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Comparison of acid-soluble collagens from the skins and scales of four carp species

Dasong Liu [a,b], Peng Zhou [a,b,*], Tiancheng Li [a], Joe M. Regenstein [c]

[a] State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, China
[b] Synergetic Innovation Center of Food Safety and Nutrition, Wuxi, Jiangsu Province 214122, China
[c] Department of Food Science, Cornell University, Ithaca, NY 14853-7201, USA

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ABSTRACT

The present study characterized and compared the acid-soluble collagens (ASC) from skins and scales of grass carp, silver carp, bighead carp and black carp. All ASC were mainly characterized as type I collagen with the triple helical structures well maintained. ASC from bighead carp and silver carp showed similar peptide hydrolysis patterns using V8 protease, and ASC from grass carp and black carp also showed similar patterns which were different from those of ASC from the former two species. Maximum transition temperature of all ASC ranged from 34.1 to 36.4 °C. Both ASC from one species and mixed ASC from different species could assemble into collagen fibrils. D-periodicities of fibrils assembled from mixed ASC were slightly smaller than those of fibrils assembled from ASC from one species. Therefore, these results suggested that the skins or scales of the four carp species could be processed together to produce collagen.

1. Introduction

Collagen with the unique triple helical structure is one of the main structural proteins in vertebrates, and it is widely used in the food, pharmaceutical, biomedical and cosmetic industries due to its excellent biocompatibility and biodegradability, and weak antigenicity (Lee, Singla, & Lee, 2001; Tzaphlidon, 2004). Purified collagens could self-assemble into native-like collagen fibrils in *vitro* with high tensile strength and stability, suggesting their potential application to the development of novel biomimetic materials.

Traditionally, the skins and bones of cows and pigs have been the main sources of collagen. However, the outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease have led to safety concerns among consumers of collagen and collagen-derived products from these land-based animals (Binsi, Shamasundar, Dileep, Badii, & Howell, 2009). Additionally, collagens obtained from pigs and non-religiously slaughtered animals are unacceptable in the Islamic and Jewish communities (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Recently, much attention has been paid to the by-products from fish processing as potential alternative sources for collagen production, such as skins, scales, bones and swim bladders (Duan, Zhang, Du, Yao, & Konno, 2009).

With the increasing demand for processed fish products and also the rapid development of fish processing industry in China, more by-products are being generated, which, if not appropriately used, may cause environmental problems. Thus, the use of these fish processing by-products as alternative sources for collagen production can both increase the economic return to the fish industries and avoid environmental problems. Previous studies have reported the extraction and characterization of collagens from many fish species, such as balloon fish skin (Huang, Shiau, Chen, & Huang, 2011), rohu and catla scales (Pati, Adhikari, & Dhara, 2010), striped catfish skin (Singh, Benjakua, Maqsood, & Kishimura, 2011).

China is the largest freshwater fish producer in the world. Grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*) and black carp (*Mylopharyngodon piceus*) are the most abundant freshwater fishes cultivated in China. These four major carp species were sometimes handled together by fish processors and the processing by-products from different species were often not separated from each other, therefore, it is very important to determine whether the processing and handling of these by-products together for ASC production is appropriate.

* Corresponding author. 1800 Lihu Ave., Jiangnan University, Wuxi, Jiangsu Province 214122, China. Tel./fax: +86 510 85912123.
E-mail address: zhoupeng@jiangnan.edu.cn (P. Zhou).

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In previous studies, collagens from these four carp species have been investigated separately, and comparison of results from these separate studies showed great variations in the biochemical properties of collagens, for example, the denaturation temperature varied significantly in the range of 24.6–35.9 °C (Jia et al., 2012; Li et al., 2008; Rodziewicz-Motowidło et al., 2008; Yao et al., 2012; Zhang, Duan, Tian, & Konno, 2009; Zhang, Duan, Ye, & Konno, 2010; Zhang et al., 2007). However, as the extraction procedures and the analysis methods used in those separate studies varied, it is uncertain whether such variations in the biochemical properties of collagens from these four carp species result directly from the differences in the extraction procedures and the analysis methods. Therefore, comparison and interpretation of results from different research groups and their application in the food industry should be done with caution. Thus, the objective of this study was to extract and characterize the ASC from the skins and scales of bighead carp, silver carp, black carp and grass carp with carefully designed extraction procedures and analysis methods to evaluate the feasibility of using skins or scales from these four carp species together as the alternative sources for ASC production.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade unless otherwise stated. *Staphylococcus aureus* V8 protease (EC 3.4.21.19), type I collagen from calf skin were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The protein marker used for electrophoretic analysis of ASC contained the following proteins : 212 kDa, myosin from rabbit muscle; 158 kDa, MBP-β-galactosidase from *Escherichia coli*; 116 kDa, β-galactosidase from *E. coli*; 97.2 kDa, phosphorylase B from rabbit muscle; 66.4 kDa, serum albumin from bovine (New England Biolabs, Inc., Ipswich, MA, USA). The protein marker used in the studies of peptide hydrolysis patterns contained recombinant proteins with molecular weight of 37, 50, 75, 100, 150, and 250 kDa (Bio-Rad laboratories, Inc., Hercules, CA, USA).

2.2. Fish samples

Live farmed fishes of each species, namely, bighead carp with weights ranging from 2 to 3 kg, silver carp 2–3 kg, grass carp 3–4 kg, and black carp 4–5 kg, were purchased from a local market in Wuxi, Jiangsu province in April and brought back to the laboratory in water within 0.5 h. The fish were stunned by a sharp blow to the head with a wooden stick in a cold room at 4 °C, and the skins and scales were removed by hand using a scalpel and then washed with cold distilled water. Then, the cleaned samples were immediately subjected to collagen extraction.

2.3. Preparation of collagen from the skins and scales

ASC were prepared according to the methods of Nagai and Suzuki (2000) and Kittiphattanabawon et al. (2005) with slight modifications. All procedures were done in a cold room at 4 °C and the solutions were stirred with magnetic stirrers (IKA Werke GmbH & Co. KG, Staufen, Germany) during pretreatment and extraction.

To remove non-collagenous proteins and pigments, the skins and scales were first soaked in 20 volumes of 0.1 M NaOH for 36 h. The alkaline solution was changed every 12 h. After being washed with distilled water, the alkaline treated skins were then suspended in 20 volumes of 10% (v/v) butyl alcohol for 24 h to remove fat with the solution being changed every 12 h; while the alkaline treated scales were suspended in 10 volumes of 0.5 M EDTA for 72 h to remove the calcium with a change of solution every 24 h.

The resulting residues were extracted with 40 volumes (skins) or 10 volumes (scales) of 0.5 M acetic acid for 72 h. The suspensions were centrifuged at 10,000 g for 20 min at 4 °C using an Avanti J-E centrifuge (Beckman Coulter, Inc., Danvers, MA, USA) and the supernatants were collected. The insoluble components were re-extracted under the same conditions for 48 h and centrifuged as described above. The supernatants of these two extracts were combined and salted out by adding NaCl to a final concentration of 2.0 M. After 12 h, the precipitates were collected by centrifugation at 10,000 *g* for 20 min and then re-dissolved in 0.5 M acetic acid. The resulting solution were dialyzed against 0.1 M acetic acid followed by distilled water using a dialysis bag with a molecular weight cut-off of 7 kDa (Shanghai Green Bird Science and Technology Development Co., Shanghai, China) and then lyophilized using the Labconco freeze dryer (Labconco Corp., Kansas, MO, USA).

2.4. Characterization of collagen

2.4.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done according to the method of Laemmli (1970) with a 4% stacking gel and 5% resolving gel. The ASC were dissolved in 1% SDS to a final concentration of 2 mg/mL, and then mixed with the sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 2% (w/v) SDS and 25% (v/v) glycerol) at a ratio of 1:1 (v/v). Then 3 μL of sample solution was loaded in each well. The electrophoresis was done with a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, Inc.). After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 6.8% (v/v) acetic acid for 4 h, and then destained with 5% (v/v) methanol and 7.5% (v/v) acetic acid until clear bands could be observed.

2.4.2. Amino acid analysis

A 100 mg sample of ASC was hydrolyzed with 8 mL of 6 M HCl in an evacuated and sealed vial at 110 °C for 24 h. After pre-column derivatization with o-phthalaldehyde and fluorenylmethyl chloroformate, the amino acid analysis was done with an Agilent 1100 Series high performance liquid chromatography system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a C18 ODS HYPERSIL column (250 mm × 4.6 mm i.d., 5 μm particle size; Agilent Technologies, Inc.). The content of each amino acid was calculated based on the area of the corresponding peak on elution curves of the sample and standard (Sigma–Aldrich Co.). The results were expressed as residues per 1000 total residues.

2.4.3. Peptide hydrolysis patterns

Peptide hydrolysis patterns of ASC were studied according to the method of Satio, Kunisaka, Urano, and Kimura (2002) with slight modifications. ASC were dissolved in 0.1 M sodium phosphate buffer (pH 7.2, containing 0.5% (w/v) SDS) to obtain a concentration of 2 mg/mL (Solution A). *S. aureus* V8 protease was dissolved in the same buffer at a ratio of 1:2000 (w/v) (Solution B). Then, 100 μL of solution A and 10 μL of solution B were mixed and incubated at 37 °C for 25 min. The reaction was terminated by placing the test tubes in boiling water for 3 min. The resulting peptides were separated by SDS-PAGE using a 4% stacking gel and 7.5% resolving gel.

2.4.4. Differential scanning calorimetry (DSC)

Measurements were done using the Q2000 Series DSC (TA Instruments, Inc., New Castle, DE, USA). ASC were dissolved in 0.05 M acetic acid at a ratio of 1:40 (w/v) for 24 h at 4 °C. The samples (9.5–10.5 mg) were accurately weighted into aluminum pans and sealed, and then scanned over the temperature range of 20–50 °C at a scanning rate of 1 °C/min. An empty sealed pan was used as the