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CHARACTERISTICS OF POLYSACCHARIDES AND PROTEIN ASSOCIATED WITH THEM FROM DRIED AND FRESHLY COLLECTED RED ALGA *Tichocarpus crinitus*

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Polysaccharides isolated by successive extraction with water at 20 and 80°C from freshly collected and dried alga T. crinitus *were compared. It was shown that the yield of polysaccharides from freshly collected alga was 40–44%; from dried material, less than 25%. It was found that the amount of extracted polysaccharides and their molecular weights decreased upon storage of dried alga for three years. Polysaccharides isolated from freshly collected and dried alga had identical structures and were a mixture of* κ/β- *and a new X-type of carrageenan. It was shown that protein, the amount of which reached 24% in the extracts obtained at 20°C, was strongly bound to the carrageenan. The amino-acid compositions of the proteins associated with the polysaccharides isolated at 20 and 80°C were identical and had an elevated content of serine, glutamic acid, glycine, and alanine.*

Keywords: *T. crinitus*, carrageenan, storage time, protein.

The alga *Tichocarpus crinitus* (Tichocarpaceae) contains carrageenan and is widely distributed in seas of the Far East. We have shown previously that the structures of gelling polysaccharides isolated from *T. crinitus* are κ/*β*-carrageenans [1]. The structure of non-gelling polysaccharides is based on a repeating disaccharide unit consisting of 3-*β*-D-galactopyranoside-2,4-disulfate and 1,4-bound 3,6-anhydro-*α*-D-galactopyranoside [2].

The previous studies were carried out using alga samples that were collected at one site of the Sea of Japan and stored in a dried state for a long time. Because the carrageenans isolated from the alga have sulfate groups and, therefore, can undergo autohydrolysis, the question of the influence of raw material storage times on the structure and properties of the polysaccharides isolated from it arises.

Our goal was to compare polysaccharides isolated by successive extraction with water at different temperatures from both freshly collected and dried specimens of *T. crinitus* stored for several years.

Alga *T. crinitus* was collected in July 2004, 2007, and 2008 in Rice Bay of Peter the Great Bay in the Sea of Japan during their growing season at a depth of 1.5 m. Polysaccharides were isolated by successive extraction with water at 20 and 80°C. Table 1 presents their yields. The resulting fractions were analyzed for the presence of polysaccharides by the phenol–H_2SO_4 method determining the total monosaccharide (MS) content. The amount of polysaccharides extracted from freshly collected alga was 1.5–2 times greater than from the dried specimen. The yield of polysaccharides obtained at 20°C from both freshly collected and dried alga was less than 2%.

Chemical analysis of polysaccharides isolated at 20°C from freshly collected alga showed a high content (up to 24.5%) of protein and equal amounts of galactose and glucose. The presence of the latter monosaccharide could be explained by the isolation of starch, a reserve polysaccharide of the algal cell wall. Polysaccharides isolated at 80°C contained only galactose and 3,6-anhydrogalactose, the main monosaccharides from which the disaccharide units of the carrageenans are constructed.

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TABLE 1. Characteristics of Polysaccharide Fractions Isolated from Freshly Collected and Dried Alga *T. crinitus*

Collect./anal. time, raw matl.	Extraction temp., °C	Yield, %	MC,* %	Protein, %	MW, kDa
2004/2004,	20	0.16	N.d.	24.5	N.d.
freshly collect.	80	44.4	59.5	6.8	450
2004/2007,	20	0.6	N.d.	N.d.	N.d.
dried	80	25.2	68.5	8.3	236
2008/2008,	20	0.9	15.0	18.0	N.d.
freshly collect.	80	41.0	61.3	6.7	680
2008/2008,	20	1.2	N.d.	N.d.	N.d.
dried	80	31.0	62.7	5.4	700

*MC is the total monosaccharide content determined by the phenol–H_2SO_4 method. N.d.: not detected.

TABLE 2. Amino-Acid Composition of Proteins in Polysaccharides Isolated from *T. crinitus* at 20 and 80°C, %*

Amino acid	20°C	80°C	Amino acid	20°C	80°C
Ser	7.36	11.19	Pro	2.52	3.93
Glu	5.95	5.05	Orn	2.51	3.92
Gly	5.71	7.06	Tyr	2.21	2.11
Ala	5.34	5.12	Ile	1.69	1.81
Asp	4.86	4.53	His	1.62	1.46
Thr	4.25	4.97	Tau	0.93	0.34
Ley	4.38	3.29	Met	0.54	0.53
Arg	3.9	3.05	Lys	0.35	0.80
Lys	3.41	2.81	1-Mehis	0.32	0.64
Phe	2.58	2.62	Cys	0.27	0.71

*Of total protein content in polysaccharide.

The molecular weight (MW) of polysaccharides, the value of which for carrageenans used in food items should be at least 100 kDa [3], is known to be an important physicochemical characteristic of them. The MW of carrageenans determines also the degree of their biological activity [4]. In addition, polyionic polysaccharides such as carrageenans can undergo autohydrolysis during storage of the raw material. This reduces their MW. Therefore, we determined the MWs of the carrageenans isolated from freshly collected and dried alga and stored from one month to three years. A viscosimetric method was used to determine the MWs. The viscosity of diluted solutions of carrageenans was measured in aqueous NaCl (0.1 M). Molecular weights were calculated using the Mark–Houwink–Kuhn equation $[\eta] = KM^{\alpha}$, where $[\eta]$ is the characteristic viscosity and K and α are constants, the values of which for this polymer–solvent system were taken as $K = 3 \times 10^3$ and $\alpha = 0.95$ [5]. Table 1 shows that polysaccharides isolated from freshly collected *T. crinitus* had the highest MWs. The MW of polysaccharides from dried alga stored for more than three years was approximately halved whereas their MW did not change after storage for one month (Table 1).

Polysaccharides that were isolated at 80°C were fractionated by aqueous KCl (4%) into gelling (KCl-insoluble) and non-gelling (KCl-soluble) fractions and analyzed using Fourier-IR spectroscopy, which is an effective and relatively simple method for evaluating structural features of carrageenans [6, 7]. We found previously that KCl-insoluble polysaccharides isolated from the vegetative form of *T. crinitus* were κ/β-carrageenans [1]. IR spectra of KCl-insoluble polysaccharides from both freshly collected and dried alga contained absorption bands characteristic of κ/β-carrageenan. The absorption band at 930 cm^{-1} belonged to 3,6-anhydrogalactose; at 846, to a C-4 sulfate of galactose; at 893, to unsulfated galactose [8, 9]. IR spectra of KCl-soluble polysaccharides corresponded to that found by us earlier for an X-type carrageenan [2]. The spectra exhibited absorption bands at 935 cm^{-1}, characteristic of 3,6-anhydrogalactose; at 848, of a C-4 sulfate; and at 852 and 811, of galactose sulfates [2]. Thus, according to IR spectroscopy, storage of dried *T. crinitus* for three years did not change the structures of the polysaccharides isolated from them.

Protein, the amount of which in the extracts obtained at 20°C reached 24%, occurred in polysaccharides isolated from freshly collected and dried *T. crinitus* according to chemical analysis (Table 1). Despite the facts that practically all

polysaccharides isolated from alga contain protein and that its content can be up to 30%, its composition and the mode of protein–polysaccharide bonding are practically unknown [10]. Also, the presence of protein has a significant effect on the manifestation of polysaccharide biological activity.

Amino-acid analysis indicated that the proteins in polysaccharides isolated from dried *T. crinitus* at the different temperatures were identical (Table 2). The dominant amino acids were serine (Ser), glutamic acid (Glu), glycine (Gly), and alanine (Ala). Also, the dominant amino acids of proteins in the algal cell wall are glutamic and aspartic acids according to the literature. Thus, the content of these acids in *Palmaria palmate* and *Porphyra tenera* are 14 and 19%, respectively, of the total amount of amino acids [11].

Protein occurring in the *T. crinitus* polysaccharides that were extracted at 20°C was characterized by a high content of serine and glutamic acid whereas that in polysaccharides that were isolated at 80°C had a high content of serine and glycine (Table 2). The protein also contained essential amino acids lysine (Lys), leucine (Leu), threonine (Thr), arginine (Arg), histidine (His), methionine (Met), isoleucine (Ile), and phenylalanine (Phe).

The lectin TCL was isolated earlier from *T. crinitus*. Its amino-acid composition was characterized by a high content of serine, threonine, proline, alanine, and glutamic acid [12]. It is not yet known if the proteins associated with the polysaccharide are similar in nature to TCL.

Polysaccharides were purified of protein using methods such as fractional precipitation by acetic acid and the Sevag method. The analytical results showed that the Sevag method was most effective and that it reduced the protein content by 1.3 times.

Peptide-hydrolyzing enzymes such as protease and collagenase were also ineffective. Treatment of carrageenan by proteinase reduced the protein content by 1.5 times relative to the starting amount. The protein content in polysaccharide treated by collagenase was practically unchanged.

Protein was separated by ion-exchange chromatography from polysaccharides that were isolated at 20°C, which contained the greatest amount of protein. The polysaccharide and protein were not separated over a polystyrene/divinylbenzene sorbent. Therefore, chromatography was performed over the anion-exchange resin Amberlite 17. Polysaccharides were eluted successively by phosphate buffer, an NaCl gradient in phosphate buffer, and aqueous NaOH (2 M). The phosphate buffer and NaCl gradient eluted polysaccharide fractions in which the polysaccharide:protein ratio differed little and was 1:1.20 and 1:1.08, respectively. Subsequent elution by NaOH solution isolated a fraction enriched in polysaccharide in which the protein content decreased by 2.5 times. However, polysaccharide was not completely separated from protein.

The results from chromatography and enzymatic hydrolysis led to the conclusion that the polysaccharide was bonded rather strongly to the protein, probably through a covalent bond. The possibility of forming a covalent bond between polysaccharides isolated from algae and the proteins in them has been discussed [13, 14]. It was hypothesized that amino acids such as proline, histidine, and serine are involved in the bonding [15].

Thus, the results indicated that the structure of carrageenans isolated from dried *T. crinitus* did not change during storage. However, their MWs decreased (from 450 to 236 kDa). Polysaccharides isolated at 20°C from freshly collected alga contained 24% protein. The amino-acid compositions of proteins isolated at 20 and 80°C were practically identical and were characterized by a high content of serine, glutamic acid, glycine, and alanine.

EXPERIMENTAL

Alga. The alga *T. crinitus* was washed with tap water to remove salts and was dried in air.

Treatment of Alga with Base. Dried alga was stored in NaOH solution (0.05 M) at 20°C for 12 h, washed with tap water to remove traces of base, and dried in air.

Isolation of Polysaccharides and Their Fractionation. Alga was ground, treated with water (1:60 ratio for dried alga; 1:10, for freshly collected), and extracted at 20°C for 24 h. The resulting extract was filtered, concentrated, and precipitated with EtOH (three times the volume). The resulting precipitate was separated by centrifugation (4,000 rpm) and lyophilized to afford the total polysaccharide fraction (Σ-20). The remaining alga was treated with water and extracted for 3 h on a water bath at 80°C with constant stirring. The resulting extract was filtered and then centrifuged (4,000 rpm) for 20 min. The precipitate was treated with water and extracted again. The supernatants were combined. A part (one half) of the supernatant was precipitated with EtOH (three times the volume). The precipitate was separated by filtration and lyophilized to afford the

total polysaccharide fraction (Σ-80). Polysaccharides were separated into gelling and non-gelling fractions using aqueous KCl (4%) as before [15].

Analytical Methods. The total carbohydrate content was determined by the phenol–H_2SO_4 method using D-galactose as a standard [16]. Neutral monosaccharides were determined as polyol acetates using GC on a 6850 chromatograph (Agilent, Germany) equipped with an HP-5MS capillary column (30 m × 0.25 mm) with phenylmethylsiloxane (5%) (Agilent, Germany) and a flame-ionization detector at 150–230°C (temperature change rate 3°/min) [17].

The content of 3,6-anhydrogalactose was found by total reductive hydrolysis [18]; the amount of sulfates, by a turbidimetric method [19]; protein content, by the Lowry method [20].

Amino-Acid Analysis. Carrageenan (1 mg) was hydrolyzed by HCl solution (6 N, 1 mL) in a sealed ampul for at 105°C for 24 h. The ampul was cooled and rinsed with EtOH (3 mL). The solution was evaporated with EtOH to dryness three times in order to remove the excess of acid. The dry solid was dissolved in a small amount of distilled water and worked up four times with $CHCl_3$ (1 mL). The aqueous layer was evaporated to dryness in a rotary evaporator at 60°C. The dry solid was dissolved in water (1 mL) and analyzed on a Biochrom-30 automated amino-acid analyzer (Biochrom, Great Britain) using an Ultrapak column (8 μm, 4.6 × 200 mm, Li^+-form). The instrument detection limit was 9 pmol. Reference standards (Sigma, USA) were used for the calculations.

Sevag Method. An aqueous solution of carrageenan (1%) was treated with $CHCl_3$ and *n*-BuOH (each 0.2 of the polysaccharide solution volume). The resulting solution was shaken for 2 min. The $CHCl_3$ fraction was evaporated to dryness.

Fractional Precipitation. Carrageenan (100 mg) was dissolved in distilled water (20 mL). The resulting solution was titrated with acetic acid (0.1 M) until a precipitate formed. The solution was left for one day at 4°C. The precipitate was separated by centrifugation for 10 min at 2,000 rpm.

Treatment of Carrageenan by Enzymes. A solution of carrageenan (15 mL, 1.8%) was prepared in Tris-HCl buffer (0.05 M, pH 7.5), treated with enzyme solution (0.6 mL, 3 mg/0.6 mL Tris-HCl buffer), and stored for 3 h at 37°C. The enzyme was inactivated by holding the mixture at 80°C for 15 min. The solution was passed through an ultrafilter membrane (pore size 30 kDa) (Millipore, USA). The polysaccharide was precipitated by EtOH (three times the volume).

Protease (Biomedical Inc.) was isolated from *Streptomyces caespitosus*. Collagenase was graciously supplied by the Marine Biochemistry Laboratory, PIBOC FEB/RAS.

Cation-exchange Chromatography. Carrageenan (2 mg) was dissolved in acetate buffer (2 mL, 0.01 M, pH 4.0), placed on a column (Amersham Pharmacia Biotech, Sweden; Mono S 10/100 GL; 10 × 0.46 cm), and eluted by a linear NaCl gradient (2 M) in acetate buffer (0.01 M). The collected fractions (1.5 mL) were analyzed for carbohydrates by the phenol–H_2SO_4 method [16] and for protein by measuring absorption at 280 nm on a μQuant spectrophotometer (Bio-Tek, USA).

Anion-exchange Chromatography. Carrageenan (2 mg) was dissolved in acetate buffer (2 mL, 0.01 M, pH 4.0), placed on a column (Amersham Pharmacia Biotech, Sweden; Mono Q 10/100 GL; 100 × 10 mm), and eluted by a linear NaCl gradient (5 M) in phosphate buffer (0.05 M). The collected fractions (2.0 mL) were analyzed for carrageenan using a color reaction with Taylor reagent [21] and for protein as indicated above.

Chromatography over Amberlite 17 Anion-exchange Resin. Carrageenan (100 mg) was dissolved in phosphate buffer (50 mL, 0.05 M, pH 7.5), placed on a column (250 × 40 mm) that was eluted successively by phosphate buffer (0.05 M, pH 7.5), a linear NaCl gradient (2 M) in phosphate buffer (0.05 M), and NaOH solution (2 M). The resulting fractions (2.0 mL) were analyzed for carrageenan. The collected fractions were purified of spectator salts by ultrafiltration through a membrane (pore size 3 kDa) (Millipore, USA), concentrated, and lyophilized.

Determination of Carrageenan Solution Viscosity. Viscosity was measured in a modified Ubbelohde viscosimeter (SKB Pushchino, Russia) with capillary diameter 0.3 mm and concentration range 0.5–2.0 mg/mL in NaCl solution (0.015 M). The flow time of the solutions at 25°C were measured to an accuracy of ±0.1 s. Characteristic viscosity was calculated by extrapolation of a plot of $(\ln \eta_{rel})/C$ to zero concentration.

Fourier-IR Spectroscopy. IR spectra of samples were recorded in films on a Vector 22 Fourier-transform spectrophotometer (Bruker) with 4 cm^{-1} resolution. Spectra were normalized to the absorption band for the monosaccharide ring at ~1070 cm^{-1}.

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