Materials Science and Engineering C 33 (2013) 174–181

Contents lists available at SciVerse ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Studies on fish scale collagen of Pacific saury (*Cololabis saira*)

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ARTICLE INFO

Article history:
Received 25 April 2011
Received in revised form 14 July 2012
Accepted 12 August 2012
Available online 19 August 2012

Keywords:
Fish scale
Collagen
Fibril
Pacific saury
Denaturation temperature
Gel

ABSTRACT

We purified and characterized Type I collagen from the scales of the Pacific saury (*Cololabis saira*) and compared it with collagen from other organisms. Subunit composition of *C. saira* collagen ($2\alpha1+\alpha2$) was similar to that of red sea bream (*Pagrus major*) and porcine collagen. *C. saira* collagen did not form a firm gel after neutralization of pH in solution. The temperature of denaturation (24–25 °C) of *C. saira* collagen was slightly lower than that of *P. major* collagen (26–27 °C). The contents of proline and hydroxyproline were lower in red sea bream and Pacific saury collagen than in porcine collagen. Circular dichroism spectra and Fourier-transformed infrared spectra showed that heat denaturation caused unfolding of the triple helices in all three collagens.

1. Introduction

Although there is considerable progress in fabrication of artificial materials including various nanomaterials [1–3], materials from natural resource keep their values in practical applications [4–6]. The Pacific saury (*Cololabis saira*) is a popular edible fish caught and commonly eaten in Japan. Catch of this species in the Japanese fishery is approximately 200–300 million tons per year (http://www.maff.go.jp/j/tokei/pdf/gyogyou_seisan_10c.pdf, data were written in Japanese). A large portion of the fish catch comes from the area of the Oyashio Current near the northern part of Japan [7,8].

The scales of *C. saira* are discarded as waste in the fisheries industry because they can be easily removed from the body onboard the fishing vessel. However, these scales have a number of potential uses. We are interested in developing a novel way to use the proteins, polysaccharides, and other biological polymers contained in this bioresource that traditionally is discarded. In this study, we isolated collagen from the scales of Pacific saury. This species has not been as well studied as other edible fishes such as salmon, cod, flounder, and sea bream [9,10].

Fish scales are classified into several categories [11]. The scale is a very special tissue that exhibits distinct species- or group-specific heterogeneity in morphology and characteristics [11]. For example, scales of Chondrichthyes are placoid, and scales of Osteichthyes are classified into several types such as cosmoid, ganoid, leptoid (ctenoid, and cycloid). Red sea bream (*Pagrus major*) have ctenoid scales, whereas Pacific saury have cycloid scales.

The surface of a fish scale is an osseous layer consisting of randomly oriented collagen fibrils with many hydroxyapatite ($Ca_{10}(O_4)_6(OH)_2$) crystals. Thin layers of the oriented collagen fibrils are piled up to form the fibrillary plate under the osseous layer [12]. The direction of the fibrils in each thin layer differs from each other. This plywood-like hierarchical supramolecular structure provides the high mechanical strength of scales, and it is of great interest to researchers in the field of biomaterials [12–17]. Fish collagen is present not only in scales but also in skin, bones, fins, and cartilage [9,10]. Scales are completely inedible, whereas fish skin is sometimes eaten. Therefore, fish scale collagen is the focus of studies aimed at using fish collagen for various purposes, including food production, industrial use, medical use, and promoting more effective use of marine bioresources.

Although collagens from various fish species have been extracted from scales and skin and characterized [12–23], this study is the first to describe Type I collagen from the Pacific saury. We purified and characterized Type I collagen from the scales of *C. saira* and compared it with red sea bream and porcine collagen.

2. Materials and methods

2.1. Materials

The Eishin Kasei Company, Rausu, Hokkaido Japan provided the scales of *C. saira*. Red sea bream were purchased from a local fish market in Sakai-city, Osaka, Japan. Porcine Type I collagen solution (0.6% (w/v), pH 3.0, Collagen BM, Nitta Gelatin, Osaka, Japan), Pepstatin A (M9T3556,

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http://dx.doi.org/10.1016/j.msec.2012.08.025

Nacalai tesque, Kyoto, Japan), a protein assay kit (500-0113,4,5, BioRad, Hercules, CA, USA), phenol (V0G3204, Nacalai tesque), ninhydrin (TFN5028, Wako, Osaka, Japan), buffer solution for amino acid analysis (TFN5028, Wako), standard solution for amino acid analysis (EPM1075, Wako), hydroxylysine (5-hydroxy-DL-lysine hydrochloride)(KWH4600, Wako), hydroxyproline (L-hydroxyproline)(KLL4255, Wako), cellulose tubes (5015-19, Membrane Filtration Products, Inc., Braine-l'Alleud, Belgium), molecular weight markers (LC5801.pps, Invitrogen, Carlsbad, CA, USA), and other chemicals and salts of specific grade were used. Isotonic phosphate buffered saline (PBS) consisted of 0.8 g/l NaCl, 0.02 g/l KCl, 0.02 g/l KH_2PO_4 (anhydrous), and 0.115 g/l Na_2PO_4 (anhydrous).

2.2. Scanning electron microscope (SEM) observation of the fish scales

Scales from both fish species were washed thoroughly in distilled water and lyophilized in a freeze dryer (FD-1, Eyela, Tokyo, Japan). Samples were coated with platinum using ion-sputtering equipment (E1010, Hitachi, Tokyo, Japan). Fine structures on the outer and inner sides of the scales were observed using a Hitachi SU1510 SEM [24,25].

2.3. Extraction of collagen from the scales

Collagen was extracted from the scales at 4 °C to minimize protein denaturation. Scales were washed and lyophilized as described above, weighed, and then incubated for 1 week in a solution consisting of 50 mM Tris HCl (pH 7.5) and 20 mM sodium ethylenediaminetetraaceticacid (Na-EDTA). Scales were washed again with distilled water and incubated for 2 days in another solution consisting of 500 mM acetic acid and 0.5% (w/v) pepsin. NaCl was added to the solution to generate a final concentration of 0.7 M. The solution was incubated overnight. After addition of pepstatin A (5 μl/ml), the mixture was incubated overnight and then centrifuged for 30 min at 10,000 rpm (CX-210, Tomy, Tokyo, Japan). The precipitate was collected and dissolved in 500 mM acetic acid solution to obtain a collagen solution. Protein concentration was measured by the Bradford method using a protein assay kit to estimate the yield of the preparation.

2.4. SEM observation of collagen gels

Collagen solution of either *P. major* or *C. saira* (10 ml) in 500 mM acetic acid was dialyzed for 1 week at 4 °C in a cellulose tube against 1 l of 1 mM HCl solution to exchange the solvent. The collagen solution was mixed with a 1 mM HCl/10-fold concentrated PBS/distilled water (5:1:4 volume ratio) to neutralize the pH, and it was incubated at 20 °C to form collagen fibrils. The collagen gel in the test tube was incubated for 3 h at 4 °C in PBS containing 2.5% (w/v) glutaraldehyde to crosslink the protein. The sample was washed for 1 h at 4 °C in PBS, incubated for 1 h in ethanol at 4 °C, and then incubated twice for 20 min each at 37 °C in *t*-butyl alcohol. The gel was wrapped in plastic film, frozen for 10 min at −80 °C in a deep freezer, and lyophilized in a freeze dryer overnight. The sample then was coated with platinum and observed by SEM as described above [24,25].

2.5. Amino acid analysis

Red sea bream collagen, and Pacific saury collagen were extracted as described above, lyophilized for 3–4 h, and then weighed. An acid solution consisting of 6 M HCl and 0.1% (w/v) phenol was added to the sample (3 mg/1 ml) in a glass vial with a mininert valve. After the atmosphere in the vial was degassed and twice replaced with nitrogen gas, the sample was hydrolyzed under nitrogen for 24 h at 110 °C in an oil bath. The hydrolyzed sample was dissolved in 0.1 M HCl solution and transferred to a vacuum flask. Acid was removed by five repeated cycles of evaporation in vacuum followed by redissolution in distilled water. The sample then was dissolved in 20 mM HCl solution, and the concentration was adjusted to 0.1 nmol/μl. The sample was filtered to remove dust, introduced into a vial, and used for the amino acid analysis, which was performed using an Amino Acid Analyzer (L-8500A, Hitachi). The ninhydrin reaction was used to measure the concentration of amino acids in the samples. Briefly, ninhydrin reagent (500 μl), the buffer solution (500 μl), and the sample solution (5 μl) were mixed and heated for 3 min at 100 °C. Absorbance at 570 nm was measured to estimate the concentration of amino acids using a UV–visible spectrophotometer (U-2000, Hitachi). A standard amino acid solution was used to calibrate the ΔA_{570} value. Porcine collagen was also hydrolyzed by the same procedure, and then used as a control.

2.6. Circular dichroism (CD) spectra

CD spectra of the collagen samples were measured using a CD spectrophotometer (J-720, Jasco, Tokyo, Japan) [24]. Collagen was diluted in 0.2 M sodium phosphate buffer (pH 7.4) to a concentration of 0.1% (w/v). The sample was placed in a quartz cuvette (light path length: 1 mm; S10-UV-1, GL Science, Tokyo, Japan). The temperature was raised in 2 °C intervals from 10 °C to 40 °C, followed by incubation for 5 min before each measurement. An incubator (LP-3100, Advantec, Tokyo, Japan) was used to control the temperature.

2.7. Fourier-transformed infrared (FTIR) spectra

Native collagen solution was incubated for 30 min at 80 °C to prepare heat-denatured collagen. Aliquots (1–4 ml) of native collagen solution (0.1–0.6% (w/v)) and heat-denatured collagen were dried in a culture dish ($\varphi = 3$ cm) for several days at 10 °C to prepare dry films. The dry films were peeled off the dish and used to measure FTIR spectra at 22 °C using a spectrophotometer (NICOLET 8700, Thermo Scientific, Waltham, MA) with a detector (DTGS KBr) and a sample window of BaF_2. The scan was repeated 1024 times to average the data in the range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} (data interval 1.928 cm^{-1}).

3. Results

3.1. Structure of the fish scale surface

Fig. 1A shows a red sea bream scale (left) and a Pacific saury scale (right); the former is colorless (white) and the latter is blue. SEM images of the outer surface (Fig. 1B, C) and inner surface (Fig. 1D, E) of a red sea bream scale and of the outer surface (Fig. 1F, G) and inner surface (Fig. 1H, I) of a Pacific saury scale also are shown. For both fish species, characteristic growth rings (annual ring-like structures) are visible on the outer side of the scale (Fig. 1B, C, F, G) and parallel collagen fibril-like structures without growth rings are visible on the inner side of the scale (Fig. 1D, E, H, I). Many sawtooth-like projections (ctenii) are arrayed in a line at the edge of the growth rings in the ctenoid scale of the red sea bream (Fig. 1C), but they are absent in the cycloid scale of the Pacific saury (Fig. 1G). This is the typical morphological difference between a ctenoid scale and a cycloid scale.

3.2. Extraction of collagen

Yield of extracted collagen from the scales was relatively variable depending on the batch of preparation: 7–26% in red sea bream and 4–15% in Pacific saury. Purity of the Type I collagen was high, as indicated by the SDS-PAGE [26] results shown in Fig. 2. A thick α1 subunit protein band and another closely associated α2 subunit protein band of less thickness were observed in collagen from both species of fish and in porcine collagen. These bands represent the typical composition of