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A Study on Assessment Of Propriety Of Fish Scale Collagen.

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ABSTARCT

Collagen has become of great interest not only as an industrialized resource, butalso as a new functional material of high potential in various fields. Acid soluble collagen (ASC) was successfully extractedfrom the scales of a common fresh water fish, *Labeo rohita* (rohu) and characterized for it functional properties. The maximum yield of ASC was obtained from fish scales of *L. rohita* with 31 ± 0.3% (based on dry weight). Proximate analysis of ASC showed that it has higher protein content (89 ± 0.51%), but lowest moisture content (7.52 ± 0.73), ash content (0.63 ± 0.65%), and fat content (0.47 ± 0.38%). The characterization of ASC was investigated by UV-vis spectroscopy, Fourier transform infrared spectroscopy (FTIR). Crystallographic characterization by X-Ray diffraction (XRD) and morphological characterization by scanning electron microscopy (SEM) confirm the extraction of pure collagen which has flake like structure. The result showed that the ASC extracted from fish scale has good quality and could be more effectively and widely used in the various industries.

Keywords: Collagen, fish scale, extraction, characterization.

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INTRODUCTION

Collagen is the most abundant fibrous animal protein polymer, accounting for almost 25 to 30% of total protein in animal body [1, 2]. It is the structural macromolecule of the extracellular matrix (ECM) involved in the formation and to maintain the flexibility and strength of connective tissues, such as, bones, skin, joints, ligaments, muscles, nails, hairs etc. Collagen plays a dominant role in maintaining biological and structural integrity of ECM and undergoes constant remodeling for physiological functions [3]. Till now, collagen in genetically distinct forms have been identified as type I to type XXVIII; among them, I, II,III, and V are the main types that make up the essential part of collagen in bone, cartilage, tendon, skin, and muscle [4]. Structurally, collagen is composed of three similarly sized triple helix polypeptide chains. Each chain contains about 1000 amino acid residues and has an average length of 300 nm and a diameter of 1.4 nm. Collagen has a repetitive primary sequence of which every third residue is glycine. The sequence of the polypeptide chain can be described as Gly-X-Y, in which X and Y are often found to be proline and hydroxyproline forming a left-hand super helix with the other two chains [5].

Collagen is ductile and is used in different fields, such as leather and films, cosmetics, biomedical and pharmaceutical industries, and in food [6-8] industries due to its special characteristics, such as biodegradability and weak antigenecity [9]. The physical and chemical properties of fish collagen are different from those of mammalian collagen [10]. Thus fish waste may be an effective alternative source for collagen production, especially from aquatic animals including fresh water and marine fish and mollusks have received increasing attention [11-13]. Collagen peptide extracted from fish scales are consisting of small peptide molecules. Its absorption in the small intestine is superior to other collagen products due to smaller molecular size and leads to more efficient collagen synthesis in different parts of the body such as joint tissue, bone, blood vessels and skin dermis. Consequently, this product is used for supplements to lessen the pains and aches due to arthritis, artheriosclerosis and other signs of aging [14, 15]. In spite of such advantages, fish collagens have not been extensively studied for medical uses mainly due to their low denaturation temperature (T_d) [16]. T_d has direct correlation with imino acids (hydroxyproline and proline) content in collagen [17]. It is also reported that the T_d of fish collagens of marine sources is below 30°C [18], which limits their application in native form at physiological temperature of human body [19]. Interestingly, T_d of collagen from fresh water fish origin is relatively higher than that of marine sources [18]. Thus, the objectives of this paper are to isolate and characterize acid soluble collagen from fresh water fish scales of *Labeo rohita* (rohu), and to evaluate its physico-chemical properties.

MATERIALS AND METHODS

Collection and pretreatment of samples

The fish wastes of *L. rohita* with an average weight of 100–200 g were freshly collected from Guntur, Andhra Pradesh, India. The fish scales were removed with hands packaged in zip locked polyethylene bags, iced, and quickly transported to the laboratory (5°C). The scales were washed twice before being dried withchilled potable water to clean the dust, dirt, sand and other extraneous matter, finally dried and kept at −20°C until further use. All the chemicals and reagents used in this work were of analytical grade.

Extraction of acid soluble collagen (ASC)

The extraction of ASC was performed at 4°C following the method of Matmaroh et al. [20]. Fish scales (100 g)were ground prior to collagen extraction. Small pieces of fish scales were then suspended in 0.1 N NaOH for 6 h atthe ratio of 1:10 (w/v) with continuous stirring to remove noncollagenous proteins and pigment. The solution was changed every 3 h. Treated scales were washed withchilled water to achieve a neutral pH. Demineralizationof fish scales was carried out using 0.5 M Na_2EDTA (pH 7.4) for 48 h at the ratio of 1:10 (w/v) andthe solution was changed every 12 h. Demineralized fishscales were then subjected to ASC extraction with 0.5 M acetic acid for 48 h. The mixture was filtered through two layers of cheese cloth. The supernatant was subjected to salting out by adding NaCl to obtain the concentration of 2.5 M in the presence of 0.05 M Tris(hydroxymethyl)amino methane (pH 7.0). The precipitated matter was collected by centrifugation at 20,000 × g for 1 h. The pellet was dissolved in a minimum volume of 0.5 M acetic acid at the ratio of 1:9 (w/v) and subsequently dialyzed in 20 volumes of 0.1 M acetic acid, followed by distilled water. All processes were carried out at 4°C. Collagen was lyophilized using a freeze-dryer (Alpha 1-2 LD Plus freeze

dryer; Martin Christ GmbH, Osterode, Germany) in –50°C for 24 h. The collagen was stored at –80°C until use. The collagen thus obtained was as ASC.

Physiochemical properties

After ASC extraction, quality factors were determined according to national and international standards.

Collagen yield measurement

The yield of acid soluble collagen was calculated based on dry weight of starting material as fish scales after pretreatmentby using the following equation [21]:

Yield of collagen (%) = (weight of lyophilized collagen [g] / initial weight of dry fish scales after pretreatment [g]) × 100

Proximate analysis:

The determinations of the moisture, ash, total crude protein contents, and fat contentsof extracted collagen were carried out using the following AOAC [22] procedures: moisture (%) was measured by drying samples in an oven at 103°C for 8 h; crude fat (%) was determined gravimetrically; crude ash (%) was obtained by incineration in a muffle furnace at 580 °C for 8 h; and crude protein (N x 5.95) (%) was measured by the Kjeldahl method after acid digestion.

Characterization of biopolymer

UV-vis spectroscopy analysis

The UV-Vis absorption spectrum of acid soluble collagen was recorded using a Shimadzu spectrophotometer UV-8500 UV 8500 II (Techomb) in the range of 200–400 nm.

Fourier transform infrared (FTIR) spectroscopy analysis

Fourier transform infrared (FTIR) (Jasco-FTIR 410 0typeA, Japan) spectrum was recorded to detect the chemical and structural nature of freeze dried acid soluble collagen samples (ASC). 2 mg of sample was mixed with 100 mg of KBr and was clamped into salt disc of 10 mm diameter for reading spectrum further by using KBr for pelleted forms of samples. The spectrum of ASC was performed at 4000–400 cm^{-1} at room temperature and automatic signals were recorded at a resolution of 4 cm^{-1}. The effective peaks were obtained and compared with that of standard collagen.

X-Ray Diffraction analysis of collagen

Further characterization of ASC was done using X-ray diffraction technique. It was carried out in an X-ray diffractometer (X'Pert Pro A Analytical) operated at 45 kV voltage and 40 mA current. The pattern was recorded by Cu Kα radiation in a θ–2θ configuration.

Microstructure analysis by SEM

The surface microstructures of collagens were analyzed by scanning electron microscope (FEI Quanta 200 SEM), to evaluate the structure of isolated collagen. The surface of dried collagens was coated with gold in vacuum using sputter coater, and was photographed.

Statistical Analysis

All determinations were carried out in triplicates and data were analyzed by ANOVA followed by Tukey's multiple comparisons test for significant differences. Values were considered significant at $p<0.05$.

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RESULTS AND DISCUSSION

Analysis of ASC properties

Table 1 revealed the proximate analysis of the acid soluble collagen isolated from fish scales. ASC possessed very huge amount of protein content (89± 0.51%), low moisture content (7.52 ± 0.73%), and trace amounts of ash (0.63 ± 0.65%) and fat (0.47 ± 0.38%). The total yield of collagen was known to vary with the type of extraction. In this case, the yield of ASC was about 31 ± 0.3% based on the lyophilized dry weight which was higher to that extracted from Jelly fish (25-35%) [23], Baltic cod (21.5%), and *Crassostrea gigas* (11%) [24]. Thus, it suggests that the process wastes of rohu were potential source of alternative natural collagen. The presence of collagen in the fish scales, which are considered fish processing by-products or waste, was also supported by the findings of Zhang et al. [10], who reported a high amount of collagen in fish scales and bones.

Table 1: Proximate analysis of ASC

Factors	Fish scale collagen
Yield	31 ± 0.3%
Moisture	7.52 ± 0.73%
Protein	89 ± 0.51%
Ash	0.63 ± 0.65%
Fat	0.47 ± 0.38%

UV-vis Spectra analysis

The UV absorption spectrum of ASC at the wavelength ranges 200–400 nm was showed in Fig (1). In the present study, ASC extracted from scales of *L. rohita* showed a distinct maximum absorption at 234 nm which is similar to the studies of Liu et al. [25], which was in accordance with the characteristic absorption of collagen. The band near 230 nm is attributed to n → n* transition of C=O in the polypeptides chains of collagen [26]. In n → n* transition, the lone pair electron density on oxygen atom of peptide bond is transferred to antibonding orbital of the carbonyl of the subsequent peptide bond [27]. The results suggested that the groups CO, COOH, $CONH_2$ were accessible in polypeptides chains of collagen.

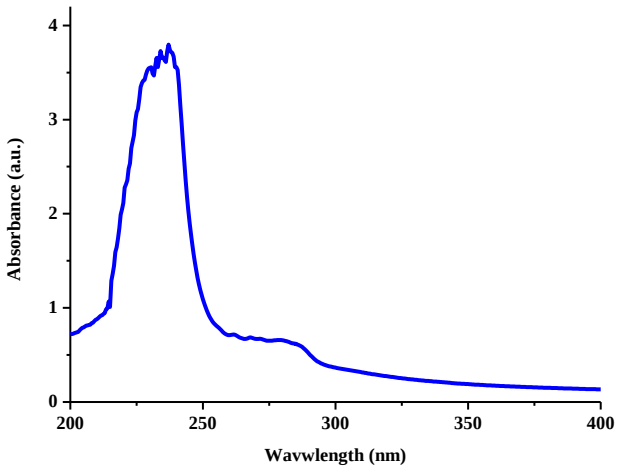

Fig 1: UV-vis absorption spectrum of fish scale collagen

FTIR measurement

The chemical state of the collagen was analyzed by a Fourier transform infrared (FTIR) spectrometer. Fig (2) shows the FTIR spectrum of acid soluble collagen extracted from fish scale. There are several characteristic bands for protein structure, such as amide A, amide B, amide I, amide II, amide III, among them

amide I and amide II bands are two major bands of the protein infrared spectrum. The peak for amide A and amide B were detected at 3371 cm^{-1}, 3032 cm^{-1} due to N-H stretching vibration, indicates the existence of hydrogen bonds in the collagen sample. Usually free N-H vibration occurs in the range of 3400 to 3440 cm^{-1} which shifts to lower frequencies in case of NH group participate in a hydrogen bond formation; it suggested that more NH groups of the ASC were involved in the hydrogen bonding in peptide structure. The amide I band detected at 1637 cm^{-1}, is mainly associated with the C=O stretching vibration (70-85%) and is directly related to the peptide backbone conformation [28], and it is a sensitive marker of the peptide secondary structure [29]. The main amide II and amide III is observed at 1510 cm^{-1}, 1303 cm^{-1}, respectively due to C-N stretching and N-H bending vibrations [30, 31]. The result of FTIR measurement indicates the isolation of fish scale collagen that can be accounted for Type I collagen with helical protein structure.

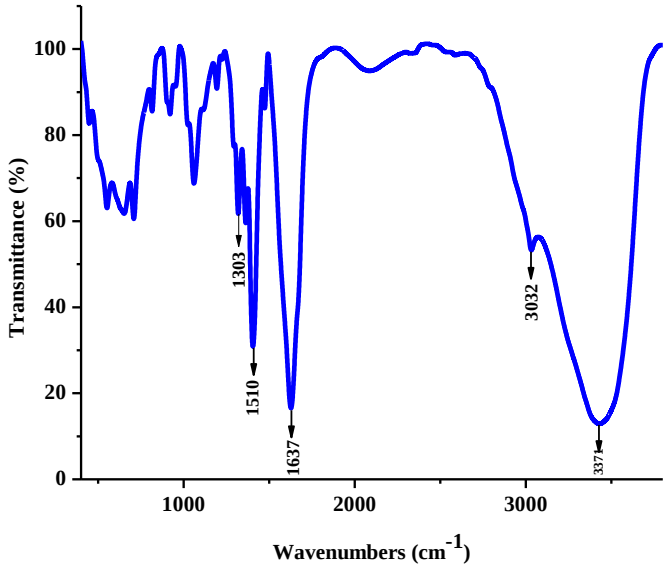

Fig 2: FTIR spectrum of ASC

XRD analysis

The crystallinity of the lyophilized acid soluble collagen was analyzed using an X-ray diffraction (Fig. 3). The X-ray spectrum shows two diffraction peaks at the diffraction angles (2θ) about 7.05° and 19.39°. The first diffraction peak was sharp butthe second one was wide, polymer31which were related to the characteristic is typical of pure collagen and these features proved collagen a quite amorphous polymer [32].

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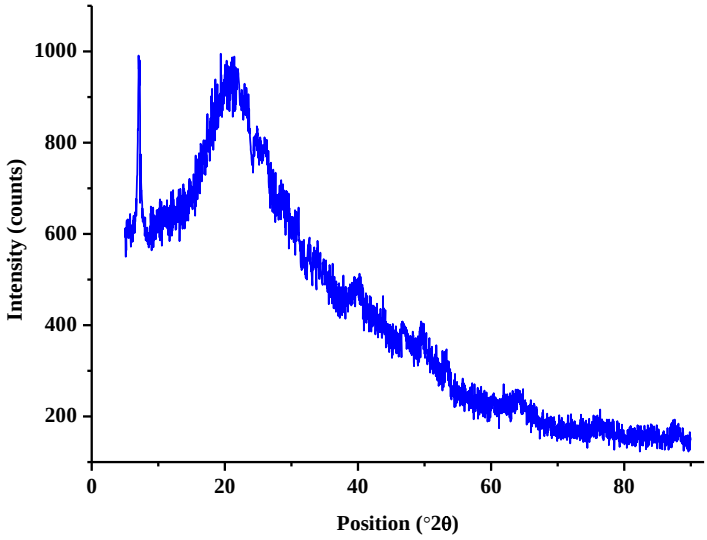

Fig 3: XRD pattern of acid soluble collagen (ASC)

SEM micrograph

The surface morphology of lyophilized pure acid soluble collagen was observed by a scanning electron microscopy. SEM micrograph shows a randomly dispersed fiber structures (Fig. 4). This fibers arrangement exhibits an irregular morphology with some parts more compact white other parts are discontinuous particle like structure.

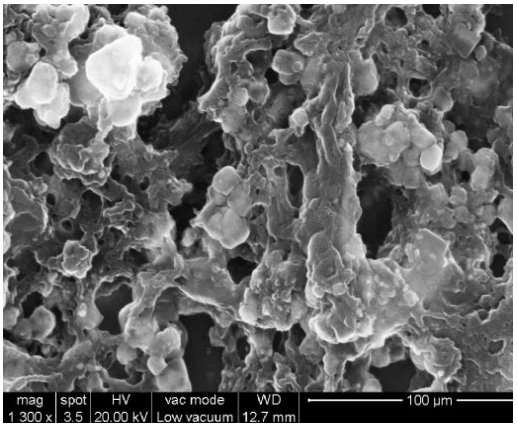

Fig 4: SEM micrograph of ASC extracted from fish scale

CONCLUSION

Fish scale is an economically and technologically viable substrate from which acid-soluble collagen was extracted. The scales of fresh water fish *L. rohita* was used to extract ASC and characterized by UV-vis, FTIR, SEM and XRD. Results showed that satisfactory yield of ASC (31%) by using this alternative source. However, the extraction technique which was used in this study should be adjusted in order to produce collagen of determined characteristics and to obtain new knowledge about many species of collagens. In the future, the biological characteristics of the extracted collagen from fish will be examined under vitro and vivo conditions and compared with mammalian collagen. In conclusion, the results of this work demonstrate the feasibility of using the obtained collagen indifferent industrial applications.

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