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A simple method for isolation of fish skin collagen- biochemical characterization of skin collgagen extracted from Albacore Tuna (*Thunnus Alalunga*), Dog Shark (*Scoliodon Sorrakowah*), and Rohu (*Labeo Rohita)*

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ABSTRACT

A simple method has been developed for the isolation of Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PDC) from the skin of Albacore tuna (Thunnus alalunga), Dog shark (Scoliodon sorrakowah), and one among Indian Major Carps ie, Rohu (Labeo rohita). Biochemical characterization of the collagen extracted was carried out. On wet weight basis the yields of ASC and PDC from shark skin were 8.96% & 7.68% respectively and that from Rohu was ASC 4.13% & PDC 3.68% respectively. The yield of collagen from Tuna skin was ASC 13.97%. No PDC was obtained for tuna skin. Proximate analysis showed all collagens had protein as a major constituent with trace amount of ash and fat. 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. A comparison of these collagens with calf skin type 1 collagen indicated the same and α_1,α_2 and β chains were the major components of these collagens. γ components were also found in lesser amounts in these collagens. The results of the present study indicated that comparing the three species Dog shark skin had good yield of collagen and it could be served as an alternative source of collagen for different biomedical applications.

Keywords Fish Skin Collagen• Extraction• Characterization • Amino acid composition

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INTRODUCTION

Collagen forms the major fraction of connective tissues such as skin, bone, tendon, the vascular system of animals and the connective tissue sheaths surrounding muscle [1]. Its contents vary, depending on fish species [2, 3]. Type 1 collagen has been found as the major collagen in the skin, bone and fins of various fish species [4]. The physical and chemical properties of collagen differ depending on the tissues such as skin, swim bladder & the myocommata in muscle. Fish collagen is heat sensitive due to labile cross links as compared to mammals; the hydroxyproline content is lower, varying from 4-10% [5]. However, different fish species containing varying amounts of collagen in the body tissue that reflect the swimming behavior and it influences the textural characteristics of fish muscle [6]. Most fish collagens have been found to consist two α - chain variants, which are normally designated as α-1 and α-2 [7, 8]. These chain variants, though having approximately the same molecular weight (95,000 Da) can be separated by

SDS PAGE due to their different affinity for SDS. α - 2 have a higher affinity for SDS and consequently exhibit a higher mobility than α 1 [9]. In addition to differences in molecular species, fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) vary significantly among fish species [10-12]. The amount of imino acids, especially hydroxyproline, depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens and gelatins [10, 13]. Collagens derived from fish species living in cold environments have lower contents of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. This is because of the involvement of hydroxyproline in inter-chain hydrogen bonding, which stabilizes the triple helical structure of collagen. Collagen film proved as a promising carrier for anticancer drug delivery system and ophthalmic drug delivery system because of its inertness, structural stability and good biocompatibility [14, 15]. The objective of the study was to develop a method to isolate collagen from the skin of Albacore tuna (*Thunnus alalunga*), Dog shark (*Scoliodon sorrakowah*), and one among Indian Major Carps ie, Rohu (*Labeo rohita*) which are generally discarded as waste in fish processing industry.

MATERIALS AND METHODS

2.1 Chemicals

All chemicals were of analytical grade. Type 1 collagen from calf skin, pepsin from stomach mucosa, high molecular weight markers, collagen hydrolysate were from Sigma chemical Co. Sodium dodecyl sulphate (SDS), Coomassie brilliant blue R-250 & N,N,N',N'-tetramethylethylenediamine (TEMED) were procured from Bio-Rad laboratories.

2.2 Raw material

The species used for the study were **Albacore tuna** (*Thunnus alalunga*), **Dog shark** (*Scoliodon sorrakowah*), and one among Indian Major Carps ie, **Rohu** (*Labeo rohita*).The skin in the iced condition was procured from Polakkandom market, Cochin, Kerala, India.

2.3 Proximate analysis

The raw skin of the three species and their collagens (both acid soluble and pepsin digestible collagens) were subjected to proximate analysis including moisture, ash, fat and protein contents, according to the method of AOAC (1995)[6].

2.4 Pretreatment of the skin

Acid Soluble Collagen (ASC) & Pepsin Digestible Collagen (PDC) were extracted from Shark Skin, Tuna Skin & Rohu skin. All the extraction procedures were carried out at 4^0C. The source material was minced and mixed with 30 volumes of 0.1N sodium hydroxide and kept stirred for 24h over a magnetic stirrer to remove non collagenous protein. The treated mass was strained through a coarse sieve. The process was repeated twice and the residue was washed twice with 30 volumes of chilled distilled water.

2.5 Collagen extraction

The residue was homogenized in a Polytron homogenizer with 30 volumes 0.5M acetic acid for one minute and the same was stirred over a magnetic stirrer for 24 h. The supernatant after centrifugation (3000 rpm, 20 min) was collected. The residue was once again extracted with acid as above and the combined supernatant was taken as acid soluble collagen (ASC).

The 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 (enzyme / tissue ratio 1:100) was added to this and kept stirring for another 24h. The supernatant after centrifuging was taken as pepsin digestible collagen (PDC).

Crystalline sodium chloride was added to both supernatants to the level of 10% and stirred for 24 h to precipitate the collagen. The precipitate was suspended in Tris-glycine buffer (50 mM containing 0.2M 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 power.

2.6 Amino acid analysis

Collagen samples were hydrolyzed in 6N HCl at 120^0C for 24h. After cooling the test tubes the contents were filtered using Whatman No 1 filter paper. The tubes were rinsed with distilled water and filtered. The filtrate was evaporated in a vacuum flash evaporator. Then deionized water was added into the tubes and continued evaporation

until the contents were acid free. The process was repeated for three times and the free amino acids were dissolved in 0.05M HCl and filtered using 0.45 micro syringe, then injected in to Shimadzu HPLC using the method [17].

2.7 Tryptophan estimation

Tryptophan was estimated after alkali hydrolysis by colorimetry [18].

2.8 UV–Vis measurement

Collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The solution was then subjected to UV–Vis measurement. Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 220–600 nm with a scan speed of 50 nm/min at room temperature.

2.9 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic patterns of different species of collagens were analysed according to the method [19]. The samples were dissolved in 50 g/L SDS solution. The mixtures were then heated at 85^0C for 1 h, followed by centrifugation at 8500g for 5 min to remove undissolved debris. Solubilized samples were mixed with the sample buffer (0.5 mol/L Tris–HCl, pH 6.8 containing 40 g/L SDS, 200 mL/L glycerol in the presence or absence of 100 mL/L βmercaptoethanol) with the ratio of 1:1 (volume ratio). The mixtures were loaded onto a polyacrylamide gel made of 75 g/L separating gel and 40 g/L stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, gels were fixed with a mixture of 500 mL/L methanol and 100 mL/L acetic acid for 30 min, followed by staining with 0.5 mL/L Coomassie blue R-250 in 150 mL/L methanol and 50 mL/L acetic acid for 1 h. Finally, they were destained with a mixture of 300 mL/L methanol and 100 mL/L acetic acid for 1 h and destained again with the same solution for 30 min. High molecular weight protein markers were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as standard collagens.

2.10 Statistical analysis

All experiments were done in triplicates. Mean values with standard deviations (SD) were reported.

RESULTS AND DISCUSSION

3.1 Proximate composition

Table 1 shows the protein, moisture and ash content of the skin of the three selected fish skins and table 2 that of the extracted collagens. Generally skin of cartilaginous fishes which include shark and rays are low in lipid content. This lean species store majority of their fat in liver whereas skin of clupeid and scombroid species (sardines, mackerels and tuna) is rich in lipid. The extracted collagen contained negligible amounts of ash and fat. Extracted collagens from skin had low contents of ash and fat, indicating the efficacy of removal of both inorganic matter and fat. Collagen samples had low moisture contents, with protein content ranging from 88.8% to 91.72%.

Table 1. Proximate composition of skin

	Shark (%)	Rohu (%)	Tuna(%)
Moisture	68.38 ± 0.43	76.54 ± 0.45	56.54 ± 0.09
Protein	27.73 ± 0.36	18.84 ± 0.06	20.54 ± 0.26
Fat	0.16 ± 0.02	2.93 ± 0.05	18.32 ± 0.11
Ash	4.19 ± 0.03	2.03 ± 0.04	4.39 ± 0.03

Values were given as mean ± standard deviation of triplicate.

Table 2. Proximate composition of extracted collagen

	Moisture	Protein	Fat	Ash
Tuna ASC	7.53 ± 0.30	91.08 ± 0.71	0.64 ± 0.01	0.74 ± 0.02
Rohu ASC	8.78 ± 0.06	89.94 ± 0.75	0.33 ± 0.03	0.43 ± 0.02
Rohu PDC	6.66 ± 0.03	91.72 ± 0.53	0.45 ± 0.02	0.50 ± 0.02
Shark ASC	9.13 ± 0.14	88.80 ± 0.59	0.37 ± 0.01	0.76 ± 0.02
Shark PDC	8.32 ± 0.17	90.80 ± 0.12	0.42 ± 0.04	0.80 ± 0.01

Values were given as mean ± standard deviation of triplicate

3.2 Collagen yield

Table 3 shows the yield of the collagen. The yield of collagen in shark skin was higher compared to Tuna and Rohu skin. The skin was not completely solubilized with 0.5 M acetic acid even with two repetitions of extraction except for tuna skin. This result suggested a high amount of cross-links at the telopeptide region as well as other inter-molecular cross-links, leading to low solubility of collagen in acid [20].

Table 3. Collagen yield

Collagen type	Yield(%)
Tuna Skin ASC	13.97
Rohu skin ASC	4.13
Rohu skin PDC	3.68
Shark skin ASC	8.96
Shark skin PDC	7.68

3.3 Amino acid compositions of collagens

Table 4 shows the Amino acid compositions of collagens. Amino acid analysis showed higher content of glycine in all forms of collagen extracted which accounted to one third of the total amino acids. Higher contents of alanine, imino acids - hydroxyl proline and proline which are characteristics of collagen could be obtained in the present study also. The collagens were found to contain no tryptophan or cysteine. They were also very low in methionine, tyrosine and histidine, like other collagens [10, 21]. Generally, glycine is about one-third of the total amino acid residues, hydroxyproline about one fifth and alanine about one-ninth in collagen samples.

Table 4 Amino acid compositions of collagens

	Tuna ASC	**Rohu ASC**	**Rohu PDC**	**Shark ASC**	**Shark PDC**
Alanine	118	130	131	109	108
Arginine	46	53	54	52	55
Aspartate	41	43	42	43	40
Cysteine	0	0	0	0	0
Glutamate	74	62	62	76	78
Glycine	332	328	330	315	321
Histidine	9	7	7	8	7
Isoleucine	9	8	7	21	18
Leucine	18	22	21	24	23
Lysine	25	24	24	26	29
Hydroxylysine	8	6	6	8	4
Methionine	11	11	11	12	12
Phenylalanine	14	18	20	15	14
Hydoxyproline	78	66	68	95	91
Proline	99	115	117	98	109
Serine	43	41	41	32	32
Threonine	23	22	22	23	22
Tyrosine	2	1	1	2	1
Valine	28	29	29	25	26

Values were given as mean ± standard deviation of triplicate

3.4 Tryptophan analysis

No tryptophan could be estimated in the collagen samples.

3.5 Ultraviolet Spectra

From UV–Vis spectra of the extracted collagens, an absorbance near 200-240 nm with high intensity was observed with no absorption peak at 280 nm. The results indicated high efficacy of non-collagenous protein removal. Collagen commonly has a low amount of tyrosine, which could absorb UV-light at 280 nm [22]. The absorbance in this region is similar to those of collagens from channel catfish skin [23], walleye Pollock [24], and largefin longbarbel catfish [20]. Peptide bonds found in the protein also absorb at 205-230nm. The absorbance at 280nm is mainly because of tryptophan, tyrosine & phenyl alanine. Tryptophan is completely absent in collagen and have negligible amount of tyrosine. Previous research indicates that collagen commonly have a low amount of tyrosine which can absorb UV-light at 280 nm [22]. For these reasons, the extracted protein is collagen. Figures 1 to 6 depict various UV spectra analysis plots for the samples.

Figure 1. Ultraviolet Spectra analysis of pure collagen from calf skin

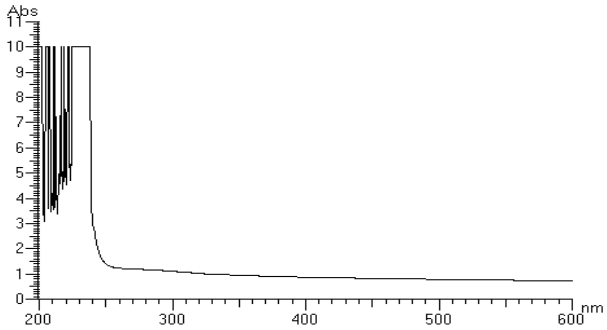


Figure 2. Ultraviolet Spectra analysis of tuna ASC

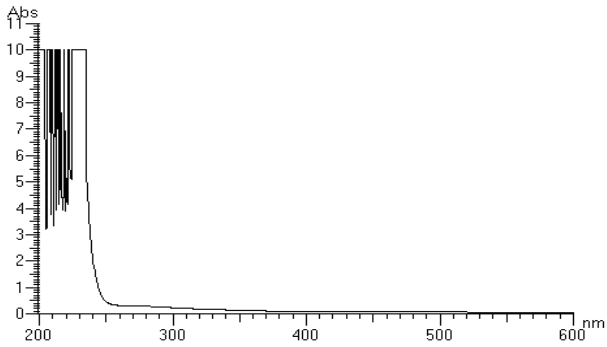


Figure 3. Ultraviolet Spectra analysis of Rohu ASC

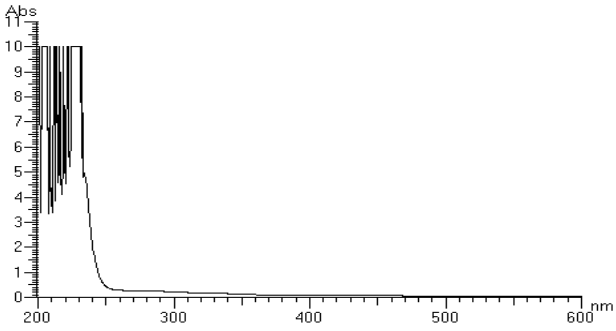


Figure 4. Ultraviolet Spectra analysis of Rohu PDC

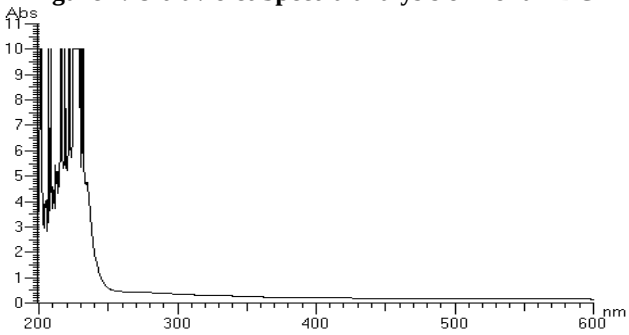


Figure 5. Ultraviolet Spectra analysis of shark ASC

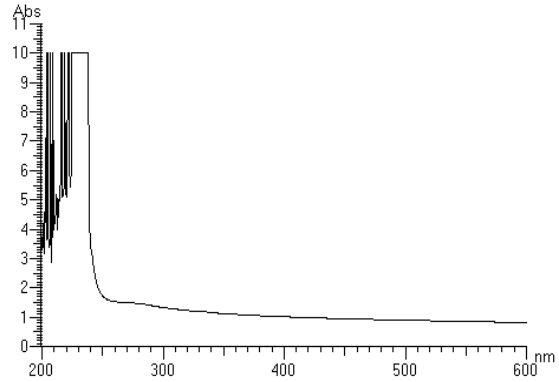
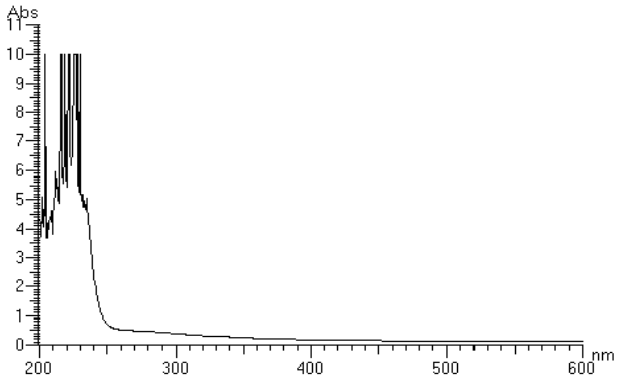


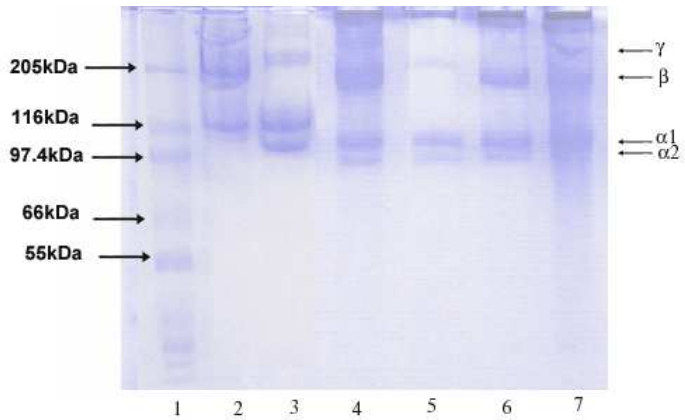
Figure 6. Ultraviolet Spectra analysis of Shark PDC



3.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Plate 1 shows the molecular weight pattern of Collagens against the high molecular weight marker. The protein patterns of ASC & PSC were analyzed by 7.5% resolving gel and it was found that the major constituents of both ASC &PDC consisted of α chains ($\alpha_1\alpha_2$), β, γ chains. These patterns were similar to the type 1 collagen from calf skin (lane 7), and also in accordance with those of collagens from most other fish species previously reported [8, 25]. Type I collagen consists of two identical α_1 chains and one α_2 chain [1, 26]. Fish skin and bone have been reported to contain type I collagen as the major collagen [27-29].The skin collagens of big eye snapper[30], brown banded bamboo shark [31], Nile perch [32], ocellate puffer fish [2], back drum seabream, sheep shead seabream [32], brown backed toadfish [33],Walleye Pollock [24], and large fin long barbel catfish [20] all consisted of two a chains (α_1&α_2), β and γ components.

Plate 1 Molecular weight pattern of Collagens



Lane 1.High molecular weight marker, Lane 2. Shark ASC, Lane 3.Shark PDC, Lane 4.Tuna ASC Lane 5.Rohu ASC, Lane 6.Rohu PDC, Lane 7. Type 1 collagen from calf skin.

CONCLUSION

The acetic acid soluble & pepsin digestible collagens from the skin of three verities of fishes viz Albacore tuna (*Thunnus alalunga*), Dog shark (*Scoliodon sorrakowah*), and Rohu (*Labeo rohita*) were extracted and characterized. The result showed that the pepsin can act as a tool for obtaining a greater yield without having a noticeable effect on the triple helical structure except in the case of tuna skin. All the collagens were of typical amino acid composition of type 1 collagen. All collagens showed maximum absorption at 200-235nm with no absorption at 280. No differentiation could be observed in the collagens from the three species regarding ($\alpha_1\alpha_2$), β, γ chains indicating their type 1 nature. The amino acid pattern, SDS-PAGE and the absorbance at 200-240 nm of collagens extracted in the present study indicates that the process of extraction yielded pure collagen with a purity of greater than 99%.

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