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# Isolation and characterization of acid-soluble collagen from the scales of marine fishes from Japan and Vietnam

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A B S T R A C T

Acid-soluble collagen (ASC) was successfully extracted from the scales of lizard fish (*Saurida* spp.) and horse mackerel (*Trachurus japonicus*) from Japan and Vietnam and grey mullet (*Mugil cephalis*), flying fish (*Cypselurus melanurus*) and yellowback seabream (*Dentex tumifrons*) from Japan. ASC yields were about 0.43–1.5% (on a dry weight basis), depending on the species. The SDS–PAGE profile showed that the ASCs were type I collagens, and consisted of two different α chains, α1 and α2, as well as a β component. ASC of horse mackerel from Vietnam contained a higher imino acid level than that from Japan. ASC denaturation temperature ($T_d$) ranged from 26 to 29 °C, depending on fish species and imino acid content ($p < 0.01$). Maximal solubility of individual collagens was observed at pHs 1–3. Collagen solubility decreased sharply at NaCl concentrations >0.4 M, regardless of fish type.

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## 1. Introduction

Collagen is the fibrous protein constituent of skin, bone, tendon and other connective tissues in land-based animals. There are 29 genetically distinct collagens present in animal tissues. Among them, type I collagen is the most abundant, and has found wide application in various fields, such as food, biomedical and pharmaceutical products, and in the beauty and cosmetic industries (Sobral et al., 2001; Duan, Zhang, Du, Yao, & Konno, 2009).

Generally, the main sources of commercial collagen have been cows and pigs. However, concerns regarding bovine and porcine health, such as the emergence of bovine spongiform encephalopathy and foot and mouth disease, have led to a declining supplying of mammalian collagen in recent years (Kittiphattanabawon, Benjakul, Visessaguan, Nagai, & Tanaka, 2005). Therefore, fish collagen is a realistic alternative to mammalian collagen (Nagai, Araki, & Suzuki, 2002; Nomura, Sakai, Ishii, & Shirai, 1996).

Marine fish species and their processed products are very important foodstuffs in Japan (Nagai and Suzuki, 2000a; Nagai and Suzuki, 2000b). Lizard fish, horse mackerel, grey mullet, flying fish and yellowback seabream are the raw materials used to produce *surimi* in Japan (Shimizu, 1987; Yamanaka & Tanaka, 2007). Horse mackerel is also used for preparing raw fresh *sashimi* and *sushi*, which are popular foods in Japan (Yamanaka & Tanaka, 2007). In Vietnam, marine fish also plays an important role in the development of seafood as an economic product. Lizard fish and horse mackerel are the raw materials used to produce fish balls in Vietnam (CTU, http://www.ctu.edu.vn/colleges/aquaculture/aquafishdata. Accessed 31.01.13). However, during fish processing, a large amount of byproduct, such as skin, scales and bone, which accounts for 50–70% of fish weight, is discarded (Kittiphattanabawon et al., 2005). Utilisation of marine waste, including scales, is necessary from the viewpoints of both environmental conservation and the development of new industries.

Collagen from the scales of carp (*Cyprinus carpio*) (Duan et al., 2009), spotted golden goatfish (*Parupeneus heptacanthus*) (Matmaroh, Benjakul, Prodpran, Encarnacion, & Kishimura, 2011), sardine (*Sardinops melanostictus*), red seabream (*Pagrus major*) and Japanese sea bass (*Lateolabrax japonicus*) (Nagai, Izumi, & Ishii, 2004) and deep-sea redfish (*Sebastes mentella*) (Wang et al., 2008) has been isolated and characterised. From the literature, it can be predicted that collagen from different fish species differs in molecular composition and functional properties. However, no information exists on scale collagen of commercially important species in Japan and Vietnam, such as horse mackerel and lizard fish. Moreover, sea water temperature is also presumed to affect fish scale collagen properties. Jongjareonrak, Benjakul, Visessaguan, and Tanaka (2005) reported the effects of environmental and body temperatures on the collagen properties of bigeye snapper skin. However, comparative studies of the effect of habitat temperature on the collagen properties of fish scales are lacking.

Thus, the objective of this study was to characterize acid-soluble collagen (ASC) from the scales of lizard fish and horse mackerel caught in Japan and Vietnam and grey mullet, flying fish, and yellowback seabream from Japan. The effects of environmental temperature on fish scale properties were also studied by analysing

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differences between lizard fish and horse mackerel caught in Japan and Vietnam.

## 2. Materials and methods

### 2.1. Preparation of fish scales

Scales of lizard fish (*Saurida* spp.), grey mullet (*Mugil cephalis*) and yellowback seabream (*Dentex tumifrons*) were collected on November, 2011 in Miyazaki Prefecture, Japan. Horse mackerel (*Trachurus japonicus*) and flying fish (*Cypselurus melanurus*) were collected on January, 2012 in Nagasaki Prefecture and Chiba Prefecture, Japan, respectively. Scales from lizard fish and horse mackerel were also collected from a frozen seafood company on January, 2012 in Nha Trang City and Kien Giang Province, Vietnam, respectively. The scales obtained in Japan were transported to our laboratory under iced condition, and the samples in Vietnam were frozen before airfreighted under frozen condition. The scales were washed with chilled distilled water before being cut into small pieces with scissors, placed in polyethylene bags and then stored at −20 °C until use. The storage time was 3 months or less.

### 2.2. Determination of protein and ash contents

Moisture, ash and protein contents in the scales of marine fishes were analysed according to the AOAC methods (2000). A conversion factor of 5.95 was used for calculating protein content (Wang et al., 2008).

### 2.3. Extraction of collagen from fish scales

Collagen was extracted following the method of Nagai and Suzuki (2000a) with slight modifications. Fish scales were removed from the non-collagenous protein with 0.1 M NaOH for 6 h at a sample/NaOH solution ratio of 1:8 (w/v). The NaOH solution was changed after 3 h and washed fully in cold distilled water until a neutral pH was achieved. Demineralization of the scales was achieved by treating with 0.5 M $Na_2$EDTA (ethylenediaminetetraacetic acid disodium salt) solution (pH 7.5) at a sample/EDTA solution ratio of 1:10 (w/v) for 24 h, and then washed with cold distilled water. After pretreatment, scales were extracted with 0.5 M acetic acid at sample/acid ratio of 1:2.5 (w/v) for 4 days. The extract was centrifuged at 20,000*g* for 1 h using a centrifuge machine (SUPREMA 21; Tomy Seiko Co., Ltd, Tokyo, Japan). The supernatant was salted out by adding NaCl to obtain a final concentration of 2.5 M in the presence of 0.05 M Tris (hydroxymethyl) aminomethane at pH 7.0. The resulting precipitate was collected by centrifugation at 20,000*g* for 30 min. The resultant pellet was dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid and distilled water and then lyophilized. All of the preparation procedures were carried out at below 4 °C.

### 2.4. Yield of extracted ASC

The yield of collagen was determined following the method of Wang et al. (2008). The hydroxyproline content of extracted solutions and fish scale were determined by HPLC. The extract yield (YD) was calculated using the following equation:

$$\text{YD}\ (\%) = \frac{\text{Hydroxyproline content in extract(mg/L)} \times \text{Volume of extract(L)}}{\text{Hydroxyproline content in fish scale(mg/g)} \times \text{dry weight of fish scale(g)}}$$

### 2.5. Analysis of amino acid composition

Twenty milligrams of ASC was hydrolysed in 6 M HCl at 110 °C for 22 h under vacuum. The hydrolysate was neutralized with 6 M and 0.6 M NaOH, and filtered through a cellulose membrane filter (0.45 μm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was used for amino acid analysis using an amino acid analysis system (Prominence; Shimadzu, Kyoto, Japan) equipped with a column (Shim-pack Amino-Li, 100 mm × 6.0 mm i.d.; column temperature, 39.0 °C; Shimadzu) and pre-column (Shim-pack ISC-30/S0504 Li, 150 mm × 4.0 mm i.d.; Shimadzu). Amino acids were detected using a fluorescence detector (RF-10AXL; Shimadzu).

Tryptophan content of ASC was determined according to the method of María, Julio, Javier, Francisco, and Manuel (2004) with slight modifications. Ten milligrams of ASC was dissolved in 3 ml of 4 M sodium hydroxide, sealed in hydrolysis tube under nitrogen, and incubated at 100 °C for 4 h. The hydrolysate was neutralized with 12 M HCl, diluted to 25 ml with 1 M sodium borate buffer (pH 9), and filtered through a cellulose membrane filter (0.45 μm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was used for tryptophan analysis using a fluorescence detector (RF-10AXL; Shimadzu).

Sulphur containing amino acid of ASC was analysed according to the method of Gehrke, Schisla, Absheer, and Zumwalt (1987) with slight modifications. One milligram of sample was dissolved in 0.5 ml of the oxidation solution (including 88% formic acid and 30% hydrogen peroxide at ratio of 9:1 (v/v), incubated at room temperature for 1 h), and kept on ice for 24 h. After 24 h, the sample was added 0.075 of 48% HBr, dried in vacuum condition, and hydrolysed in 6 M HCl at 110 °C for 22 h under vacuum condition. The hydrolysate was neutralized with 6 M and 0.6 M NaOH, and filtered through a cellulose membrane filter (0.45 μm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was used for sulfur containing amino acid analysis using an amino acid analysis system.

### 2.6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE of collagen from scales was determined according to the method of Laemmli (1970) with slight modifications. Collagen samples were dissolved in 0.1 M acetic acid solution and then mixed with sample buffer (0.5 M Tris–HCl, pH 6.8, containing 10% (w/v) SDS and 20% (v/v) glycerol) in the presence of 10% (v/v) mercaptoethanol at a collagen/sample buffer ratio of 1:2 (v/v). Each sample (10 μg) was loaded onto the polyacrylamide gel (7.5%) and electrophoresed at a constant current of 20 mA. After electrophoresis, the gel was fixed with 25% (v/v) methanol and 5% (v/v) acetic acid for 30 min, and then stained with 0.1% (w/v) Coomassie blue R-250 in 30% (v/v) methanol and 10% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers (Sigma Chemical Co., St. Louis, MO, USA) were used to estimate the molecular weight of proteins.

### 2.7. Solubility of collagen

Collagen solubility was measured in 0.1 M acetic acid at various NaCl concentrations and pHs according to the method of Montero, Jimenez-Colmenero, and Borderias (1991) with slight modifications. Collagen samples were dissolved in 0.1 M acetic acid with gentle stirring at 4 °C to obtain final concentrations of 3 and 6 mg/ml.

To determine the effect of NaCl concentration on collagen solubility, 5 ml of collagen solution (6 mg/ml) was mixed with 5 ml of NaCl in 0.1 M acetic acid at various concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 M). The mixtures were stirred gently for 30 min at 4 °C and centrifuged at 20,000*g* for 30 min at 4 °C. Protein content in the supernatants was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as the protein standard. Relative solubility of the collagen samples

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