EVALUATION OF ANTIOXIDANT FUNCTIONALITY OF COLLAGEN HYDROLYSATE FROM FISH SKIN - A CLEAN AND SAFE ROUTE OF VALORISATION OF MARINE WASTE

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KEYWORDS:

Antioxidant, collagen, gastric stability, hydrolysis, nutraceutical.

INTRODUCTION:

The fish production in Karnataka state has increased significantly over the years, leading to a rise in fish waste. However, this waste can be utilized to produce value-added products such as enzymes, bioactive peptides, biopolymers and collagen (Shahidi et al., 2019). Collagen, a crucial structural protein found in metazoans, is an important component of the extracellular matrix that exhibits several desirable properties like low-immunogenicity, gel-forming ability, high viscosity, and good emulsion formation, making it highly sought after in food, cosmetic, and pharmaceutical industries. The global collagen market is expected to grow annually at a rate of 9%, reaching a value of 8.36 billion USD in 2020 2028 (http://www.grandviewresearch.com). Bovine and porcine sources have been the preferred choice for its industrial production so far. However, due to prion-related diseases, the search for alternative sources has received attention and marine waste proved to be an excellent alternative as it is both economically cheap and environmentally viable. Collagen hydrolysate, a lower molecular weight product obtained from collagen hydrolysis, displays improved properties than its native form (He et al., 2013, Chi et al.,2014, Jemil et al., 2014). It possesses diverse bioactivities, of which, antioxidant bioactivity is of particular interest because of its direct application in nutraceutical and food industries. It indicates the ability of hydrolysed collagen to scavenge free radicals, thereby preventing many life-style diseases. The nutraceutical industry, therefore, has witnessed huge demand for health-promoting foods with antioxidant peptides/hydrolysate. To cater to this need, collagen hydrolysate derived from skin of *Rachycentron canadum* (cobia) was tested for its antioxidant activity and

gastric stability. This fish found in Atlantic Ocean, Indian coast and Caribbean Sea is preferred for its flesh and the skin is mostly discarded after filleting, that in turn adds to the existing problem of waste mismanagement. The current project focused on generation of collagen hydrolysate from cobia fish skin using papain and evaluation of its antioxidant activity and gastric stability.

OBJECTIVES:

The study primarily aimed at extracting collagen from cobia fish skin waste using acid and pepsin method followed by preparation of collagen hydrolysate using papain. Further, antioxidant activity and gastric stability of the hydrolysate were determined by DPPH method and *in vitro* simulation method respectively.

The study was designed with the following specific objectives.

1. To extract collagen from cobia fish skin using acid and pepsin solubilisation method

2. To enzymatically hydrolyse the extracted collagen using papain

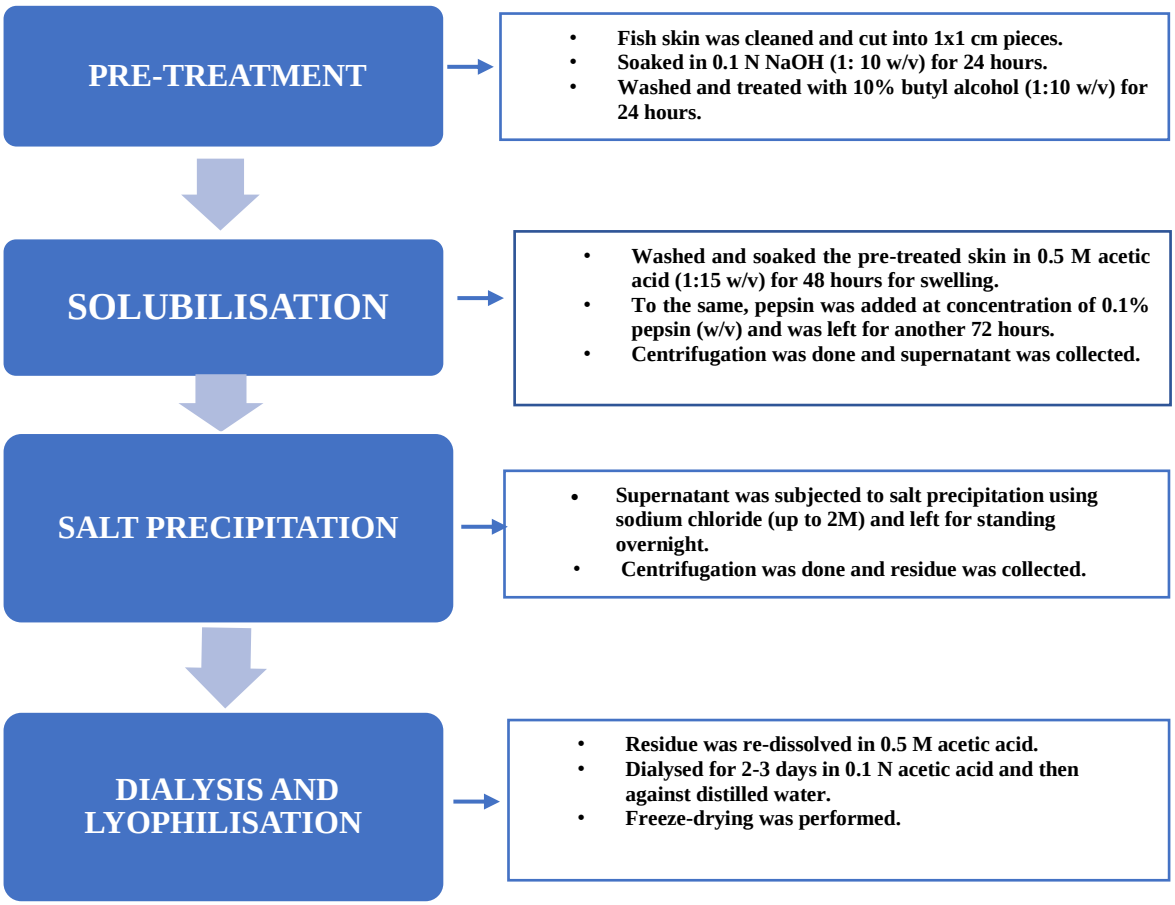
3. To determine the *in vitro* antioxidant activity of the hydrolysed collagen

4. To evaluate the *in vitro* gastric stability of the collagen hydrolysate

MATERIALS AND METHODOLOGY:

Chemicals and enzymes: Butyl alcohol, ethanol, glacial acetic acid, hydrochloric acid, sodium chloride and sodium hydroxide used were of analytical grade. Papain (12 caseinolytic activity units/mg), pepsin (porcine stomach mucosa- EC 3.4.23.1) (1000 units/mg dry matter), N-acetyl cysteine (NAC), sodium dodecyl sulphate (SDS), 1,1-diphenyl-2-picrylhydrazine (DPPH) and ascorbic acid were procured from Himedia, Mumbai. OPA (opthalaldehyde) was obtained from SRL labs (Sisco Research Laboratories pvt. Ltd, Mumbai). Skin of cobia fish was collected from local fish markets of north and south Bengaluru.

Extraction of collagen: Collagen was extracted from cobia (*Rachycentron canadum)* skin waste as per the following flow chart (Nalinnanon et al., 2007).



All the above procedures were performed at 4°C and collagen yield was calculated on wet weight basis as:

Yield % = (weight of lyophilized collagen/ wet weight of fish skin) *100

Enzymatic hydrolysis of collagen using papain: Papain with activity of 12 caseinolytic Activity (CA) units/ml (The amount of enzyme that liberated 1µg of tyrosine under the standard assay conditions was considered as one unit of caseinolytic activity) was dissolved in 0.2 M phosphate buffer and then used for enzymatic hydrolysis of collagen from cobia skin. The hydrolytic conditions were adopted after optimisation as mentioned in table 1 (Benjakul et al., 2018).

Table 1- Optimum conditions for hydrolysis of collagen with Papain

Enzyme	Enzyme/substrate ratio (w/w)	pH	Temperature	Time of hydrolysis
Papain	1:12	7.0	40°C	4 hours

The collagen was kept in shaker incubator set at 40°C for 4 hours for hydrolysis with papain and the reaction was terminated by keeping at 100°C for 10 minutes. Once it reached normal temperature, it was centrifuged at 10,000 rpm for 15 minutes at 4°C and supernatant was collected and stored for further assays.

Determination of degree of hydrolysis (DH%) using opthaldehyde (OPA) assay: OPA/NAC reagent (100mL) was prepared by combining 10mL of 50mM OPA (in methanol) and 10mL of 50mM NAC, 5mL of 20% (w/v) SDS, and 75mL of borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminium foil to protect from light and allowed to stir for at least 1h before use. The OPA assay was carried out by the addition of 10µL of sample to 1.2mL of OPA reagent. The absorbance of this solution was measured at 340 nm with an UV spectrophotometer. The absorbance values for the interaction of amino groups with OPA were taken after 2 min standing for unhydrolysed collagen and after 10 min standing for hydrolysed collagen. The total amount of amino groups was determined in protein samples by incubation with 6N HCl at 110 °C for 24 h. The DH was calculated using the following equation (Spellman et al., 2003).

$DH\% = (NH_2)\ Tx - (NH_2)\ To\ /(NH_2)\ Total - (NH_2)\ To * 100$

where (NH_2) Tx is the free amino group content of collagen hydrolysate (µmol/mL), (NH_2) To is the free amino group content of unhydrolysed collagen sample (µmol/mL) and (NH_2) Total is the total amount of free amino groups in collagen sample (µmol/mL). Leucine (stock-2mg/mL) was used as standard to determine free amino group content.

Antioxidant activity of the collagen hydrolysate by DPPH method: Collagen hydrolysate obtained was tested for its antioxidant activity using method described by Wu et al., (2018) with slight modifications. Briefly, 100 µl of collagen hydrolysate (from stock of 200 µg/ml in milli-q water) was mixed with 100 µl of DPPH solution (1mM in 95% ethanol). Ascorbic acid was used as positive control (200 µg/ml in milli-q water). Blank control was DPPH alone without sample. The assay was performed in 96 well plate, kept for 30 minutes of incubation in dark and absorbance read at 517 nm using microplate reader.

The DPPH radical scavenging activity was calculated using the following formula (Wu et al., 2018).

DPPH radical scavenging activity (%) = [(Abs Blank-Abs sample) /Abs blank] × 100

Validation of gastric stability of the hydrolysate: The collagen hydrolysate with significant antioxidant activity was tested for its gastric stability using *in vitro* simulated gastric system according to Ritian et al., (2018) with slight modifications. Simulated gastric juice containing 0.2% (w/v) NaCl, 0.08% (w/v) pepsin, 0.7% (v/v) HCl in milli-q water was prepared and collagen hydrolysate was dissolved in 1:5 w/v ratio and kept in shaker set at 37°C for 4 hours. Pepsin was inactivated by keeping in boiling water bath for 10 minutes and centrifuged at 10,000g for 30 minutes (Ritian et al., 2021). The supernatant was collected and used for antioxidant assay.

Antioxidant assay of the above gastric simulated collagen hydrolysate was performed in the same way as described above.

RESULTS AND CONCLUSIONS:

Collagen yield: The mean yield of collagen from cobia fish skin using acid-pepsin method was found to be 9.8% on wet weight basis.

Hydrolysis of collagen using papain: Papain (12CA units/mL) was the protease enzyme chosen for hydrolysis of collagen and conditions for hydrolysis was optimised after many trials based on review of literature.

Determination of degree of hydrolysis using OPA assay:

Substrate ratio of 1:12 w/w, pH 7.0 and temperature of 40°C across different time points (1hr to 5hrs) was followed. Maximum DH% of 12.7% was obtained at 4hrs as indicated in Figure 1 and the same conditions were considered for further investigations.

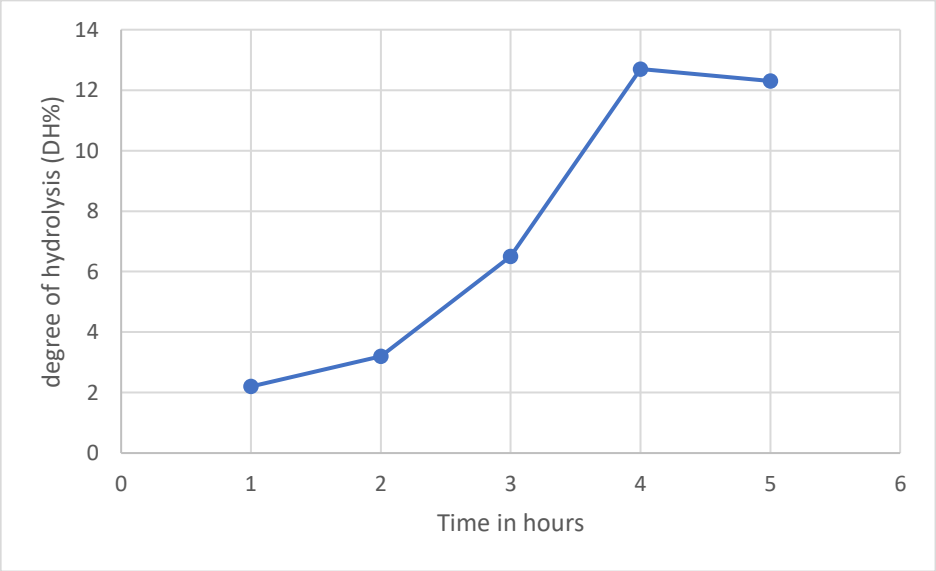


Figure 1- Degree of hydrolysis of collagen from 1 to 5 hours with Papain

Antioxidant activity and Gastric stability:

Antioxidant activity was determined using DPPH assay and the radical scavenging activity was found to be

35.9%,46.6% and 77.6% respectively for unhydrolyzed collagen, hydrolysed collagen and hydrolysed collagen after gastric simulation. Ascorbic acid was used as positive control and its radical scavenging activity was 89%.

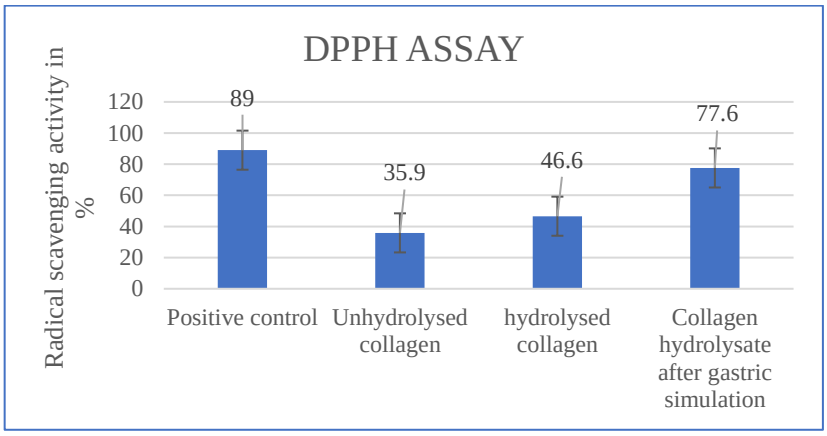


Figure 2- Radical scavenging activity (%) of unhydrolyzed collagen, hydrolysed collagen, hydrolysed collagen after gastric simulation and positive control (ascorbic acid)

Conclusions: The study demonstrated extraction of collagen from cobia fish skin using the acetic acid-pepsin method. An average collagen yield of 9.8% was obtained based on wet weight of the skin. The extracted collagen was subsequently subjected to hydrolysis using papain in optimised conditions. The optimum condition was determined based on highest degree of hydrolysis achieved, which was found to be 12.7%. To evaluate antioxidant activity of the collagen hydrolysate, DPPH assay was conducted both before and after gastric simulation. The results were compared with a positive control (ascorbic acid) and with unhydrolyzed collagen. The hydrolysed collagen exhibited higher radical scavenging activity (46.6%), compared to unhydrolyzed collagen (35.9%). Interestingly the hydrolysed collagen's radical scavenging activity remained high at 77.6% after gastric simulation. This indicates that the collagen hydrolysate derived from fish skin possesses substantial antioxidant activity even after gastric simulation. It can be concluded from the study that the collagen hydrolysate has good potential as an antioxidant ingredient.

INNOVATION IN THE PROJECT:

An innovative approach for the sustainable exploration of marine resources is demonstrated via this project. To extract collagen hydrolysate and assess its antioxidant effects, this study used fish skin, which is frequently thrown away as waste in fisheries sector. In addition to addressing the problem of waste management, this creative idea offers a practical way to access a useful ingredient with potential health advantages. The practical application of fish skin is innovative, but so is the environmentally responsible and sustainable strategy used throughout the process.

Through the conversion of waste into a useful resource, the project offers a strategy to achieve circular bioeconomy. Previous studies in this background so far focussed only on the antioxidant activity of collagen and its hydrolysate. This project has made an effort to extend the understanding till evaluation of its gastric stability, which is one of the important parameters to decide its direct application in nutraceutical industry for oral consumption. Thus, the initiative fits with the rising demand for eco-friendly and natural ingredients for attaining health benefits and meets the demand for sustainable and clean-label components by offering a collagen hydrolysate made from fish skin. Additionally, recycling of fish skin may economically benefit the fishing industry and coastal communities.

SCOPE FOR FUTURE WORK:

There is ample scope for research and exploration of collagen hydrolysate derived from fish skin for its antioxidative ability as a clean and safe route for the valorisation of marine waste. The study can be further extended to understand the underlying molecular mechanisms of the antioxidant activity displayed by collagen hydrolysate and its effect on cellular antioxidant defence mechanisms. To further evaluate collagen hydrolysate's potential as a functional food ingredient or nutraceutical, research into its bioavailability and pharmacokinetics is necessary. Its applicability for different uses can be determined by researching its absorption, distribution, metabolism, and excretion as well as stability under various physiological situations. Collagen hydrolysate's safety must be guaranteed and future studies should include thorough toxicity analyses (acute and chronic). The creation of collagen hydrolysate formulations and products is another exciting field of research. Investigating its potential uses in the food, pharmaceutical and cosmetic industries may result in the creation of functional food items or nutritional supplements offering a workable and environment friendly method of valorising marine waste.

REFERENCES:

1. Benjakul, S., Karnjanapratum, S., & Visessanguan, W. (2018). Production and characterization of odorless antioxidative hydrolyzed collagen from seabass (*Lates calcarifer*) skin without descaling. *Waste and biomass valorization*, *9*, 549-559.

2. Chi, C. F., Cao, Z. H., Wang, B., Hu, F. Y., Li, Z. R., & Zhang, B. (2014). Antioxidant and functional properties of collagen hydrolysates from Spanish mackerel skin as influenced by average molecular weight. *Molecules*, *19*(8), 11211–11230. https://doi.org/10.3390/molecules190811211

3. He, H. L., L. D and M. C. B. (2013). Review on the angiotensin-I-converting enzyme (ACE) inhibitor peptides from marine proteins. *Applied Biochemistry and Biotechnology,* 169, 738–749.

4. http://www.grandviewresearch.com (accessed on 13 December,2022).

5. Jemil, I., J. M., N. R. et al., (2014). Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from fish meat fermented by *Bacillus subtilis*. *Process Biochemistry 49: 963–972. 2014*, *49*, 963–972.

6. Nalinanon, S., Benjakul, S., Visessanguan, W & Kishimura, H. (2007). Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry*, *104*(2), 593-601.

7. Ritian, J., Teng, X., Liao, M., Zhang, L., Wei, Z., Meng, R., & Liu, N. (2021). Release of dipeptidyl peptidase IV inhibitory peptides from salmon (*Salmo salar*) skin collagen based on digestion–intestinal absorption in vitro. *International Journal of Food Science & Technology*, *56*(7), 3507-3518.

8. Shahidi, F.; Varatharajan, V.; Peng, H.; Senadheera, R. Utilization of marine by products for the recovery of value-added products. *J. Food Bioact.* 2019, *6*, 10–61.

9. Spellman, D., McEvoy, E., O'cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International dairy journal*, *13*(6), 447-453.

10. Wu, R., Wu, C., Liu, D., Yang, X., Huang, J., Zhang, J., ... & He, H. (2018). Antioxidant and anti-freezing peptides from salmon collagen hydrolysate prepared by bacterial extracellular protease. *Food Chemistry*, *248*, 346-352.