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Isolation and biochemical characterization of vital protein collagen: fish waste valorization of Arabian Sea fish *Sardinella longiceps* and its fluorescence quenching property

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ABSTRACT

Introduction and Aim: There has been a practice of dumping fish wastes or converting them into low market value products; the scope of this work relies on the fact that isolation of vital collagen in an efficient way from fish wastes by a valorization technique to convert it into a value-added product, hence decreasing environmental pollution load with increasing economic impact.

Materials and Methods: The acid-soluble type-I collagen (ASC) from fish bone, skin, fins and head of Indian oil sardine (*Sardinella longiceps*) was isolated and characterized. The protein content of isolated ASC was found to be 1.15 mg/g, 1.53 mg/g, 1.24 mg/g and 1.13 mg/g respectively. The ASC was isolated with a yield of 1.8%, 2.5%, 1.2% and 2.1% respectively.

Results: From the FTIR spectrum, it can be confirmed that ASC contains five amide bands with triple-helical nature of the polypeptide chain. The SEM analysis of the ASC surface exhibited densely packed fibers of collagen. The ASC exhibited the maximum absorption peak at 225 nm. The maximum solubility of ASC was observed at pH 4 and 4% of NaCl concentration. The study further extended to investigate the quenching property of collagen with Rhodamine B, which was confirmed by photoluminescence (PL) analysis. There is a decrease in absolute quantum yield ranging from 21 to 1.75%.

Conclusion: The quenching property of collagen was established by the PL assay of collagen mixed with Rhodamine B dye solution, which is a known fluorophore. So these materials might be used as biopolymer hybrid materials in the place of inorganic/organic quenching materials.

Keywords: Acid soluble collagen; Indian oil sardine; photoluminescence; Rhodamine B; quenching.

INTRODUCTION

All over the world, fishing is an important occupation for the need of nutritional food in the form of fish proteins. The main edible part is the muscle, whereas, the head, skin, fins, scales, and viscera are considered as fish wastes (1). “Fish waste to wealth” is a visible solution to handle fish processing waste and convert into useful products such as animal feed, fishmeal, fish oil, pet food, fish silage and fertilizers etc., (2). In spite of this, a large quantity of fish wastes are produced causing disposal problems leading to environmental pollution. Therefore, research has been carried out to develop methods to convert these wastes into value-added products. The proteins in fish can be divided into three groups, of which collagen (30% of the total protein content of animal body) forms a major component of the connective tissue; it is vital for mechanical protection of tissues, organs and physiological regulation of the cellular environment. Glycine, proline and hydroxyproline are the major amino acids present in collagen. These three amino acids are very important for the formation of the specific triple helical structure and stabilization of the collagen molecule through hydrogen bonding. Fish collagen has a similar amino acid sequence to that of mammal collagen, and it is abundant, cheaper and does not cause any infectious diseases and ethnic problems. Collagen has a variety of applications in various fields of biomedical, cosmetics, and food industry, etc., (3, 4, 5). Hence, the present study was primarily focused on the isolation of acid soluble collagen (ASC) from the bone, skin, fins and head of Indian oil sardine (*Sardinella longiceps*) which belongs to the Clupeidae family. It is one of the most important commercial fish available at the Arabian Sea of west coast of India.

Enormous reports are available associated with the study of quenching property of protein and polysaccharides with dye. However, there is a lack of scientific evidence for the quenching property of collagen with Rhodamine B dye. Therefore, the isolated acid soluble skin collagen was further subjected to study the fluorescence quenching property of collagen by using PL analysis. Fluorescence is a property in which light is absorbed and remitted within a few nanoseconds at a lower energy level. Fluorescence used in biological studies generally considered as a non-destructive way of tracking or analyzing biological molecules such as protein-protein, ligand-receptor, protein-nucleic acid and ligand-lipid interactions.

MATERIALS AND METHODS

Indian oil sardines were collected from the fish market at Mangalore. The bone, skin, fins and the head portions of the fish were removed and washed with cold distilled water and stored at -20°C until further use. Chemicals used in the study such as disodium ethylenediamine tetra acetic acid (0.5M), butyl alcohol (10%), acetic acid (0.5M), sodium chloride (2.5M), tris (hydroxymethyl) aminomethane (0.05M), Folin-Ciocalteu reagent (FC), acrylamide (30%), bisacrylamide (0.8%), bovine serum albumin (BSA) (0-50µg/mL), sodium dodecyl sulphate (4%), ammonium persulphate (10%), glycerol (20%), glycine (0.192M), Rhodamine B were procured from Merck®, India.

Isolation of acid-soluble collagen (ASC) from the bone, skin, fins and head of Indian oil sardine

The acid-soluble collagen was isolated from the different parts of Indian oil sardine (*Sardinella longiceps*) using the method reported by Nagai and Suzuki (6) with a slight modification. The fish bone, skin, fins and head were removed and weighed. The samples were washed with cold distilled water for a few minutes and ground. Collagen was extracted with 0.1M NaOH for 48h at a sample/alkali solution ratio of 1:10 (w/v). The process was repeated at every 5h to ensure complete removal of non-collagenous matter. The residue was washed with cold distilled water until the pH value was close to neutral. The bone and fin samples were found to contain more calcium so those were decalcified with 0.5M disodium EDTA solution (pH 7.5) for 5 days. The fat was removed with 10% butyl alcohol at a sample/alcohol ratio of 1:10 (w/v) overnight, after that, the samples were washed with cold distilled water. The residue was extracted with 0.5M acetic acid at a sample/acid ratio of 1:4 (w/v) for 3 days. The insoluble matter was re-extracted with the same solution at sample/acid ratio of 1:2.5 (w/v) for 2 days. Each extract was filtered by cotton cloth and then centrifuged at 10000 rpm for 30 minutes. The supernatant was salted out by adding NaCl to a final concentration of 2.5M in the presence of 0.05M Tris-HCl, pH 7.0, stirred well and kept overnight to precipitate the collagen. The resultant precipitate was collected by centrifuging at 10000 rpm for 30 minutes. Pellet was dissolved in 0.5M acetic acid and dialyzed against 0.1M acetic acid and distilled water respectively. Amicon Ultra centrifugal filters (Millipore) 100kDa cut off filters were used for concentrating the proteins. The dialyzed collagen solution (10mL) was taken in an Amicon centrifugal tube and it was centrifuged 5000 rpm for 15 minutes at 4°C. The retentate was collected and then lyophilized. It was labeled as concentrated acid-soluble collagen (ASC) and stored at 4°C.

Collagen yield (dry basis) was determined by the following formula:

$$\text{Collagen yield} = \frac{(\text{Weight of final collagen sample in g})}{(\text{weight of sample in g})} X100$$

Protein estimation

The protein concentration of ASC samples were determined by using Bradford method. Bovine serum albumin (concentration range: 0-50µg/mL) was used as a standard. Standard and samples were taken; Bradford reagent was added and kept for incubation at room temperature for 10 minutes. Absorbance was measured at 600 nm. The standard graph was plotted by taking a concentration of protein along the X-axis and absorbance along the Y-axis. From the standard graph protein concentration of the samples were determined.

Collagen solubility tests

The collagen samples were dissolved in 0.5M acetic acid to obtain a final collagen concentration 3 mg/mL and the mixture was stirred at 4°C until collagen was completely solubilized (7).

Effect of pH and effect of NaCl

Collagen solution (3mL) in 0.5M acetic acid was added to a centrifuge tube (50mL) and the pH was adjusted with either 6N NaOH or 6N HCl to obtain a final pH ranging from 2, 4, 6, 8, 10 and 12. The volume of the solution was made up to 5mL with distilled water. To find the effect of NaCl, collagen solution (3mL) in 0.5M acetic acid was mixed with 5 mL of cold NaCl in 0.5M acetic acid at various concentrations (0%, 2%, 4%, 6%, 8%, 10% and 12% w/v). In both the experiments, solution was stirred gently for 30 minutes at 4°C and centrifuged at 10,000 rpm. Protein content in the supernatant was determined by Lowry's method using BSA as standard. The relative solubility of collagen was calculated in comparison to pH rendering the highest solubility and the salt concentration exhibiting the highest solubility.

Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis (SDS-PAGE)

Protein patterns of the collagen samples were analyzed using SDS-PAGE according to the method of Laemmli, using 10% resolving gel and 6% stacking gel. Collagen samples were dissolved in 0.1M acetic acid with a 2 times sample buffer (0.125M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol, 1% Bromophenol blue and 10% (v/v) β-mercaptoethanol). The samples were heated to 93°C-95°C for 4 minutes prior to loading onto the 10 % gel. The gel was subjected to electrophoresis at 50V for 5 minutes until the collagen subunits had passed through the stacking gel and then at 100V for 4 h (till the migration reached the end of the resolving gel). The proteins were visualized with Coomassie blue R-250. High molecular weight markers (205kDa to 29kDa) (Sigma Chemical co., St. Loius, Mo, USA) were used to estimate the molecular size of proteins. Commercial collagen, Type-I calfskin collagen was used as a standard.

Instrumental analysis

UV-Visible spectroscopy

The UV-Visible absorption spectrum of ASC was measured by a spectrophotometer (Spectroquant Pharo 300) at different wavelengths (200-800 nm).

Fourier Transform Infrared Spectroscopic analysis

Structural confirmation of isolated ASC was carried out by using Fourier Transform Infrared Spectroscopic analysis (Shimadzu FTIR instrument (Model: IR Prestige- 21, Japan within a scan range of 4000-550 cm^{-1}.

Scanning Electron Micrograph

Scanning Electron Micrograph (Carl Zeiss Microscopy Ltd) analyzed the surface morphology of isolated collagen. The lyophilized collagen sample was cut using a punch and fixed to an adhesive carbon stub. The sample was sputtered with gold particles under vacuum to increase electrical conductivity and operated at 5 kV.

Photoluminescence (PL) analysis

Different concentrations (0.01%, 0.25%, 0.5%, 1% and 2%) of acid soluble skin collagen was added to 1% Rhodamine B solution and it was stirred at 4°C, 350 rpm for an hour on a magnetic stirrer. Photoluminescence (PL) study was carried out using a luminescence spectrometer (F-7000 "Hitachi") which has large stigmata concave grating of 900 lines/mm with a scan speed 15, 60, 240, 1200 nm/min. The Quantum yield was determined by Fluor Tools.com software.

RESULTS

Preparation of acid soluble collagen and protein estimation

Collagen was isolated from fish bone, skin, fins and head of Indian oil sardine (*Sardinella longiceps*). The isolated ASC sample was found to be odorless and whitish fiber-like appearance as shown in Fig.1.

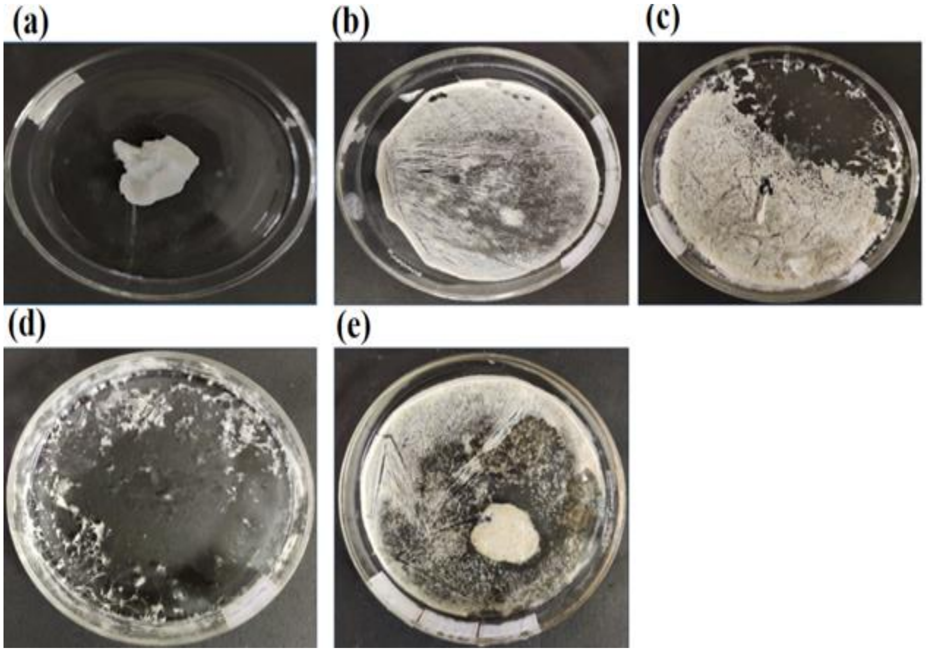

Fig. 1: ASC isolated from *Sardinella longiceps* (a) Standard calfskin collagen, (b) Bone ASC, (c) Skin ASC, (d) Fin ASC, (e) Head ASC.

The yield of ASC from fish bone, skin, fins and head was found to be 1.8%, 2.5%, 1.2% and 2.1% respectively, on the basis of lyophilized dry weight. The ASC of skin showed a higher yield compared to the bone, fins and head. Similar report was reported elsewhere (8). The protein content was estimated by the Bradford method and was found to be 1.15 mg/g, 1.53 mg/g, 1.24 mg/g and 1.13 mg/g for ASC isolated from bone, skin, fins and head respectively. Protein concentration was higher in skin when compared to bone, fins and head. The pH of isolated collagen from fish bone, skin, fins and head was found to be 3.5, 3.4, 3.4 and 3.5.

Solubility of ASC

Effect of pH and NaCl concentration on collagen solubility

The change in the solubility of ASC, with the change in the pH is given in Fig. 2(a). A sharp increase in solubility observed when the pH was increased up to pH-4 and the solubility decreased gently with increasing pH from 6 to 10.

The absorbance of the solution increased gradually when the concentration of NaCl increased up to 4% as shown in Fig. 2(b). This could be directly related to the amount of dissolved ASC in solution.

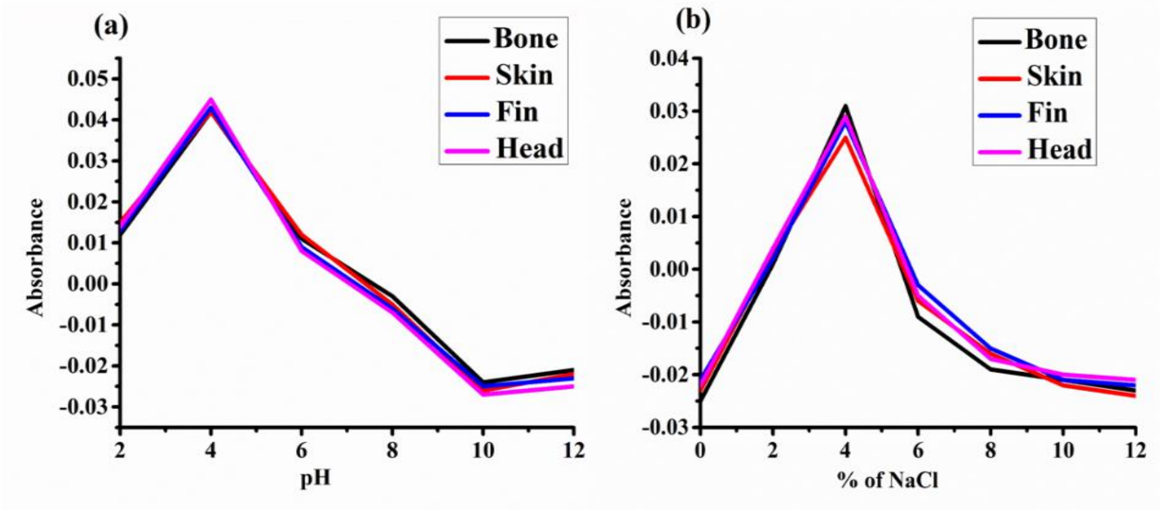

Fig. 2: (a) Solubility of ASC isolated from bone, skin, fins and head of *Sardinella longiceps* at different pH, (b) Solubility of ASC isolated from *Sardinella longiceps* at different NaCl concentrations.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) of ASC

The ASC samples from *Sardinella longiceps* were analyzed by polyacrylamide gel electrophoresis in the presence of SDS using 10% gel with reference to calfskin collagen. Generally, the electrophoresis pattern of ASC consists of α chains (α1- and α2-chains at a ratio of approximately 2:1) and high-molecular-weight components including β chain (dimmers) and γ chain (trimmers) components, as well as their cross-linked molecules (7). These patterns were similar to the type-1 collagen from calfskin. The electrophoresis patterns as shown in Fig. 3. In this study, for the isolated ASC, three bands were observed with different molecular weight. For standard calfskin collagen, the bands were observed at 138.038kDa, 161.182kDa, 208.15kDa. For the isolated bone collagen, the bands were observed at 134.04kDa, 158.48kDa and 207.173kDa. Skin collagen showed bands at 132.32kDa, 162.132kDa and 207.182kDa, fin collagen showed bands at 134.54kDa, 159.133kDa and 207.186kDa. Head collagen showed bands at 133.15kDa, 154.53kDa and 207.184kDa.

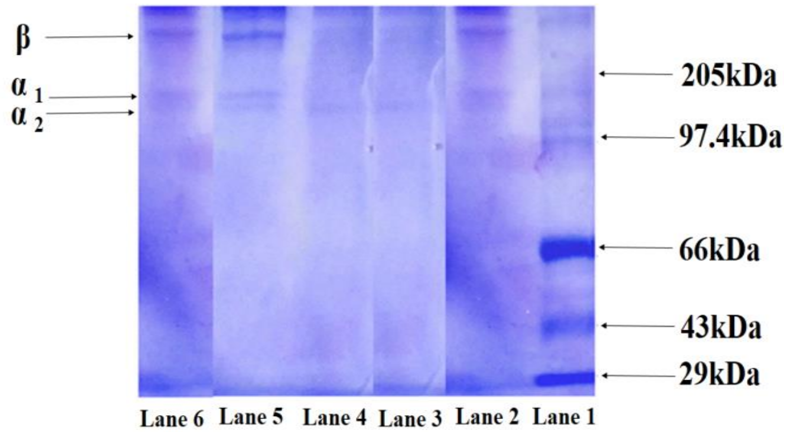

Fig. 3: SDS–Polyacrylamide gel electrophoresis of isolated ASC. Lane 1 contains a molecular weight marker with a range of 29kDa-205kDa, Lane 2 contains standard type-1 calfskin collagen, Lane 3, 4, 5 and 6 contains ASC isolated from bone, skin, fin and head of *Sardinella longiceps*.

UV-Visible spectral analysis of ASC

A sharp peak was obtained at around 225 nm for isolated ASC with respect to standard calfskin collagen along with a small peak at 204 nm as indicated in Fig. 4.

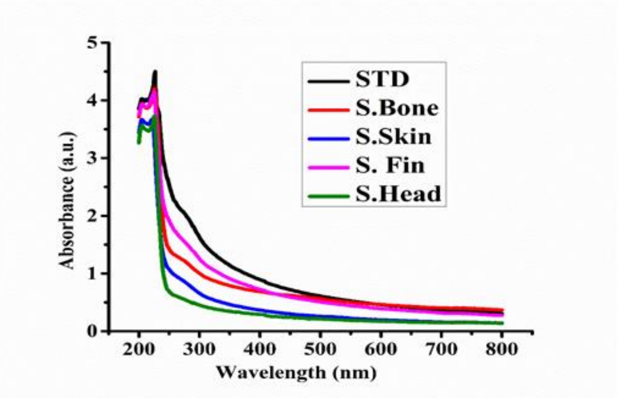

Fig. 4: UV-Visible spectra of standard calfskin collagen and ASC isolated from *Sardinella longiceps*

FTIR analysis of ASC

FTIR analysis was carried out to investigate and identify the various functional groups present in isolated ASC as shown in Fig. 5. These studies revealed that amide A band occur in the range of 3315 cm^{-1}, amide B band occurs in the range of 2926 cm^{-1}. Amide I band position of collagen was found at 1645 cm^{-1} for bone, skin and head collagen. The band observed at 1641 cm^{-1} for fin collagen compared to the normal absorption range of the amide I in the range of 1600-1700 cm^{-1} (9). The amide II bands position were detected at 1552 cm^{-1} for isolated bone, skin and head. The band observed at 1531 cm^{-1} for fin collagen compared to the normal absorption range of the amide II in the range of 1550-1600 cm^{-1} and amide III bands were found at 1246 cm^{-1} for isolated bones, skin, fins and head collagen. The revealed value is similar to that of standard Type-I calfskin collagen where, amide A band found at 3315 cm^{-1}, amide B band observed at 2926 cm^{-1}, amide I, II and III band observed at 1645 cm^{-1}, 1546 cm^{-1} and 1230 cm^{-1} respectively. Hence the collagen isolated from *Sardinella longiceps* have similar characteristics as that of type-I collagens from other sources.

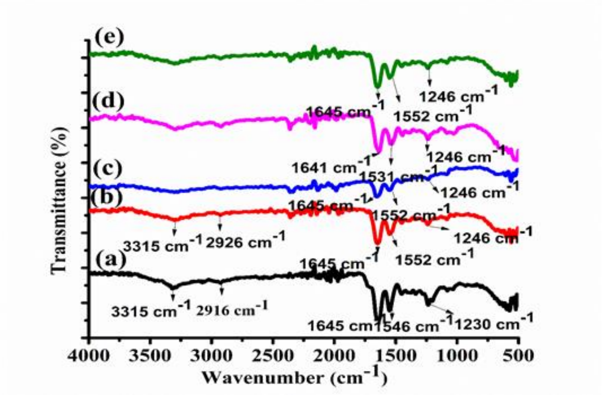

Fig. 5: Fourier transform infrared spectroscopy of ASC isolated from *Sardinella longiceps* (a) Standard calfskin collagen, (b) Bone ASC, (c) Skin ASC, (d) Fin ASC, (e) Head ASC.

SEM analysis of ASC

The micrographs indicate that the isolated collagen was fibrillar in nature and densely packed as shown in the Fig. 6. The diameters of collagen fibers were measured on micrographs and found to be close to 10μm thickness.

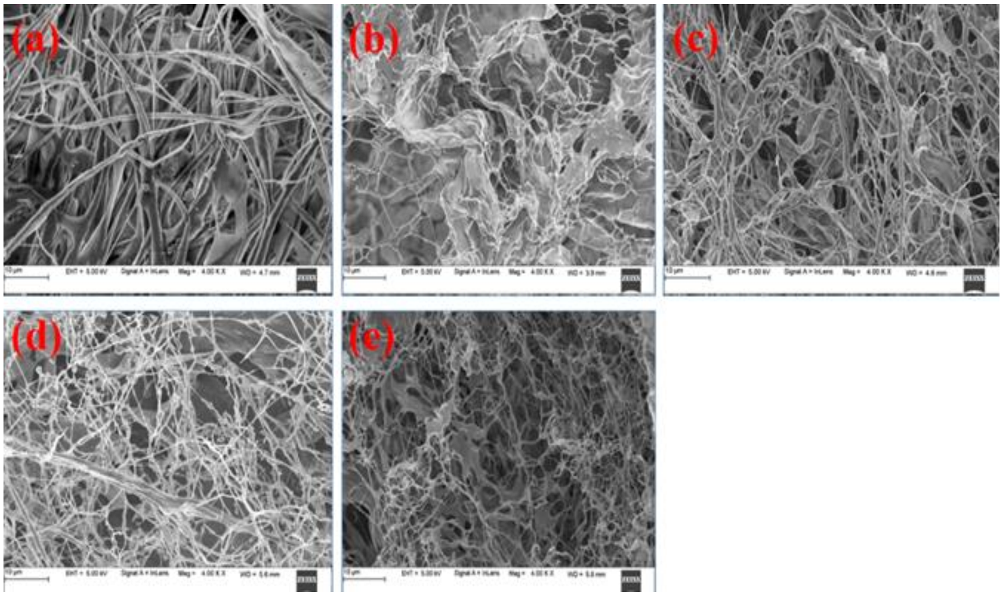

Fig. 6: SEM micrographs depicting the surface morphology of ASC from *Sardinella longiceps* (a) Standard collagen, (b) Bone ASC, (c) Skin ASC, (d) Fin ASC, (e) Head ASC.

Photoluminescence (PL) measurement

Further, the study was extended to confirm the quenching property of the collagen by adding collagen with Rhodamine B dye. It was used as florescent dye and its PL intensity was quenched by the addition of collagen. Collagen fluorophore usually absorbs in the UV region with emission in the range of 400 nm. In order to understand the quenching behavior of collagen, it was excited at 400 nm. Rhodamine showed emission spectra at the wavelength of 586 nm. The different concentrations of collagen solutions were added to the Rhodamine B solution, the emission spectra recorded at the wavelength 620 nm is depicted in Fig. 7.

The absolute quantum yield was decreased from 21 to 1.75% as shown in Fig. 8. This sudden decrease in intensity was due to the quenching effect exhibited by collagen.

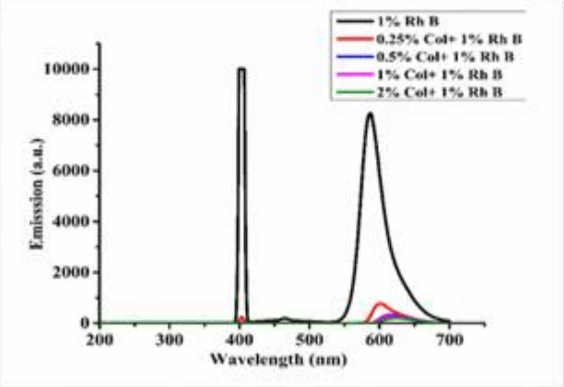

Fig. 7: Photoluminescence spectra of collagen/ Rhodamine solution

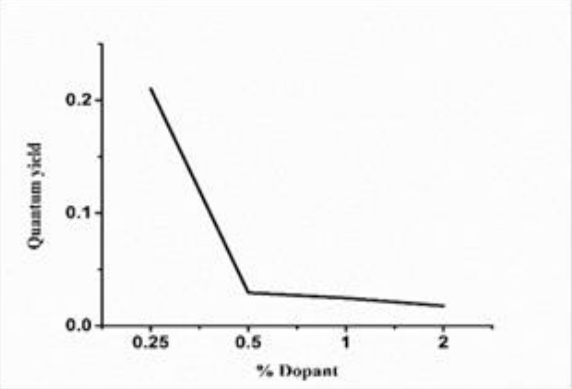

Fig. 8: The proportional decrease in the quantum yield with the dopant % increase.

DISCUSSION

The variation in the collagen yield was mainly due to the presence of a lower degree of cross-linking among the collagen molecule. The solubility of the collagen in acid solutions would increase when the collagen molecules are weakly cross-linked among each other in the telopeptide region. The differences in the collagen yield also depends on the type of fish species, age, and collagen structure and physical properties such as temperature, extraction method, extraction time, the influence of acetic acid and pH effected on the collagen yield (10). The comparative yields (%) of the collagen obtained from different types of fishes are given in Table 1. The data shows that the yield of collagen obtained from Indian oil sardine was higher than other marine fishes. Indian oil sardine fish community is available abundantly at the Arabian sea and is the main catch for domestic use and exporting leaves behind a substantial amount of waste; could be exploited for the isolation of collagen and its derivatives as high-end value-added products which contribute to minimizing pollution, at the same time valorization of fish waste.

Table 1: The yield (%) of collagen from different types of marine fishes

Sl. No.	Fish	% ASC collagen				Reference
		Skin	Bone	Fin	Head	
1.	Indian oil sardine *(Sardinella longiceps)*	2.5	1.8	1.2	2.1	Present work
2.	Rainbow trout (*Onchorhynchus mykiss*)	----	1.16	----	---	11
3.	Carp *(Cyprinus carpio)*	----	1.02	----	---	10
4.	Black drum	2.3	----	----	---	12
5.	Bigeye snapper	----	1.59	----	---	13
6.	*Brama australis*	1.5	----	----	---	14
8.	*Priacanthus tayenus*	----	1.59	----	---	13
9.	*Sepiella inermis*	0.58	----	----	---	15

The basic amino acid residues such as glycine and arginine groups bind with Bradford reagent and giving the greenish-blue color of high absorptivity imparting accuracy in the estimation.

Acidic nature of the isolated collagen was due to the presence of acidic medium and the percentage of hydroxyl proline is higher in the collagen composition. The free -OH groups impart acidity to the medium hence lowering the pH of the collagen to moderately acidic pH.

As per earlier reports (7), the solubility of collagens is pH dependent. Collagens showed high solubility at acidic pH (2-5). The increasing solubility (pH 2-5) was due to the increasing number of protons or proteins available in hydroxyproline a unique amino acid residue present in collagen fibers (16). High acid solubility was a characteristic of collagen that makes it an attractive commercial source.

The initial increase in the solubility with the concentration of NaCl could be due to the "salting in" effect. Above this concentration, a sharp decrease in soluble protein concentration was observed due to the salting out effect (11, 17). The outcome advocated that the extraction process at these conditions yield collagen in pure form.

The SDS results suggested that ASC was most likely to be classified as type-I collagen. Similar results were reported previously for collagen protein (8, 15). The ASC isolated from fish bone, skin, fins and head collagen subunit molecular weights (α1, α2, and β chains) revealed to be slightly lower than standard calfskin collagen. This difference could be due to the dissimilar source of the collagens, namely resources from aquatic animals and mammalian animals (8).

The previous report for Carp fish has been shown to have a major peak at 223 nm (10). Also, Na (1988) (18) had demonstrated that the UV absorbance spectra of collagen were in the range between 220-230 nm and they increased upon unfolding. Usually, the peptide bonds found in the protein absorb at 205-230 nm with an absorption peak at 280 nm. However, the absence of an absorption band at 280 nm in the present sample clearly indicated complete removal of non-collagenous protein residues from isolated collagen. The absorbance at 280 nm accounted for the presence of tryptophan, tyrosine & phenylalanine. The results obtained clearly indicated that tryptophan was completely absent in the collagen sample. However, the contribution from tyrosine might be negligible due to its involvement in the folding. For these reasons, the extracted protein could be concluded as collagen.

Earlier reports have shown that, an amide A band was observed at 3306 cm^{-1} for type-I collagen, and it was associated with -NH- stretching vibration. This could be attributed to the presence of hydrogen-bonded hydroxyl groups. Amide B band was observed at 2964 cm^{-1} and it was related to an asymmetrical stretch of -CH_2-. Amide I band at 1640 cm^{-1}, which was associated with the -C=O- stretching vibrations and hydrogen bonds coupled with -COO-, usually occurs within the range of 1600–1700 cm^{-1}. Amide II band observed at 1532 cm^{-1} was responsible for the -NH- bending coupled with -CN- stretching vibrations, with a characteristic absorption peak near 1550 cm^{-1}. The amide II band position was shifted to the lower frequency at 1531 cm^{-1} (fin ASC), which also showed the existence of hydrogen bonds in collagen. Amide III band generally appears at 1200–1360 cm^{-1}, and could arise from the C–N stretching, N–H bending vibration as well as the wagging vibration of CH_2 groups of the glycine backbone and the proline side chains. Moreover, absorption bands around 1390–1455 cm^{-1} were also found, which extensively corresponded to pyrrolidine ring vibration of hydroxyproline and proline. The region of amide I, amide II and amide III are known to directly related with the triple-helical shape of a polypeptide (8, 19, 20).

The micrographic structures showed the surface of the collagen as rough and uneven. The regular porous structure of collagen was clearly visible. The cross-linkage between the fiber networks could be mediated by dispersion forces, entropic, electrostatic bonds, hydrogen bonds and hydrophobic interactions (9, 15, 21).

The quenching of fluorescence was clearly observed by the substantial decrease in the emission intensity as the concentration of collagen increased in Rhodamine B solution (1%). Some of the researchers studied on the florescence quenching of Rhodamine B by using various chemicals such as triethyl amine and n-butyl amine and even silver and gold nanoparticles as quenchers (22-24). Many organic and inorganic fluorescence-quenching materials found to have applications especially in the PCR technique to detect the particular DNA sequence. The PCR probe consists of a fluorescent reporter and quencher where fluorescence quenching refers to any process, which decreases the fluorescence intensity by reducing the quantum yield of a given fluorophore. The quencher absorbs the fluorescence emitted by the reporter fluorochrome over a wide range of wavelengths. Quenching molecules accept energy from the fluorophore and dissipate their absorbed energy as heat, which remain dark. As a result of these properties, quenchers have become very useful as energy acceptors in PCR and fluorescence resonance energy transfer (FRET) pairs (23). In the PCR technique, quencher fluorescence can increase background noise due to overlap between the quencher and reporter fluorescence spectra eg: TAMRA (carboxy tetramethyl rhodamine). Dark quenchers offer a solution to this problem because they do not show an emission bandwidth. However, due to toxicity, there is limited use of synthetic fluorophores in PCR. To overcome this issue, researchers have focused on the use of biopolymers. Therefore, collagen could be used as a quencher in place of TAMRA due to its quenching property. This property of collagen is mainly due to the sorption of smaller peptides present in the collagen chain. It could be understood that the intermolecular energy transfer between collagen with Rhodamine B molecule tends to decrease the intensity of fluorescent material (25). Usually, TAMRA (carboxy tetramethyl rhodamine) is being used as a florescence quencher in the PCR technique could be easily replaced by highly abundant natural material collagen.

CONCLUSION

Acid soluble collagen (ASC) from bone, skin, fins and head of Indian oil sardine was extracted and characterized. The amount of protein was determined by the Bradford method using bovine serum albumin as standard. Characterization of collagen was carried out using various instrumental techniques such as FTIR, UV, SEM and SDS-PAGE. The maximum solubility of ASC was found to be at pH 4 and 4% of NaCl concentration. The yield of collagen obtained from *Sardinella longicepes* is comparatively higher than other marine fishes. As this fish, community is abundantly available at the Arabian Sea and is the main catch for domestic use and exporting, leaving behind a substantial amount of waste; so can be exploited for the isolation of collagen and its derivatives as high-end value-added products. This might contribute to minimizing pollution to use in various biomedical applications. Further, the quenching property of collagen was established by the PL assay of collagen mixed with Rhodamine B dye solution, which is a known fluorophore. So these materials might be used as biopolymer hybrid materials in the place of inorganic/organic quenching materials.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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