ORIGINAL PAPER

CAPITALIZATION OF MARINE RESOURCES FROM BLACK SEA BY OBTAINING AND CHARACTERIZING COLLAGEN FROM *GREY MULLET*

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Abstract. *The Black Sea offers multiple capitalization possibilities for the medical and pharmaceutical field. The harness of marine bioresources for obtaining pharmaceuticals products have been highlighted in recent times. For the first time, the collagen from Grey Mullet fish has been extracted from the Black Sea. Acid soluble collagen (ASC) from the skin wastes of Grey Mullet from the Black Sea was successfully isolated and characterized as type I collagen. For collagen extraction, the acid method with acetic acid 0.5M showed better results than those using HCl 0.1M. Various physico-chemical determinations have been performed to characterize the collagen. For obtaining structural data FTIR and UV-DC spectral analyses have been used. In conclusion, we can say that the collagen hydrogel obtained from fish skin can be used to obtain biomaterials for pharmaceutical field use.*

Keywords: *Collagen type I, Grey Mullet.*

INTRODUCTION

Seaweed are known and utilized from the most ancient times in food, medicine and the soil fertilization. The Black Sea offers multiple opportunities to capitalize on it in order to apply it on the medical and pharmaceutical domain [1]. For harnessing marine resources offered by the Black Sea for medical purposes, the quality of marine habitats along the Romanian shore has been taken into consideration [2], in the sense that the possible infestations with pollutants from the coastal waters [3].

All the prevention and protection measures according to the legal requirements have been applied in order to assure human health [4]. There have been concerns about how to obtain nutritional principles from marine organisms [5]. Another area to harness marine algae was to isolate active principles of pharmaceutical interest [6]. Several studies have been conducted in the characterization of macrophages that can be used to obtain topical pharmaceutical formulations [7]. Another direction of researches was trying to utilize the marine algae biomass in agriculture [8].

The collagen, as a macromolecular structure was found in both vertebrates and invertebrates organisms, especially in mammals.

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The main role of collagen is a structural one; there are different types according to their specific organization [9]. For this reason, collagen hydrogels have been chosen as a main biological material in tissue regeneration activities. The richest source of obtaining collagen is the skin and bones of terrestrial animals like the pig and cow. Because of the risks of bovine spongiform encephalopathy and foot and mouth disease in recent years the collagen sources had to be reconsidered [10, 11]. That's how new natural sources of collagen and modern technology for its production appeared [12]. It has been studied both as undenatured with applications in cosmetics, food and pharmaceutical industries and as denatured collagen with applications in food and biomedical industries [13, 14]. According on the source of collagen, there are different technologies for obtaining collagen- based macromolecules [15].

For the first time, there has been a successful extraction of collagen from *Grey Mullet* found in the Black Sea. In the present paper we show the biotechnological processes for obtaining soluble collagen from the skin of *Grey Mullet* and its characterization in order to analyze his uses in various pharmaceutical areas

2. MATERIALS AND METHODS

2.1 OBTAINING COLLAGEN FROM FISH

For the collagen extraction, fish from *Grey Mullet* species have been used, that were found in the Black Sea in January 2017. Grey Mullet fishes with weights between 380-405 g and lengths from 30-48 cm (see Table 1) have been acquired from the fish market – Pescaria Constanta.

Table 1. The weight and size of fishes from which the samples were collected.

Species: Grey Mullet from the Black Sea	Weight (g)	Size (cm)
Sample 1	0.360	30
Sample 2	0.380	33
Sample 3	0.385	37
Sample 4	0.390	39
Sample 5	0.400	39
Sample 6	0.405	40

The fish have been transported in freezers to the Physical Chemistry Laboratory of the Faculty of Pharmacy from "Ovidius" University of Constanta, where the entire extraction process has been made through the acid method. The collagen extraction from fish wastes, scales and fins of Grey Mullet and sturgeon has been made with the acid method, with acetic acid 0.5M and HCl 0.1M. The extraction method was composed of two technological processes: Process 1– Pretreatment to obtain the material which will be used for obtaining collagen hydrogels; Process 2 – The exact extraction by which the collagen was isolated.

1. Pretreatment. In this stage the fish was cleaned with distilled water and unscaled. The fins were cut off, and then the skin was removed through peeling. The collected wastes contained: skin, scales and fins have been washed several times with distilled water and weighed. The operations have taken place at room temperature of 21-23 °C.

2. **The main extraction** has been composed from a succession of technological stages: skinned fish; demineralization and deproteinization of fish skin,; acid soluble collagen extraction (ASC); filtering the mixture; collagen precipitation; centrifuging the precipitate samples; lyophilization – this stage was achieved only for samples which wanted to be obtained as powder.

2.2 CHARACTERIZATION OF NATIVE COLLAGEN EXTRACTED FROM GREY MULLET

For the characterization of the *Grey Mullet* collagen obtained from the Black Sea there have been multiple physicochemical analyses, using standardized methods: the content of dried substance, the content of ash, the total nitrogen, protein substance, fat substance, pH.

The total nitrogen content has been determined using the Kjeldahl method, according SR ISO 5397/1996.

The percentage of protein substance has been calculated on the basis of the coefficient the transformation coefficient of nitrogen in protein substance).

The determination of dried substance content has been made according to the standard gravimetric methods, by drying at constant mass at 105^0C, according Standard 6615/2-74

pH has been determined by using standard potentiometric method 8619/3-81.

The total ash sulphate content determination has been made according to the standardized methods from SR ISO 4047/1995.

Spectral analyses methods

The spectral method chosen was UV-DC in order to obtain information about collagen structure. In this regard the circular dichroism was determined and the IR spectra were obtained through FTIR analyses. The circular dichroism is an important method for the analysis of the helix structure and the extent of distortion of the sample utilized in biology studies. The UV-DC spectra was obtained using a spectropolarimeter Jasco J-810, utilizing a quartz tank with the optic road of 0.02 cm, a scanning speed of 50nm/min with a step of 0.2 nm and a response time of 2 s, in the 250-195 nm domain. The spectra represent the mediation of 4 consecutive measurements at room temperature (25^oC).

The FTIR analysis was made with the Spectrometer FT/IR 4200 Jasco with a wavelength of 7800-350 cm^{-1}; the accuracy of the wavelength ±0.01 cm^{-1}, the maximum resolution 0.5 cm^{-1}.

The spectrometer has a system with a single beam and a radiation source of great intensity. The analyzed samples were in the form of collagen powder obtained after 3 cycles of lyofilization carried out in a 36 hour interval.

3. RESULTS AND DISCUSSION

3.1. THE COLLAGEN CHARACTERIZATION AND EXTRACTION

In the present study, the possibility of obtaining marine originated collagen from Grey Mullet skin was evaluated through 2 different methods of acid extraction: with HCl 0.1 M and with acetic acid 0.5 M.

In Table 2 the results and observations are presented for each stage of the working procedure.

Table 2. The technological stages of the procedure to obtain collagen from fish .

Nr. of stages	The type of operational processes	The acid extraction method with acetic acid 0.5M	The acid extraction method with HCl 0.1M
Stage 1	*Deskineed fish*	The operation was performed at room temperature *20±2 ^{0}C.* From Grey Mullet in the Black Sea we obtained fish skin and was necessary to remove as much as possible the fish meat see Figs 1 and 2 .	
Stage 2	*Demineralization Deproteinization*	It is made with 10% NaCl solution for 24 hours at room temperature in glass jars after initial treatment with 10% NaOH and washing with distilled water. Removing meat fractions occurs. The material (skin, bones and fins) chopped was treated with NaCl 10%. A solution-immersed mixture was obtained (Fig. 3).	
Stage 3	*Soluble collagen extraction*	Treat with 0.5 M acetic acid in a ratio of 1: 7 for 72 hours All processes were carried out at the 4 °C with continuous stirring.	Treat with 0.1 M HCl 0.1M in a ratio of 1: 7 for 72 hours All processes were carried out at the 4 °C with continuous stirring.
		The supernatant presented viscous aspect with white opalescent color. The extraction was realized in 3 different stages. At 24 hours each the supernatant was removed and replaced with fresh acetic acid solution or HCl	
Stage 4	*Filtration*	The final resulted collagen filtered products after passing the mixtures trough sieve and filter paper were brought together at the end of the operations. An opalescent mixture was obtained.	
Stage 5	*Collagen precipitation*	The filtered obtained products were treated with NaCl 2.6 M which realized the precipitation of the collagen hydrolyzed.	The opalescent mixture were treated with 3 M NaCl to precipitate the collagen hydrolyzate.
Stage 6	*Centrifugation*	The collagen products were separated through centrifugation 30 minutes, to 5200 rotation /min (Figs. 4 and 5).	
Stage 7	*Cross-linking*	The collagen cross-linking was obtained with tannic acid 2%. The obtained collagen has a white milky aspect (see Fig. 6).	

Figure 1. *Grey Mullet* fishes.

Figure 2. *Grey Mullet* skin.

Figure 3. *Grey Mullet* skin waste.

In Figs. 7 and 8 the hydrolyzed collagen obtained products through the 2 procedures are presented. We can see the difference in aspect of the 2 final products. The collagen obtained by acetic acid method has a translucent gelled aspect while the one obtained with HCl is white and opalescent- opaque.

Figure 4. The hydrolyzed collagen using centrifugation.

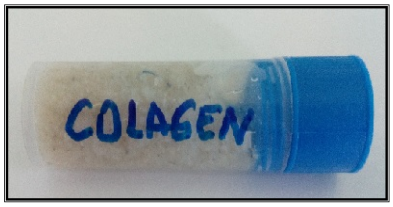

Figure 5. Uncrossed-linked collagen obtained after centrifugation.

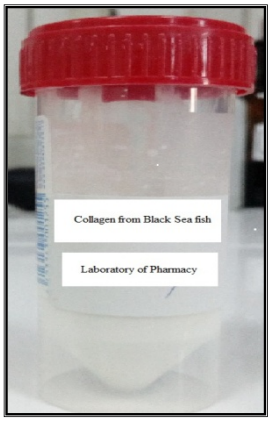

Figure 6. The cross-linked hydrolyzed collagen.

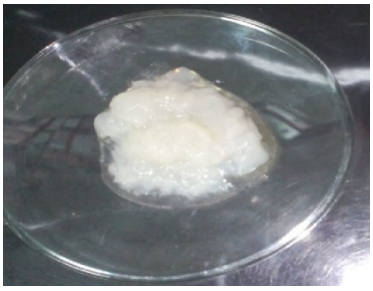

Figure 7. The hydrolyzed collagen obtained from Grey Mullet with HCl 0.1 M.

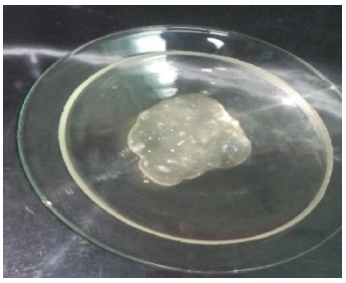

Figure 8. The hydrolyzed collagen obtained from Grey Mullet with acetic acid 0.5 M.

Results obtained for physico-chemical characteristics of hydrolyzed collagen are shown in Table 3.

Table 3. Physico-chemical characteristics of the hydrolysed collagen.

CARACTERISTICS	COLLAGEN EXTRACTED with 0.5 M ACID ACETIC acid	COLLAGEN EXTRACTED with 0.1 M HCL
Dried substance %	1.68	1.16
Ash %	1.47	1.02
Total nitrogen mg %	9.71	6.73
Protein substance mg %	54.57	37.86
pH	3.5	2.5

Results were better with acetic acid 0.5 M, results were sustained by the values obtained on the physico-chemical determinations of the initial collagen.

The results are in according with literature data presented on collagen obtained from other fish species. [16,17] Thus, the collagen obtained by extraction with Acid acetic 0.5 M, was relatively higher in protein substance(54,57%) than HCl extraction (37.86%)and have 3.5 pH, compared to 2.5 for collagen extracted with hydroclorhidric acid, recommending 0.5M acetic acid as solvent for collagen extraction by acid method.

3.2. CIRCULAR DICHROISM IN UV

To prevent distortion, we have to check the temperature. Despite the associated complications about the comparison and collection of the data, UV-DC remains an important method of structural data analysis. The obtained spectra for skin collagen from fish out of the Black Sea are presented in Fig. 9. Together in Fig.10 a spectra for the appreciation of the collagen obtained out of calf skin dichroism is presented.

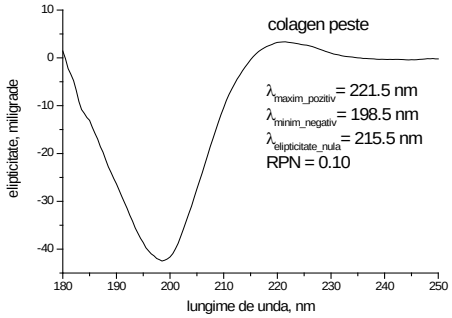

Figure 9. Results obtained for the Grey Mullet collagen dichroism.

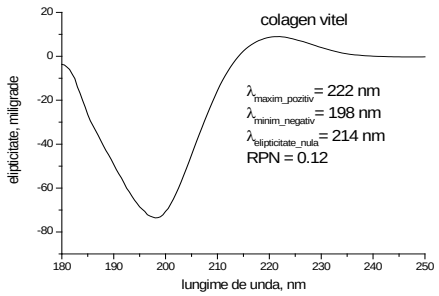

Figure 10. Results obtained for the calf collagen dichroism.

The circular dichroism in UV (UV-DC) measures the differential absorption of the circular polarized light that appears when this kind of light passes through a substance whose molecules possess structural symmetry. Protein amide bonds found in the ordonated regions helix α and folded structures β have optic activity due to orientation. The sample concentration had to be sufficiently low (<0,125 $mg.mL^{-1}$) in order to avoid the detector saturation. From Figs. 9 and 10 we can highlight the following:

- Values close to the minimum wavelength were obtained (198.5 nm at fish collagen and 198 nm for calf collagen);
- Close values were also obtained:
  - Maximum 221.5 nm for ellipticity point zero (215.5 nm), for fish collagen;
  - Maximum 222 nm for ellipticity point zero (214 nm) for calf collagen;
- Close values were obtained for Rpn parameter value 0.10 for fish collagen and 0.12 for calf collagen.

This results show that during the extraction the triple helix structure of the collagen wasn't affected and both the collagen hydrogel obtained from fish and calf can be utilized for biomaterials

3.3. THE FTIR SPECTRAL ANALYSIS

The FTIR spectroscopy was used to study the changes in the secondary collagen structure [18, 19]. The IR spectra of the hydrolyzed collagen obtained from Grey Mullet skin presents absorption bands situated in amide band as you can see in the FTIR spectra presented in Fig. 11. The main absorption bands of the amide A, I, II and III have been situated between: 3326 - 1643 and 1527 - 1238 cm^{-1}. Amide I, II and II are found (1650 and 1200 cm^{-1}) which is fine according with scientific literature [18, 19].

Absorption of the amide band I is most used in IR spectroscopic analysis to confirm the secondary protein structure. The amide band II results from the combination of -C-N bonds with the -N-H bond (in the context of peptide-specific deformations).

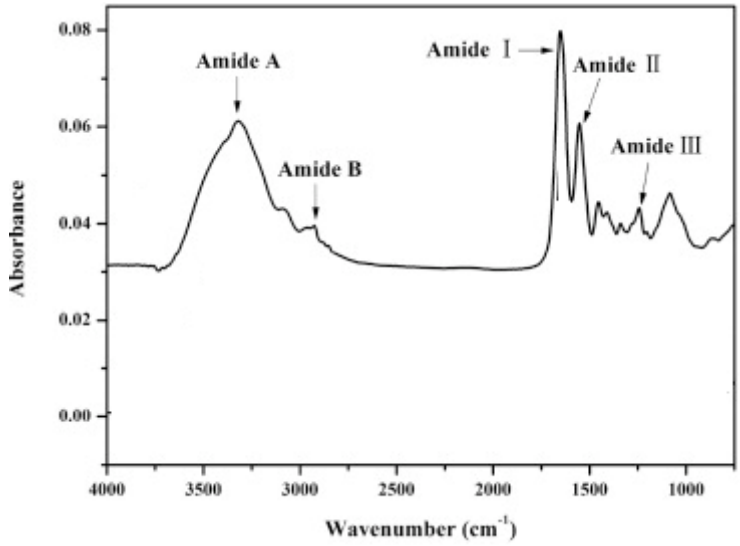

Figure 11. FTIR spectra of colagen from the skin of *Grey Mullet* extracted.

The amide I, II, and III bands are known to be responsible for the degree of molecular order found in collagen, and to be involved in the formation of its triple helical structure, which results from C═O stretching, and N–H bending and C–H stretching, respectively. An absorption ratio of approximately 1, between the amide III and the 1450–1454 cm^{-1} band, indicates that triple helical structure is intact concordant with literature data [16]. Radios of 0.91 and 1.11 were obtained for the hydrolyzed collagen, indicating the triple helical structures were maintained. The triple helix collagen structure extracted through the acid method from fish skin was confirmed from the absorption report between 1238 (amide III) and 1527 cm^{-1} which is approx. 1 concordance with literature data [17]. The same method has been used frequently in many scientific reports [18-21], which indicates that FTIR was a simple and practical method [22-24] to evaluate the triple helix structure of the collagen.

4. CONCLUSIONS

From the present study it can conclude the following: collagen in the skin of Grey Mullet was successfully isolated and classified as type 1 collagen; from physicochemical analysis the content of protein and total nitrogen from *Grey Mullet* skin extracted with acetic acid 0.5M was highlighted and was higher than the one extracted with HCl 0.1 M .We can conclude that the biotechnological extracting process with acetic acid is preferred to the HCl one; circular dichroism determination has shown that the triple helix structure of collagen has not been affected and the hydrolyzed collagen obtained from fish skin presents similar properties to the one extracted from calf skin; FTIR analyses have confirmed that the hydrolyzed collagen from fish maintains its triple helix structure; The hydrolyzed collagen obtained from *Grey Mullet* can be used with great success in obtaining various semisolid pharmaceutical formulations with reduced costs.

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