ORIGINAL PAPER

STUDY ON THE COLLAGEN FROM SKIN OF MARINE FISH FROM THE BLACK SEA

MELAT CHERIM[1], ERGUN – YUKMEL RASIT[2]

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Abstract. *Collagen is a protein with an important role in the body. Harvesting it from natural bio-resources offered by the Black Sea is the general objective of this study. The Black Sea offers multiple possibilities for raw materials rich in bioactive compounds. The directions in which studies have been performed to capitalize on these natural bioresources are highlighted. Of the Gray Mullet marine fish, collagen hydrogels were obtained through various extraction treatments (acid treatment, enzymatic treatment, combined treatment).The paper presents the extraction yields achieved by the applied treatments. The study shows the results obtained through multiple physico-chemical analyzes performed to characterize the collagen obtained (determination of total nitrogen, moisture content, protein content, pH, ash content, fat content and mineral content, solubility). The conclusions of these results lead to the idea that this product containing collagen hydrolyzate is suitable for use as a biomaterial.*

Keywords: *collagen from skin, marine fish, Gray Mullet.*

1. INTRODUCTION

Collagen mainly has a structural role and there different types according to their specific organization in distinct tissues [1]. For this reason, collagen hydrolysates have been chosen as a major biological material for biomaterials [2] as well as for tissue regeneration approaches [3]. The most common source of collagen is the skin and bone of terrestrial animals such as the cow and pig. Because of the risks of bovine spongiform encephalopathy and foot-and-mouth disease in recent years, the collagen source had to be reconsidered [3, 4].Thus, new natural sources of collagen and modernized methodologies for their production appeared [4, 5]. Marine ecosystems have habitats that have been studied so as to assess their specificity [6, 7], considering that they are home to a rich resource for the pharmaceutical industry [8, 9]. Literature on the matter has studies on the marine ecosystems of the Romanian Black Sea seaside [10, 11] and the monitoring of the quality of electrolyte-rich marine waters [12, 13]. A particular interest is represented by the studies on the existence of pollutants in the marine ecosystem [14, 15]. Considerations on heavy metal content [16-20] and existing PAHs [21, 22] in marine water from the Romanian Black Sea coastline have been taken into account. There are studies on the protection measures against pollutants in order to obtain resources for obtaining products with a low level of toxicity [23, 24]. Studies that take into account the meteorological and microbiological aspects of the Romanian Black Sea coast [25-

[1]Ovidius University of Constanta, Faculty of Pharmacy, 900527 Constanta, Romania.
E-mail: melat_kem@yahoo.com.
[2]University Politehnica of Bucharest, Faculty of Electrical Engineering, 060042 Bucharest, Romania.
E-mail: ergunel@gmail.com.

28] are also important. For the pharmaceutical industry in the Black Sea, a rich source of raw materials has been highlighted by many studies. In this respect we can highlight the seaweed [29-31] for which there were studies on polyphenol content and implicit antioxidant activity [32-36], the content of vitamins [37], the content of other important bioactive compounds [38, 39]. The Black Sea marine resource offers a wide range of active nutritional principles from marine organisms [40-43]. From various marine fish, collagen [4, 5] was extracted from which various pharmaceutical preparations were obtained by combining it with seaweed [42-44] for various medical and cosmetic purposes. For the obtaining of pharmaceutical preparations, the multiple methods used in clinical trials [47-50] should be considered.

This study is part of the series of marine harvesting with the intent to obtain collagen from *Gray Mullet* fish in the Black Sea. Fish collagen has a great potential for use because of its undeniable advantages [51, 52]:

- it is easier to extract and provides better yields than the mammalian skin;
- it presents a relatively low risk of unknown pathogens;
- the possibilities of distortion are lower.

Although collagen extracted from fish does not form high viscosity gels, they are very convenient for certain applications, such as micro-encapsulation or obtaining light-sensitive coatings.

2. MATERIALS AND METHODS

2.1. EXTRACTION OF GRAY MULLET SKIN COLLAGEN FROM THE BLACK SEA

Materials used for extraction of collagen

The selection of samples for the isolation of fish collagen aimed to harness the resources of the Black Sea. In the proposed study, collagen extraction was carried out on *Gray Mullet* (chefal) fish from the Black Sea.

Methods used for extracting collagen from fish skin

As extraction methods, literature shows different collagen isolation biotechnologies, depending on applied treatments:

- alkaline and acidic treatments,
- acidic treatments [5],
- enzymatic treatments,
- combined treatments [40].

Extraction by enzyme treatment with pepsin

The collagen supernatant in 0.5 M acetic acid was digested with pepsin (activity 1:10 000) 10% (w / v) for 24 hours with continuous stirring. The mixtures were then centrifuged at 9,000 g / min for 30 min. The obtained supernatants were precipitated by addition of NaCl to a final concentration of 0.9 mol / L.

Collagen solubility test

The optimal solubility test was carried out by dissolving in 0.5 M acetic acid at different pH and NaCl concentrations. Collagen samples were dissolved in 0.5M acetic acid with gentle stirring at 4 °C for 12 hours to give the final concentration of 3M, 2.6M and 0.9M in NaCl.

2.2. PHYSICO - CHEMICAL ANALYSIS OF EXTRAS COLLECTIONHYDROLYSIS IN GRAY MULLET

Physico-chemical methods consist of the following analyzes such as: total nitrogen, protein, fatty substance, pH, dry matter content, ash content.

Preliminary physicochemical methods aimed to determine the appearance, color and moisture. The samples analyzed were: collagen extracted from *Gray Mullet* skin through acid treatment with 0.5 M acetic acid and collagen extracted with 0.1 M HCl.

Effect of pH

6 mL of collagen solution was transferred to the centrifuge tube and the pH was adjusted with 6 N HCl to obtain a final pH range of 1 to 10. The sample solution was brought up to 10 mL with distilled water and the same pH was adjusted. The solution was gently agitated for 30 minutes at 4 °C and centrifuged at 10000 x g for 30 minutes at 4 °C.

The relative solubility of collagen was calculated in comparison to the determination of the pH with the highest solubility.

Effect of NaCl

5 mL of collagen solution in 0.5 M acetic acid was mixed with 5 mL of cold NaCl in acetic acid of varying concentrations (0-12% w / v) to obtain final concentrations of 1-6 % (w/v). The mixture was gently agitated at 4 °C for 30 min and centrifuged at 10.000 x g for 30 min at 4 °C. Relative solubility was calculated as compared to the one with the highest soluble salt concentration.

3. RESULTS AND DISCUSSION

3.1 RESULTS OF EXTRACTION TREATMENTS

Skin Removal. The operation was performed at room temperature, 20 ± 2 °C. From the Black Sea, *Gray Mullet* fish skin was obtained that required total removal of fish meat. *Gray Mullet* skin has a fine, slightly glossy, tear-resistant appearance (Fig. 1). Following the removal of the scales and skin, the following quantities were obtained, shown in Table 1.

Table 1. The proportion of skin and cartilage residues in *Gray Mullet* samples

Gray Mullet		
Initial mass	Skin mass	Wings mass + scales Mass
2320 g	352.63 g	236.46 g

For the *Gray Mullet* sample of the total sample weight of 2320 g, the values varied between 352.63 g, for fish skin and 236.46 g for wings and scales. (Fig.2).

Figure 1. *Gray Mullet* skin.

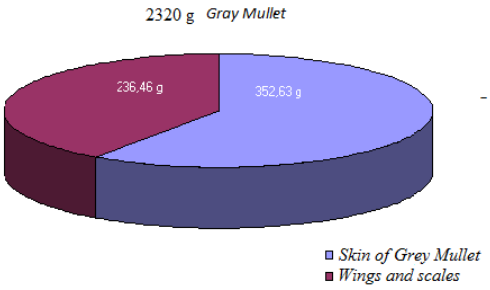


Figure 2. The percentage of Gray *Mullet* skin in total sample mass.

The biotechnological process of collagen acid extraction comprises several steps that are included in the technological scheme of Fig. 3.Enzyme treatment with pepsin is described in Fig.4

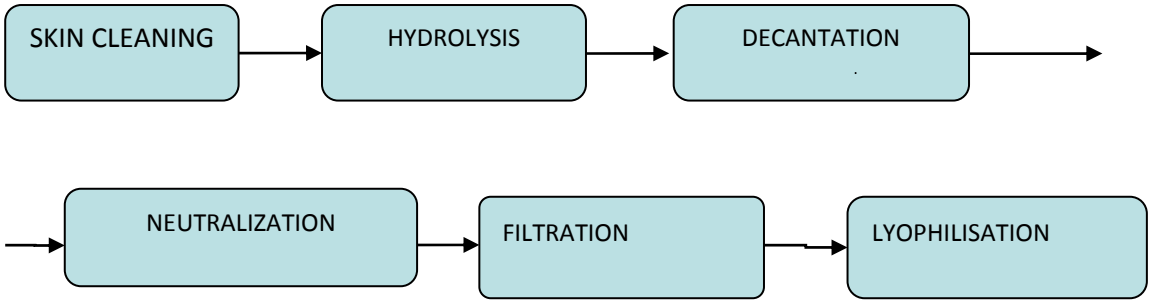


Figure 3. Collagen Acid Extraction Biotechnology Process Scheme.

Collagen soluble in PSC pepsin was collected by centrifugation at 12.000 g / min for 20 min.

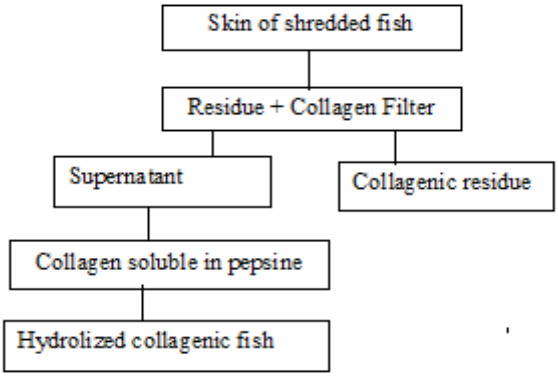


Figure 4. Pepsin-soluble collagen recovery (PSC) recovery scheme, hydrolyzate preparation and analytical determinations.

This was then dissolved in 0.5 M acetic acid and dialyzed in 50 volumes of 0.1 M acetic acid for 24 hours, followed by dialysis in the same volume of distilled water for another 24 hours.

The dialysate was lyophilized and was designated pegylated solubilized collagen (PSC).
PSC yield was calculated from the dry weight percentage of PSC as compared to the dry weight of the initial skin used. The extraction of the collagen was performed in triplicate and the yield value was the mean of the triple determinations.

3.2 CALCULATION OF EXTRACTION YIELD

The ASC and PSC yields were calculated from the percentage of dry weight of extracted collagen (Mo) as compared to the wet weight of the fish used (M):

$$\text{Yield } \% = Mo/ M * 100$$

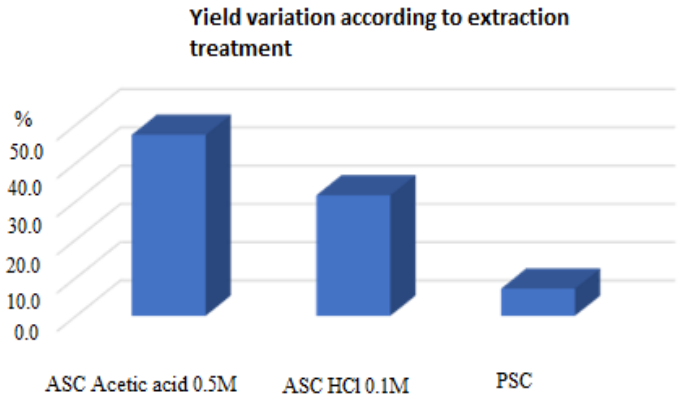


Figure 5. Yield variation according to the extraction treatment.

The results of the yield of skin collagen by acid treatment with 0.5 M acetic acid and 0.1 M HCl are shown in Fig. 5 and Table 2.

Table 2. Collagen yield depending on the extraction treatment.

ASC Acetic Acid 0.5 M	ASC HCl 0.1M	PSC
47.3%	31.5%	7.10

The results showed that pepsin improves collagen extraction efficient because it can specifically create regions of telopeptide of collagen, results which are consistent with literature data [48].

In addition, by hydrolysis of the non-triple helix domain, non-collagen proteins are easier to remove and thus the collagen becomes slightly solubilized in acidic solution and the antigenicity caused by the telopeptides is reduced, resulting in a collagen of greater purity, with the possibility to be used in various applications [47].

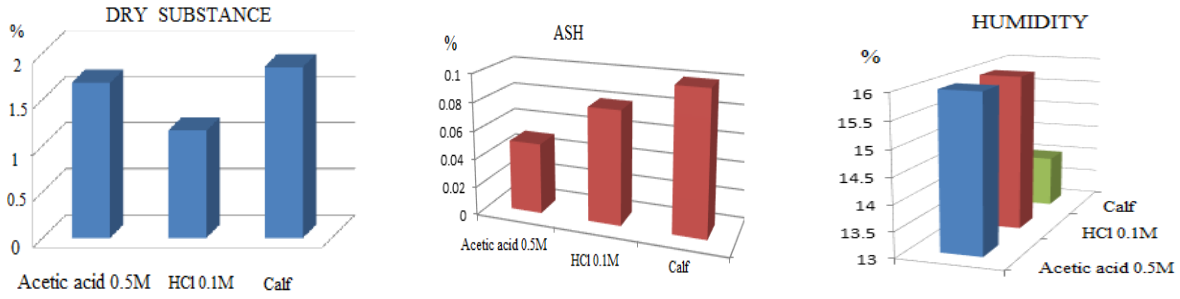
3.3 RESULTS FROM PHYSICO-CHEMICAL ANALYSIS OF COLLAGENIC HYDROLYSATE EXTRACTED FROM THE GRAY MULLET

In order to characterize the collagen obtained from the Black Sea *Gray Mullet*, several physical-chemical analyzes were performed. Tests that have been performed to characterize extracted collagen have been selected and adapted so that reliable results can be obtained and to ensure the quality and safety of the research. Table 3 presents the results obtained for collagen extracted from *Gray Mullet* species in the Black Sea by two procedures, compared with that extracted from calfskin.

Table 3. Physico-chemical characteristics of collagen hydrogel.

Characteristics	Fish collagen extracted with 0.5 M acetic acid	Fish collagen extracted with 0.5 M HCl 0.1 M	Bovine collagen
Appearance	white gelatinous	white opaque	translucent gelatinous
Color	White-yellow	White-yellow	White-yellow
Humidity [%]	16.0	16.0	14.0
Dry matter [%]	1.68	1.16	1.85
Ash 600-800 °C [%]	0.05	0.08	0.10
Total nitrogen mg (Khieldal) [%]	9.71	6.73	15.76
Proteic substance(N x 5.62) [%]	54.57	37.86	88.59
pH	3.5	2.5	2.39

The graphical representation of the percentages of the comparative physico-chemical characterization of calf and fish collagen, obtained through different acidic treatments for the dry matter content, ash and moisture content is presented in Fig. 6.



a) **b)** **c)**

Figure 6. Physico-chemical comparative parameters of fish and calf collagen: dry matter (a), ash (b) and moisture content (c).

In Figs. 7 and 8 the comparative values for total nitrogen and protein content are given.

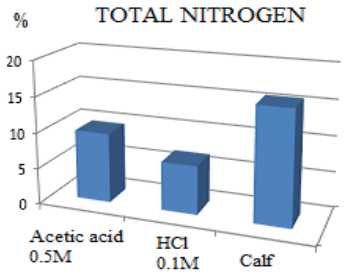


Figure 7. Physico-chemical comparative parameters of fish and calf collagen.

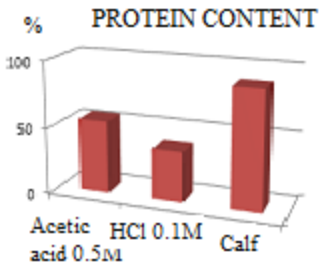


Figure 8. Physico-chemical comparative parameters of fish and calf collagen.

pH variation and mineral content values for the three types of collagen hydrolysate are shown in figures 9 and 10.

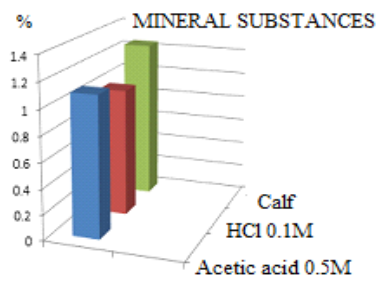


Figure 9. Physico-chemical comparative parameters of fish and calf collagen Mineral substances content.

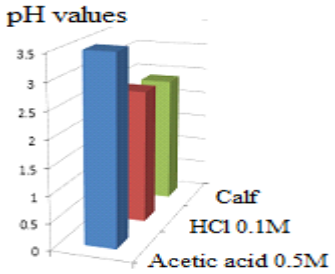


Figure 10. Physico-chemical comparative parameters of fish and calf collagen pH variation.

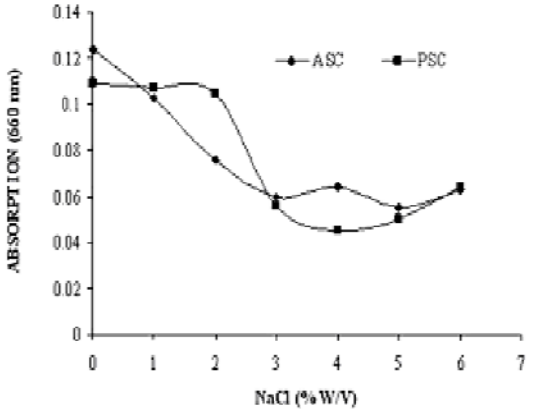


Figure 11. Solubility of ASC and PSC at different concentrations of NaCl.

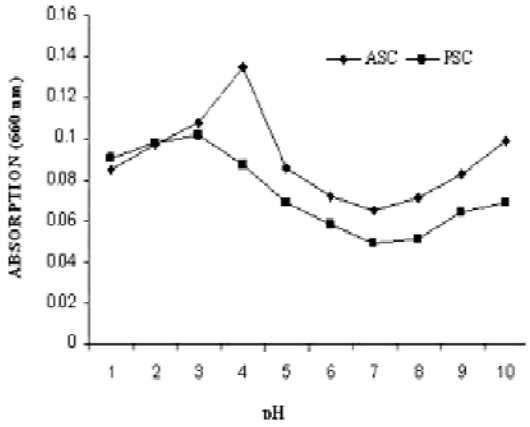


Figure 12. Solubility of ASC and PSC depending on pH.

The values of fish collagen are similar to those of the calf, with obvious differences in protein content, where values of 88, 59% for calves and 54.57% for fish were registered. Determination of ASC and PSC solubility in fish, depending on the concentration of NaCl, and of pH, are presented in Figs. 11 and 12.

4. CONCLUSIONS

From this study we can draw the following conclusions:

- *Gray Mullet* skin collagen was successfully isolated through acid treatment with 0.5 M acetic acid, 0.1 M hydrochloric acid, as well as through enzymatic treatment with pepsin;
- It can be concluded that the 0.5 M acetic acid extraction biotechnology process is preferred to the 0.1 M HCl extraction;
- The results showed that pepsin efficiently improves collagen extraction as it is able to specifically create regions of collagen telopeptide, results which agree with literature data [47, 48];
- The optimal NaCl concentration used to precipitate collagen ranged from 0.9M to 3M;
- Extraction yields varied between 47.3% for collagen extracted through acid treatment with 0.5M acetic acid, 31.5% for hydrochloric acid extraction and 7.10% for pepsin extraction. Extracted collagen has been characterized through rapid analysis methods, but also through complex physicochemical methods of high accuracy;

– Thus, rapid characterization methods can be highlighted: determining of total nitrogen, protein, pH, ash content, fatty substances, and mineral content, which show that the purity of collagen from the initial hydrogel makes it suitable for its use as a biomaterial.

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