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FORMATION OF SOLUBLE CHITOSAN–CARRAGEENAN POLYELECTROLYTE COMPLEXES

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The formation process of soluble chitosan (C)/κ-carrageenan (K) complexes was studied. It was shown that soluble complexes were obtained preferentially by mixing the starting components at given ratios whereas soluble complexes were formed only over a narrow range of starting-component ratios by titration of K with C. The formation of C/K polyelectrolyte complexes was confirmed by gel-permeation chromatography and centrifugation in a Percoll gradient. The formation process of C/K complexes depended on the C molecular weight and the concentrations and ratios of starting polysaccharides. It was shown that unbound components in addition to the complex remained in the mixture upon mixing carrageenan with high-molecular-weight C (C-HM) in 1:1.5 and 1:30 ratios (mol/mol) whereas low-molecular-weight C (C-LM) was bonded completely to the polyanion.

Keywords: polyelectrolyte complexes, chitosan, carrageenan.

Chitosan and carrageenan, polysaccharides from marine hydrobiota, exhibit a multifaceted biological activity.

Chitosan (C) is a linear polysaccharide, the polymeric chain of which is constructed of β-1,4-bonded glucosamine and *N*-acetylglucosamine units. This polysaccharide is usually obtained by basic deacetylation of chitin, which is a structural component of the cell wall of many crustaceans and insects [1].

Carrageenan is a sulfated polysaccharide consisting of D-galactose units and its derivatives bonded by $\beta(1\rightarrow4)$ and $\alpha(1\rightarrow3)$ glycoside bonds. The structures of about 20 so-called idealized types of carrageenan that differ in the content of 3,6-anhydrogalactose and the location and amount of sulfates have been established [2].

Chitosan and carrageenan can be used to produce various polyelectrolyte complexes (PECs) owing to their polyionic nature. Chitosan–carrageenan PECs that were obtained as films [3], microcapsules [4], and gels [5] were reported. These were examined mainly as vectors for delivery of various biologically active substances and components for controlled release of drugs. A soluble form of chitosan–carrageenan complexes could expand their spectrum of application.

Systematic studies by various research groups found that the principal parameters affecting the size and solubility of PECs were the polyelectrolyte concentration, polymer charge density, solution ionic strength, and content of low-molecular-weight salts in it. According to the generally accepted theory of complex formation [6, 7], the basis of which is their stoichiometry, PECs are usually divided into two categories. These are highly aggregated complexes with stoichiometry close to 1:1 that exist in solution as large colloidal particles and soluble complexes that are formed with an excess of the longer chain polyelectrolyte [6].

Soluble non-stoichiometric complexes can be formed over a relatively narrow range of ratios under the following conditions. 1) One of the components should have weakly ionic groups. 2) The molecular weights of the polyelectrolytes should differ substantially. 3) Low-molecular-weight salts should be present in the solution [6]. Chitosan is chemically a weak polybase. Depolymerization of it produces rather easily samples with different polymer chain lengths. Carrageenan, in turn, is a negatively charged high-molecular-weight polysaccharide. All this provides hope that soluble chitosan–carrageenan complexes can be produced if certain conditions are observed.

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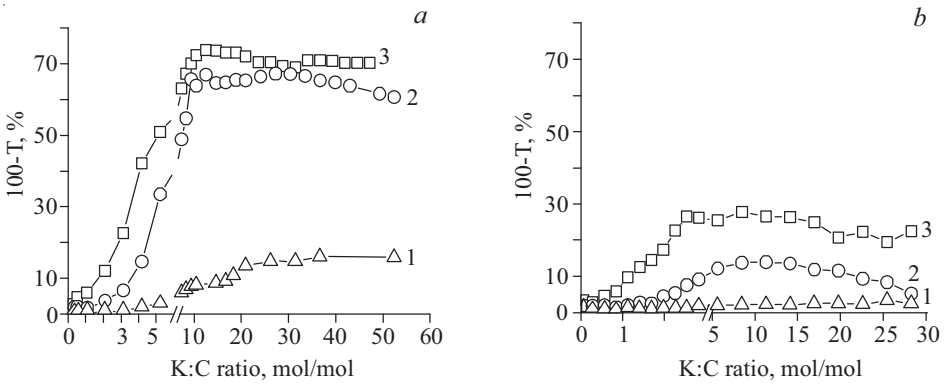

Fig. 1. Turbidity of K:C-HM mixtures as functions of the ratio of starting components that were obtained by method I (*a*) and II (*b*) for C_K = 0.1 mg/mL (1), 0.5 (2), and 1 (3).

The goal of the present work was to select the conditions for forming soluble chitosan–carrageenan complexes and to study the effect on the complexation process of the concentrations of starting components, their ratio, and the molecular weight of chitosan.

We used two chitosan (C) samples of different polymer lengths with molecular weights 70 kDa (C-HM) and 11 kDa (C-LM) and degree of *N*-acetylation 1 and 11%, respectively, as the polycation. The polyanion was κ-carrageenan (K) with molecular weight 367 kDa that was isolated from the red alga *Chondrus armatus*.

It was shown earlier [8] that C:K complexes that were obtained with a K concentration >0.4% were gels. Therefore, we used solutions of K with concentrations 1 mg/mL and less to prepare the K:C PECs.

Turbidimetric titration based on recording the turbidity of the system, which was proportional to the molecular weight and number of particles in the reaction medium and was calculated using the intensity attenuation of light passing through it, was a simple and sensitive method for following the PEC formation process [9].

According to the literature, non-stoichiometric soluble PECs, as a rule, are produced by two methods. In the first, a solution of a polyelectrolyte is treated dropwise successively with a solution of the second PEC component (method I) [10]; in the second, given volumes of polyelectrolytes are mixed in certain ratios (method II) [11].

Figure 1*a* shows the turbidity of a K:C-HM mixture that was produced by method I as a function of the ratio of starting components.

The turbidity almost did not change upon adding a solution of C to a solution of K at low concentration (0.1 mg/mL, Fig. 1*a*, curve 1) until the K:C-HM ratio was 1:3 mol/mol. This could indicate that soluble complexes formed. Further addition of C-HM increased the turbidity of the test system, indicating that the PEC particles enlarged and formed a precipitate. At higher K concentrations (0.5 mg/mL, Fig. 1*a*, curve 2 and 1 mg/mL, Fig. 1*a*, curve 3), the turbidity increased sharply already with a K:C-HM ratio of 1:2 mol/mol, reaching a maximum for K:C-HM = 1:10. Further changes were not observed. This indicated that the system had come to equilibrium.

We used a competitive binding method to prove that the C:K complex had formed. This method was based on release of the dye tropeolin 000-II from its complex with C [12]. The polyanion K released tropeolin from its complex with C and bound to it upon adding K to the tropeolin–C complex. This proved that the C:K complex formed at low concentrations (data not presented).

The nature of the turbidity functions for mixtures obtained by method II (Fig. 1*b*) differed from that for complexes obtained by method I. At low K concentrations (0.1 mg/mL, Fig. 1*b*, curve 1), the turbidity of the mixtures practically did not change. This indicated that soluble K:C-HM complexes formed over the whole studied (from 1:0.1 to 1:30 mol/mol) range of mole ratios.

At a K concentration of 0.5 mg/mL (Fig. 1*b*, curve 2), the range of formation of soluble complexes decreased sharply (from 1:0.1 to 1:1.5 mol/mol). Insoluble complexes formed already at a K:C-HM ratio of 1:2 mol/mol. This was observed as an increase of the solution turbidity that increased up to a K:C-HM ratio of 1:10 mol/mol. Increasing the C-HM content in the complex further caused the turbidity of the mixture to gradually decrease so that a soluble complex was formed at K:C-HM = 1:30.

At initial K concentration of 1 mg/mL (Fig. 1*b*, curve 3), like in the mixtures prepared by method I (Fig. 1a, curve 3), primarily insoluble complexes were formed. However, the particles enlarged up to K:C-HM = 1:5, after which the turbidity was practically constant.

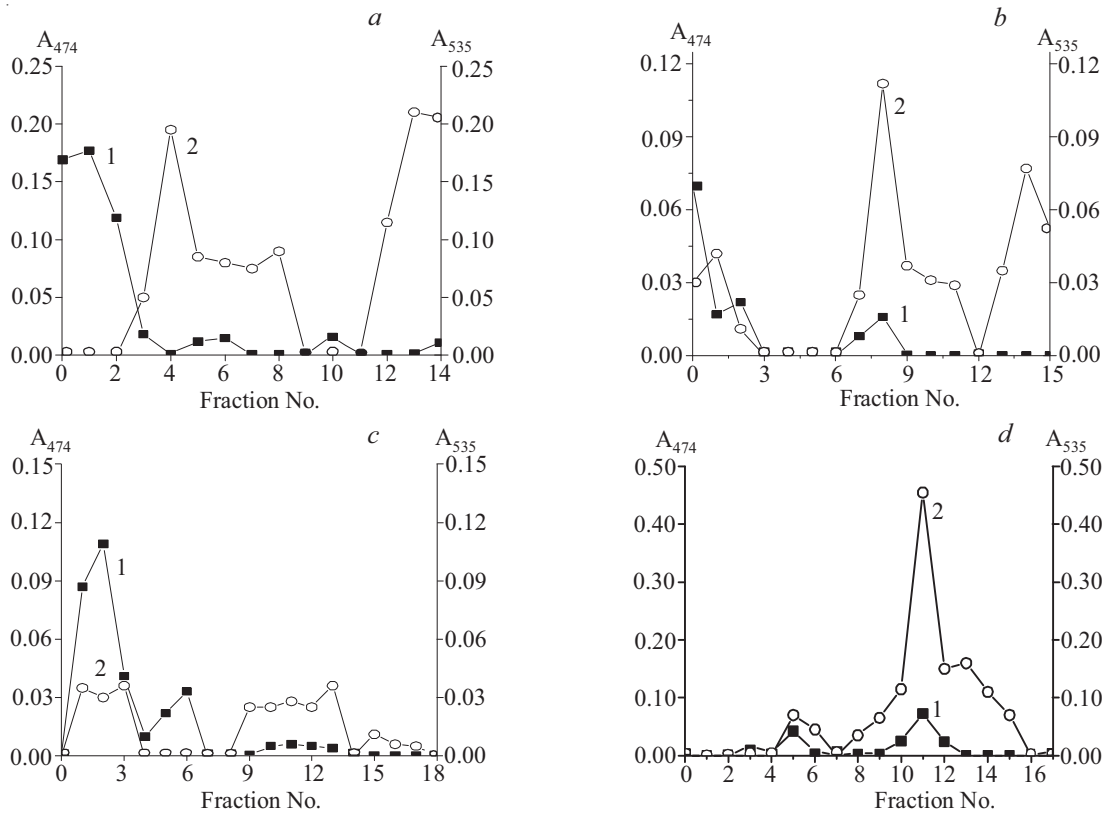

Fig. 2. Centrifugation in Percoll gradient of starting polysaccharides (*a*); K:C-HM mixtures (mol/mol) for 1:1.5 (*b*) and 1:30 (*c*); and a K:C-LM mixture (mol/mol) for 1:1.5 (*d*); content of C amino groups (A_{474nm}) (1); content of K sulfate groups (A_{535nm}) (2).

Turbidimetric analysis of K:C-LM (17 kDa) mixtures that were obtained by method II showed that C-LM, like C-HM, formed soluble complexes over the whole range of studied ratios with K at a concentration of 0.1 mg/mL. At a higher concentration (0.5 mg/mL), the general nature of the curve was analogous to that for mixtures with C-HM. Soluble complexes were formed at ratios from 1:0.1 to 1:12.5 mol/mol and from 1:100 to 1:210 mol/mol (data not presented).

Thus, it was found from turbidimetric analysis that the complex formation process depended on the preparation method. Soluble complexes were obtained by mixing given ratios of starting components (method II) whereas titration of one component by the other (method I) formed soluble complexes over a very narrow range of ratios of starting components.

The turbidimetric method enabled the formation of PEC particles to be followed quickly. However, it did not provide information on the completeness of binding of the complex components. This was an important parameter in the formation process. The use of methods for detecting complexation directly that monitor the completeness of binding of the components in the PEC has great significance for further studies of the complex biological activity. Therefore, gel-permeation chromatography and rapid centrifugation in a Percoll gradient were used to study the PEC formation process. PECs obtained by method II at various ratios of starting components were analyzed using these methods.

Figure 2 shows results from centrifugation in a Percoll gradient of the starting complex components (Fig. 2*a*) and their mixtures with K:C-HM ratios 1:1.5 mol/mol (Fig. 2*b*) and 1:30 (Fig. 2*c*).

It can be seen (Fig. 2*a*) that C-HM was a homogeneous polysaccharide that settled in the lower part of the gradient. Under the centrifugation conditions, K was separated into two fractions in the upper and lower parts of the gradient.

The elution curves of K:C-HM mixtures (Fig. 2*b* and 2*c*) differed from those of the starting polysaccharides (Fig. 2*a*). The coincidence of the elution curve maxima for C-HM and K in their mixture indicated that a K:C-HM complex formed. Two types of complexes in the lower and middle parts of the gradient formed with a K:C-HM ratio of 1:1.5 mol/mol (Fig. 2*b*). Part of the K remained free (fractions 12–15). Increasing the amount of C in the mixture to a ratio of 1:30 mol/mol (Fig. 2*c*) did not lead to complete binding of the polyanion. In this instance also two types of complexes formed in the lower (fractions 1–3) and middle (fractions 9–14) parts of the gradient. In addition, unbound K (fractions 16–18) and C-HM (fractions 5–6) remained.

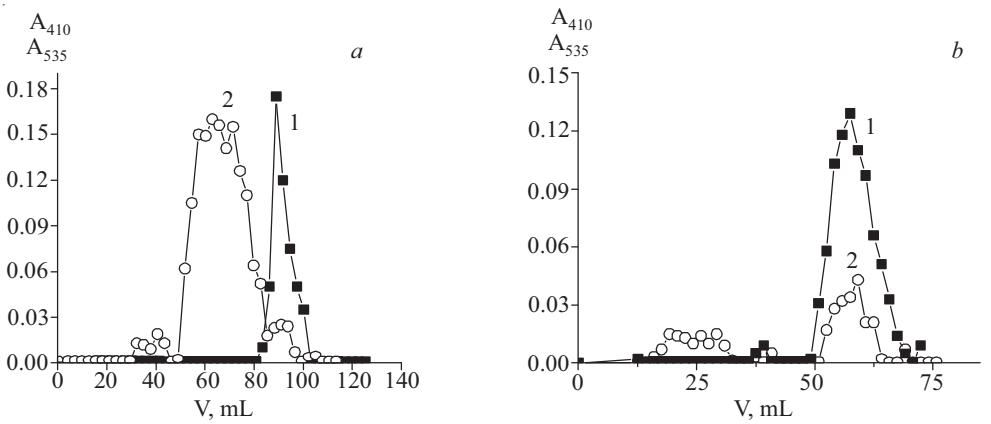

Fig. 3. Gel-permeation chromatography of starting polysaccharides (*a*) and K:C-HM mixture (mol/mol) for 1:30 (*b*); content of C-HM amino groups (A_{410nm}) (1); content of K sulfate groups (A_{535nm}) (2).

Gel-permeation chromatography over ToyoPearl HW-65 gel was used to confirm the results from gradient centrifugation. Figure 3 shows the results from gel-permeation chromatography of the starting polysaccharides and the complex with a K:C-HM ratio of 1:30 mol/mol.

Figure 3*a* shows that the molecular weight of C-HM was homogeneous whereas that of K (Fig. 3*a*) showed a slight dispersion. The coincidence of the maxima on the elution curves for C and K in their mixture indicated that a complex formed (Fig. 3*b*). Free components were also observed in the mixture.

Thus, the results from gel-permeation chromatography confirmed those from centrifugation and indicated that a K:C-HM complex formed. Our results showed that, regardless of the ratio of starting components, complete binding of C-HM and K was not observed. The reason for this could be specifics of the molecular structures of the starting polysaccharides. The presence of 3,6-anhydrogalactose in K reduced the flexibility of the molecule. This could create steric hindrance to binding with C-HM. In turn, as noted earlier, C-HM is a rather rigid linear polymer that can form associates in solution [12] in which several binding sites can be unavailable to the polyanion. A complicated reaction of C-HM similar to this was observed during complexation with another polyanion, a lipopolysaccharide [13]. Moreover, complete binding of the components in the complex was achieved on going to C of lower molecular weight [13].

In our instance, we observed complete binding of K to C-LM. Figure 2*d* shows results from centrifugation of a 1:1.5 mol/mol K:C-LM mixture at K concentration 0.5 mg/mL. It can be seen that C-LM was completely bound to K to form complexes in the middle part of the gradient (fractions 4–6 and 8–15).

Thus, we obtained soluble K:C complexes. The complexation process depended on the preparation method and concentration and ratio of starting components. Soluble K:C complexes were formed at K concentration 0.1 mg/mL over the whole studied (from 1:0.1 to 1:30 mol/mol) range of ratios of starting components. At higher K concentrations, soluble complexes formed over a narrower range of ratios. Results of gel-permeation chromatography and centrifugation in a Percoll gradient showed that the C molecular weight affected the binding process. C-LM was completely bound to K, in contrast with C-HM.

EXPERIMENTAL

Chitosan. We used two C samples, one of which was obtained by basic deacetylation of chitin (C-HM); the other, by chemical depolymerization using the mild degradation agent H_2O_2 (C-LM). The methods for determining the molecular weights of the polycations were published previously [1].

Isolation of κ-Carrageenan (K). K was isolated by extraction with hot water from *C. armatus*. The methods for isolation and structure determination of K were published [14].

Analytical Methods. The K content was determined by reaction of polysaccharide sulfate group with Taylor's blue (1,9-dimethylmethylene blue) [15]. The optical density was measured at 535 nm on a μQuant spectrophotometer (Bio-Tek Instruments Inc., USA). The C content was determined by reaction of amino group with trinitrobenzenesulfonic acid (TNBS) [16]. The optical density was measured at 410 nm on the μQuant spectrophotometer.

Preparation of Complexes. Method I. Solutions of K in phosphate-salt buffer (0.01 M, pH 7.2) at concentration 0.1, 0.5, or 1 mg/mL were titrated by C solution in the same buffer at concentration 40, 20, or 4 mg/mL by successive addition of portions (5 μL) with stirring on a magnetic stirrer until the solution optical density was constant (10–20 min). The absorbance of the mixture was determined on a Cecil spectrophotometer (England) at 630 nm [10]. The reference solution was a solution of K with concentration corresponding to the K concentration in the mixture with C.

Method II. A solution of K in phosphate-salt buffer (100 μL, 0.01 M, pH 7.2) at concentration 0.2, 1 or 2 mg/mL was placed into a cell. Then, a solution of C (0–100 μL) at concentrations from 0.2 to 20 mg/mL and buffer were added so that the volume in each cell was 200 μL. The absorption of the mixture was determined on the μQuant spectrophotometer at 630 nm [11, 17].

Determination of Solution Turbidity. Solution turbidity (τ) was calculated using the formula

$$\tau = 100 - e^{-A} \times 100,$$

where A is the optical density at the given wavelength.

Gel-permeation Chromatography. C (3 mL, 3 mg/mL) or K (2 mL, 1 mg/mL) or a K:C mixture (5 mL) prepared by method II was placed onto a column of ToyoPearl HW-65 gel (l = 90 cm, d = 1 cm, V = 70.65 mL) and eluted by phosphate-salt buffer (0.01 M, pH 7.2). Fractions (1 mL) were collected. The C content in a fraction was determined by the qualitative reaction of C amino group with TNBS. The color reaction of sulfate group with Taylor's blue was used as a test for the presence of K.

Centrifugation in a Percoll Gradient. Percoll (Sigma, 26 mL, 30%) in NaCl solution (0.15 M) was placed into a 28-mL centrifuge tube. A sample of K, C, or their mixture (2 mL) was layered on the Percoll and centrifuged in an angled-rotor Heraeus Biofuge Stratos (Germany) at 20,000 g for 60 min. After centrifuging, the tube contents were removed through the top using a peristaltic pump. Fractions (1.5 mL) were collected. The density of the Percoll solution in each fraction was calculated using the refractive index determined on a RF-4 refractometer (LOMO). The presence of C in the fractions was determined by the reaction of polysaccharide amino group with TNBS; of K, by the reaction of sulfate group with Taylor's blue.

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