

Conference Paper

Technology and Properties of Chondroitin Sulfate from Marine Hydrobionts

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Abstract

Chondroitin sulfate was isolated from the cartilage tissue of salmon (*Salmo salar*), northern skate (*Raja hyperborean*) and black-shark (*Galeus melastomus*). The polysaccharide content is from 67.1% to 82.2% in the samples. FTIR spectra of the obtained samples contain peaks at wave numbers characteristic of chondroitin sulfates (1550, 1350–1300, 1160–1120 и 822 cm^{-1}). The thermal properties of chondroitin sulfates were studied by differential thermogravimetry. It was shown that the weight loss of the samples in the temperature range of 40–600 °C occurs in several stages. At the first stage, desorption of physically bound water occurs to a temperature of 150–200 °C, which can be explained by the difficulty in breaking hydrogen bonds between water molecules and the polar functional groups of chondroitin sulfate. Thermal decomposition of the bulk of chondroitin sulfate samples is observed at temperatures from 230 to 530 °C. The presence of peaks on the TGA curves in the temperature range 236–330 °C is associated with the release of sulfate groups and carboxylic acids (thermal destruction of uronic acid and pyranose units). When samples are heated above 350–400 °C, thermal oxidative degradation of residual carbon and sulfur compounds develops. The mass loss rates in each region were determined and the activation energies of each event were calculated. The nature of the cartilage tissue from which chondroitin sulfate is isolated affects the rate of destruction. Thus, in the temperature range 236–244 °C, the lowest destruction rate is observed for samples of chondroitin sulfate from salmon cartilage.

Keywords: chondroitin sulphate, marine hydrobionts, biotechnology, physical and chemical properties.

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1. Introduction

The natural chondroitin sulfate polysaccharide is a sulfated glycosaminoglycan whose macromolecules consist of alternating monomer units of sulfated N-acetyl-D-galactosamine and D-glucuronic acid (see Figure 1) [1]. Due to the polar functional groups of chondroitin sulfates (CS), various chemical bonds with inorganic components can form, and, as a result, biocompatibility and degradability in the biological environment.

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Currently, natural glycosaminoglycans are widely used in the production of fortified foods, preventive and medical preparations. [2, 3].

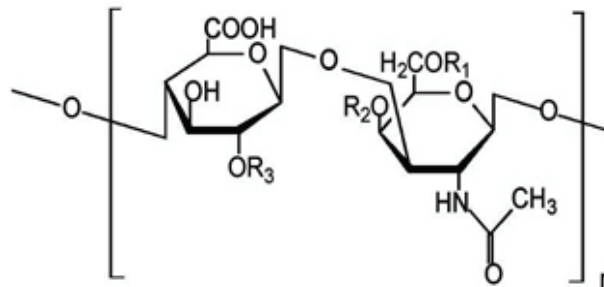


Figure 1: Monomer unit of chondroitin sulfate.

Chondroitin sulfate is a high molecular weight polysaccharide that is a specific component of cartilage tissue. In tissues, chondroitin sulfate is present as a polysaccharide-protein complex, i.e. proteoglycan, where the polysaccharide chains are covalently linked to the protein core. [1]. Modern methods of producing chondroitin sulfates are multi-stage extraction processes. According to the technologies described in the literature, the main stages of chondroitin sulfate preparing include defatting of raw materials, alkaline and enzymatic hydrolysis, the sedimentation of chondroitin sulfate from solution, additional purification of the preparation, drying [4, 5]. Chondroitin sulfates can be isolated from various sources: trachea, nasal septum [6], keel cartilage of chickens [7], cartilage of sharks and other fish [8].

The most promising raw material for the production of chondroitin sulfate is waste from the processing of hydrobionts, because they contain a significant amount of bone-cartilage tissue. About 50% of the fish mass is waste (head, fins, skin, internal organs), which can be further used as raw materials for the extraction of biologically active substances, such as glycosaminoglycans.

The purpose of this work is to isolate chondroitin sulfate from the cartilage tissue of marine aquatic organisms and to study the effect of raw materials on the chemical and thermal properties of the resulting polysaccharide.

2. Methods and Equipment

Chondroitin sulfate was isolated from the cartilaginous tissue of salmon (*Salmo salar*), northern stingray (*Raja hyperborean*) and blackmouth shark (*Galeus melastomus*) according to the technology described in the patent [9] and improved in [10, 11].

The crushed raw material was degreased by washing with acetone three times, and then dried. Then, to isolate the polysaccharide, alkaline cartilage was hydrolyzed

in a sodium hydroxide solution ($C_{NaOH} = 0.2 \text{ mol/dm}^3$) at a temperature of $37 \pm 1 \text{ }^\circ\text{C}$ for three hours. Such alkaline hydrolysis conditions allow the dissolution of alkali-soluble substances and prevent the destruction of chondroitin sulfate. After the reaction mixture was neutralized to $\text{pH } 7.0 \pm 0.1$, the insoluble precipitate was separated and the filtrate was sent for enzymatic hydrolysis. Enzymatic hydrolysis was carried out using an enzyme preparation from King crab hepatopancreas at a temperature of $50 \pm 1 \text{ }^\circ\text{C}$ for 5 hours. The resulting hydrolyzate was centrifuged, and then filtered through a membrane with a pore size of $0.45 \text{ }\mu\text{m}$. The isolation of the polysaccharide from the solution was carried out by the precipitation method with the addition of an excess of the precipitant -- 96% ethanol -- to the solution. The deposition time was at least 20 hours. To separate chondroitin sulfate, the suspension was centrifuged; the precipitate was washed with ethanol and dried in an oven at a temperature $50 \pm 5 \text{ }^\circ\text{C}$.

The chemical composition of chondroitin sulfate samples was determined by standard methods [12]. The quantitative determination of the mass fraction of water in chondroitin sulphate was carried out by evaporation at $100\text{-}105 \text{ }^\circ\text{C}$, the amount of total nitrogen was determined by the Kjeldahl method on an automatic system for determining total nitrogen/protein Kjeltex Auto System 1043 («Tecator», Sweden).

The content of minerals (sulfates) was determined by the titrimetric method [13]. Previously, a sample of chondroitin sulfate was heated in 30% solution of hydrogen peroxide (H_2O_2) in the presence of NaOH until complete decomposition of the substance. Then the solution was dried in an oven. For analysis, the dry residue was dissolved in distilled water and sulfates were determined titrimetrically.

The mass fraction of chondroitin sulfate in the preparations obtained was previously estimated by the weight method from the mass fraction of total nitrogen. The disaccharide unit of chondroitin sulfate contains N-acetyl-D-galactosamine, therefore, for each structural unit there is one nitrogen atom. Knowing the amount of nitrogen in the sample, you can determine the amount of chondroitin sulfate. It should be borne in mind that the samples contain a certain amount of protein, so the total nitrogen value will be added from the protein nitrogen and chondroitin sulfate nitrogen. The content of chondroitin sulfate (CS) in the samples was calculated by the formula:

$$CS(\%) = \frac{35.9 \cdot (5.3 \cdot N - 100)}{5.3 - 35.9}, \quad (1)$$

Where 35.9 is a coefficient characterizing the amount of nitrogen in chondroitin sulfate disaccharide, 5.3 is a coefficient characterizing the amount of nitrogen in collagen, the most characteristic connective tissue protein [14], N is the amount of total nitrogen in the sample (%), 100 is the value of the total amount of sample (%).

The obtained samples of chondroitin sulfate were identified by IR spectroscopy [15, 16]. Absorption spectra were recorded on Fourier transform infrared spectrophotometer IRTracer-100 («Shimadzu», Japan) in the frequency range from 4000 to 400 cm^{-1} , with a resolution of 2 cm^{-1} . The research sample was a mixture of chondroitin sulfate and KBr in a mass ratio of 1:75. To prepare tablets, a mixture of the sample and KBr ($m = 150 \text{ mg}$) was pressed under a pressure of 650 kgf / cm^2 for 1 min at room temperature. FTIR spectra were taken immediately after pressing.

Thermal analysis of chondroitin sulfate was performed by differential thermogravimetry (DTG) using a Diamond TG/DTA thermal analysis instrument (PerkinElmer Inc., USA). Thermograms were taken under the following conditions: aluminum crucible, atmosphere – air, temperature range 40-600 $^{\circ}\text{C}$, sample heating rate of 15 $^{\circ}\text{C}$ per minute, weight of the samples was 20-25 mg. Thermogravimetric curves (TG curves) and differential thermogravimetric curves (DTG curves) were obtained.

3. Results and Discussion

The obtained preparations of chondroitin sulfate are cream (samples from sharks and salmon) and white powder (skate). Given the features of the method for the separation of chondroitin sulfate, it was assumed that the obtained samples contained protein breakdown products, high molecular weight polysaccharides, water and a mineral residue.

Minerals are a natural component of the structural elements of all cells and tissues. General ideas about the content of minerals give a mass fraction of ash. The ash content of many food products is a standard indicator. Chondroitin sulfate contains sulfate groups, therefore, the determination of ash in the sample comes down to the determination of sulfates.

The mass-average molecular weight (M_w) of chondroitin sulfate samples was previously determined by the nephelometric method. For chondroitin sulfate it was 64 kDa for salmon, 47 kDa for northern skate, and 55 kDa for black shark [11].

The chemical composition of chondroitin sulfate preparations is presented in the table. It is shown that the type of raw material affects the chemical composition of the product.

The content of chondroitin sulfate in the samples is from 67.1% to 82.2%. The polysaccharides isolated from the cartilage tissue of salmon and northern skates have approximately the same chemical composition. Chondroitin sulfate, isolated from blackmouth shark cartilage, contains the most protein (23.8%) and the least polysaccharide (67.1%).

TABLE 1: Chemical composition of chondroitin sulfate preparations from various sources.

Raw Materials	Moisture content X, %	Total nitrogen N, %	Protein, P, %	Chondroitin sulphate, %	Ash, %
Salmon (<i>Salmon salar</i>)	9.0 ± 0.5	5.6 ± 0.1	8.7 ± 0.1	82.3 ± 0.1	16.8 ± 0.1
Blackmouth shark (<i>Galeus melastomus</i>)	9.0 ± 0.5	7.8 ± 0.1	23.8 ± 0.1	67.1 ± 0.1	10.9 ± 0.1
Nothern Skate (<i>Raja hyperborean</i>)	9.0 ± 0.5	6.1 ± 0.1	11.8 ± 0.1	79.1 ± 0.1	16.3 ± 0.1

Perhaps, under these processing conditions, the process of protein hydrolysis proceeds less efficiently when chondroitin sulfate is extracted from cartilage, where it is present in the form of a polysaccharide-protein complex. As a result, the reaction mixture contains peptide fragments with $M_w = 5-50$ kDa, which makes it difficult to remove by ultrafiltration.

The obtained samples of chondroitin sulfate were identified by FTIR spectroscopy. From literature data it is known that the chondroitin sulphate must have peaks at the wave numbers 1550, 1350-1300 and 1160-1120 cm^{-1} [15, 16]. Figure 2 shows the FTIR spectra of chondroitin sulphate samples obtained from marine aquatic organisms. For comparison, we used control sample of chondroitin sulphate from the northern skate, the structure of which was previously analyzed by NMR [17].

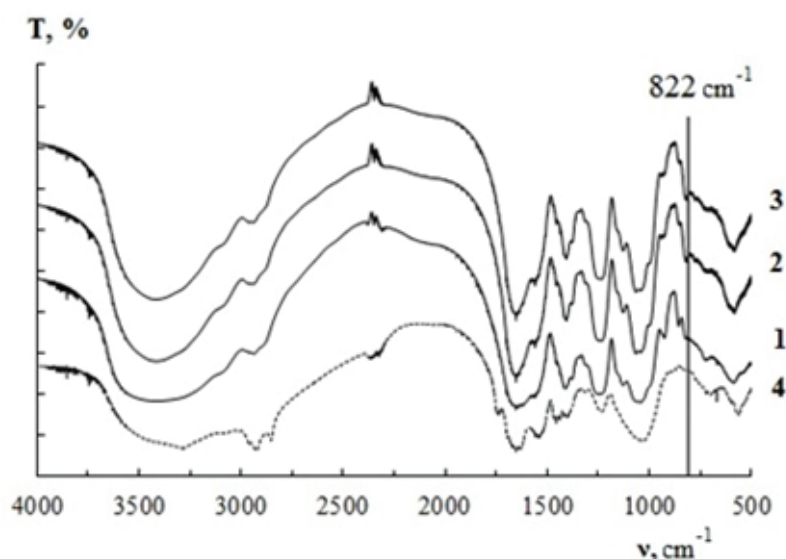


Figure 2: FTIR spectrums of chondroitin sulfate samples isolated from the cartilage of salmon (1), black shark (2) and northern skate (3). Control sample -- curve 4.

The analysis of the curves was showed that the obtained samples of chondroitin sulfate have peaks at the wave numbers characteristic of this polysaccharide. The presence of peaks at these wave numbers indicates that the spectra of the obtained preparations correspond to the spectra of chondroitin sulfate.

The source of chondroitin sulfate production can be judged by specific absorption bands [16]. Absorption at a wave number of 850 cm^{-1} indicates that chondroitin sulfate is derived from mammalian tissue; the peak at the wave number of 820 cm^{-1} is associated with chondroitin sulfate from the cartilage tissue of aquatic organisms. FTIR spectra of our chondroitin sulfate samples have a peak in the wavelength range of $825\text{--}820\text{ cm}^{-1}$.

The method of thermal analysis allows studying the structure of polymer compounds, conformational and phase transformations of macromolecules. It is known that various chemical reactions can occur during thermal decomposition: hydrogen bond breaking, conformational and phase transitions [18]. During the thermal decomposition of chondroitin sulfate, a process of thermal oxidative degradation proceeds, accompanied by the destruction of both glycoside and acetamide chemical bonds. The temperature dependences of mass change for chondroitin sulfate samples received by method of differential thermogravitation measurements are given in the Figure 3.

The analysis of the curves in Figure 3 showed that the mass loss of samples in the temperature range of $40\text{--}600\text{ }^{\circ}\text{C}$ occurs in several stages.

The first stage ($20\text{--}150\text{ }^{\circ}\text{C}$) corresponds to a decrease in mass due to water loss. The chondroitin sulfate macromolecule contains polar functional groups (Fig. 1), which have the ability to physically bind water. For chondroitin sulfate samples from salmon and black shark, the loss of physically bound water is observed at $t_{max} = 66\text{--}68\text{ }^{\circ}\text{C}$, for the northern skate at $t_{max} = 88\text{ }^{\circ}\text{C}$ (Fig. 3). The weight loss of the samples in this case is 1-7%. The desorption of bound water occurs almost to $150\text{--}200\text{ }^{\circ}\text{C}$. This can be explained by the difficulty in breaking hydrogen bonds between water molecules and the polar functional groups of chondroitin sulfate.

Under experimental conditions, the main decomposition of the macromolecular chain of chondroitin sulfate during thermal degradation occurs at $200\text{--}400\text{ }^{\circ}\text{C}$: the second, third and fourth peaks in the DTG curves.

According to the work [19], the destruction of chondroitin sulfate begins at approximately $246\text{ }^{\circ}\text{C}$ and can vary up to $367\text{ }^{\circ}\text{C}$. It is assumed that the second and third peaks are associated with the release of sulfate groups and carboxylic acids.

In the temperature range $236\text{--}244\text{ }^{\circ}\text{C}$ (II peak in Fig. 3), the destruction rate of the samples is 6.7-11.5 %/min. The lowest degradation rate is observed for chondroitin sulfate isolated from the cartilage of salmon -- 6.7 %/min, for samples of chondroitin sulfate isolated from the cartilage of black shark and stingray -- $v = 7.4$ and $8.0\text{ } \%/ \text{min}$, respectively.

The third peak ($315\text{--}330\text{ }^{\circ}\text{C}$) in the DTG curves (Fig. 3) during the decomposition of the chondroitin sulfate is associated with the beginning of the destruction of pyranose

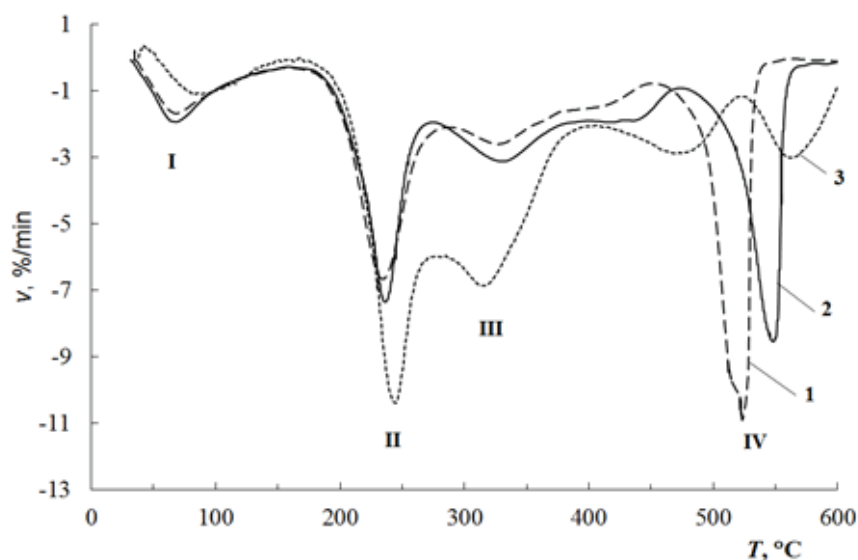


Figure 3: Temperature dependence of the mass loss rate (v) of chondroitin sulfate obtained from the cartilage tissue of salmon (1), shark (2) and stingray (3). Peaks I, II, III and IV - stages of thermal degradation of chondroitin sulfate.

units present in the chondroitin sulfate [19]. At this stage, chondroitin sulphate from the cartilage salmon also had the smallest $v = 2.6$ %/min. For chondroitin sulphate from the cartilage shark and stingray, the destruction rate is 3.1 and 6.7 %/min. respectively.

When samples are heated above 350-400 °C, thermal oxidative degradation develops, leading to a loss in sample mass of more than 50%. Heating the samples to 520-560 °C (IV peak in Fig. 3) leads to further mass loss up to the formation of a coked residue. The destruction rate at these temperatures is 3.1-10.8 %/min.

To process the thermal analysis results, the Freeman-Carroll method was used [18]. To linearize the experimental data using the Freeman-Carroll method, the kinetic curves of thermal decomposition of chondroitin sulphate are plotted in the coordinates $\ln(Rt) = f(1/T)$, which allows determining the activation energy of thermal destruction process of samples. The obtained experimental data of thermal degradation process of the studied samples were divided into three linear temperature ranges: 100-240 °C; 240-300 °C and 320-600 °C. The calculations showed that the stage of adsorption water loss for the studied samples corresponds to the effective activation energy $E = 10-11$ kJ/mol, in the temperature range 210-310 °C the activation energy is 54.1 kJ/mol, the stage of maximum destruction rate corresponds to 7.7-8.4 kJ/mol.

4. Conclusion

Chondroitin sulfate was isolated from the cartilage tissue of salmon (*Salmo salar*), northern stingray (*Raja hyperborean*) and blackmouth shark (*Galeus melastomus*), its chemical and thermal properties were studied. The highest polysaccharide content (82.2%) is in the sample obtained from the cartilage of salmon, the smallest (67.1%) – In chondroitin sulphate from the northern skate. FTIR spectrums of the obtained samples contain peaks at the wave numbers characteristic of chondroitin sulfates (1550, 1350-1300, 1160-1120 and 822 cm^{-1}).

The results of thermogravimetric analysis of chondroitin sulfates in the temperature range of 40-600 °C showed that the weight loss of the samples occurs in several stages. Under experimental conditions, thermal decomposition of the bulk of chondroitin sulfate samples is observed at temperatures from 236 to 563 °C. Several peaks are present on the DTG curves, the first of which is associated with the removal of water, and the subsequent ones, apparently, with the thermal destruction of uronic acid, pyranose, and, finally, residual carbon and sulfur compounds. The mass loss rates in each region were determined and the activation energies of each event were calculated.

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Conflict of Interest

The authors have no conflict of interest to declare.

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