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Comparison of Collagen Extracted from Skin of Double-spotted Queenfish and Malabar Grouper

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Abstract

Acid soluble collagen (ASC) and pepsin digestible collagen (PDC) from the skin of double-spotted queenfish (*Scomberoides lysan*) and Malabar grouper (*Epinephelus malabaricus*), were isolated and characterized. On wet weight basis, the yields of ASC and PDC from queen fish and grouper were 7.82, 3.92, 12.5 and 6.49% respectively. Amino acid analysis revealed that they contained glycine as a major amino acid with high contents of alanine, proline and hydroxyproline. Based on sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns and subunit compositions, all were identified to be type 1 collagens when compared with calf skin type 1 collagen. α_1, $\alpha 2$ and β chains were the major components of the presently isolated collagens. While comparing these two species, queen fish skin had good yield of collagen which could be served as an alternative source of collagen for different applications.

Keywords: Acid soluble collagen, pepsin digestible collagen, yield, type I collagen

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Introduction

Collagen is the most abundant animal protein polymer, accounting for almost 25 to 30% of total protein in the animal body (Liu et al., 2007; Bama et al., 2010). Being one of the extracellular matrix constituents of multi-cellular animals (Mizuta et al., 2005), it can be found in connective tissues and serves as a major component of bones, cartilage, skin, tendons, ligaments, blood vessels, muscles, teeth and other organs of vertebrates (Senaratne et al., 2006; Quereshi et al., 2010). Collagen contents in fishes vary, depending on fish species (Nagai et al., 2002). Studies on extraction of fish collagens have been extensively carried out recently due to its broad application in cosmetic, biomedical and pharmaceutical industries (Cliché et al., 2003).

The outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy and foot and mouth disease (FMD) have created anxiety among consumers of collagen and collagen derived products of land animal origin. Collagen from porcine sources cannot be used as component of some foods due to aesthetic and religious objections. Therefore, alternative sources, such as fish processing waste have received increasing attention for collagen extraction (Jongjareonrak et al., 2005). Several studies have described extraction of collagen from aquatic sources such as skin of ocellate puffer fish (Nagai et al., 2002), black drum and sheepshead sea bream (Ogawa et al., 2003), brown backed toad fish (Senaratne et al., 2006), Baltic cod (Sadowska et al., 2003), Nile perch (Muyonga et al., 2004), big eye snapper (Jongjareonrak et al., 2005), skate (Hwang et al., 2007), grass carp (Zang et al., 2007) deep sea red fish (Wang et al., 2007) dusky spine foot, sea chub, eagle ray and sting ray (Bea et al., 2008) large fin long barbel catfish (Zang et al., 2009) and brown banded bamboo shark (Kittiphattanabawon et al., 2010). These studies described collagens from different species, tissues and living environments which may have different biochemical properties.

Characterization of collagen from warm water species of fish needs further elaboration. The objective of the present study was to isolate and characterize collagen from the skin of two commercially important warm water species of fish, double-spotted queenfish (*Scomberoides lysan*) and Malabar

grouper (*Epinephelus malabaricus*) for better utilization of waste from fish processing industry.

Materials and Methods

All chemicals used were of analytical grade. Type 1 collagen from calf skin, pepsin from bovine gastric mucosa, high molecular weight markers and collagen hydrolysate were from Sigma Chemical Co. Sodium dodecyl sulphate (SDS), Coomassie brilliant blue R-250 & N,N,N',N'-tetra methyl ethylene diamine (TEMED) were procured from Bio-Rad laboratories.

Fresh skin of fishes, grouper and queenfish weighing 1.8 ± 0.87 kg and of total length 46 ± 3.5 cm and 4.6 ± 1.1 kg and of total length 76 ± 5.6 cm respectively were procured from local market near Cochin, Kerala. Skin were stored in ice with a skin/ice ratio of 1:2 (w/w) and transported within 1 h to the laboratory. The skin was washed with cold water (5-8°C) and cut into small pieces (2 ± 0.5 cm^2). The prepared skin samples were packed in polyethylene bags, added glaze water and kept at -20°C prior to collagen extraction.

The raw skin of these two species and their collagens (both acid soluble and pepsin digestible collagens) were subjected to proximate analysis, according to AOAC (2000).

Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PDC) were extracted from queenfish skin and grouper skin. All procedures were performed as per Hema et al. (2013). All the extraction processes were carried out at 4°C. To remove non-collagenous proteins, the skin portions were mixed with ten volumes (v/w) of 0.1 M NaOH and stirred for 5 to 6 h. The sample was then washed thoroughly with excess distilled water until the pH was neutral or slightly basic. The residue was filtered using cheese cloth and actively stirred in five volumes (v/w) of 0.5 M acetic acid for 20 h to extract acid soluble collagen. The supernatant after centrifugation (3000 rpm, 20 min) was collected. The residue was once again extracted with acid as above and the combined supernatants were taken ASC. Residue from the previous step was homogenized with 30 volumes of 0.5M formic acid for 1 min and stirred for 24 h. A solution of pepsin having activity >250 units mg^{-1} (enzyme / tissue ratio 1:100) was added to this and stirred for another 24 h. The supernatant after centrifugation was taken as PDC. Crystalline sodium chloride was added to both supernatants to the level of 10% and stirred for 24 h to precipitate collagen. The precipitate was suspended in Tris-glycine buffer (50 mM containing 0.2 M NaCl, pH 7.4) and dialyzed against the same buffer for 24 h and then centrifuged. The collagen obtained was spray dried to get fine powder.

For amino acid analysis 100 mg dry collagen sample was weighed and hydrolyzed with 10 ml 6 N HCl at 110°C for 24 h. The filtered sample was injected to the amino acid analyzer (HPLC- LC 10 AS). The amino acid composition was determined as per the method of Ishida et al. (1981) using Model Hitachi L-2130 Elite La Chrome (Tokyo, Japan) amino acid analyser connected with cation exchange column (Shodex, CX Pak, 4.6×15mm). Electrophoretic patterns of the collagens were analyzed according to Laemmli (1970) by SDS PAGE.

All experiments were done in triplicates. Mean values with standard deviations (SD) were reported. Means were compared using t-test. The significant difference between means was computed at 5% level of significance using SAS 9.3

Results and Discussion

Table 1 shows proximate composition of skin of queenfish and grouper. The protein, fat and ash contents are higher for queenfish compared to grouper. Table 2 shows the proximate values of the collagen extracted from the skin of the two species. From the table it is clear that there is negligible

Table 1. Proximate composition of skin of grouper and queenfish

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Grouper skin	70.62 ± 1.05^a	20.78 ± 0.68^b	03.58 ± 0.36^b	02.05 ± 0.11^b
Queenfish skin	64.67 ± 0.40^b	22.43 ± 0.29^a	07.76 ± 0.71^a	04.04 ± 0.09^a

Values are given as mean ± SD. Values with the same superscript letters within a column are not significantly different ($p>0.05$)

Table 2. Proximate composition of collagen extracted from skin of grouper and queenfish

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Grouper skin collagen	06.46 ± 0.24[b]	91.07 ± 0.94[a]	00.45 ± 0.12[a]	00.54 ± 0.07[b]
Queenfish skin collagen	07.39 ± 0.14[a]	90.66 ± 0.92[a]	00.61 ± 0.06[a]	00.80 ± 0.06[a]

Values are given as mean ± SD. Values with the same superscript letters within a column are not significantly different ($p>0.05$)

amount of fat and ash contents in the extracted collagen. Table 3 compares the yield of collagen from the two species and the yield is high for grouper skin ($p>0.05$).

Amino acid composition of ASC and PDC extracted from grouper and queenfish skins is given in Table 4. The analysis detected the presence of 18 amino acids and it contained high percentage of glycine, followed by alanine, proline, hydroxyproline and

Table 3. Collagen yield from grouper skin and queenfish skin

Collagen type	ASC (%)	PDC (%)
Grouper skin collagen	12.5 ± 0.63[a]	6.49 ± 0.51[a]
Queenfish skin collagen	7.82 ± 0.70[b]	3.92 ± 0.11[b]

ASC: Acid soluble collagen; PDC: Pepsin digestible collagen
Values are given as mean ± SD. Values with the same super script letters within a column are not significantly different ($p>0.05$)

Table 4. Amino acid composition of acid soluble collagen and pepsin digestible collagen from grouper and queenfish skin (expressed as residues per 1000 total amino acid residues)

Amino Acids	Queenfish skin ASC	Queenfish skin PDC	Grouper skin ASC	Grouper skin PDC
Alanine	118 ± 0.11	130 ± 0.15	131 ± 0.45	109 ± 0.71
Arginine	46 ± 0.02	53 ± 0.53	54 ± 0.67	52 ± 0.66
Aspartic acid	41 ± 0.15	43 ± 0.14	42 ± 0.21	43 ± 0.14
Cysteine	-	-	-	-
Glutamic acid	74 ± 0.21	62 ± 0.10	62 ± 0.54	76 ± 0.44
Glycine	332 ± 0.20	328 ± 0.14	330 ± 0.71	315 ± 0.84
Histidine	9 ± 0.11	7 ± 0.5	7 ± 0.45	8 ± 0.42
Isoleucine	9 ± 0.00	8 ± 0.18	7 ± 0.59	21 ± 0.84
Leucine	18 ± 0.05	22 ± 0.22	21 ± 0.55	24 ± 0.65
Lysine	25 ± 0.09	24 ± 0.09	24 ± 0.23	26 ± 0.43
Hydroxy lysine	8 ± 0.07	6 ± 0.16	6 ± 0.42	8 ± 0.28
Methionine	11 ± 0.05	11 ± 0.20	11 ± 0.12	12 ± 0.54
Phenyl alanine	14 ± 0.15	18 ± 0.14	20 ± 0.67	15 ± 0.23
Hydroxy proline	78 ± 0.14	66 ± 0.17	68 ± 0.47	95 ± 0.75
Proline	99 ± 0.10	115 ± 0.22	117 ± 0.55	98 ± 0.43
Serine	43 ± 0.12	41 ± 0.10	41 ± 0.43	32 ± 0.91
Threonine	23 ± 0.05	22 ± 0.00	22 ± 0.14	23 ± 0.21
Tyrosine	2 ± 0.11	1 ± 0.15	1 ± 0.56	2 ± 0.45
Valine	28 ± 0.17	29 ± 0.08	29 ± 0.87	25 ± 0.19

ASC: Acid soluble collagen; PDC: Pepsin digestible collagen. Values are given as mean ± SD

glutamic acid. On the other hand, histidine and tyrosine were found to be least and cysteine completely absent in the collagens. The imino acid content (proline + hydroxyproline) of queenfish and grouper skin ASC and PDC was 177, 181, 185 and 193 per 1000 residues respectively. The values are comparable to most fish collagens such as grass carp skin collagen (186 residues/1000 residues) and big eye snapper skin collagen (193 residues/1000 residues) (Kittiphattanabawon et al., 2005; Zhang et al., 2007). The variation in imino acid content amongst different species is mostly due to changes in the habitat, particularly temperature.

SDS-PAGE patterns of collagens from the skin of queenfish and grouper are shown in Fig. 1. Collagen extracted from both the species shows similar protein patterns and it was found that the major constituents of both ASC and PDC consisted of α chains (α_1 α_2) and β chains. These patterns were similar to the type 1 collagen from calf skin (lane 6), and also in accordance with those of collagens from most other fish species previously reported (Muyonga et al., 2004; Nagai et al., 2001). Type I collagen consists of two identical α chains (Pearson & Young, 1989; Wong, 1989). Fish skin and bone have been reported to contain type I as the major collagen (Ciarlo et al., 1997; Kimura & Ohno, 1987; Montero et al., 1990; Nagai & Suzuki, 2000b). The skin collagens of bigeye snapper (Kittiphattanabawon et al., 2005), brown-banded bamboo shark (Kittiphattanabawon et al., 2010), Nile perch (Muyonga et al., 2004), ocellate puffer fish (Nagai et al., 2002), back drum seabream, sheepshead seabream (Ogawa et al., 2003), brown backed toadfish (Senaratne et al., 2006), Walleye Pollock (Yan et al., 2008), and large fin long barbel catfish (Zhang et al., 2009) consisted of two α chains (α_1 & α_2) and β components. No difference could be observed in the pattern of α_1 α_2 and β chains of the ASC and PDC of skin of the two fishes in the present study.

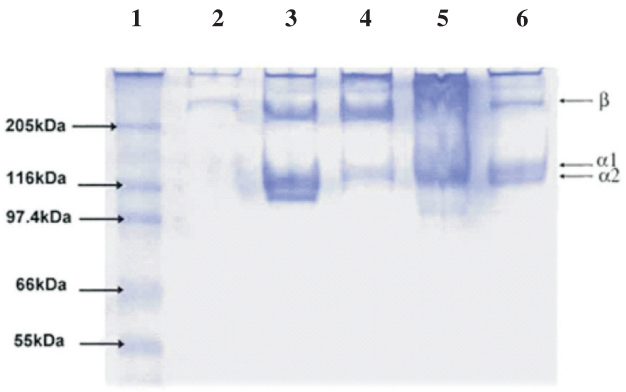

Fig. 1. SDS-PAGE pattern of extracted collagen

Lane 1. High molecular weight marker, lane 2. Queenfish skin ASC, lane 3. Queenfish skin PDC, lane 4.Grouper skin ASC lane 5. Grouper skin PDC, lane 6. Type 1 collagen from calf skin

Collagen yield from grouper skin was found to be high when compared to queenfish skin. All the extracted collagens showed composition typical of collagens. No differentiation could be observed in the collagens from the two species against standard bovine collagen indicating their type 1 nature. The amino acid pattern and SDS-PAGE of collagens extracted in the present study indicate that the process of extraction yielded pure collagen and extraction of collagen by digesting with pepsin increases the yield of total collagen from fish skin.

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