



Comparative assessment of physico-chemical characteristics and fibril formation capacity of thermostable carp scales collagen



Gaurav Kumar Pal, P.V. Suresh *

Academy of Scientific and Innovative Research, Meat and Marine Sciences Department, CSIR-Central Food Technological Research Institute, Mysuru 570020, India

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ABSTRACT

Collagen and collagen fibers have been widely documented as a potential and competitive biomaterial for medical applications. However, the searches for safe and realistic new collagen sources are still underway. Currently, fishery by-products (scales), a promising collagen source are usually discarded. In the present study, *in vitro* fibril-forming ability of the extracted fish scale collagen is reported. The aim of the investigation was to evaluate the concomitant comparison of fibril-forming abilities and characteristics of acid and pepsin soluble collagens from the scales of Indian major carp catla (*Catla catla*) and rohu (*Labeo rohita*). The extracted collagens were characterized as type I, with a total yield of 2.80–4.11% (w/w). Denaturation temperature determined for all collagens were between 35.9 and 37.7 °C. All collagens exhibited high solubility in acidic pH and low NaCl concentrations. SEM clarified the lyophilized collagens and their fibril-forming capacity. Amino acid content and radical scavenging efficacy were also analyzed for the extracted collagen. The results revealed that extracted scale collagen from a renewable biological source could be used as biomaterials in various sectors. It might be suitable for preparing collagen gel for biomedical devices or as a scaffold for cell culture because of its high stability and fibril formation capacity.

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1. Introduction

Collagen is the most abundant connective tissue structural proteins. Type I collagen is the most abundant collagen amongst 29 types of collagen with widespread use in different sectors such as food, cosmetic, biomedical, and pharmaceutical industries [1,2]. Collagen can be used as a competitive biomaterial for both soft and hard tissue repair [3,4]. *In vitro* conditions, collagen fibers have mechanical and biological properties that make them useful in tissue engineering applications (scaffolds, artificial tendons, and skin) [4,5].

Currently, porcine or bovine are the principal source of type I commercial collagen. However, the emergence of bovine spongiform encephalopathy and consumer's current levels of awareness of the adverse impact of porcine or bovine on health, have led to a decline in the use of collagen and collagen derived products from these animals [6,7]. Further, the fish processing by-products/discards such as skins, scales, fins, and bones offer the favorite raw materials for the collagen extraction, due to its bulk availability as well as a high yield of collagen [8,9]. However, fish collagen has also been considered as a new possible allergen. Hence, the application of fish collagen would be limited in food and biomedical sectors. The problem can be overcome using pepsin soluble collagen (PSC) extraction method. It is an effective approach for

removing non-helical telopeptide which is a major antigenic factor in collagen. PSC method effectively degrades the non-helical telopeptide region of collagen and reduced the risk of immune rejection [1,10–12].

It is well-known that collagen molecules in solution can spontaneously self-assemble to fibrillary structures [4]. The collagen can be self-assemble into fibrils *in vitro* when the collagen solution is adjusted to an ideal pH, temperature, and ionic strength [13]. The fish collagen low denaturation temperature can be improved to >40 °C, after fibril formation. Therefore, collagen fibers formed *in vitro* have biological and mechanical properties similar to native tissues that make them useful in tissue engineering applications [4,13–14]. The radical scavenging activity of the fish collagen is an important characteristic for the oral tolerance mechanism in the autoimmune diseases such as of rheumatoid arthritis [15]. Recently, natural antioxidants have increased the attention of the researchers because they are widely distributed and safer than the synthetic antioxidants. Very few studies have been done on the radical scavenging activity of the collagen [15,16].

India ranks second in the world freshwater aquaculture fish production. Indian major carp catla (*Catla catla*) and rohu (*Labeo rohita*) are the most commercially important freshwater fish cultivated in India and produced about 3,418,794 tons in the year 2013–2014. Processing of these carp fish generates possibly an enormous volume of non-edible by-products and waste including scales that are discarded in the fish shop and fish-processing factories. Thus, the carp processing by-products (waste), which account 30–35% (w/w) of the whole fish is utilized and poses a high level of disposal problems [7,8]. The scales of these

* Corresponding author.

E-mail addresses: gauravpal00@gmail.com (G.K. Pal), drsureshpv@cftri.res.in, drsureshpv@hotmail.com (P.V. Suresh).

carps are discarded as waste in the fisheries industry, which accounts 2.0–3.5% (w/w) of total fish weight, can be potential sources for extraction of realistic collagens. The utilization of an abundant carp processing by-products (waste) as viable sources for realistic collagen, will support to accelerate revenue for the farmers as well as efforts to decrease the volume flow of by-products (waste) [7–10].

To the best of our knowledge, the *in vitro* fibril-forming ability and antioxidant efficacy of the collagen from the scales of Indian major carp fishes, particularly those in the tropical regions was not reported in the literature. Therefore, we are interested in developing a novel way to use the carp fish scales proteins that traditionally discarded. To make better utilization of these by-products, the present study was carried out to isolate and characterize the acid soluble collagens (ASC) and pepsin soluble collagens (PSC) from the scales of Indian carps. This study also attempted to evaluate the feasibility of fish scales as a realistic alternative source of collagen for biomaterial and tissue engineering applications.

2. Materials and methods

2.1. Sample collection and preparation of Indian carp scales

By-products (skin with scales) of Indian carp catla and rohu with average body weight of 750–850 g were collected from a local freshwater fish market (Devaraja fish market, Mysuru, India). Fish were transported to the laboratory in the chilled condition and processed within 30 min. Scales from skin separated manually and washed thoroughly with chilled (~4 °C) water followed by final rinsing with chilled demineralized water. The cleaned and washed scales were packed in polyethylene bags and stored at –20 °C for storage time not longer than one month. For the removal of non-collagenous proteins, minerals, fat and pigments, the fish scales were cut into ~0.5² cm size with a scissor before treatment.

2.2. Proximate composition of Indian carp scales

The moisture, ash, crude fat and protein (Kjeldahl) contents of the scales of Indian carp (catla and rohu) were analyzed according to the standard method of AOAC (Association of Official Analytical Chemists) [17]. The 6.25 was used as converting factor for calculation of protein content. Hydroxyproline content in the fish scales was analyzed after hydrolysis with 6 mol L⁻¹ HCl at 110 °C for 24 h following the method described [18]. The collagen hydrolysate was clarified with activated carbon and filtered through Whatman filter paper No. 4. The filtrate of collagen hydrolysate was neutralized with 10 mol L⁻¹ and 1 mol L⁻¹ NaOH to obtain the pH of 6–6.5. The neutralized collagen hydrolysate (100 µL) was transferred into a test tube, and isopropanol (200 µL) was added and mixed well. After that, 100 µL of oxidant solution and 1.3 mL of Ehrlich's reagent solution were added and mixed thoroughly. After that, the mixture was heated at 60 °C for 25 min in a water bath and cooled for 5 min in a running tap water. The mixture solution was diluted to 5 mL with isopropanol and absorbance was measured at 558 nm against the blank. The standard hydroxyproline solution was prepared with concentration ranging from 10 to 60 mg/kg and used for the preparation of standard curve. Hydroxyproline content was calculated as mg/g sample.

2.3. Pre-treatment of Indian major carp scales

Non-collagenous proteins, minerals, fat and pigment were removed from the Indian major carp scales according to procedures described [10] with slight modifications. Pre-treatment of carp scale was completed in three sequential steps. The first step is the removal of non-collagenous proteins and pigments. Briefly, the fish scales pieces were suspended in 0.1 mol L⁻¹ NaOH at a solid to liquid ratio of 1:10 (w/v) with continuous gentle stirring for 48 h. The alkali solution was changed

at an interval of 24 h. Treated scales were washed with chilled (~4 °C) distilled water to achieve the neutral pH. The deproteinized fish scales were subjected to demineralization according to the method described elsewhere [12] with slight modifications. Demineralization of fish scales was performed using 0.5 mol L⁻¹ ethylene diamine tetraacetic acid disodium salt (Na₂EDTA·2H₂O) solution (pH 7.5) at a solid to liquid ratio of 1:10 (w/v) for 72 h with a change of solution at an interval of 24 h. Then, washed with chilled distilled water and the residues were defatted with 10% (v/v) butyl alcohol at a solid to liquid ratio of 1:10 (w/v) for 24 h. The defatted scales residues were thoroughly washed with the same volume of chilled (~4 °C) distilled water. Subsequently, the demineralized and defatted fish scales residues were used for acid soluble and pepsin soluble collagens extraction. All process was carried out at low temperature walk-in cold room (4 °C).

2.3.1. Extraction of acid soluble collagen (ASC)

Acid soluble collagens were prepared according to the method reported [19] with slight modifications. Briefly, the demineralized and defatted scales residues were subjected to ASC extraction with 0.5 mol L⁻¹ acetic acid at a solid to liquid ratio of 1:10 (w/v) for 72 h. The suspensions were filtered through a double fold cheesecloth. The filtrates were centrifuged at 20,000 RPM for 60 min at 4 °C using a Beckman optima-100 ultracentrifuge (Beckman Coulter, Inc., Danvers, MA, USA), and the supernatants were collected. The supernatants were subjected to salting out by adding NaCl to the final concentration of 2.6 mol L⁻¹ in the presence of 0.05 mol L⁻¹ Tris (hydroxymethyl) aminomethane (pH 7.0). The precipitated materials were collected by centrifugation at 20,000 RPM for 60 min at 4 °C and dissolved in a minimum volume of 0.5 mol L⁻¹ acetic acid. The resultant solutions obtained were subjected to dialysis against 0.1 mol L⁻¹ acetic acid for 48 h with the change of solution every 12 h followed by distilled water with the changes of water until neutral pH was obtained. The dialyzed solutions were lyophilized using a freeze-drier (Scanvac Cool Safe, Lynge, Denmark). The collagen obtained was denoted as acid soluble collagen (ASC) and stored at –20 °C until use.

2.3.2. Extraction of pepsin soluble collagen (PSC)

Pepsin soluble collagens were prepared according to the method reported [10] with slight modifications. The residual materials remained after ASC extraction was subjected to PSC extraction. The residues from ASC extraction were further extracted using 0.5 mol L⁻¹ acetic acid containing 0.5% (w/v) pepsin (1000 U/mg) at the solid to liquid ratio of 1:10 (w/v) at 4 °C for 48 h with continuous gentle stirring. After the treatment, the supernatants were collected using the method described in Section 2.3.1 for ASC preparation. Further, precipitation, dialysis, and lyophilization were also performed using the method described in Section 2.3.1 for the extraction of ASC. The dried matter obtained with the treatment of pepsin was referred to as pepsin soluble collagen (PSC). Both the acid collagens (ASC and PSC) were subjected to functional characterizations and determinations of radical scavenging activities.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Protein pattern of ASC and PSC from the scales of catla and rohu was performed according to the procedure reported [10,20] with slight modifications. Briefly, collagen samples were dissolved in 0.1 mol L⁻¹ acetic acid solution to a final concentration of 3 mg/mL. Solubilized collagen samples were mixed at 1:1 (v/v) ratio with sample loading buffer (0.65 mmol L⁻¹ Tris-HCl, pH 6.8 containing 2% SDS and 10% sucrose in the presence of 5% (v/v) β-mercaptoethanol) and heated in a boiling water bath for 3 min. Samples (10 µg proteins) were loaded onto a polyacrylamide gel made of 6% resolving gel and 4% stacking gel and subjected to electrophoresis at a constant current of 30 mA. High-molecular-

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