Chapter 2

ISOLATION & CHARACTERIZATION OF COLLAGEN FROM DIFFERENT SPECIES OF FISHES

Contents

2.1 Introduction

In recent years the utilization of waste from fish processing industry for the production of value added products has attracted substantial attention. The fish processing industry generates a large amount of waste. These wastes are a mixture of heads, viscera, skin and bone (Morrissey and Park, 2000). About 30% of such waste consists of skin and bone with high content of collagen (Gomez-Guillen *et al.*, 2002). Fish skin, which is a major byproduct of the fish-processing industry, could provide a valuable source of collagen (Badii and Howell, 2006). The solid waste from surimi processing, which may range from 50–70% of the original raw material (Morrissey *et al.*, 2005), could also be the initial material for obtaining collagen from under-utilized fish resources.

Collagen is one of the most abundant biological macromolecules of extracellular matrix where it provides the major structural and mechanical support to tissues. Native collagens from different sources find applications in biomedical and pharmaceutical industries as well as cosmetics. Denatured collagen, known as gelatin, finds applications in the food and biomedical industries. 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 (Rehn *et al.*, 2001).

Commonly, the main sources for collagen production 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 (Kittiphattanabawon *et al.*, 2005). 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. Gudmundsson and Hafsteinsson (1997); Choi and Regenstein (2000) have suggested that, the commercial use of fish skin and bones, which are normally discarded, is a good waste management practice leading to additional economic benefit.

So far, skin collagen from several fish species have been isolated and characterized such as big eye snapper skin (Jongjareonrak *et al.*, 2010), Nile perch skin (Muyonga *et al.*, 2004), Baltic cod skin (Sadowska *et al.*, 2003) and deep-sea redfish skin (Wang *et al.*, 2008). The major impediment to dissolution of collagen type I from tissue is the presence of covalent cross links between molecules. Collagen is insoluble in organic solvents. Water soluble collagen represents only a small fraction of total collagen and the amount depends on the age of the animal and type of tissue extracted. Collagen molecules present within fibrillar aggregates can be dissociated and brought into aqueous solution. However, the nature of the crosslinks prevalent in different tissues determines the particular extractant to be used and the corresponding yields.

Many protocols were tried for the extraction of collagen from fish processing discards, particularly fish skin. For isolation and characterization of collagen extracted from the skin of stripped catfish, the skin is washed with cold water at 5 to 8°C (Prabjeet *et al.*, 2011). Collagen is extracted at appropriate temperatures to obtain good quality collagen. However, according to Nagai and susuki (2000b) in isolation of collagen from fish waste material-skin, bone,

and fin, all the preparative procedures are to be done at 4°C. For extraction using pepsin-digestion, collagen extraction was done at 4°C for 24 hours before being salted with 0.5M NaCl (Nagai and susuki, 2000b). They extracted collagen from fish waste materials such as skin, bone, and fins, for three days using 0.5M acetic acid. However, the skin was not completely solubilized with 0.5M acetic acid for three days. So the residues were re-extracted with the same solution for a further two days. All residues were then solubilized and highly viscous solutions were obtained. Senar atne *et al.* (2006) who used 0.5M acetic acid combined with 10% (w/v) pepsin found that the fish skin completely got solubilized. The addition of 0.9M NaCl into collagen extract before centrifugation helped to induce salting out of collagen with better purification (Falguni *et al.*, 2010).

The physical, chemical and rheological properties of collagen depend upon their source. The properties of collagen markedly vary with the habitat, species, and part of fish being isolated (Falguni *et al.,* 2010). A number of studies have addressed properties of fish skin Collagen (Arnesen and Gildberg, 2007; Choi and Regenstein, 2000; Gomez- Guillen & Montero, 2001; Grossman and Bergman, 1992; Gudmundsson, 2002; Gudmundsson and Hafsteinsson, 1997; Holzer, 1996; Jamilah and Harvinder, 2002; Jongjareonrak *et al.*, 2006; Zhou and Regenstein, 2005) showing that their properties differ from those of mammalian collagens.

Fish collagen has lower denaturation temperatures compared to vertebrate's collagen. The denaturation temperature of mammalian collagen was higher than 30°C while most fish collagens denature at temperatures below 30°C (Ogawa *et al.,* 2003). Marine collagen had a lower denaturation temperature by about 10°C than that of the porcine skin collagen (Nagai *et al.,* 2008). This indicates that fish collagen is generally less stable than mammalian counterparts (Ogawa *et al.,* 2003). Bae *et al.* (2008) observed that the amino acid composition of collagens from cartilaginous fish differ from those of bony fish.

Collagen is composed of three triple helix polypeptide chains (2α and 2β chains). Each chain contains about 1000 amino acid residues in size and has an average length of 300 nm and diameter of 1.4 nm. Collagen triple helix is stabilized mainly by inter and intra chain water mediated hydrogen bonding as well as directs inter-chain hydrogen bonding (Brodsky and Persikov, 2005). Collagen has a repetitive primary sequence, of which every third residue is glycine (Whitford, 2005). Most fish collagens have been found to consist of two α-chain variants, which are normally denoted as α1 and α2 (Nagai *et al.*, 2001; Gomez-Guillen *et al.*, 2002). These alpha chain variants though having approximately the same molecular weight (~95,000Da) can be separated by SDS PAGE due to their different affinity for SDS (Kubo and Takagi, 1984).

Cho *et al.* (2005) reported that collagen extracted from yellow fin tuna skin showed better functional properties than those from other fish sources. Some studies have ascertained that freshwater fish can have a high collagen yield (Grossman and Bergman, 1992; Jamilah and Harvinder, 2002; Muyonga *et al.*, 2004a). Only a few studies have been conducted on warm-water fish collagen and these showed that collagen from these species had better functional properties than those from cold-water fish species (Gilsenan and Ross-Murphy, 2000; Grossman and Bergman, 1992)

Dog shark and skipjack tuna are commercially important species available along the south west coast of India. Rohu is one of the major carp species, a natural inhabitant of the freshwater sections of the rivers of India and contributes to the inland catch. At present, the fishery is sustainable for all the above species.

The objective of the present study was to isolate and characterize collagen from the skin of five different species of fishes viz. Albacore tuna (*Thunnus alalunga*), Dog shark (*Scoliodon sorrakowah*), and one among Indian Major Carps ie, Rohu (*Labeo rohita*), Queen Fish (*Scomberoides lysan*) and Grouper (*Epinephelus malabaricus*).

2.2 Materials and methods

2.2.1 Raw materials

The skins of the selected species in the iced condition were procured from Fort Cochin (9.9680°N, 76.2449°E), Kerala, India. Skin were stored in ice separately 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.

2.2.2 Collagen extraction

Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PDC) were extracted from the skin of five species of fishes. All the extraction procedures were carried out at 4°C.

2.2.2.1 Pretreatment of the skin

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.

2.2.2.2 Acid 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).

2.2.2.3 Salt precipitation and Dialysis

Crystalline sodium chloride was added to both supernatants to the level of 10% and stirred for 24h 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 collagen obtained was spray dried to get fine powder.

2.2.2.4 Percentage yield calculation

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

(Weight of final collagen sample in g) / (weight of skin sample in g) × 100

2.2.2.5 Solubility

The collagen solubility was determined by the method of Montero *et al.,* 1991 with a slight modification. The collagens were dissolved in 0.5 M acetic acid to obtain a final collagen concentration of 3mg/ml and the mixture was stirred at 4°C until collagen was completely solubilized.

2.2.2.6 Effect of pH on collagen solubility

Solubility at different pH and NaCl concentration was determined by the method of Kittiphattanabawon *et al.* (2005). Collagen solution (8 ml) was added to a centrifuge tube and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain a final pH ranging from 1 to 10. The volume of solution was made up to 10 ml by distilled water previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000g at 4°C for 30 min.

Protein content in the supernatant was determined by Lowry's method, using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

2.2.3 Proximate composition analysis

The selected skin and the collagen extract were analyzed for the proximate composition. The content of moisture, protein, fat and ash were analyzed by the method of AOAC (2000).

2.2.3.1 Determination of moisture

The moisture content was determined according to the AOAC (2000) by drying 10.0 g sample at 105°C in a thermostatically controlled hot air oven. The sample were taken in a pre-weighed petri dish and kept in oven and the reduction in weight was checked by repeatedly weighing and then cooling the sample in desiccators till the weight become constant. Moisture content was expressed as percentage.

2.2.3.2 Determination of crude protein

Micro- Kjeldahl distillation method for crude protein

One gram of the homogenized sample was accurately weighed into digestion tube. About 2.0 g of digestion mixture ($CuSO_4$ & K_2SO_4) as in the ratio of (1:8) and 10ml of H_2SO_4 (AR) were added to the sample taken in the digestion tube. The sample was digested to a clear solution in digestion unit and the solution thus obtained was made up with 100 ml of distilled water by intermittent cooling. 5ml of this sample solution was pipetted out into the Kjeldhal micro distillation apparatus, followed by the addition of 10 ml of 40% NaOH. The ammonia produced on steam distillation was absorbed into 2% boric acid solution with Tashiro's indicator. The distillate collected was back titrated against N/70 H_2SO_4. Crude protein

content in the sample was calculated by multiplying the nitrogen content by the factor of 6.25 and expressed as percentage.

2.2.3.3 Determination of crude fat

The estimation of crude fat content was done by continuous extraction of fat with petroleum ether, by soxhlet extraction method (AOAC, 2000). About 2 g of moisture free sample was accurately weighed into an extraction thimble and was placed in the extractor. The extractor was connected to a pre-weighed dry receiving flask and water condenser. The solvent in the receiving flask was evaporated completely and weighed for fat content. The result was expressed as percentage of crude fat.

2.2.3.4 Determination of ash

The ash content was measured by incineration of the sample according to AOAC, 2000). About 2.0g of moisture free sample taken in a pre-weighed clean dry silica crucible was charred on a low heat, followed by incineration in a muffle furnace at 550°C to get a white ash. Silica crucible were finally cooled in desiccators and weighed. Ash content was expressed as percentage.

2.2.4 Characterization of extracted protein

2.2.4.1 Amino acid composition analysis by HPLC

Collagen samples were hydrolyzed in 6N HCl at 120°C 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 of Ishida *et al*. (1981).

2.2.4.2 SDS-poly acryl amide gel electrophoresis

Electrophoretic patterns of different species of collagens were analysed according to the method of Laemmli (1970). The samples were dissolved in 50 g/L SDS solution. The mixtures were then heated at 85°C for 1h, 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 20mA/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 1h 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 collagen.

2.2.4.3 UV spectrophotometric analysis

Collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1mg/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.

2.2.4.4 FTIR analysis

FTIR spectra of collagens were carried out as per the method described by Muyonga *et al.* (2004b) using a Nicolet Avatar 360 ESP Infrared Spectrophotometer at a scanning range from 400 to 4000

cm^{-1} at data acquisition rate of 4 cm^{-1} per point. Spectra were obtained from tablets containing 2mg collagen samples in approximately 100 mg potassium bromide (KBr). All spectra were obtained after background substraction using the Omnic software.

2.2.5 Statistical analysis

All experiments were done in triplicates. Mean values with standard deviations (SD) were reported. Statistical calculations were performed in the SPSS 20 software package (IBM SPSS statistics, Version 20). Data analyses were performed using one way analysis of variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD test. P values ≤ 0.05 were considered statistically significant.

2.2 Result and Discussion

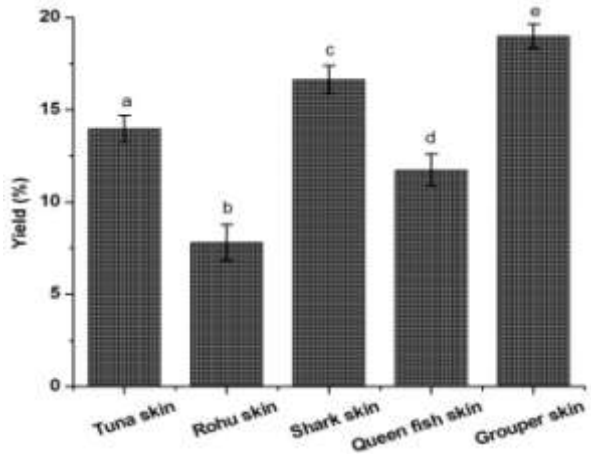
2.3.1 Collagen extraction

Acid Soluble Collagen (ASC) and Pepsin Digestible Collagen (PSC) were extracted from all the five species of fishes. The total collagen yields were 13.97% (±0.73%SD), 7.81% (±0.98% SD), 16.64% (±0.76% SD), 11.74% (±0.88% SD) and 18.99% (±0.65% SD) on dry weight basis for tuna skin, rohu skin, shark skin, queen fish skin and grouper skin respectively. Highest yield among the five species was obtained from grouper skin. One way ANOVA showed that there is significant difference in the yield of collagen from different species of fishes.

Tuna skin collagen has got completely dissolved in 0.5M acetic acid. In the case of other fishes, skin collagen was not completely solubilized with 0.5 M acetic acid, but further solubilization of the remaining residue was achieved by limited pepsin proteolysis. This result might suggest that there were lots of cross-links in fish skin collagens other than tuna at the telopeptide region, as well as at the inter-molecular cross-links, leading to a low solubility of collagen in acid (Foegeding *et al.,* 1996; Zhang *et al.,*

2007). With further limited pepsin treatment, the cross-links at the telopeptide region were cleaved without damaging the integrity of the triple helix. Therefore, a high solubility of collagen in acid was obtained after adding pepsin. The yields of ASC and PSC from different fish skins have been reported for black drum (2.3% and15.8%, respectively), for channel catfish (25.8% and 38.4% respectively), for paper nautlius (5.2% and 50% respectively) and for ocellate puffer fish (10.7% and 44.7%, respectively) (Nagai and Suzuki, 2002; Nagai *et al.*, 2002a; Ogawa *et al.*, 2003; Liu *et al.*, 2007)

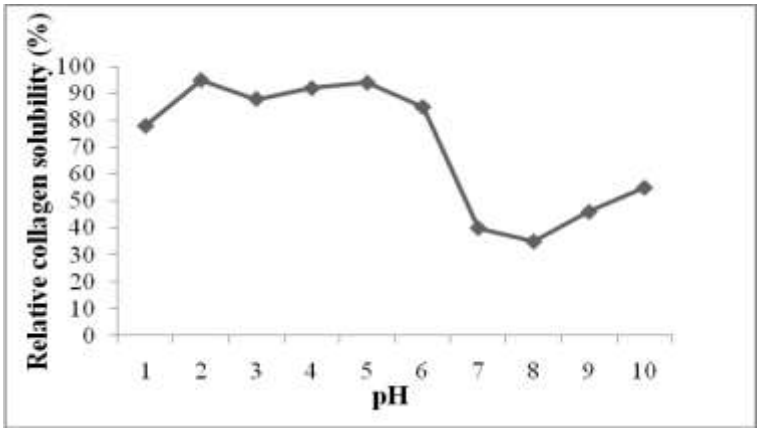
Figure 2.1 Yield of total collagen (%) from the skin of five species of fishes on dry weight basis.



2.3.2 Solubility of collagens

Highest solubility of collagens from the skin of the selected species was found at pH 2 and 5, respectively. Generally, both collagens could be more soluble in the acidic pH ranges (Foegeding *et al.*, 1996). Sharp decrease in solubility was observed at neutral pH. However, solubility was slightly decreased at very acidic pH. When pH values are above and below pI, a protein has a net negative or positive charge, respectively (Vojdani, 1996). As a consequence, more water interacts with the charged protein for water, thereby causing the protein to precipitate (Vojdani, 1996).

Figure 2.2 Relative solubility of grouper skin collagen at different pH



2.3.3 Proximate composition analysis

Table 2.1 shows the protein, moisture and ash content of the skin of the selected species of fishes and table 2.2 shows the same 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.

One way ANOVA test was used to compare difference in the means of the moisture content, crude protein, fat and ash contents of the skins of different species of fishes. This was followed by tukey's post hoc analysis to determine in more detail how the proximate composition varies in the skin of different species of fishes. Analysis shows that there were significant differences in the moisture content of all the species. But there were no significant difference in the protein contents of queen fish and tuna skin and also in the case of grouper and rohu skin. Also there were no significant difference in the ash content of tuna and shark skin and fat content of queen fish and grouper skin. All other values were significantly showing differences in the protein, fat and ash content in different fish varieties.

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 2.2).

Table 2.1 Proximate composition analysis of skin of the selected fish species

Sample	*Moisture*	*Protein*	*Ash*	*Fat*
Queen fish skin	64.65 ± 0.63^{a}	21.10 ± 0.17^{a}	7.02 ± 0.14^{b}	05.07 ± 0.16^{a}
Grouper skin	61.71 ± 0.36^{b}	18.47 ± 0.24^{b}	7.56 ± 0.58^{c}	$05.4\ 0 \pm 0.11^{a}$
Tuna skin	56.54 ± 0.09^{c}	20.54 ± 0.26^{a}	4.39 ± 0.03^{a}	08.32 ± 0.11^{b}
Rohu skin	76.54 ± 0.43^{d}	18.84 ± 0.06^{b}	2.03 ± 0.04^{d}	02.93 ± 0.05^{c}
Shark skin	68.38 ± 0.43^{e}	27.73 ± 0.36^{c}	4.19 ± 0.03^{a}	00.16 ± 0.02^{d}

All values are expressed as mean ± standard deviation, n = 3. Different superscripts in the same column indicate significant differences ($p < 0.05$)

Table 2.2 Proximate composition analysis of extracted collagen

Sample	*Moisture*	*Protein*	*Ash*	*Fat*
Tuna ASC	7.53 ± 0.30	91.08 ± 0.71	0.74 ± 0.02	0.64 ± 0.01
Rohu ASC	8.78 ± 0.06	89.94 ± 0.75	0.43 ± 0.05	0.33 ± 0.05
Rohu PDC	6.66 ± 0.58	91.72 ± 0.59	0.5 ± 0.02	0.45 ± 0.02
Shark ASC	9.13 ± 0.14	88.8 ± 0.53	0.76 ± 0.03	0.37 ± 0.08
Shark PDC	8.32 ± 0.17	90.8 ± 0.12	0.8 ± 0.02	0.42 ± 0.05
Queen fish ASC	7.53 ± 0.3	91.08 ± 0.71	0.64 ± 0.04	0.74 ± 0.04
Queen fish PDC	8.78 ± 0.06	89.94 ± 0.75	0.33 ± 0.04	0.43 ± 0.05
Grouper ASC	6.66 ± 0.52	91.72 ± 0.53	0.45 ± 0.02	0.5 ± 0.02
Grouper PDC	9.13 ± 0.14	88.8 ± 0.59	0.37 ± 0.02	0.76 ± 0.06

All values are expressed as mean ± standard deviation, n = 3.

2.3.4 Amino acid composition analysis

The amino acid compositions of collagen extracted from the various species are shown in Table 2.3. It is expressed as amino acids g/100g protein. The collagens were found to contain no tryptophan or cysteine. They were also very low in methionine, tyrosine and histidine contents, like in all other collagens.

Fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) varied significantly among fish species (Balian and Bowes, 1977; Gudmundsson and Hafsteinsson, 1997). 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 (Balian and Bowes, 1977; Kimura *et al.*, 1991; Rigby, 1968). When considering the imino acid (proline and hydroxyproline) of collagen extracted from the five species in the present study, they had a higher content when compared to, Sole (17.4%) Megrim (17.5%), Cod (15.6%), Hake (17.3%) and Squid (17.5%) (Gomez-Guillen *et al.*, 2002)

Glycine is the major amino acid in all species of collagens as reported by Senaratne *et al.* (2006) followed by proline, alanine, and hydroxyproline. In the collagen of ocellate puffer fish, glycine is the most abundant followed by alanine, proline, and glutamic acid (Takeshi *et al.*, 2002).

Table 2.3 Amino acid composition of Shark Skin Collagen (SSC), Rohu skin Collagen (RSC), Tuna skin Collagen (TSC), Queen Fish Skin Collagen (QSC) and Grouper Skin Collagen (GSC)

Amino acid	*Amino acids g/100g protein*				
	SSC	***RSC***	***TSC***	***QSC***	***GSC***
Aspartic	3.77 ± 0.38	4.43 ± 0.42	4.07 ± 0.31	3.47 ± 0.31	4.03 ± 0.42
Threonine	1.98 ± 0.17	2.30 ± 0.36	2.33 ± 0.32	2.67 ± 0.38	2.17 ± 0.25
Serine	3.53 ± 0.21	4.17 ± 0.40	4.37 ± 0.38	3.23 ± 0.35	3.80 ± 0.36
Glutamic	7.67 ± 0.21	6.27 ± 0.40	7.33 ± 0.40	6.43 ±0.32	4.90 ± 0.26
Proline	9.88 ± 0.27	11.77 ±0.85	10.23 ± 1.10	10.97 ± 0.55	12.27 ± 0.60
Glycine	32.53 ± 1.11	33.07 ± 1.24	33.43 ± 1.19	31.41 ± 1.98	31.23 ± 1.33
Alanine	11.23 ± 0.61	13.10 ±0.82	12.07 ± 0.60	12.57 ± 0.86	11.37 ± 0.47
Cystine	ND	ND	ND	ND	ND
Valine	2.60 ± 0.30	3.03 ± 0.42	2.90 ± 0.36	2.40 ± 0.36	2.73 ± 0.31
Methionine	1.03 ± 0.25	1.20 ± 0.36	1.27 ± 0.32	0.77 ± 0.15	0.90 ± 0.10
Isoleucine	1.57 ± 0.35	0.83 ± 0.06	1.10 ± 0.26	0.77 ± 0.12	1.03 ± 0.21

Leucine	2.23 ± 0.42	2.23 ± 0.38	2.03 ± 0.35	2.47 ± 0.38	2.33 ± 0.32
Tyrosine	0.13 ± 0.06	0.23 ± 0.06	0.30 ± 0.00	0.80 ± 0.10	0.37 ± 0.06
Phenylalanine	1.50 ± 0.36	1.90 ± 0.44	1.43 ± 0.23	1.37 ± 0.31	1.47 ± 0.21
Histidine	0.87 ± 0.21	0.70 ±0.10	0.87 ± 0.06	1.00 ± 0.20	1.43 ± 0.42
Lysine	2.77 ± 0.25	2.47 ± 0.38	2.57 ± 0.35	3.17 ±0.23	3.80 ± 0.44
Arginine	5.20 ± 0.46	5.53 ± 0.35	4.83 ± 0.31	4.57 ± 0.31	5.13 ± 0.45
H. Proline	9.83 ± 0.84	6.83 ± 0.67	7.90 ± 0.30	10.13 ± 1.39	9.77 ± 0.35
TOTAL	98.33	100.07	99.03	98.18	98.73
Imino acid (Pro + Hyp)	19.71	18.60	18.13	21.10	22.04

2.3.5 SDS-poly acrylamide gel electrophoresis

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 (α1 α2), 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 (Muyonga *et al.*, 2004a; Nagai *et al.*, 2001).

Type I collagen consists of two identical α1 chains and one α2 chain (Burghagen, 1999; Foegeding *et al.*, 1996; Wong, 1989). Fish skin and bone have been reported to contain type I collagen as the major collagen (Ciarlo *et al.*, 1997; Montero *et al.*, 1991; Nagai and Suzuki, 2000b).

The skin collagens of bigeye snapper (Kittiphattanabawon *et al.*, 2005), brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010), Nile perch (Muyonga *et al.*, 2004a), ocellate puffer fish (Nagai *et al.*, 2002), back drum seabream, sheepshead seabream (Ogawa *et al.*, 2003), brown backed toadfish (Senaratne *et al.*, 2006), and largefin longbarbel catfish (Zhang *et al.*, 2007) all consisted of two α chains (α1& α2), β and γ components.

Figure 2.4 SDS PAGE Analysis: lane 1. High molecular weight markers, 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.

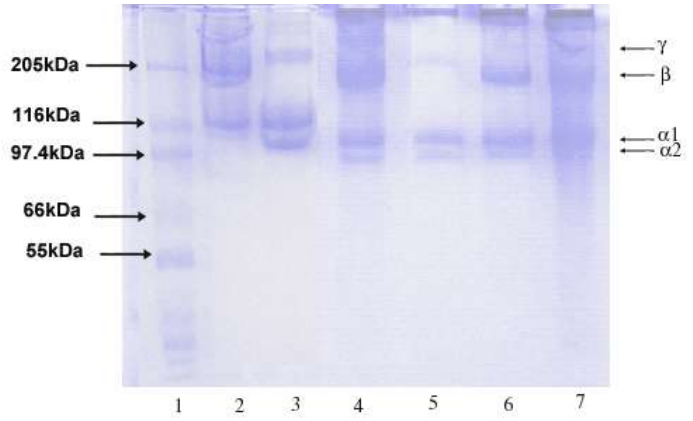
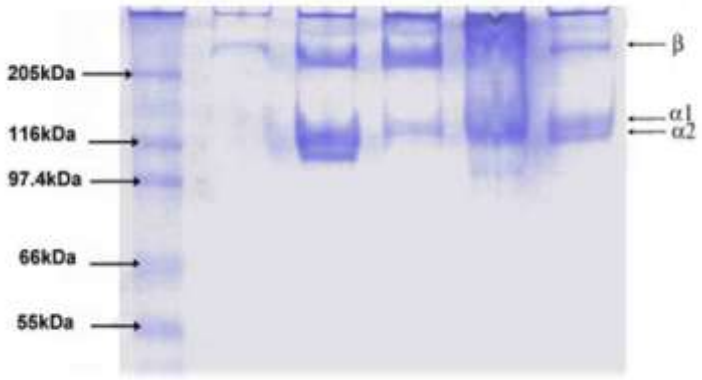


Figure 2.5 SDS PAGE Analysis: lane 1. High molecular weight marker, lane 2. Queenfish skin ASC, lane 3. Queen fish skin PDC, lane 4.Grouper skin ASC lane 5.Grouper skin PDC, lane 6. Type 1 collagen from calf skin.



2.3.6 UV spectrophotometric analysis

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. The absorbance in this region is similar to those of collagens from channel catfish skin, walleye Pollock, and large fin long barbel

catfish (Prabjeet Singh *et al.*, 2011). Peptide bonds found in the protein also absorb at 205-230nm. The absorbance at 280nm is mainly because of tryptophan, tyrosine and phenyl alanine. Tryptophan was completely absent in the extracted collagen. Also there was negligible amount of tyrosine detected. Previous researchers indicated that collagen commonly have a low amount of tyrosine which can absorb UV-light at 280 nm. Fig.2.6 to fig 2.11 depicts various UV spectra analysis plots for the samples.

Figure 2.6 UV analysis of pure collagen from calf skin

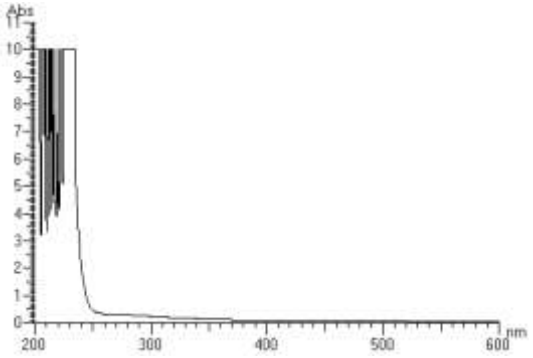


Figure 2.7 UV analysis of tuna skin collagen

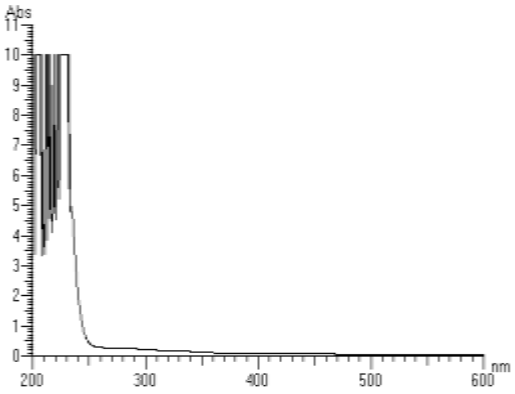


Figure 2.8 UV analysis of Rohu skin collagen

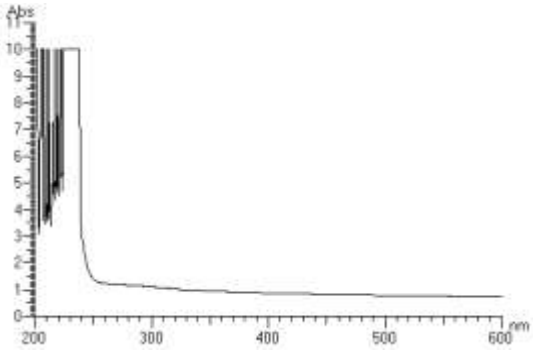


Figure 2.9 UV analysis of shark skin collagen

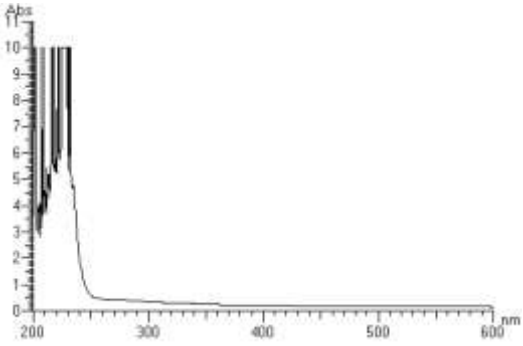
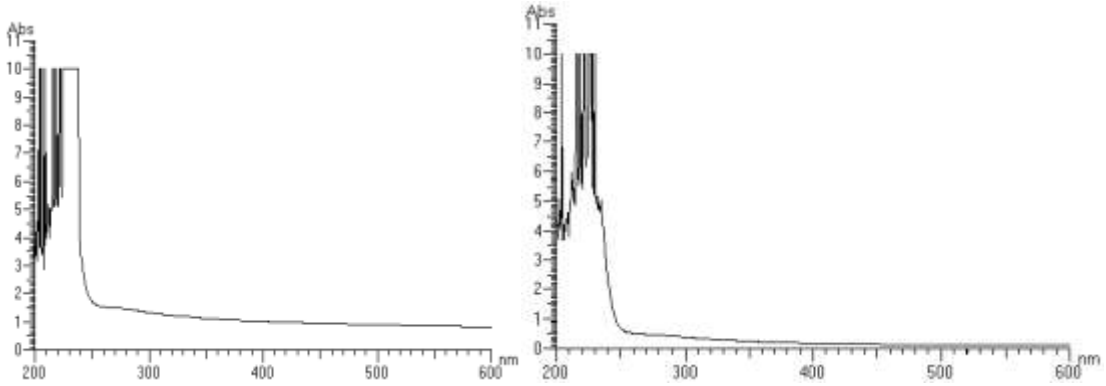


Figure 2.10 UV analysis of queen fish skin collagen

Figure 2.11 UV analysis of grouper skin collagen



2.3.7 FTIR analysis

The IR spectra obtained from the collagen extracted from the skin of different species of fishes are shown in fig. 2.12 – 2.14. The absorption spectra were obtained in the range between the wave number 400 – 4000cm^{-1}.

The analysis was used for detecting vibrational modes of individual chemical groups and bonds in the purified collagen. The spectra showed five characteristic amide bands representing amide A, amide B, amide I, amide II and amide III which are characteristic of the peptide bonds at the wavenumbers of 3413.39, 2926.45, 1644.98, 1245.79 and 1022.09 cm^{-1} respectively for shark skin. For grouper and croaker skin collagen the corresponding bands were observed in the wave numbers of 3419.17 and 3403.74 cm^{-1} for amide A band, 2916.64 and 2900.41 cm^{-1} for amide B band, 1629.55 and 1644.93 cm^{-1} for amide I band, 1205.5 and 1157.08cm^{-1} for amide II band and 1089.56 and 1063.55cm^{-1} for amide III band.

The amide B band positions found at wavenumbers in the range of 2900 to 2928 cm^{-1}, respectively, representing the asymmetrical stretch of CH_2. The amide I band with characteristic frequencies in the range from 1600 to 1700 cm^{-1} was mainly associated with the stretching vibrations of the carbonyl group along the polypeptide backbone, and was a sensitive marker of the peptide secondary structure. Amide II peak to N–H and C–N torsional vibrations, while amide III peak is associated to CH_2 residual groups from glycine and proline (Sionkowska *et al.,* 2006; Petibois *et al.,*

2006). Amide I band, amide II band and amide III band, which were known to be related to the degree of molecular order and to be involved with the triple helical structure of collagen, resulted from carbonyl group stretching, amino group bending and CH_2 stretching, respectively (Muyonga *et al.*, 2004). The FTIR spectra of skin collagen showed that the collagens extracted were of high purity.

Figure 2.12 FTIR spectra of queen fish skin collagen

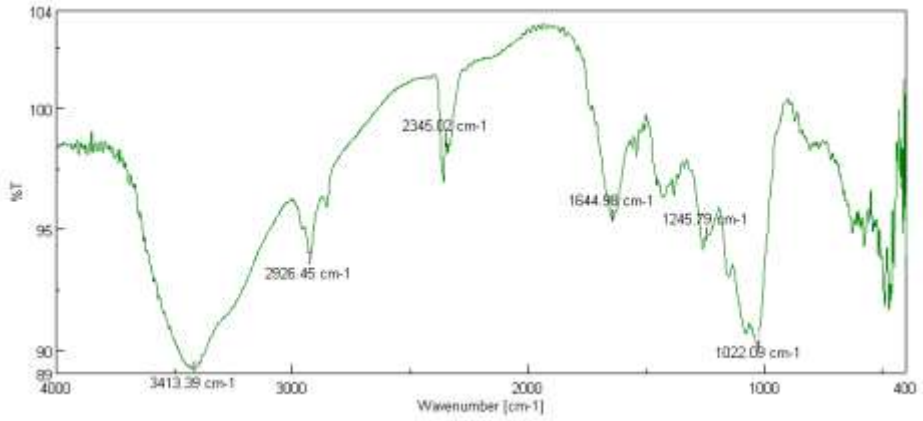


Figure 2.13 FTIR spectra of grouper skin collagen

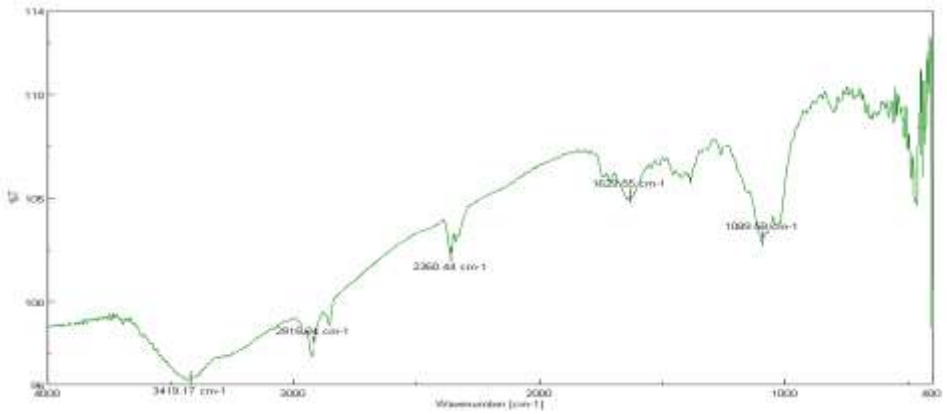
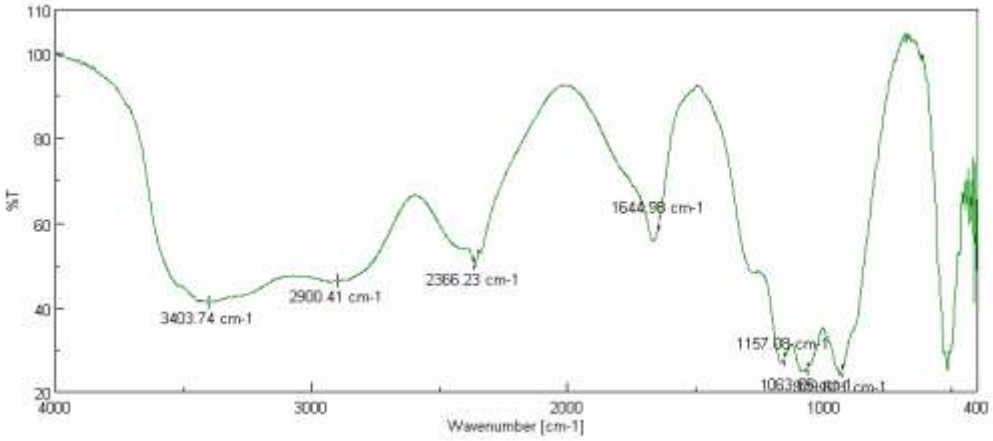


Figure 2.14 FTIR spectra of Shark skin collagen



2.4 Conclusion

A considerable quantity of acid soluble and pepsin digestible collagen were successfully extracted and characterized from the skin of five species of fishes. Pepsin aided extraction can serve as a tool for obtaining the greater yield without a marked effect on the triple-helical structure.

Extracted collagens from skin had low contents of ash and fat, indicating the efficacy of removal of both inorganic matter and fat and the protein content ranging from 88.8% to 91.72%. Further characterization studies with SDS PAGE, FTIR and UV Spectral analysis concludes that the extracted protein is collagen in pure form. HPLC analysis also confirms that the amino acid profile of extracted protein is characteristic to the protein collagen. The electrophoretic pattern reveals that the extracted skin collagen were type I nature and it consisted of two α-chains (α1 and α2).

Collagens showed high solubility at acidic pH (2–5) and the solubility markedly decreased in presence of NaCl (above 3%). High solubility of collagen is an attribute that makes it attractive as a commercial source. The results suggest that the extraction process yields collagen in pure form and could be used for wide applications in food, pharmaceutical, cosmetic and biomaterials.
