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Biochemical characterisation and assessment of fibril-forming ability of collagens extracted from Bester sturgeon *Huso huso* × *Acipenser ruthenus*

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ABSTRACT

Collagens purified from Bester sturgeon organs were characterised biochemically, and their fibril-forming abilities and fibril morphologies formed *in vitro* clarified. Yields of collagens were 2.1%, 11.9%, 0.4%, 18.1%, 0.4%, 0.8% and 0.03% (collagen dry weight/tissue wet weight) from scales, skin, muscle, swim bladder, digestive tract, notochord and snout cartilage, respectively. Using SDS–PAGE and amino acid composition analyses, collagens from scales, skin, muscle, the swim bladder and digestive tract were characterised as type I, and collagens from the notochord and snout cartilage as type II. Denaturation temperatures of the collagens, measured using circular dichroism, were 29.6, 26.8, 29.0, 32.9, 31.6 and 36.3 °C in scales, skin, muscle, swim bladder, digestive tract, and notochord, respectively. For fibril formation, swim bladder and skin collagen showed a more rapid rate of increase in turbidity, a shorter time to attain the maximum turbidity, and formed thicker fibrils compared with porcine tendon type I collagen.

1. Introduction

Collagen, in the form of elongated fibrils, contributes to the physiological functions and mechanical properties of skin, tendon, bone, cartilage and other tissues (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003). Collagen has been widely used in many industries such as the food, photographic film, cosmetic, and leather industries (Zhang, Liu, & Li, 2009). Furthermore, because of its low antigenic activity, high cell adhesion properties, biocompatibility, and biodegradability, collagen-based biomaterials have been applied in medical research, as well as tissue engineering (Parenteau-Bareil, Gauvin, & Berthod, 2010). The most common sources of collagen for biomaterials and tissue engineering are bovine skin and tendons, porcine skin, and rat tail (Parenteau-Bareil et al., 2010). However, concerns about the use of land animal collagens are growing because of the risk of common human diseases. Religious beliefs also restrict usage of porcine or bovine collagens in certain regions in the world. In recent years, as an alternative source to porcine or bovine collagen, fish collagen has received increasing attention, for its advantages of using highly abundant fish offal, and avoiding human diseases and religious objections (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005a). The biochemical nature of collagens extracted from fish skin, scales, and bones have been reported in many studies (Duan, Zhang, Du, Yao, & Konno, 2009; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Nagai & Suzuki, 2000; Singh, Benjakul, Maqsood, & Kishimura, 2011; Zhang et al., 2009).

Sturgeon is highly valued as a food fish, and is especially famous for its caviar. Contemporary sturgeon culture was begun in the 1980s, and worldwide production had increased to ca. 25,000 t by 2008 (Zhang, Shimoda, Ura, Adachi, & Takagi, 2012). Thus, sturgeon culture now forms the basis of an important and promising industry. However, the culture cost of sturgeon is higher than other fishes, because caviar production requires long culture times. On the other hand, the lack of utilisation of other parts of sturgeon constrains the development of its culture. Therefore, we believe that if skin, offal and cartilage of sturgeon are used as a source of collagen, the value of the by-products would increase, and waste disposal problems could be alleviated.

Zeng et al. (2012) have reported the structure and characteristics of collagen extracted from cobia *Rachycentron canadum* skin for the utilisation in biomaterials, which should increase the value of cobia offal. However, the important collagen property, the fibril-forming ability, and morphology for biomaterial utilisation has not been reported. The collagen molecule is distinct from other proteins as its three polypeptide chains (α-chains) form a unique triple helical structure. The molecules self-assemble into fibrils *in vitro* when collagen solution is adjusted to an appropriate temperature, pH, and ionic strength (Kadler, Holmes, Trotter, & Chapman, 1996). After fibril formation, the low denaturation temperature of fish collagen can be improved to more than 40 °C, and

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therefore it is possible for use as a biomaterial for humans (Bae et al., 2009). However, there are few studies about the fibril-forming ability of fish collagens.

Bester, a hybrid sturgeon of *Huso huso* × *Acipenser ruthenus*, is suited to aquaculture because of its rapid growth, ability to grow in freshwater ponds, and the relative ease of inducing sexual maturation (Zhang et al., 2012). This fish is now widely cultured in China (Wei, Zou, Li, & Li, 2011) and Japan. The objective of this study was to extract and assess the biochemical nature of the collagens from the Bester sturgeon. In addition, the fibril-forming ability and morphology of fibrils formed *in vitro* were investigated.

2. Materials and methods

2.1. Extraction of collagens

A live cultured Bester sturgeon (length = 0.76 m, 2.00 kg) was procured from the Nanae Fresh-Water Laboratory, Field Science centre for Northern Biosphere, Hokkaido University, Japan. The fish was deeply anaesthetized in 2-phenoxyethanol solution and gutted. Scales, skin, muscle, swim bladder, digestive tract, notochord and snout cartilage were removed and washed with chilled tap water, then stored at −30 °C until use. Samples were washed with cold distilled water (4 °C) and cut into small pieces (0.5 × 0.5 cm). Skin fat was removed over 24 h in 99.5% ethanol (three solution changes) with a sample/solution ratio of 1:10. Scales and snout cartilage samples were removed, non-collagenous proteins extracted in 0.1 M NaOH with a sample/solution ratio of 1:10 for 6 h, and then samples were decalcified in 0.5 M EDTA with a sample/solution ratio of 1:10 for 24 h. After that, skin, scales and cartilage samples were washed with cold water for 24 h. To extract collagens, the samples were stirred continuously in a solution of HCl (pH 2.0) containing 0.1% (w/v) porcine pepsin (EC 3.4.23.1, 1:10,000, Wako Pure Chemical Industries Ltd., Osaka, Japan) with a sample/solvent ratio of 1:10 (w/v) for 48 h at 10 °C. The mixtures were centrifuged at 2000*g* for 1 h to get supernatants, and the residue was re-extracted in the same conditions. The supernatants were sequentially filtered with membrane filters with pore sizes of 3.0, 0.8, and 0.47 μm (Advantec, Tokyo, Japan). Collagen in the filtrate was precipitated by adding NaCl to a final concentration of 1 M. The resulting precipitate was collected by centrifugation at 2000*g* at 4 °C for 90 min, and dissolved in an HCl solution (pH 2.0). This process was repeated three times to purify the collagen. The purified collagen was dialyzed against 50 volumes of distilled water at 4 °C for 24 h with two changes of water. The dialysate was lyophilized by freeze dryer (FDU-830, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The percentage of dry weight of collagen extracted in comparison with the wet weight of the initial tissues was calculated as the collagen yield.

2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) analysis

SDS–PAGE was performed according to the method of Laemmli (1970). The lyophilized collagens were dissolved in HCl (pH 3.0, 3 mg collagen/ml), and then mixed at a ratio of 1:2 (v/v) with the sample buffer (0.5 M Tris–HCl buffer, pH 6.8, with 4% SDS and 20% glycerol) containing 10% β-mercaptoethanol. The mixed solution was boiled for 5 min. Ten micrograms of protein were loaded in each lane. Electrophoresis was performed at 15 mA for the stacking gel and 20 mA for the 7.5% running gel. After electrophoresis, the gel was stained for 30 min using 0.1% Coomassie Brilliant Blue R250 solution and destained with a mixture of 20% ethanol, 5% acetic acid and 2.5% glycerin. Precision plus protein standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to estimate the molecular weight. Quantitative analysis of band intensity was determined by AE-6932GXES Printgraph (ATTO Co., Tokyo, Japan) with CS Analyzer ver 3.0 (image analysis system).

2.3. Amino acid analysis

Samples were hydrolysed in 6 N HCl at 110 °C for 24 h. The hydrolysates were evaporated, and the remaining materials dissolved in citric acid buffer solution, and then analysed using an automated amino acid analyzer (JLC-500 V, JEOL Ltd., Tokyo, Japan). Samples were assayed three times and the averages were used to obtain amino acid compositions. In this study, the number of cysteine residues was obtained by first determining the number of cystine residues (a dimeric amino acid) then calculating cysteine from cystine. The number of tryptophan residues was not determined.

2.4. Circular dichroism (CD) measurement

CD spectra were measured using a JASCO model 725 spectrometer (JASCO, Tokyo, Japan). The measurement was performed according to the method of Ikoma et al. (2003). Lyophilized collagens were dissolved in an HCl solution (pH 3.0) to 1 mg/ml, and placed into a quartz cell. CD spectra were measured at 190–250 nm wavelengths at 10 °C under a scan speed of 50 nm/min with an interval of 0.1 nm. Then a rotatory angle at a fixed wavelength of 221 nm was measured at 10–50 °C with a rate of 1 °C/min to determine the collagen denaturation temperature (Td).

2.5. Collagen fibril formation in vitro

Collagen molecules self-assemble into fibrils *in vitro* (fibril formation) when collagen solution is adjusted to an appropriate temperature, pH, and ionic strength. Fibril formation of collagen from the skin and swim bladder was performed according to the method of Bae et al. (2009). Lyophilized collagens were dissolved in an HCl solution (pH 3.0) to 0.3% (w/v). The collagen solution was mixed with 0.1 M Na-phosphate buffer (pH 7.4) containing NaCl at 0, 70 and 140 mM. The ratio of the collagen solution/Na-phosphate buffer was 1:2 (v/v), and the final pH of the solution was 7.4. Porcine tendon collagen (Cellmatrix Type I-A, 0.3%, Nitta Gelatin Inc., Osaka, Japan) was used as a control. With fibril formation, the transparent solution would become white. The mixed solution was placed into a cell, and the resulting fibril formation at 21 ± 1 °C was monitored by the absorbance at 320 nm as the turbidity change using a spectral monitor GeneQuant *pro* (GE Healthcare Life Sciences, Tokyo, Japan). This is the measurement on the speed of collagen fibril formation in a short time.

2.6. Measurement of degree of collagen fibril formation

Skin and swim bladder collagen fibrils as well as porcine tendon collagen fibrils were formed for 24 h at 21 ± 1 °C, which was assumed to be the endpoint of fibril formation, using the same conditions as described above. Then the sample solution was centrifuged at 20,000*g* for 20 min to precipitate fibrils, and the protein content of the supernatant was measured based on the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as a standard. Degree of collagen fibril formation was defined as the percentage of the decrease of the collagen concentration in the solution after the experiment, which means the percent of collagen molecules that formed the fibrils.