Isolation And Comparison Of Collagen Yield From Skin Of *Rhizoprionodon acutus , Scomberomorus guttatus* and *Rachycentron canadum*

Parimala Karthik[1], Bassil Aljallah[1], Bindhu.OS[1]*†

[1] *Department of Chemistry and Biochemistry, School of Sciences, Department Department of Chemistry and Biochemistry School of Sciences, Jain (DeemedtobeUniversity) Bangalore-560027*

Abstract

Fish waste generation is estimated to be about 4 million metric tons in India, which is mostly dumped into the environment indiscriminately. A sustainable way of managing this waste is to valorise it by generating products like enzymes, bio-polymers and bioactive peptides suitable for biotechnological and pharmaceutical applications. Collagen, an abundant extracellular matrix protein, is a high-value product that can be extracted from fish waste like skin. In the current study, collagen has been isolated from the skin of three different species of fish - Rhizoprionodon acutus **(**Milk shark**)**, Scomberomorus guttatus (Indo-pacific king mackerel) and Rachycentron canadum (Cobia fish). Acid and pepsin extraction methods were followed for isolation of collagen and the mean yield of collagen was calculated on a wet-weight basis. Attenuated Total Reflectance- Fourier Transform Infrared Spectroscopy (AT-FTIR) and Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) techniques were carried out for characterisation of extracted collagen. Results showed that collagen yield was 10.81%, 7.91 and 3.62% for Rachycentron canadum (Cobia fish), Rhizoprionodon acutus (Milk shark) and Scomberomorus guttatus (Indo-pacific king mackerel) respectively. Characterisation confirmed that it was type I collagen and comparable with standard mammalian type I collagen. Fish skin can thus, be an acceptable source of type I collagen which can be explored for diverse industrial applications.

Keywords: Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy, collagen, characterisation, fish skin, pharmaceutical applications

*Corresponding author.
†E-mail: os.bindhu@jainuniversity.ac.in

1 INTRODUCTION

Majority of the global population relies on edible fish to meet their protein, omega-3 fatty acids, macro and micronutrient requirements. An exponential increase in world population has thus witnessed a corresponding escalation in production of fish globally (212.8 million metric tons in 2020 from 148.1 million metric tons in 2010) (Shahbandeh,2022). India contributes to approximately 7.58% of the global fish production (https://en.gaonconnection.com) which has led to enormous generation of fish waste by fish processing and catching industries. India alone generates more than four million metric tonnes of fish waste (Yuvaraj et al., 2016). Sustainable disposal of this waste is a great challenge, as inappropriate dumping can add to existing environmental pollution. Valorisation of fish waste is a positive step in this direction which aims to convert the waste into value-added products like enzymes, collagen protein, bioactive peptides, biopolymers etc., (Shahidi et al., 2019).

Collagen protein is a valorised product that can be extracted from fish waste. Collagen confers rigidity and integrity to tissues and the extracellular matrix comprises primarily of this protein (about 30% vertebral protein is collagen) (Voet et al., 2006). Data reveals the occurrence of 29 types of collagen (I to XXIX) (Owczarzy et al., 2020) of which its type I category is predominantly present in the skin. It is also the most sought-after biomaterial and hence has found utility in different domains of biomedical industries (Lafarga and Hayes, 2014). Desirable functional properties exhibited by collagen such as enhanced water absorption capacity, emulsion formation & stabilisation and gel forming ability has favoured its application in food industry. It has found application in biomedical industry for the treatment of osteoporosis, tissue regeneration, as a vehicle for drugs and a substitute for human skin, blood vessels and ligaments (Kim and Mendis, 2006; Gómez-Guillén et al., 2011). Recent studies have examined the role of peptides from collagen with bio-functional activities like antimicrobial, antioxidant, anti-hypertensive etc., (Schmidt et al., 2016). Such wide range of applications have led to an escalating demand for this structural protein. The global market demand for collagen was 8.36 billion USD in 2020 and is expected to grow annually at a rate of 9% from 2020–2028 (http://www.grandviewresearch.com). Hitherto, it was produced from bovine and porcine sources to meet the growing industrial requirement, but threat of prion related diseases and for religious reasons, search for alternative sources of collagen gained momentum (Wang et al., 2014). Previous studies in this context has proved the suitability of marine waste as an excellent alternative source, environmentally and economically viable option for collagen production. The observations made by Nagai and Suzuki (2000) endorsed the use of fish waste such as skin, bone and fin as suitable sources for collagen production. Research has focussed on different extraction

procedures for isolation of collagen from fish waste based on their solubility in neutral, acidic solutions and acid solution with added enzymes. Acetic acid (0.5M) and pepsin aided extraction methods have been successfully used in collagen isolation from skin of channel catfish (Liu et al., 2007), brownbanded bamboo shark (Kittiphattanabawon et al., 2010), eel fish (Veeruraj et al., 2013), Nile Tilapia (Tang et al., 2018) so on. Recently, ultrasound treatment has also been explored for extraction along with traditional methods as it is found to lower the duration of processing and enhance yield (Kim et al., 2012). Various characterisation procedures are employed for confirmation of collagen types. Tylingo et al., (2016) characterised collagen extracted from Clarias gariepinus (African catfish), Salmo salar (Salmon) and Gadus morhua (Baltic Cod) skin using Attenuated total reflectance-Fourier Transform Infrared Spectroscopy (AT-FTIR) and Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) techniques to confirm its type I nature. Similar studies were carried out for collagen from skin of bluefin tuna (Tanaka et al., 2018) and Malaysian catfish (Kiew et al., 2013).

The present work was designed to prepare and characterise collagen from Rhizoprionodon acutus (Milk shark), Scomberomorus guttatus (Indo-pacific king mackerel) and Rachycentron canadum (Cobia fish) skin discarded during filleting. The mean percent yield of lyophilised collagen of the three species was compared.

2 MATERIALS AND METHODS

2.1 Chemicals/enzymes

Analytical grade chemicals like ammonium persulphate, butyl alcohol, coomassie Blue R-250, glacial acetic acid, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydroxide and N, N, N', N'-tetramethylethylenediamine (TEMED) were used. Pepsin (porcine stomach mucosa -EC 3.4.23.1) (1000 units/mg dry matter) and broad range protein molecular weight marker (3.5 to 200 Kda) were procured from Himedia. Rat tail collagen Type I was a kind gift received from Dr. Colin Jamora's laboratory (IFOM-InStem Joint Research laboratory). Skin of Cobia, Indo-pacific king mackerel and Milk shark fish was obtained from local fish markets of north and south Bengaluru.

2.2 Isolation of collagen from skin of Rhizoprionodon acutus, Scomberomorus guttatus and Rachycentron canadum

2.2.1 *Pre-treatment*

The fish skin procured was cleaned, cut into 1cm x 1cm pieces and repeatedly washed with distilled water. Non collagenous proteins were removed by pre-treating with 0.1N sodium hydroxide (1:10 weight/volume) for one day in refrigerated condition. It was followed by rinsing with distilled water several times till it attained neutral pH and then, treated with 10% butyl alcohol (1:10 w/v) for 24 hours at 4C to remove fats and pigments. Subsequently, it was washed several times with cold distilled water.

2.2.2 *Extraction of acid-soluble collagen*

Acid soluble collagen was extracted by the method of Kittiphattanabawon et al. (2010). The pre-treated skin was immersed in 0.5 M acetic acid in 1:15 (weight/volume) ratio for 72 h, stirring continuously. It was followed by filtration using sieve and cheese cloth and the filtrate was subjected to salt precipitation using sodium chloride (up to 2.6 M concentration).The precipitate was collected, 0.5 M acetic acid was used to dissolve the same and dialysis was carried out with 0.1 M acetic acid for one day and with distilled water for two days. Lyophilisation of the dialysate was performed and the product obtained was represented as “acid soluble collagen” (ASC).

2.2.3 *Extraction of pepsin-soluble collagen*

Isolation of pepsin-soluble collagen was done using the method of Nalinanon et al. (2007) with slight modifications. The residue obtained after acid extraction was further treated with pepsin (20 Kunits/gram of residue) in acetic acid in 1:15 (w/v) ratio. It was left in refrigerated condition for three days, stirring continuously and then filtered using sieve and cheesecloth. The filtrate was subjected to subsequent steps of salt precipitation, dialysis and freeze drying as in ASC extraction. The collagen thus obtained was labelled “pepsin soluble collagen, PSC”.

2.2.4 *Yield calculation*

The total collagen mean yield for both ASC/PSC on wet weight basis was calculated as indicated by Zeng et al., (2012).

% yield of collagen (on basis of wet weight)= (Weight of lyophilised collagen/weight of the wet skin)*100

2.3 Characterisation of collagen

2.3.1 *Attenuated Total reflectance – Fourier Transform Infrared Spectroscopy (AT-FTIR)*

FTIR is a technique that helps in identification of specific functional groups of an organic molecule. These groups show characteristic vibrational frequencies when an Infrared signal is passed through them and the expected frequencies can be converted into a specific spectrum.

The sample is placed in a holder in the path of the IR source. A detector reads the analogue signal and converts the signal to a spectrum. A computer is used to analyse the signals and identify the peaks. Some spectrometers come with an attachment known as an ATR (attenuated total reflection), in which the Infrared beam is focussed through an internal reflection element (IRE). The sample must have a lower index of refraction than the IRE to achieve total internal reflection and after reflection on the sample/IRE interface, the infrared radiation passes through the sample and data from the same is recorded.

ATR-FTIR data from fish collagen sample and standard type I collagen was obtained using an FTIR spectrometer (FTIR- BRUKER, ALPHA, 200619 MODEL) across the range of 4,000-600 cm-1 at 4 cm-1 resolution with 32 scans per minute. Analysis of the spectra obtained was done using ORIGIN software.

2.4 Sodium Dodecyl Sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was conducted as per the method of Laemmli (1970). The samples were first solubilised in 5% SDS and then mixed with sample buffer (tris–HCl 0.5 M, pH 6.8 containing 20% glycerol, 4% SDS and 10% (v/v) beta mercaptoethanol) in 1:1 (v/v) ratio. Samples and broad range protein molecular weight marker were loaded into wells of a polyacrylamide gel (7.5% separating gel and 4% stacking gel) and electrophoresis was performed at 50 volts till it crossed stacking gel and at 100 volts for resolving gel. The gels were fixed in methanol: water: acetic acid (5:4:1 ratio) fixing solution for half an hour and then stained with Coomassie blue staining solution. The gel was then de-stained with acetic acid (10%). The electrophoretic banding pattern was compared to rat tail type I collagen.

3 RESULTS & DISCUSSION

3.1 Isolation of collagen

Collagen was extracted from the skin of Rhizoprionodon acutus (Milk Shark), Scomberomorus guttatus (Indo-pacific king mackerel) and Rachycentron canadum (Cobia) using acetic acid and pepsin solubilisation methods. It appeared white (Figure 1) and was odourless. The

mean yield of collagen (total collagen from both acid and pepsin extraction methods) of the three species of fish, namely Rhizoprionodon acutus (Milk Shark), Scomberomorus guttatus (Indo-pacific king mackerel) and Rachycentron canadum (Cobia) is given in Table 1.

FIGURE 1

Lyophilised collagenfrom Rachycentron canadum (Cobia) skin

Values are presented as mean % yield ± standard deviation from three independent trials.

Total collagen yield by both acetic acid and pepsin solubilisation methods was highest in Rachycentron canadum (Cobia) skin (10.81%) and least in Scomberomorus guttatus (Indo-pacific king mackerel) (3.62%). This result was comparable to collagen yield obtained from brownstripe red snapper skin- 13.7% (Jongjareonrak et al., 2005) and cobia skin-12.4 (Zeng et al., 2012) from acetic acid and pepsin solubilisation methods. The terminal ends are non-

TABLE 1
Mean percent yield of lyophilised collagen from three fish species

Species of fish	Mean % yield ± SD (wet weight basis)
Rhizoprionodon acutus (Milk Shark)	7.91 ± 0.18
Scomberomorus guttatus (Indo-pacific king mackerel)	3.62 ± 0.02
Rachycentron canadum (Cobia)	10.81 ± 0.13

helical regions and collagen is highly cross-linked at these telopeptide parts, which decreases its solubility in acid solution (Foegeding, Lanier, & Hultin, 1996).The use of pepsin will aid in cleaving these telopeptide regions of collagen and increasing its solubility (Balian & Bowes, 1977). Accordingly, in the study conducted, it was inferred that addition of pepsin in acetic acid solution aided in almost complete solubilisation. Differences observed in the collagen yield among the three species could be attributed to variations in the degree of cross-linking.

3.2 Characterisation of collagen

3.2.1 *Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (AT-FTIR)*

Collagen was characterised using the AT-FTIR technique. This spectroscopic method is easy to perform, accurate and requires a small sample. It captures changes in motions like bending (spinning, rolling, scissoring and wagging) and stretching in the dipole moment of a polyatomic molecule (Nielsen, S., 2010). AT-FTIR was used to analyse collagen from the three fish species and spectral analysis of collagen revealed characteristic Amide A, B, I, II and III peaks are indicated in Figure 3. The wavenumbers of these peaks are indicated in Table 2.

The amide A peak wavenumber was found to be 3290.252 cm^{-1}, 3294.332 cm^{-1} and 3304.531 cm^{-1} for collagen from Rhizoprionodon acutus (Milk Shark), Scomberomorus guttatus (Indo-pacific king mackerel) and Rachycentron canadum (Cobia) respectively. Amide B peak wavenumber was 2937.361 cm^{-1}, 2921.042 cm^{-1} and 2925.122 cm^{-1} for Rhizoprionodon acutus (Milk Shark), Scomberomorus guttatus (Indo-pacific king mackerel) and Rachycentron canadum (Cobia) respectively. Amide I, II and III wavenumbers were between 1625.75 to 1631.867, 1578.832 to 1593.11 and between 1236.139 to 128.179 respectively among the three species. Results from the present work corroborated with the peak wavenumbers illus-

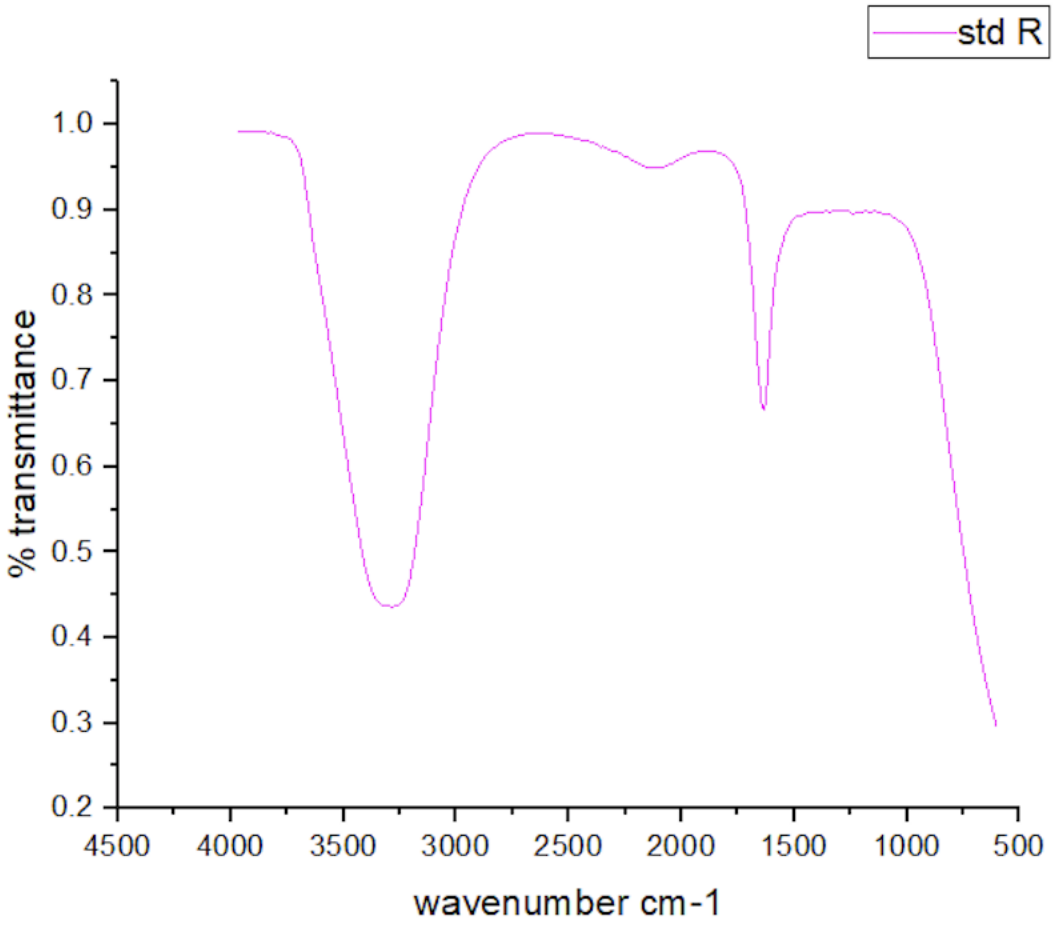

FIGURE 2

AT-FTIR of type I collagen from rat tail (standard)Std R- type I collagen from rat tail

TABLE 2

Wavenumbers of amide A, B, I, II and III peaks of collagen from skin of Milk shark, Indo-pacific king mackerel and Cobia

Indicative peaks	Milk shark	Indo-pacific king mackerel	Cobia
Amide A	3290.252 cm^{-1}	3294.332 cm^{-1}	3304.531 cm^{-1}
Amide B	2937.361 cm^{-1}	2921.042 cm^{-1}	2925.122 cm^{-1}
Amide I	1625.748 cm^{-1}	1631.867 cm^{-1}	1629.827 cm^{-1}
Amide II	1593.11 cm^{-1}	1593.11 cm^{-1}	1578.832 cm^{-1}
Amide III	1238 cm^{-1}	1236.139 cm^{-1}	1238.179 cm^{-1}

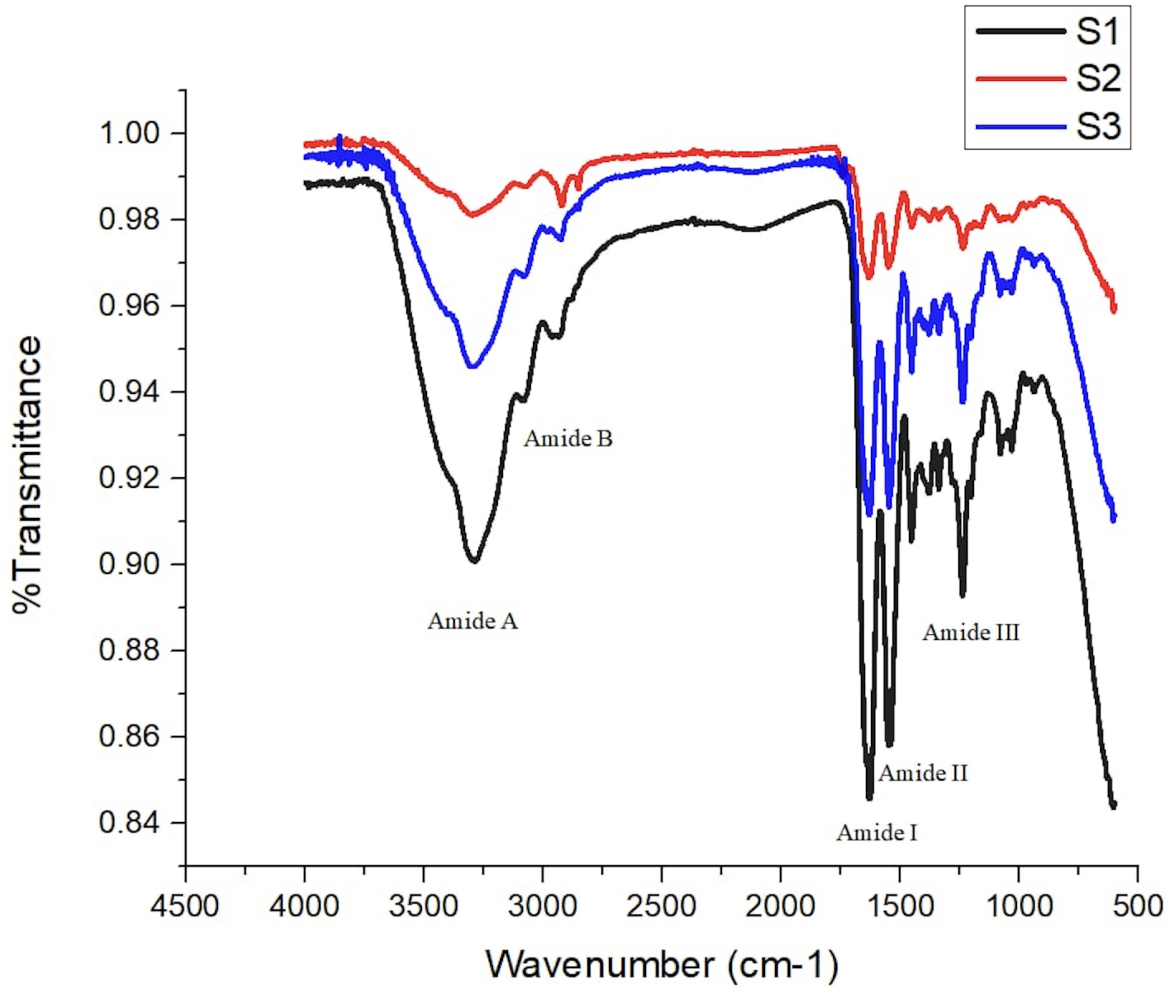

FIGURE 3

S1- Rhizoprionodon acutus (Milk Shark), S2- Scomberomorus guttatus(Indo-pacific king mackerel) and S3- Rachycentron canadum (Cobia)

trated by Devita et al.,(2021) (Table 3) where they have also discussed the motion of polyatomic molecule associated with bonds.

All three species showed similar spectra of collagen and were comparable with rat tail type I collagen. Slight differences were seen in ranges between the 1100-1600 region, which is the fingerprint region and is species-specific. Amide III wavenumber is between 1236 and 1238, which indicates that the triple helical structural integrity is maintained (Muyonga et al., 2004).

3.3 SDS-PAGE profile

The electrophoretic pattern of acid-solubilised and pepsin solubilised collagen from Milk shark and Cobia and that of rat tail type I collagen is indicated in Figure 4.

TABLE 3

Compilation of indicative peaks, corresponding wavenumbers and associated bonds in Type I collagen

Indicative peaks	Wavenumbers of collagen type I	Associated bonds
Amide A	3400-3440 cm^{-1}	N-H stretching vibration, shift near 3300 cm^{-1} indicates presence of hydrogen bonds
Amide B	2940-2920 cm^{-1}	Asymmetrical stretch of CH_2
Amide I	1600-1700 cm^{-1}	C=O stretch/hydrogen bond coupled with $COO-$
Amide II	1550-1600 cm^{-1}	N-H bending vibration coupled with CN stretching vibration
Amide III	1236-1250 cm^{-1}	Indicates helical structure of collagen

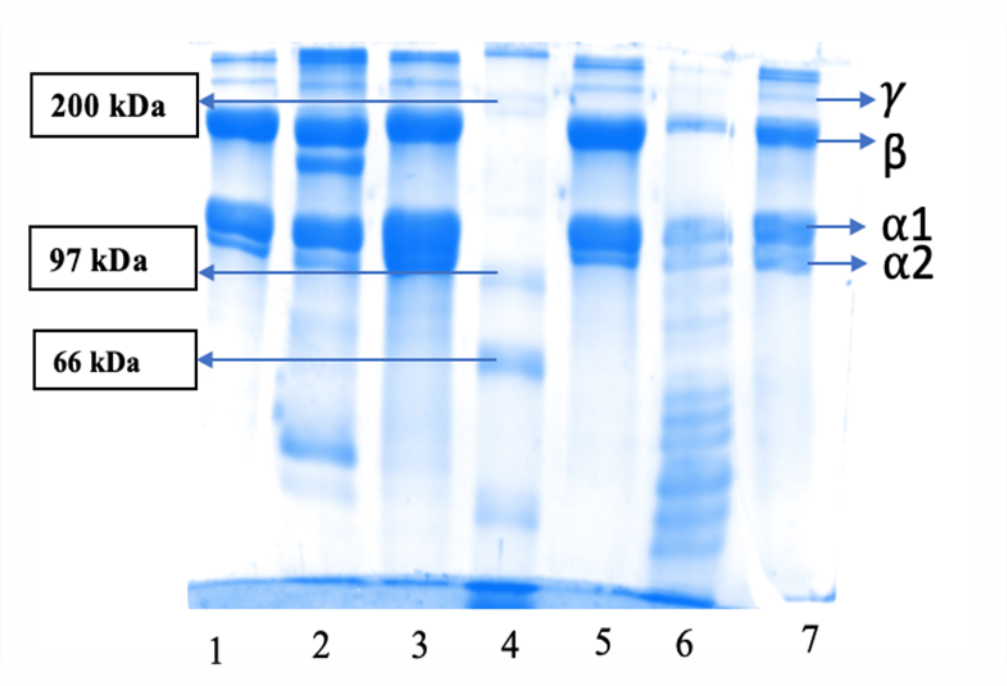

FIGURE 4

1^{st} lane- Acid solubilized collagen (ASC) from shark; 2^{nd}lane- Pepsin solubilized collagen (PSC) from shark;3^{rd} lane- Acid& pepsin solubilized collagen from Cobia; 4^{th} lane-molecularweight marker;5^{th} lane-Acid solubilized collagen from Cobia; 6^{th}lane-Pepsin solubilized collagen from cobia; 7^{th} lane- rat tail type1 collagen

Electrophoretic banding pattern of all the collagen samples showed a1 (monomer), a2 (monomer), b (dimer) and g (trimer) bands. Molecular weight of a1 and a2 was in the range of 100-120 Kda and that of b was around 200 Kda. The banding pattern of acid soluble collagen was like rat tail type I collagen. The intensity of a1was twice that of a2 which suggests the occurrence of two a1 and one a2 monomers. According to previous data, collagen type I comprises of two similar a1chains and a a2 chain (Rochdi et al., 2000, Sun et al., 2017). Pepsin solubilized collagen from Cobia showed additional lower molecular weight bands indicating pepsin's hydrolytic effect on collagen's terminal regions. The banding pattern of collagen and its subunit composition observed in the present work was same as results obtained from Giant Croaker (Nibea japonica) skin (Tang et al., 2018) and bigeye snapper (Priacanthus tayenus) (Nalinanon et al., 2007). Hence, collagen isolated from Milk shark, Indo-pacific king mackerel and Cobia skin is type I.

4 CONCLUSION

The study revealed that collagen could be efficiently isolated from skin of Rhizoprionodon acutus (Milk Shark), Scomberomorus guttatus (Indo-pacific king mackerel) and Rachycentron canadum (Cobia) fish species by acetic acid and pepsin extraction methods. Among the three species, the mean collagen yield (wet weight basis) was highest in Rhizoprionodon acutus (Milk Shark) (10.81%) while it was 7.91% and 3.62% for milk shark and Indo-pacific king mackerel respectively. The extracted collagen was of type I as demonstrated by AT-FTIR and SDS-PAGE techniques. Hence, fish skin can be a potential alternative source for isolation of collagen type I.

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