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Research Article

ISOLATION, CHARACTERIZATION, AND DETERMINATION OF ANTIOXIDATIVE PROPERTIES OF PROTEIN EXTRACTED FROM ROHU (*LABEO ROHITA*) FISH SKIN

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ABSTRACT

Objective: Isolation, characterization and analysis of antioxidant activity of protein extracted from *Labeo rohita* skin.

Methods: The present work aimed to characterize protein isolated from Indian major carp *L. rohita* skin. Protein isolation was carried out by the salting-out method and the protein content was estimated with bovine serum albumin. Isolated protein was subjected to Fourier-transform infrared analyzer to identify the presence of $-NH_3$ group to be claimed as protein. It was also subjected to high-performance liquid chromatography (HPLC) analyzer and run against standard collagen (Sigma), the molecular weight of the protein was determined through mass spectroscopy (MS) to know the protein structure more precisely the protein isolate was studied under a scanning electron microscope (SEM). Antioxidative activity of the crude protein sample was studied through 2,2-diphenyl-1-picrylhydrazyl, ferric reducing antioxidant potential (FRAP) assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay.

Results: The protein isolated was 23% of the dry weight of fish skin. In HPLC analysis, the isolated protein gives peaks that are similar to that of standard collagen, and in MS, its molecular weight was near about 300 kDa, which is the molecular weight of collagen type 1. The SEM image shows a crystal structure of protein isolates. Results of antioxidative property show that fish skin protein isolates have good antioxidative activity also.

Conclusion: The isolated protein was collagen and it can be used as a replacer of the renowned market available collagen.

Keywords: Protein, Collagen, Fish skin, 2,2-Diphenyl-1-picrylhydrazyl, ABTS, FRAP.

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INTRODUCTION

Collagen is the main protein constituent found in the skin of every higher animal. Collagen has numerous applications in pharmaceuticals, cosmetics, and the food industry [1]. The collagen protein is a long, stiff, and triple-stranded helix, in which peptide chains are wrapped around one another to form a rope-like helix structure. These peptides are extremely rich in glycine and proline, which are very important amino acids [2]. Collagen is mainly extracted from the skins of ruminant and porcine livestock and poultry [3,4]. However, nowadays, interest has moved toward fish, jellyfish, marine sponge, and squid [5-8]. Collagen is extracted mainly from sea fish, whereas 41.24% of fish are found in freshwater. This high amount of fish skin is discarded as waste during fish processing, can be utilized for the production of collagen. Hence, an attempt has been made to isolate and characterize the protein from *Labeo rohita* skin and to identify the extracted protein, whether it is collagen or not through Fourier transform infrared (FT-IR), scanning electron microscope (SEM), high-performance liquid chromatography (HPLC), and mass spectroscopy (MS) study. Furthermore, the antioxidative properties of the crude protein were analyzed through 2,2-diphenyl-1-picrylhydrazyl (DPPH), FRAP, and ABTS assay.

METHODS

Preparation of samples

Fish skins were collected from the local market. It was washed thoroughly to remove dust particles and any sort of scales with first under tap water and then with distilled water. It was then cut into smaller pieces and kept at −20°C until further use.

Isolation of protein

The skin was dried and dissolved in n-hexane in 1:10 (w/v) ratio for 24 h to remove the lipid part from the skin. Protein was extracted from fish skin through the salting-out method [9] with modification. Fish skins were stirred with 0.1 N $NaHCO_3$(1:10) for 6 h followed by centrifugation at 6000 rpm for 20 min. The pellet was collected and again stirred with 0.5 N CH_3COOH (1:10) for 24 h and centrifuged at 6000 rpm for 20 min. The supernatant was collected and protein was extracted, which was by salting out with NaCl until the final concentration of the solution becomes 0.7 N/L. The precipitate was collected as crude protein and lyophilized for further use.

Characterization of fish skin protein

FT-IR analysis

FT-IR analysis of the lyophilized samples was carried out to identify the functional group present in the sample. As the sample was claimed to be protein, the presence of $-NH_3$ group is a parameter to establish it as protein. The presence or absence of $-NH_3$ is detected through FT-IR analysis. FT-IR analysis of the lyophilized sample was carried out on FT-IR – 4600 type A, serial no-D060661786 with triglycine sulfate detector. Spectrum was recorded between wavenumber 0 and 7800.65 cm^{-1}.

HPLC analysis

The identification of the crude protein sample was carried out in an HPLC analyzer. The HPLC apparatus was from Waters, model no. 2695. The detection was carried out using C_{18} column and 2487 dual detector, with a run time of 1 ml/min and a wavelength of 280/210/370 nm. The same was done for standard collagen (Sigma) and compared with the sample.

MS analysis

MS analysis was carried out to detect the molecular weight of the crude protein sample. The test was performed by gradient method in an MS apparatus from Waters, model no. XEVO G2-XS QTof, BEH C_{18} column, and photodiode array (PDA) detector with a run time of 1 ml/min at

254 nm absorbance. The sample had given peaks and that is similar to the molecular weight of collagen.

SEM image analysis

Scanning electron microscopy of the crude protein was done in a SEM with gold coating and the SEM images resemble with that of collagen.

Antioxidative property analysis

Antioxidative properties of the crude sample were determined to evaluate the radical scavenging activity of crude protein through DPPH, FRAP, ABTS [10], and reducing power assay [11].

DPPH free radical scavenging activity

A volume of 2 mL of a methanolic solution of the crude protein was put into test tubes and 2 mL of 1 mM DPPH solution was added. The tubes were kept in the dark for 1 h. Absorbance at 517 nm was measured with a spectrophotometer (JASCO V-630) and compared to an ascorbic acid calibration curve. The results were expressed as mg ascorbic acid/g of sample. The percentage inhibition of the DPPH radical was calculated using the following formula:

$$I\% = \frac{(A_0 - A)}{A_0} \times 100$$

Where, I = DPPH inhibition (%), A_0 = absorbance of control sample (t = 0 h), and A = absorbance of a tested sample at the end of the reaction (t = 1 h).

Determination of FRAP (ferric reducing/antioxidant power) activity

The antioxidant activity by FRAP assay was carried out by the method of the ferric reducing ability of plasma (FRAP) assay of Benzie and Strain, 1996, with slight modifications. Twenty microliters of sample and 180 µL of FRAP reagent were incubated at 37°C for 40 min in the dark. The absorbance of the resulting solution was measured at 593 nm in an ultraviolet spectrophotometer (JASCO V-630). The change in absorbance between the final reading (4 min reading) and blank reading (0 min reading) of the sample and the same of standard ascorbic acid was selected for the calculation of FRAP value which is calculated using the formula:

FRAP value = (sample changes from 0 to 4 min/standard changes from 0 to 4 min) × FRAP value of standard

ABTS (2,2'-azinobis-3-ethylbenzenethiazoline-6-sulfonic acid) decolorization method

The antioxidant activity by the ABTS method was performed using a volume of 20 µL (diluted 1:10) of crude protein extract added to 2 mL of ABTS solution, and the mixture was kept at room temperature for 10 min.

Table 1: Proximate composition of *Labeo rohita* skin

Component	Amount (%)
Moisture	45.25±0.27
Ash	2.70±0.01
Carbohydrate	2.15±0.02
Protein	24.50±0.03
Fat	25.40±0.02

All tests were performed in triplets. Data are expressed as Mean ± SD (standard deviation)

Table 2: Antioxidative property of FPI

Test performed	Amount
DPPH	47.36%
FRAP	10 µg of AAE/g
ABTS	35.57%
Reducing power	1.030–1.313

FPI: Fish protein isolate, DPPH: 2,2-Diphenyl-1-picrylhydrazyl

The absorbance was measured at 734 nm with a spectrophotometer (JASCO V-630). The values were compared with those of the butyl hydroxytoluene standard curve. The result was calculated using the following formula:

$$\%\ \text{Antioxidant activity} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c and A_s are the absorbance of control and sample, respectively.

Reducing power assay

It was measured according to the method of Oyaizu. In a test tube, 2.5 ml crude protein extract, 2.5 ml sodium phosphate buffer (0.2 M), and 2.5 ml potassium ferricyanide were mixed and incubated in a water bath for 20 min at 50°C. After that, 2.5 ml trichloroacetic acid was

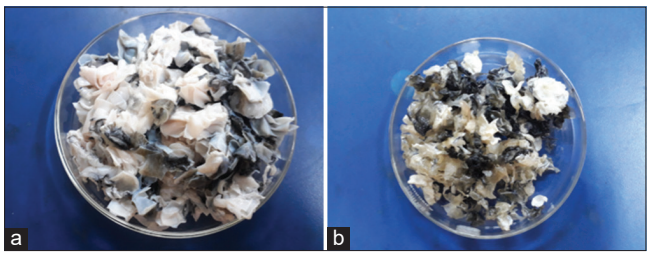


Fig. 1: (a) Fish skin collected from the local market. (b) Scales are isolated from the fish skin

Fig. 2: Crude protein powder

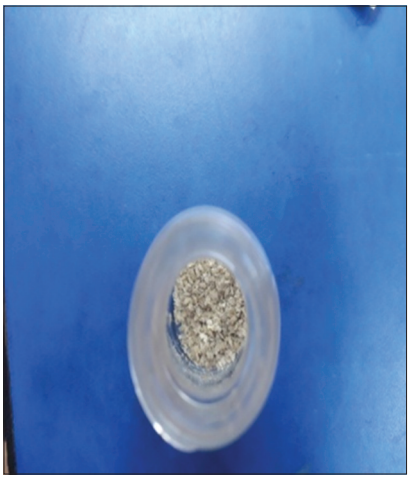


Fig. 3: Close view of crude protein powder

added. The mixture was centrifuged for 10 min and the supernatant was taken into a test tube, in which 5 ml distilled water and 1 ml ferric chloride were added. Absorbance was measured at 700 nm. An increase in absorbance of the reaction mixture indicates an increase in reducing the power of the test sample.

RESULTS

Proximate composition of fish skin

Fish skins were subjected to proximate composition to reveal the major nutrient component of the skin. The results of the proximate composition analysis are mentioned in Table 1. Rohu skin contains about 24.50 ± 0.03% of protein which is quite a good amount. Fig. 1a and b shows the picture of fish skin collected from the market and after the scale is removed from the skin, respectively.

Protein yield

From 40 g of fish skin, sample 9.8 g crude protein was recovered and hence the protein yield is 24.5%. Figs. 2 and 3 imply the picture of lyophilized and powdered protein samples.

FT-IR analysis

The FT-IR spectrum of Rohu fish skin protein is represented in Fig. 4. The FT-IR spectrum exhibits a bands of amide A (associated with N-H groups and involved in hydrogen bonds of peptide), amide B (related to the asymmetric stretching of C-H), amide I (associated with stretching vibrations of the C=O and an indication of secondary structure of peptides), amide II (attribute stretching of the carbonyl group coupled to a carbonyl group), and amide III (stretching vibration between C-N and bending vibration of N-H) at different wavelength. The standard amide II band is supposed to be occurred at 1550 cm^{-1} and 1600 cm^{-1}[12]. In case of Rohu skin, it also shows a band near 1533 cm^{-1}. The FT-IR spectrum of collagen protein reported in the previous work [13] indicated that the band for amide I is in between 1644 and 1653 cm^{-1}, amide II is in 1541 and 1548 cm^{-1}, and for amide III, it is 1237–1239 cm^{-1}, band of amide A is in between 3304 and 3315 cm^{-1}, and for amide B, it is 2922 and 2940 cm^{-1}. Protein isolated from Rohu skin exhibits bands in nearly wavelength of 1640.16 cm^{-1} and 1734.66 cm^{-1}, that is, for amide I, at 1257.36 cm^{-1} for amide III, at 3486.67 cm^{-1}, 3254.29 cm^{-1}, and 3047.94 cm^{-1} for amide A, and at 2859.92 cm^{-1}, 2947.66 cm^{-1}, and 2921.63 cm^{-1} for amide B. The FT-IR spectrum indicates that the isolated protein gives a similar wavelength of collagen.

HPLC analysis

HPLC analysis of Rohu skin is given in Fig. 5. It gives the retention time (RT) for different protein chains of a_1 (I), a_2 (I), a_3 (II), a_1 (III), a_3 (IV), and a_2 (V). Standard RT for the protein chains is 13.7, 13.9, 12.7, 13.0, 20.1, and 12.3 min, respectively [14]. Rohu skin protein isolate gives RT of 11.174, 11.319, and 15.389 min, which is close to that of standard collagen. Depending on the HPLC result, it can be stated that the protein can be of collagen.

MS analysis

The mass spectrum of the protein isolate is mentioned in Fig. 6. X-axis of a mass spectrum indicates mass-charge ratio and Y-axis represents the signal intensity of the ions. The mass spectrometric analysis produces fragments of molecular weights which is called peak list. The mass of these peptide fragments is calculated and compared to that of a standard. Collagen I possesses α_1 and α_2 subunit at a molecular weight of 116.5–126 KDa and 255.5–281.1 KDa, respectively, and a β subunit approximately at 417.3 KDa [15]. The molecular weight of Rohu skin protein isolate falls in this range.

SEM analysis

SEM image of the protein represents in Fig. 7 and it gives a crystalline structure of the protein. In the previous work [16], SEM image showed

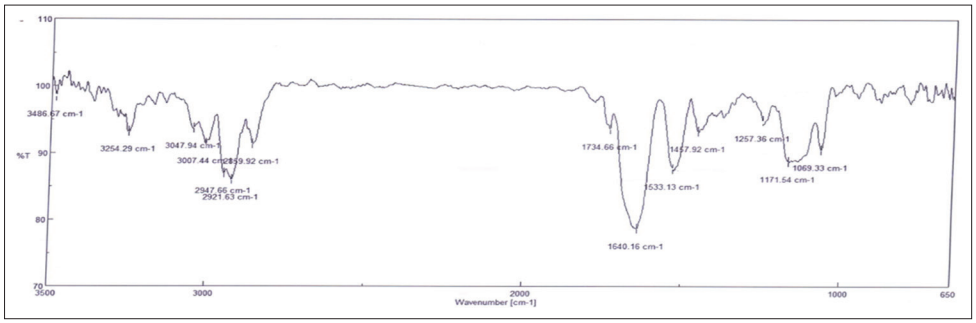
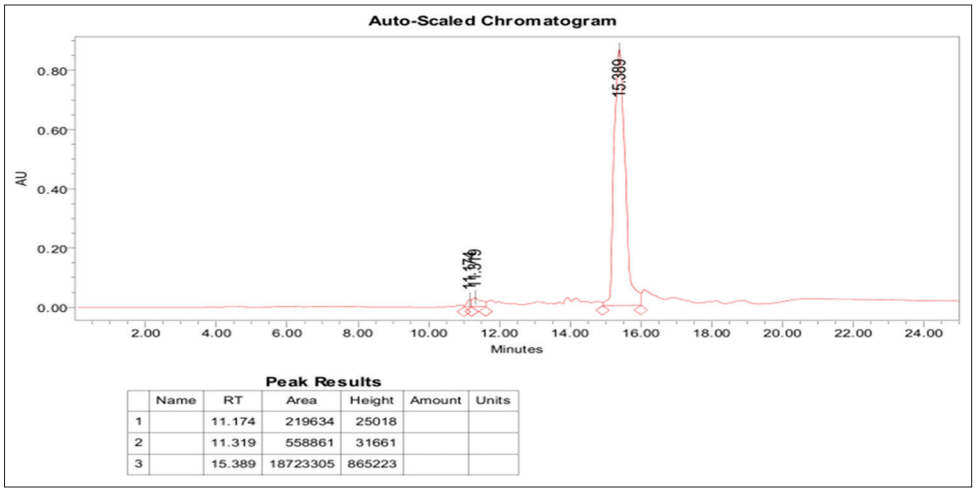


Fig. 4: Fourier transform infrared spectrum of Rohu skin protein isolate



Peak Results

	Name	RT	Area	Height	Amount	Units
1		11.174	219634	25018		
2		11.319	558861	31661		
3		15.389	18723305	865223		

Fig. 5: High-performance liquid chromatography of Rohu skin protein isolate

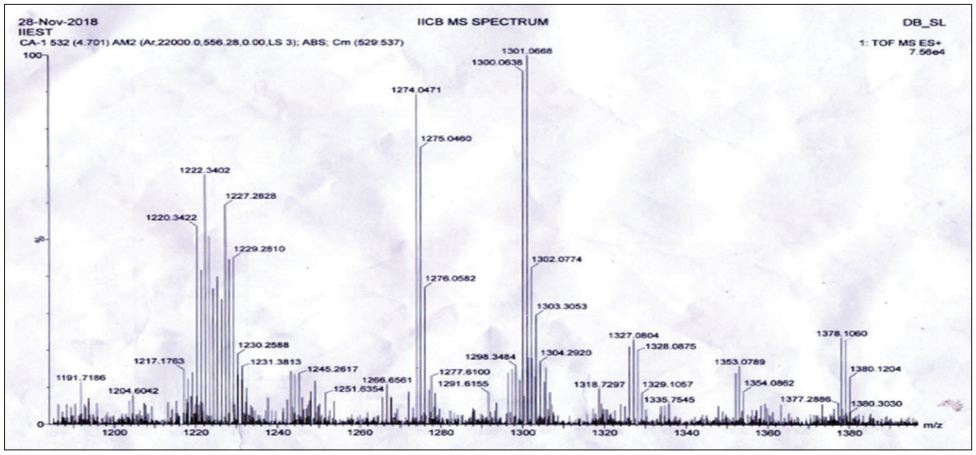


Fig. 6: Mass spectrum of Rohu skin protein isolate

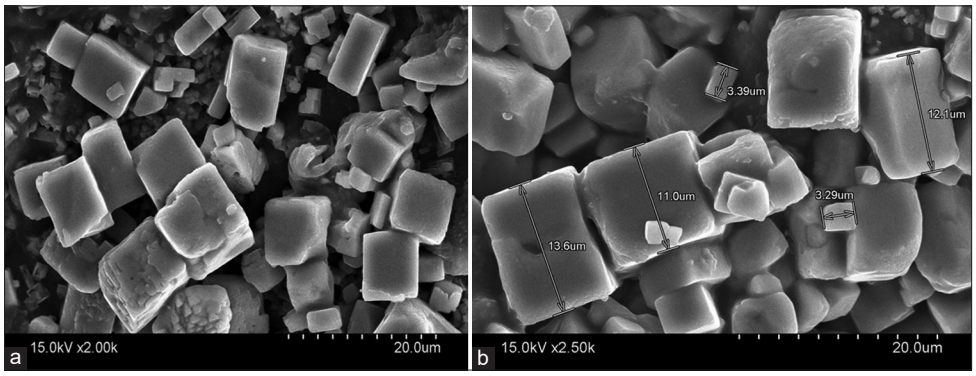


Fig. 7: (a and b) Scanning electron microscope of Rohu skin protein isolate

the fibrous nature of collagen. As the sample is in powder form it is showing a crystalline structure in SEM image.

Antioxidative property analysis

The antioxidative property of the protein isolate is mentioned in Table 2.

CONCLUSION

Based on the tests performed *Labeo rohita* skin isolates can be claimed as type 1 collagen. Antioxidative properties were evaluated to know whether the FPI is oxidatively stable or not through different tests. The FPIs proved to have good antioxidative properties through DPPH, FRAP, ABTS, and reducing power assay. The results of all the tests performed lead to conclude that FPI can be used as an alternative source of collagen and can be used both in food formulation and pharmaceutical as a replacer of conventional collagen source.

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AUTHORS' CONTRIBUTIONS

Both the authors had contributed equally to the work. Nabanita Ghosh is a Ph.D. Research Scholar and D. K Bhattacharyya is the Principal Investigator of the work.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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