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Isolation of acid and pepsin soluble collagens from the skin of Indian mackerel *Rastrelliger kanagurta* (Cuvier, 1817)

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Abstract
The objective of this study was to isolation and compare the acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from the skins of Indian mackerel. Collagen was extracted from the skin of *Rastrelliger kanagurta*, the fish from marine water. Acid soluble collagen (ASC) and Pepsin soluble collagen (PSC) were extracted from mackerel skin. The yield of ASC and PSC from mackerel skin was recorded as 9.32% and 7.68% respectively; higher yield was recorded in ASC method. It was found that the major constituents of both ASC and PSC consisted of alpha chains (α1, α2) and beta (β) chains. Molecular weight pattern from mackerel skin collagen is shown in various bands, these bands with molecular weight in between 135 kDa to 180 kDa in ASC and PSC pattern. The proximate composition of collagen extracted from mackerel skin i.e. moisture, crude protein, fat and ash content in collagen was recorded as 9.17%, 87.74%, 1.23% and 1.86% respectively.

Keywords: Acid soluble collagen (ASC), Pepsin soluble collagen (PSC), Molecular weight pattern, proximate compositions

Introduction
Collagen, the main structural protein of metazoans, is present in several types of tissues such as skin, bone, cartilage, etc. [1]. The word "collagen" is derived from the Greek words 'kolla' and 'genos' meaning glue and formation, respectively [2]. It has got a variety of biomedical and pharmaceutical applications. Their applications include treatment of pain associated with osteoarthritis, hypertension, use in tissue engineering, implants in human inhibition of angiogenic diseases, etc. [2]. Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein [3]. Collagens have been used widely as materials in the pharmaceutical, cosmetic, biomedical and food industries [4]. So far, skin and bone collagen from several fish species have been isolated and characterised [5-7]. Collagen of fish skins studied in recent years were mainly from marine species, such as Black Drum (*Pogonia cromis*) [8], brown stripe Red Snapper (*Lutjanus vitta*) [6]. Isolation and characterization of collagen from fresh water fish, however, was rarely reported, except for the Nile perch (*Lates niloticus*) [9]. Collagen has a wide range of applications in leather and film industries, pharmaceutical, cosmetic and biomedical materials and food [10-12]. For biomedical and cosmetic applications it is crucial to extract collagen with denaturation temperature closed to the value of denaturation temperature of mammalian collagen. This is why thermostable collagen from the marine sources has been studied [13]. The presence of collagen in all connective tissue makes it one of the most studied biomolecules of the extracellular matrix. This fibrous protein species is the major component of skin and bone and represents approximately 25% of the total dry weight of mammals [14] Biomedical and pharmaceutical applications of collagen include the treatment of hypertension, urinary incontinence and pain associated with osteoarthritis, use in tissue engineering for implants in humans, inhibition of angiogenic diseases, such as diabetes complications, obesity, and arthritis [2]. Commonly, the main sources for collagen product ion are pig skin, cattle skin and bone. The outbreak of BSE, TSE and foot and mouth diseases has resulted in justified anxiety amongst users of cattle collagen [15]. As a consequence, much attention has been paid to alternative sources of collagen, especially from fish skin and fish bone from the seafood processing industries [16]. Developed a simple method 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 Rohu (*Labeo rohita*) [17].

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) collagens and by extracting from the skin, bone and muscle of a trash fish, leather jacket (*Odonus niger*) with three different extraction methods [18]. Therefore, the objective of the present study was to extract Indian mackerel collagen and its characterization.

Materials and Methods

The selected species of Indian mackerel (*Rastrelliger kanagurta)* in the iced condition was procured from, Mirkarwada fish landing centre in post monsoon season, Maharashtra, India. Fish was stored in chilled box and transported to the laboratory and kept at -20 °C prior to further study. The selected skin and the purified collagen were analysed for the proximate composition. The meat was analyzed for proximate composition which includes moisture, crude protein, fat and ash by following standard methods of AOAC [19]. The moisture content was estimated by moisture analyzer (Milton Model – Mm113.11). Crude protein was estimated by using Kjeldahl method (Kel Plus Classic DX, Pelican, India). Fat content was determined by Soxhlet method (Sosc Pus-SCS 2, Pelican, India). Ash content was estimated by using muffle furnace (Classic Scientific, India) [19]. All used chemicals were of analytical grade. All results were calculated in percentage on wet weight basis.

Collagen extraction

The extraction of Acid Soluble Collagen (ASC) and Pepsin Soluble Collagen (PSC) was done according to the methods [20] with certain modifications from the skin of *Rastrelliger kanagurta*. All the extraction procedures were carried out at 4 °C.

Sample preparation for collagen extraction

Frozen fish was thawed under tap water, washed with cold water (5-8 °C) and then the skin was removed the skin from flesh and cut into small pieces (2 ± 0.5 cm^2). The prepared skin sample was used for the extraction collagen.

Pre-treatment of the skin

To remove non-collagenous protein and pigments, the skin of *Rastrelliger kanagurta* was mixed with 20 volumes of 0.1N sodium hydroxide and kept stirred for 24 hrs, with the alkaline solution being changed every 12 hrs. The treated mass was strained through a coarse sieve. The process was repeated twice and the residue was washed twice with 20 volumes of chilled distilled water.

Acid Extraction

The residue was homogenized in a homogenizer (VELP Scientifica, OV5 homogenizer, made in Europe) with 20 volumes 0.5M acetic acid for one minute and the same was stirred over a magnetic stirrer (Labline, made SUNBHIM) for 24 h. The supernatant after centrifugation (4000 rpm, 15 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 20 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 soluble collagen (PSC). Crystalline sodium chloride (NaCl) was added to both supernatants to the level of 10% and stirred for 24 hrs 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 24h and then centrifuged. The liquid precipitate was dried in vacuum oven dryer at 45 ^{0}C for 24 hrs. After drying, grind the sample to get fine powder.

Percentage yield calculation

Collagen yield (dry basis) from the skin was calculated by the following formula:

$$\text{Collagen yield (\%)} = \frac{\text{(Weight of final collagen sample in g)}}{\text{(Weight of skin sample in g)}} \times 100$$

SDS-poly acryl amide gel electrophoresis

Electrophoretic patterns of collagens obtained from mackerel skin were analysed according to the method [21] with certain modifications. The samples (2mg/ml) were dissolved in 50 g/L SDS solution. The mixtures were then heated at 85°C for 1h, followed by centrifugation at 8500 rpm 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 of 100 mL/L β mercaptoethanol) 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 20mA. 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 distained with a mixture of 300 mL/L methanol and 100 mL/L acetic acid for 1h and destained again with the same solution for 30 min. High molecular weight protein markers (10-245kDa) were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as standard collagen.

Results and Discussion

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from mackerel skin. The yield of ASC and PSC mackerel skin were recorded as, 9.32 ± 0.54 and 7.68 ± 1.25% respectively. Higher yield was recorded in ASC extraction method as shown in (Table 1 and Fig.1). The proximate composition of mackerel fish ASC and PSC i.e. moisture, protein, fat and ash content was recorded as 9.17 ± 0.61, 87.74 ± 0.15, 1.23 ± 0.36, 1.86 ± 0.49% and 09.8 ± 0.96, 85.1 ± 0.32, 01.06 ± 0.15, 01.92 ± 0.20% respectively as shown in (Table 2 and Fig. 2 and 3). Fig. 4 shows the electrophoretic patterns of collagens against the high molecular weight marker. The protein patterns of ASC and PSC were analysed by 10% resolving gel and 5% stacking gel. Molecular weight pattern from mackerel skin collagen is shown in various bands, these bands with molecular weight of in between 135kDa to 180 kDa in ASC and PSC pattern. Lane 1 and 3 marker standard protein, carbonic anhydrous (30 kDa), Ovalbum (45 kDa), Serum albumin (66 kDa), Phosphorylase – b (96 kDa), β- galactosidase (120 kDa), myosin (200 kDa), lane 2 and lane 4 is ASC and PSC respectively. The yield obtained from ASC method for tuna skin, rohu skin and shark skin was 13.97%, 4.13% and 8.96% respectively as per 17. Similarly, in the same study, the yield obtained from rohu skin and shark skin by PDC method was 3.68% and 7.68% respectively. However, in the present study the yield from ASC method and PSC method was 9.32% and 7.68% respectively. Yields of ASC and PSC from *Lates*

calcarifer scales on a dry weight basis were found 0.38% and 1.06% g/100 g, respectively. PSC showed that higher yield than ASC was reported in *L. calcarifer* [22] and collagen content of tilapia was higher than of collagen yield 15% obtained through alcalase hydrolysis condition as per 23. In this present study, the recovery of ASC and PSC was 9.32 and 7.68%, respectively. Therefore, the low extraction yield of both ASC and PSC was more likely governed by the high cross-links of collagen. The result of proximate composition of mackerel skin, ASC and PSC collagen were given in Table 2 and Fig. 2 and 3. The results showed that moisture level was relatively low (9.17%), the protein and fat levels were 87.74% and 1.23% respectively and ash content was 1.86%. Based on proximate analysis, moisture content in mackerel skin collagen was near to other study which shark was 9.13% and moisture content of mackerel skin collagen tuna was higher than of moisture 7.53% as per 17. Protein content in mackerel skin collagen was near to other study which shark was 88.80% and protein content of mackerel skin collagen tuna was lower than of protein 91.08% as per 17. Fat content in mackerel skin collagen was lower than other study which shark was 0.37% and protein content of mackerel skin collagen tuna was lower than of protein 70.64%. Moreover, the ash content of mackerel skin collagen was higher than tuna and shark skin collagen was 0.74% and 0.80% respectively as per 17. Extracted collagens from skin had low contents of ash and fat, indicating the efficiency of removal of both inorganic matter and fat. Collagens sample have low moisture contents and high protein content. The molecular weight pattern of collagens against the high molecular weight marker. The protein patterns of ASC and PSC were analysed by 10% resolving gel and 5% stacking gel and it was found the major constituents of both ASC and PSC consisted of alpha chains (α_1, α_2) and beta (β) chains. Molecular weight pattern from Mackerel skin collagen is shown in various bands, these bands with molecular weight of in between 135kDa to 180 kDa in ASC and PSC pattern. These pattern were similar to the type I collagen of shark and tuna as per 17 and also in accordance with those of collagen from most other fish species previously reported by 9, 24 and 15.

Table 1: Yield of mackerel skin collagen extracted by ASC & PSC method

Type of collagen	Yield of collagen (%)
ASC	9.32 ± 0.54
PSC	7.68 ± 1.25

Values are given as mean ± standard deviation of triplicate

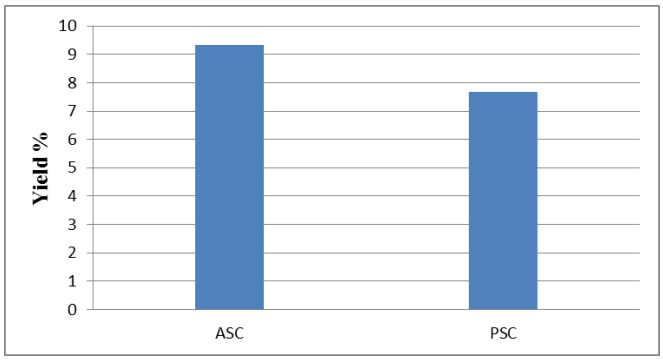

Fig 1: Yield of mackerel skin collagen extracted by ASC & PSC method

Table 2: Proximate composition of mackerel, skin, ASC and PSC

S. No	Proximate composition (%)	Mackerel	Skin	ASC	PSC
1	Moisture	72.24±1.20	69.45±0.7	9.17±0.61	09.8±0.96
2	Protein	19.14±1.43	16.20±0.6	87.74±0.15	85.1±0.32
3	Fat	08.19±0.85	6.15±0.25	01.23±0.36	01.06±0.15
4	Ash	01.42±0.45	07.65±1.2	01.86±0.49	01.92±0.20

Values are mean ± standard deviation of triplicate

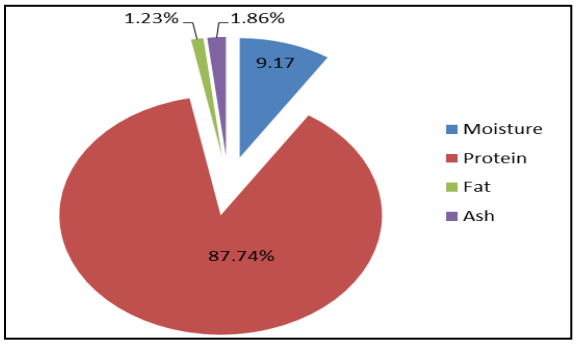

Fig 2: Proximate composition of ASC

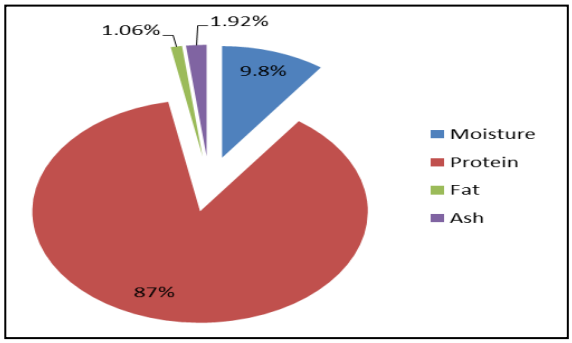

Fig 3: Proximate composition of PSC

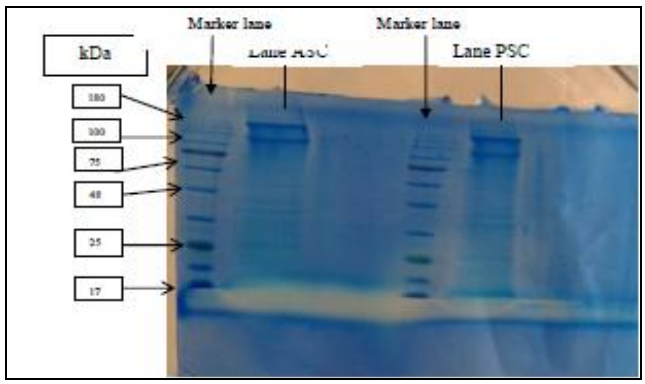

Fig 4: SDS- PAGE patterns of ASC and PSC from the skin of mackerel

Conclusion

The mackerel proximate composition was recorded moisture, crude protein, fat and ash content 72.24%, 19.14%, 8.19% and 1.42% respectively. Acid soluble collagen (ASC) and Pepsin soluble collagen (PSC) were extracted from mackerel skin. The yield of ASC and PSC from Mackerel skin was recorded, 9.32 ± 0.54 and 7.68 ± 1.25% respectively, higher yield was recorded in ASC collagen extraction method. The proximate composition of mackerel fish ASC and PSC i.e. moisture, protein, fat and ash content was recorded as 9.17 ±

0.61, 87.74 ± 0.15, 1.23 ± 0.36, 1.86 ± 0.49% and 09.8 ± 0.96, 85.1 ± 0.32, 01.06 ± 0.15, 01.92 ± 0.20% respectively. Molecular weight pattern from mackerel skin collagen is shown in various bands, these bands with molecular weight of in between 135kDa to 180 kDa in ASC and PSC pattern.

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