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Collagen Isolation from Arctic Marine Organisms and Their Industrial Processing Wastes

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Abstract

The results of the isolation of collagen hydrolysates from tissues of two Arctic marine organisms are presented. Extraction and use of marine organism collagen is a part of complex waste less processing of sea catches. Possible ways of preparing water-soluble collagen hydrolysates from different sources were studied. It is established that for preparing soluble collagen hydrolysate from skin of cod *Gadus morhua* acid hydrolysis in 0.3% acetic acid is suitable. Purification of solution by means of ultrafiltration gives a pure collagen hydrolysate with mass fraction of the main substance about 98%. Collagen of muscular skin bag of holothuria *Molpadia borealis* is almost insoluble in acid and alkali solutions. The major mass of collagen remains in an insoluble residue. The analysis of infrared spectrum transmission and chemical analysis of the general carbohydrates and collagen in different fractions showed that as a result of acid and alkaline processing of raw materials the glycosaminoglycans (GAG) and some quantity of collagen are extracted, their main quantity was determined in alkaline extracts. For extraction of soluble collagen from insoluble residue it is required enzymatic by pepsin in acidic medium. Properties of holothurian collagen and fish collagen are different. The preparation of water-soluble collagen derivatives requires using enzymatic hydrolysis.

Keywords: collagen, hydrolysate collagen, glycosaminoglycans, ultrafiltration, enzymic hydrolysis

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1. Introduction

Connecting tissue of living organisms, in particular, aquatic organisms, performs the important structural and protective function and forms a basic framework (stroma) and outside covers (term) of all bodies. It consists generally of proteins - collagen and elastin.

Collagen is a fibrous protein that provides strength and elasticity of connecting tissue. Mechanical characteristics of collagen are associated with its primary and space structures [1, 2]. Now 28 types of collagen that differ from each other on the amino-acid

sequence and degree of modification are described. There is a set of other proteins containing in the structure the domain with a triple collagenic spiral except collagenic proteins, but they are ranked to "collagen-like" proteins [3].

In the industry, collagen is extracted principally from cattle and pig skins. Now unpopularity of this source is motivated with religious restrictions, existence of associated diseases, such as, for example, cow spongy encephalopathy, and the appropriate risk of these diseases for people. Collagen of sea origin is safer and is an important alternative to collagen of land animals. For this reason, many types of research are carried out on preparing collagen from sea sources [4, 5]. Proteins of connecting tissues can be extracted from fishes (skin, bones, scales, fins) and invertebrates (Echinodermata, Crustacea, Jellyfishes) [4].

For hydrobionts of the Northern fishing basin data on the protein content of different parts of the body are available [6], but data on the content of collagen are practically absent. Extraction and use of marine organism collagen can solve a problem of decrease in waste products. Manufacturing of fish products is followed by a large number of protein-containing waste making from 30 to 70% of feedstock. Recently the quantity of the fish products made of skin-off fillet increased, waste in most cases is exposed to utilization.

Preparing of soluble collagen (collagen hydrolysate, gelatin) from fish skin is known long ago. Traditionally from fish skin partially degraded collagen - gelatin is extracted by hot water and applied in the food, medical, cosmetic industry.

Scope of collagen applications extends at an increase in its solubility therefore recently a large number of publications are devoted to preparing collagen soluble in acids [3, 7, 8] and almost completely hydrolyzed to amino acids and peptides.

Soluble products of partial or full hydrolysis of collagen are of considerable interest to medicine. Recent results of researches (2010-2017) of a rather favorable collagen hydrolysate action in experiments with animals and clinical experiments are presented in the review article [9]. Improvement of antioxidant capacity, the rejuvenating result, prevention and processing of osteoporosis and osteoarthritis, improvement of healing of small wounds, anti-tumor effect, decrease in risk of cardiovascular diseases, anti-inflammatory and other effects of low-molecular products of collagen cleavage are shown.

Holothurians are considered as a potential source of collagen and its water-soluble products. Several species of holothurians such as *Molpadia arctica*, *Molpadia borealis* and *Cucumaria frondosa* are widespread in the northern seas [10].

From scientific publications methods of collagen preparing from holothurians in the form of insoluble fibers [11] and a soluble collagen hydrolysates [12] are known. Authors [12] used the two-level scheme of preparing a soluble collagen hydrolysate. The first stage includes the release of crude collagen fibers by washing of the crushed fabric of muscular skin bag with water and EDTA solution in 0,1 M Tris-HCl at pH 8.0 and alkali treatment for removal of not collagenic substances (proteins). The second stage is collagen hydrolysis by pepsin solution, dissolution of the prepared substance in 0,5 M acetic acid and its purification with dialysis. This method is used to get a soluble collagen hydrolysate from different types of holothurians such as *Stichopus monotuberculatus* [13], *Stichopus japonicas* [14], *Parastichopus californicus* [15], *Holothuria parva* [16], *Stichopus vastus* [17], etc.

The purpose of our work is the improvement of technology of preparing water-soluble collagen (a collagen hydrolysate) from marine organisms (fishes and holothurians).

2. Objects and Methods of Researches

In work skin covering of a cod *(Gadus morhua morhua)*, being waste by fillet production, provided from LLC Biomarin (Murmansk), and a muscular skin bag of a holothuria *M. borealis* harvested in forwarding conditions on site of trade (The central trench of the Barents Sea) on May 2017, used as collagen-containing raw materials.

When preparing partially hydrolyzed collagen some known from scientific literature ways were applied.

Method 1 [7]. Collagen-containing raw materials (skin of cod) were purified of scales, muscular tissue cuts and fat, crushed the meat grinder with an opening diameter equal 3,5 mm and washed out water 3 times 30 minutes each. After washing raw materials were mixed with 3% acetic acid solution at mass ratio 1:5 and stored at periodic mixing of 15 hours. The solution was filtered through two layers of nylon fabric and collagenic dispersion was dried up.

Method 2 [18]. The purified raw materials were homogenized with 4 water volumes at 5 ˚C within 2 min., extracted 3 times on 2 h each with 10 volumes of 1% NaCl solution, washed-out water, homogenized with 15 volumes of 0,1 M NaOH solution within 2 min. and centrifuged at 5000 rpm within 15 min. Washing with 0,1 M NaOH solution was repeated. A precipitate was suspended in 50 volumes of water, mixture pH established by 7.0 by addition 0,1 M HCl solution. The precipitate was separated on a sieve, washed-out water and homogenized in 100 volume of 0,01 M HCl solution within 3 min. After centrifuging within 5 min. at 3000 rpm suction liquid was merged and neutralized by 0,1

M NaOH solution to pH 7.0. The fibrous collagen precipitate was filtered, washed-out 3 times by water and dried up.

For comparison, the commercial drug collagen hydrolysate ``Collagen Hydrolysaat'' from skin of cod *Gadus morhua* manufactured by Van Ork Voeding (Netherlands), and gelatin sample from cod skin, produced at chemistry department of MSTU were used.

Separation of solid and liquid fraction after extraction was carried out by means of the Avanti J-25 centrifuge (Beckman, the USA).

To solution ultrafiltration the UP-1 installation (NPP Biospektr, the USSR) with the hollow fiber device UVA-2-5 having molecular weight cut-off (MWCO) range of 5 kDa was applied.

Drying of all prepared samples was carried out in the lyophilic dryer HETO HD 8 (Sweden).

In work, the enzyme preparation "Hepatopancreatin" made from hepatopancreas of the Red King crab *Paralithodes Camtschaticus* [19] and pepsin were used.

The mass fraction of collagen was calculated on 4 hydroxyproline mass fraction after carrying out acid hydrolysis of proteins [20] by a method [21].

The molecular weight of the soluble and fermented collagen was determined by size exclusion HPLC on a liquid chromatograph LC-10AVP (Shimadzu Corp., Japan) with the column TSKgel α-4000 (Tosoh, Japan) and the detector SPD-10A_{VP} (205 and 280 nm) in 0,15 mol/dm^3 NaCl solution. For graduation a set of proteins with the known molecular weight of Sigma (USA) used.

The sum of GAG was determined by Dishe's technique [22]. As the standard for plotting of standard curve the chondroitin sulfate preparation from GreenLand Health (People's Republic of China) with a mass fraction of the main substance 90% was used.

The content of mineral substances (ashes) was determined by a standard method [23].

Registration of infrared (IR) spectra of samples was carried out in the range of wave numbers 400-4000 cm^{-1} on FTIR spectrophotometer IRTracer 100 (Shimadzu, Japan) in KBr tablets ("Fluka", the USA).

3. Experimental Results and Discussion

After scientific literature analysis on a subject of collagen preparing from different types of raw materials the technology of collagen extraction from fish processing waste [7, 18] was taken as a basis. In it the collagen hydrolysate is extracted from fish skin by

dissolution and partial hydrolysis in acetic acid solution. The filtered solution of collagen is neutralized and dried up.

The collagen hydrolysate preparation prepared on method 1 [7] contained collagen (32.4%) and a large number of ashes (12.3%). Method 2 [18] allows making rather bigger yield of collagen-containing products with higher concentration of collagen (73.8%) and smaller content of mineral substances (7.7%).

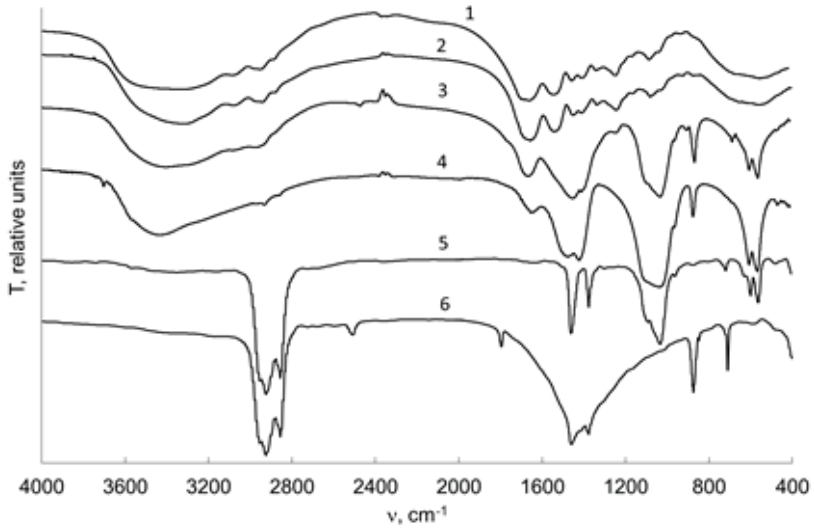

Figure 1: IR transmission spectra of cod skin collagen samples: 1 -- collagen hydrolysate of Van Ork Voeding; 2 - method 1 after ultrafiltration; 3 - method 2; 4 - method 1 without ultrafiltration. IR spectra of salts: 5 - $Ca_3(PO_{4)2}$; 6 - $CaCO_3$.

The collagen prepared in these two ways has a high mass fraction of ashes. In IR spectra sharp peaks of absorption at 563, 608, 878, 965, 1031, and 1461-1611 cm^{-1}, caused, apparently, by presence of mineral connections, in particular, of carbonates and calcium phosphates are visible (Figure 1). We applied an ultrafiltration method with use of hollow fiber membrane module having molecular weight cut-off (MWCO) range of 5 kDa to low-molecular substance cleaning of preparation. As a result ashless sample was prepared (mass fractions of collagen 98.1%, ashes 0.65%). The IR spectrum of this sample was identical to cod skin gelatin one.

The method 1 improved by the use of ultrafiltration stage is tested now at manufacturing of collagen hydrolysate on pilot plant of LLC Biomarin in Murmansk.

The reduction of allows increasing solubility of collagen hydrolysate. For example, processing of raw materials during 3 h at 50 °C by enzyme with collagenolytic activity from Kamchatka crab hepatopancreas reduces molecular weight (Figure 2).

The second object studied as a collagen source - sea cucumbers whose muscular skin bag according to literary data contains up to 70% of collagen [13]. When developing a method of collagen extraction we considered also a possibility of utilization of GAG

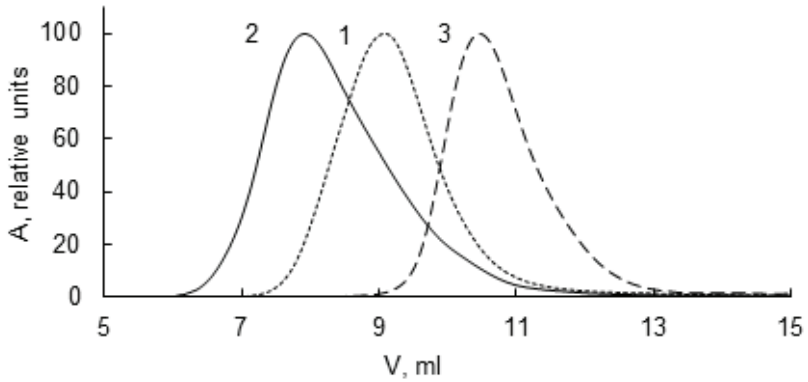

Figure 2: Chromatograms of collagen samples prepared from cod skin: 1 - the commercial drug ``Collagen Hydrolysaat'' (MW 27.6 kDa), 2 - method 1 (MW 185.0 kDa) and 3 - method 1 + enzymic processing 3 h at 50 °C (MW 12.4 kDa). A - optical density at 205 nm, relative units, V - the volume of eluting, ml.

whose mass fraction makes about 15%. Therefore, to a stage of acid collagen extraction we added a stage of alkaline extraction of acidic polysaccharides.

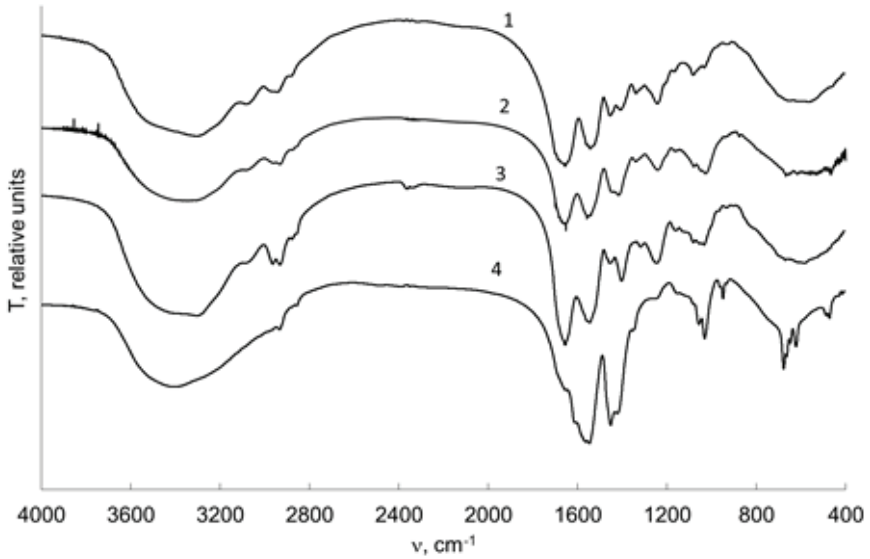

Figure 3: IR spectrums of transmission of samples: 1 - gelatin from cod skin; 2 - residue after extraction muscular skin bag of holothuria *M. borealis*; 3 - alkaline extract; 4 - acid extract.

The scheme included raw materials pretreatment (washing in water, crushing, filtering on paper or grid) and the chemical treatment consisting of consecutive washings of raw materials with acetic acid solutions for extraction of collagen and alkali solution for extraction of acidic polysaccharides. Solutions were cleared of low-molecular substances on the ultrafiltration membrane, partially boiled out under vacuum on the rotational evaporator and dried up in the sublimation dryer. The preparations presumably containing collagen and complex of polysaccharides were received. The insoluble residue was washed out and dried up. The sequence of chemical treatments than was changed and received also two soluble samples and an insoluble residue.

Results of a fraction and residue researches are given in Figures 3 and 4.

IR spectra of the samples prepared after the first and second alkaline extraction are close to the gelatin IR spectrum that can be explained with partial dissolution of collagen in alkaline condition (Figure 4).

At the first and second acid treatment, the IR spectra of the samples prepared from acid filtrate do not correspond to collagen spectrum though contain collagen quantities.

Mass fractions of GAG and collagen in each fraction are given in Figure 4.

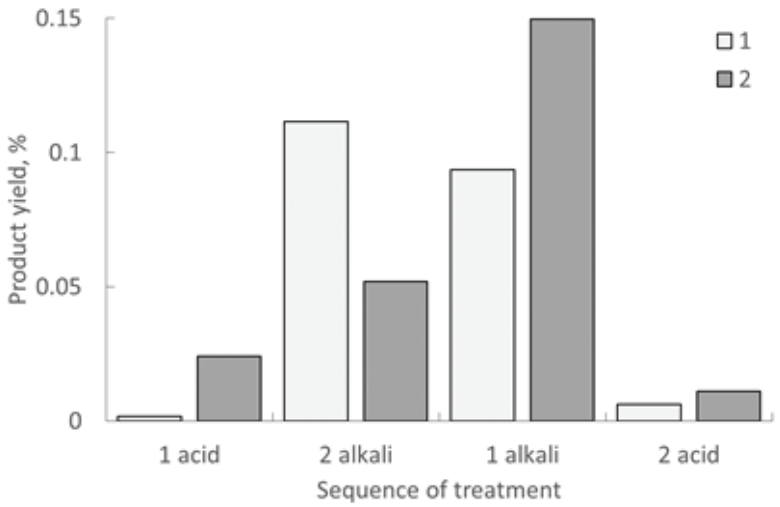

Figure 4: Distribution of GAG (1) and collagen (2) in the fractions prepared by extraction from a muscular skin bag of sea cucumber.

Our experiments showed that in both cases the most part of collagen is not dissolved in alkaline, and acidic medium and remains in a precipitate, unlike collagen from cod skin. The IR spectrum of an insoluble residue practically repeats IR spectrum of collagen (Figure 3). The mass fraction of collagen in precipitate was 23.4%.

This result testifies to the special structure of holothurian collagen. For preparing water-soluble collagen from tissues of sea cucumber probably stronger acid or enzymic hydrolysis is required. As shown in [24], fibrilla of crude holothurian collagen on both ends is surrounded with C-and N-end telopeptida that does collagen to less soluble in acidic medium. Such cross bonds can be removed, for example, with pepsin, without change of triple spiral integrity [15].

For preparing a soluble collagen hydrolysate the precipitate was processed by pepsin as it is described in [25]. The resulting solution was centrifuged, neutralized for collagen precipitation, washed-out water and dried up freeze-dried. The yield of soluble collagen was 80% of dry precipitate mass. The product IR spectrum completely corresponded to a collagen one.

4. Conclusion

The possibility of preparing soluble collagen from different marine organisms - fishes and holothurians - is studied.

The possibility of manufacturing soluble collagen hydrolysate of collagen from cod skin and its cleaning by ultrafiltration of low-molecular substances, including, mineral salts, is shown.

It is established, acid and alkaline processing of sea cucumber fabrics does not allow extracting completely collagen, its main quantity remains insoluble. For its transfer in a water-soluble state the additional hydrolysis, for example, by an enzyme is required.

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Conflict of Interest

The authors have no conflict of interest to declare.

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