CSo (Fig. 6). These results strongly
424 suggested that the good DPPH radical scavenging activity of CSo is partially due to
425 the intermediate and final products of non-enzymatic browning reactions. Considering
426 that the sample mainly consisted of oligosaccharides, the caramelization reaction is
427 likely to be the major reaction during high-temperature hydrolysis.

428

429 *3.3.3 TFC of CSo*

430 The intermediate products of the non-enzymatic browning reaction generated
431 with sugar dehydration, sugar fragmentation, or amino acid degradation (Nie et al.,
432 2013) included small molecules, such as furans, ketones, and phenols (Liu et al., 2012;
433 Su et al., 2011). As flavonoids are a type of phenol and are effective antioxidants, the
434 TFC of CSo was determined, assuming that these were generated during the
435 non-enzymatic browning reaction. In Fig. 7, the absorbance at 437 nm indicates the
436 amount of flavonoid-like products that reacted with $AlCl_3$. These results showed that
437 CSo had a considerably higher absorbance than H-CSo (Fig. 7), suggesting that CSo
438 had flavonoid-like products that are probably produced with the non-enzymatic
439 browning reaction, and the products had been efficiently removed using the
440 purification steps of CSo to H-CSo.

441 The CSo used was composed of a mixture of CS oligosaccharides and other
442 bioactive compounds. The CSo showed strong free radical scavenging activity and
443 high intestinal absorptivity (unpublished observation). Thus, its oral administration as
444 an antioxidant may be beneficial for human health. However, the effect of the final
445 browning product (melanoidin) on human health is controversial (Bastos & Gugliucci,
446 2015); hence, melanoidin intake should be limited even if it has antioxidant activity
447 (Bastos & Gugliucci, 2015). The intermediate and final compounds of the
448 non-enzymatic browning reaction should be measured and quantified to ensure their
449 safety at higher doses.

3.4 Evaluation of in vitro *cytotoxicity*

To further confirm the suitability of Pa-CP and CSo as functional antioxidant food ingredients, their cytotoxicity in a mouse L929 fibroblast cell model was measured. The previous report had already shown that CS oligosaccharides promoted cell proliferation (Li et al., 2019). However, the potential cytotoxicity of CSo, including the intermediate and final products of the non-enzymatic browning reaction, was of concern. The metabolism of mouse L929 fibroblasts was measured using the CCK8 assay after culturing the cells in the presence of high concentrations of CSo and Pa-CP (1 mg/mL). The cellular activities of both the CSo and Pa-CP groups were similar to that of the control group after 48 h of incubation (Fig. 8), indicating that CSo and Pa-CP were not cytotoxic even at high doses. The metabolic activity of cells grown in the presence of Pa-CP was consistent with that reported in previous studies, which showed the absence of cytotoxicity in antioxidant peptides from marine by-products (Pal & Suresh, 2016; Sila & Bougatef, 2016).

Collectively, the results indicated the potential of the skate-derived Pa-CP and CSo as antioxidants in functional foods without any apparent cytotoxic effects. Although high intestinal absorptivity of both Pa-CP and CSo is expected, their functions should be further studied using *in vivo* models.

4. Conclusions

The antioxidant components of skate cartilage were investigated to maximize the

use of resources and to improve the efficiency of the fishery processing industry. High free radical scavenging activities in Pa-CP and CSo derived from skate by-products were measured. The antioxidant activity of Pa-CP was related to their MW, with the <3 kDa-MW Pa-CP fraction having stronger antioxidant activity than that of larger peptides. Pure CS (H-CSp and H-CSo) showed almost no free radical scavenging activity, whereas thermally-hydrolyzed CSo showed good free radical scavenging activity because of the non-enzymatic browning products produced during thermal hydrolysis. CSo possibly includes flavonoid-like products that have antioxidant properties. CSo and Pa-CP were not cytotoxic and are likely to have high intestinal absorptivity. Therefore, these products have a potential to be valuable antioxidants in functional foods. These results might be significant for achieving zero-waste during marine processing and improving the efficiency of the skate-processing industry. Future studies should examine the *in vivo* effects of dietary CSo and Pa-CP as antioxidants.

Acknowledgments

This study was supported in part by a 2019 Grant-in-Aid for the Research and Development for the Recycle Technology from the Hokkaido Local Government Office. Authors thank the Editage service (www.editage.jp) for English language editing.

Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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600
601
602

603 **Figure legends**

604 **Fig. 1.** Alcian blue (A) and Coomassie Brilliant Blue (B) stained Tricine-SDS-PAGE.
605 M, protein markers; CSp, chondroitin sulfate polysaccharides; CSo, thermally
606 hydrolyzed chondroitin sulfate oligosaccharides; Tr-CP, trypsin hydrolyzed collagen
607 peptides; Ch-CP, chymotrypsin hydrolyzed collagen peptides; Pa-CP, papain
608 hydrolyzed collagen peptides.

609

610 **Fig. 2.** DPPH (A) and ABTS (B) radical scavenging activity of collagen peptides (CP).
611 Values are expressed as the mean and standard errors (n = 3). Vc, vitamin C; Tr-CP,
612 trypsin hydrolyzed CP; Ch-CP, chymotrypsin hydrolyzed CP; Pa-CP, papain
613 hydrolyzed CP.

614

615 **Fig. 3.** Molecular weight distribution of collagen peptides (CP). Tr-CP, trypsin
616 hydrolyzed CP; Ch-CP, chymotrypsin hydrolyzed CP; Pa-CP, papain hydrolyzed CP.

617

618 **Fig. 4.** ABTS radical scavenging activity of papain hydrolyzed collagen peptides
619 (Pa-CP) separated into fractions by molecular weight. The columns and bars show the
620 mean values and standard errors, respectively (n = 3). Different letters indicate
621 statistically significant differences ($p<0.01$, Tukey-Kramer test). Pa-CP Whole, whole
622 Pa-CP; Pa-CP >10, Pa-CP >10 kDa; Pa-CP 3–10, Pa-CP between 3–10 kDa; Pa-CP <3,
623 Pa-CP <3 kDa.

624

Fig. 5. DPPH (A) and ABTS (B) radical scavenging activity of different types of chondroitin sulfate (CS). Values are expressed as means ± standard errors, respectively (n = 3). CSo, thermally hydrolyzed CS oligosaccharides; H-CSo, high-purity CS oligosaccharides; CSp, CS polysaccharides; H-CSp, high-purity CS polysaccharides.

Fig. 6. UV-absorbance (A) and brown color (B) intensity of chondroitin sulfate (CS) oligosaccharides and papain hydrolyzed collagen peptides. CSo, thermally hydrolyzed CS oligosaccharides; H-CSo, high-purity CS oligosaccharides; Pa-CP, papain hydrolyzed collagen peptides.

Fig. 7. Total flavonoid content of chondroitin sulfate (CS) oligosaccharides. The columns and bars show the mean values and standard errors, respectively (n = 3). CSo, thermally hydrolyzed CS oligosaccharides; H-CSo, high-purity CS oligosaccharides.

Fig. 8. Cytotoxicity of thermally hydrolyzed chondroitin sulfate (CS) oligosaccharides and papain hydrolyzed collagen peptides. Values are expressed as means ± standard errors (n = 10). EFW, endotoxin-free water; CSo, thermally hydrolyzed CS oligosaccharides; Pa-CP, papain hydrolyzed collagen peptides. Sample concentration was 1 mg/mL.

Fig. 1

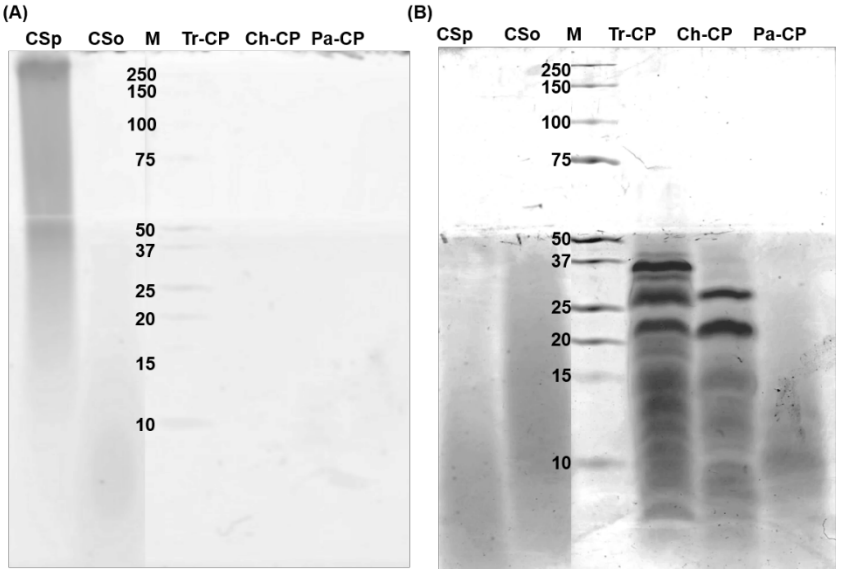

(A)
CSp
CSo
M
Tr-CP
Ch-CP
Pa-CP
250
150
100
75
50
37
25
20
15
10
(B)
CSp
CSo
M
Tr-CP
Ch-CP
Pa-CP
250
150
100
75
50
37
25
20
15
10

Fig. 2

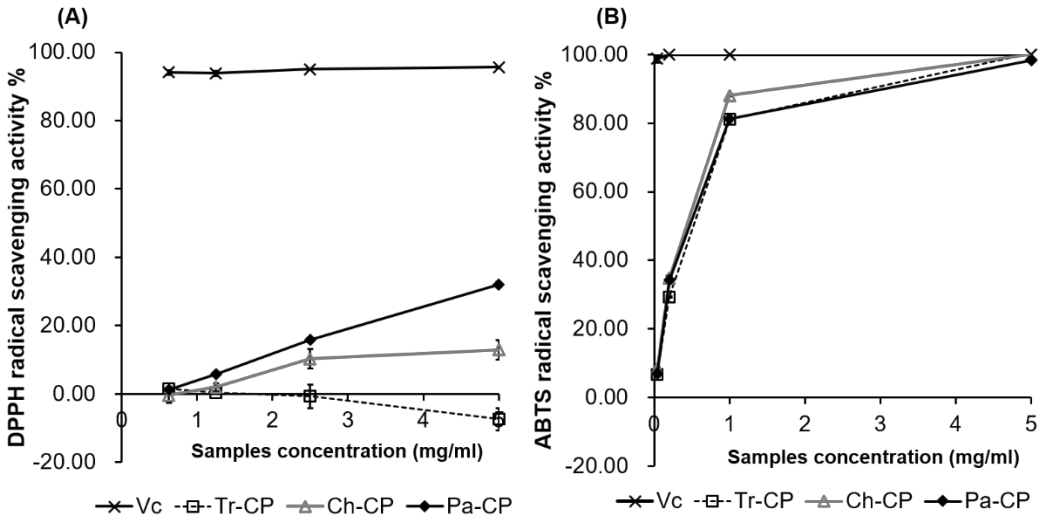

(A)
DPPH radical scavenging activity %
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
0
1
2
3
4
5
Samples concentration (mg/ml)
Vc
Tr-CP
Ch-CP
Pa-CP
(B)
ABTS radical scavenging activity %
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
0
1
2
3
4
5
Samples concentration (mg/ml)
Vc
Tr-CP
Ch-CP
Pa-CP

Fig. 3

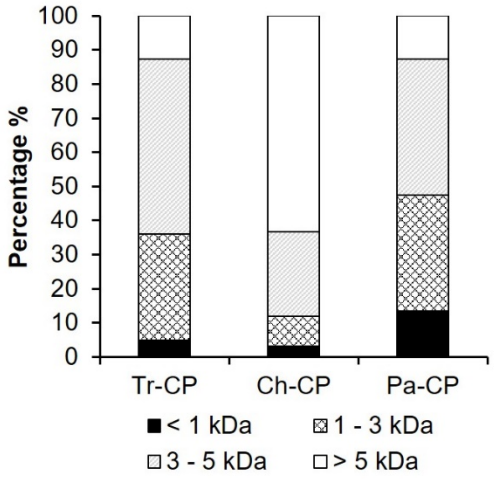

Percentage %
100
90
80
70
60
50
40
30
20
10
0
Tr-CP
Ch-CP
Pa-CP
< 1 kDa
1 - 3 kDa
3 - 5 kDa
> 5 kDa

Fig. 4

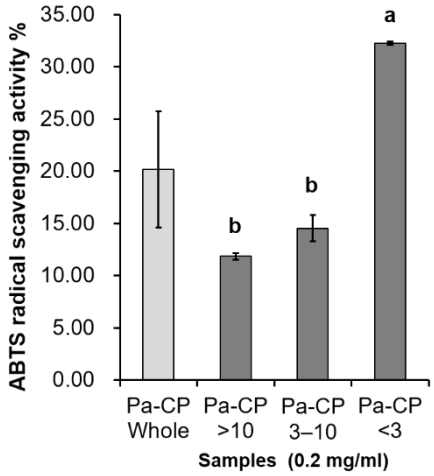

ABTS radical scavenging activity %
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
a
b
b
Pa-CP Whole
Pa-CP >10
Pa-CP 3–10
Pa-CP <3
Samples (0.2 mg/ml)

Fig. 5

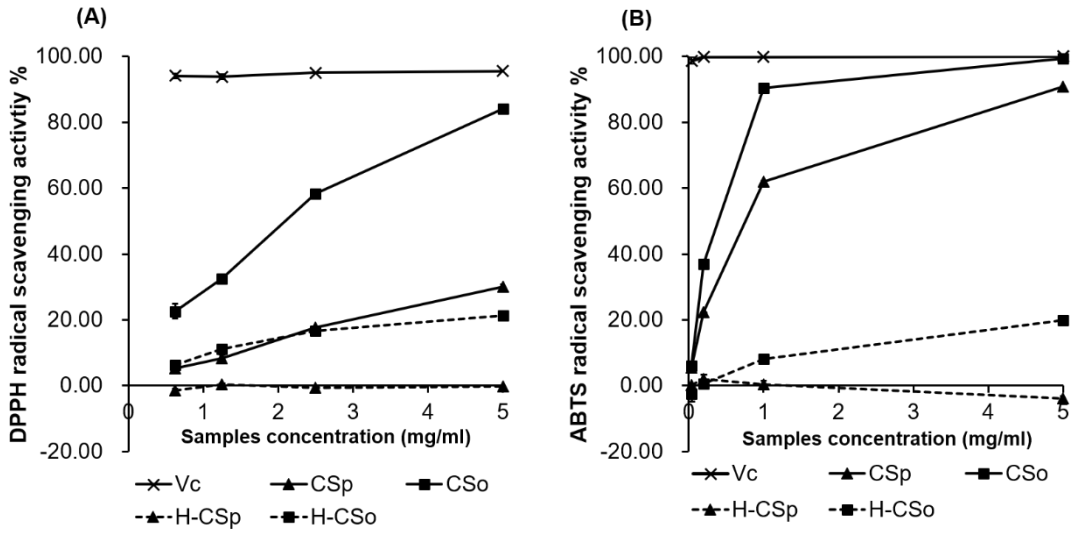

(A)
DPPH radical scavenging activtiy %
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
0
1
2
3
4
5
Samples concentration (mg/ml)
Vc
CSp
CSo
H-CSp
H-CSo
(B)
ABTS radical scavenging activity %
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
0
1
2
3
4
5
Samples concentration (mg/ml)
Vc
CSp
CSo
H-CSp
H-CSo

Fig. 6

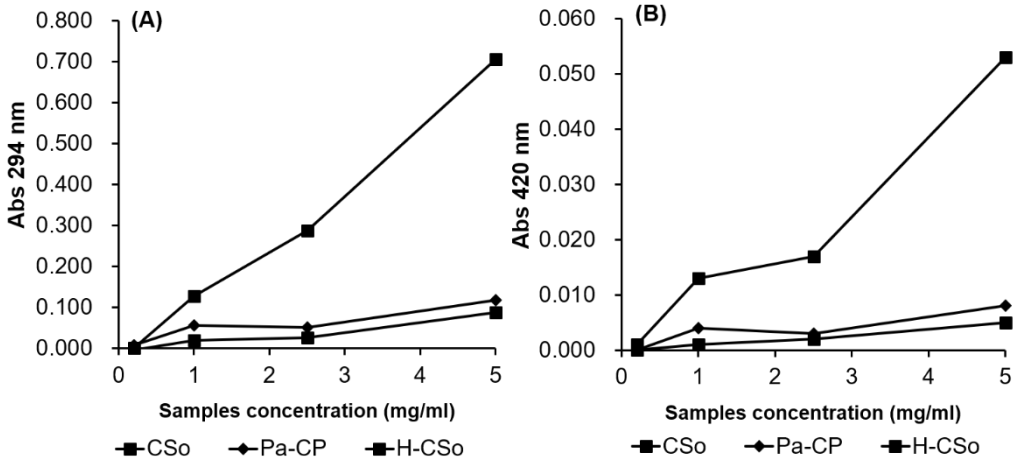

(A)
Abs 294 nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
1
2
3
4
5
Samples concentration (mg/ml)
CSo
Pa-CP
H-CSo
(B)
Abs 420 nm
0.060
0.050
0.040
0.030
0.020
0.010
0.000
0
1
2
3
4
5
Samples concentration (mg/ml)
CSo
Pa-CP
H-CSo

Fig. 7

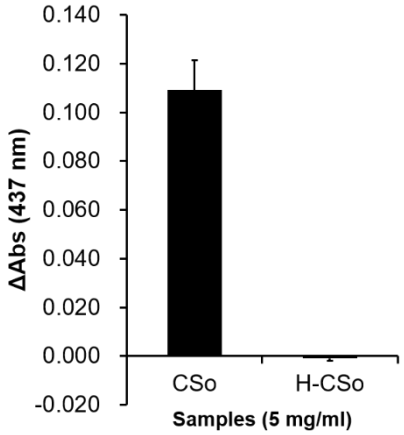

0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
-0.020
ΔAbs (437 nm)
CSo
H-CSo
Samples (5 mg/ml)

Fig. 8

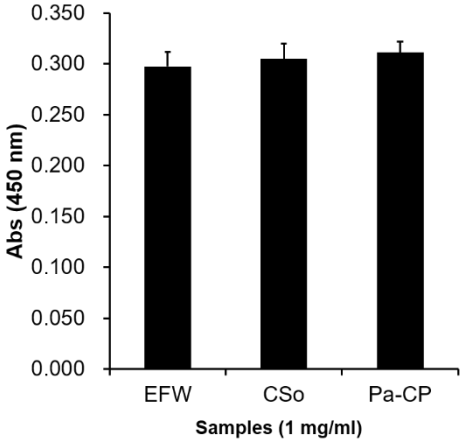

0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
Abs (450 nm)
EFW
CSo
Pa-CP
Samples (1 mg/ml)

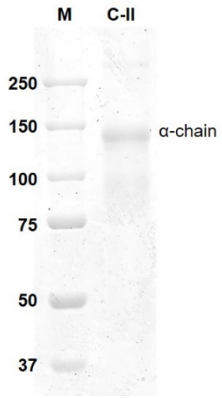
Figure legends

Supplementary Fig. 1. SDS-PAGE analysis of skate type II collagen with Coomassie Brilliant Blue stain. M, protein markers.

Supplementary Fig. 2. Tricine-SDS-PAGE analysis of H-CSp and H-CSo. No peptides were stained using Coomassie Brilliant Blue stain in H-CSp and H-CSo. M, protein markers; H-CSp, High-purity chondroitin sulfate polysaccharides; H-CSo, High-purity chondroitin sulfate oligosaccharides. Line 1, 2 and 3, Coomassie Brilliant Blue stain; line 4 and 5, Alcian blue stain.

914 Supplementary Fig. 1.

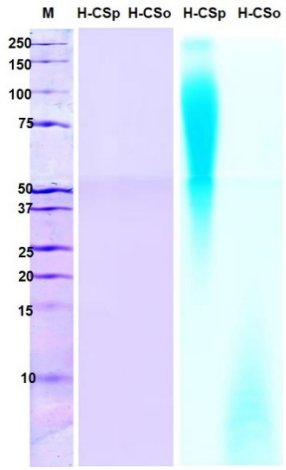
915



916

917

Supplementary Fig. 2.



Highlights

- Japanese skate processing industry produces large amounts of by-products (mainly cartilage).
- Chondroitin sulfate (CS) and type II collagen were extracted from the by-products.
- Papain hydrolysis of collagen produced antioxidant peptides.
- Thermal hydrolysis of CS produced browning products with antioxidant properties.
- These substances can possibly be absorbed by the intestines and are suitable as food.