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APPLICATION OF CARRAGEENAN BEADS FOR CHROMATOGRAPHIC PURIFICATION OF PROTEINS

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SUMMARY

Three different kinds of κ-carrageenan beads were prepared and applied for the purification of enzymes: (1) κ-carrageenan beads containing sulphate groups were applied for the chromatographic purification of crude urokinase, and were found to be a useful carrier for the purification of the enzyme; (2) by using a column packed with the cross-linked and desulphated κ-carrageenan beads, urease, asparaginase and bovine serum albumin were molecular-sieved according to their molecular weight; and (3) κ-carrageenan beads containing sulphate groups could be used as a matrix for binding tanin via Fe^{3+}; the tannin–κ-carrageenan beads could be used as protein adsorbents.

INTRODUCTION

κ-Carrageenan is a polysaccharide prepared from seaweeds and is used as an emulsifier, stabilizer and thickener in a variety of foods. It is composed of unit structures of β-D-galactose sulphate and 3,6-anhydro-α-D-galactose, and gels when cooled below 10°C. This gelating behaviour is similar to agar or agarose.

In a previous paper[1], we investigated conditions for the gelation of κ-carrageenan in detail, and found that this polysaccharide is a new versatile matrix for immobilization of enzymes and microbial cells. That is, the polysaccharide is easily induced to gel by contact with metal ions, amines, amino acid derivatives and water-miscible organic solvents. Further, κ-carrageenan shows a unique ability to bind with and stabilize proteins, especially milk proteins. Its binding is primarily due to half-ester sulphate groups, which are strongly anionic, being comparable with sulphuric acid[2]. Therefore, it was considered that if the κ-carrageenan could be made in bead form, the gel beads may be used as a carrier in ion-exchange chromatography for purification of biomaterials.

Further, we considered that if the sulphate groups of κ-carrageenan beads can be removed, the resultant desulphated κ-carrageenan beads may be used as a carrier for gel filtration.

It is well known that tannin is used as a protein precipitating agent and forms a complex with ferrous ion, and also that ferrous ion forms a complex with sulphate

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groups. So, we supposed that κ-carrageenan beads with sulphate groups can be used as a carrier for binding tannin through ferrous ion, and the tannin–κ-carrageenan beads may be used as a specific adsorbent for proteins.

We have prepared three different kinds of κ-carrageenan beads and applied them to the purification of enzymes. In this paper these results are presented.

MATERIALS AND METHODS

Materials

κ-Carrageenan was obtained from Sansyo (Osaka, Japan). The molecular weight of the polysaccharide is around 100,000–800,000, and the sulphate content is 20–30% of the unit weight. Chinese gallotannin and Nessler's reagent were purchased from Katayama (Osaka, Japan). Bovine serum albumin was purchased from Daiichi Kagaku (Tokyo, Japan). Crystalline asparaginase from *Escherichia coli* was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Crude urokinase was obtained from Godo Shusei (Tokyo, Japan). Glucoamylase was the product of Tanabe Seiyaku (Osaka, Japan). SP-Sephadex C-25 was purchased from Pharmacia (Uppsala, Sweden). Amberlite IR-120 was purchased from Organo (Tokyo, Japan). Agar was purchased from Wako Junyaku (Osaka, Japan). Agar beads were prepared according to the methods of Hjertén[3]. All other chemicals used were reagent grade.

Measurement of enzymic activities

Activities of asparaginase and urokinase were assayed according to the methods of Tosa *et al.*[4] and Ploug and Kjeldgaard[5], respectively. Urease activity was assayed as follows. A reaction mixture of 1.0 ml of urease solution, 2.0 ml of 0.1 *M* Tris–HCl buffer (pH 7.2) containing 1 m*M* EDTA and 0.1 ml of 4 *M* urea solution was incubated at 30°C for 10 min. After reaction, 0.1 ml of the reaction mixture was transferred to test-tubes containing 1.0 ml of 1 *M* NaOH, and the liberated ammonia in the reaction mixture was measured colorimetrically by the nesslerization method.

Preparation of carrageenan beads

In 240 ml of physiological saline solution, 12 g of κ-carrageenan were dissolved at 80°C, and then the temperature of the solution was brought to 50°C. Polyoxyethylene mono-oleate (3 g) was dissolved in organic solvent containing 360 ml of toluene and 120 ml of carbon tetrachloride, and then the solution was warmed to 50°C. The two solutions were mixed with Omnimixer (Sorvall, U.S.A.) at 1500 rpm for 10 min at 50°C to give an emulsion. The emulsion was dispersed into 2% KCl solution under stirring. The obtained gel beads were sufficiently washed with acetone and 2% KCl solution, and then sieved in the wet state in the range 60–170 mesh.

Cross-linking and desulphurization of carrageenan beads

Cross-linked and desulphated carrageenan beads were prepared according to the method of Porath *et al.*[6], performed for the preparation of agar and agarose gel beads. That is, 7.75 g of carrageenan beads were suspended in 100 ml of 2 *M* KOH, and 100 mg of sodium borohydride and 5 ml of epichlorohydrin were added to the suspension. The mixture was stirred at 60°C for 30 min, and then the reaction mixture was filtered. The gel was thoroughly washed with 2% KCl solution and resuspended

in 100 ml of 2 *M* KOH. To the suspension, 100 mg of sodium borohydride were added, and then the mixture was autoclaved at 120°C for 30 min. After reaction, the cross-linked and desulphated beads were sufficiently washed with pnysiological saline solution.

Preparation of tannin–carrageenan beads

κ-Carrageenan beads (10 g) were suspended in 100 ml of 0.01 *M* Tris–HCl buffer (pH 8.0) containing 0.2 *M* $FeCl_3$, and then the suspension was stirred at low speed for 60 min at 30°C to give yellow-coloured beads. The beads were resuspended in 25 ml of 2 % KCl solution containing chinese gallotannin at a concentration of 1 %, and then the suspension was stirred at low speed for 10 min at 30°C. After reaction, the obtained tannin–carrageenan beads were thoroughly washed with 2 % KCl solution, ethanol and again with 2 % KCl solution.

Preparation of other tannin–matrix complexes

Other complexes of tannin and matrices containing sulphate groups were prepared by the same method as tannin–carrageenan beads. Immobilization of tannin on cellulose was carried out according to the method of Watanabe *et al.*[7].

RESULTS AND DISCUSSION

Purification of urokinase with carrageenan beads

The κ-carrageenan beads containing sulphate groups were applied to the chromatographic purification of enzymes and found to be a useful carrier for purification of urokinase. That is, crude urokinase was easily purified by this carrageenan bead column (Fig. 1). Urokinase was clearly separated from other proteins. This separation may be due to ion–ion interactions between the sulphate groups of κ-carrageenan beads and charged groups of urokinase. The main fraction of enzyme activity was found in fraction Nos. 33–41. The activity yield was 57.8 % and the specific activity was calculated to be 25,130 units per milligram of protein. By this chromatography the enzyme was purified *ca.* five-fold. These values for specific activity and activity yield are similar to those obtained when Amberlite CG-50[8] and SP-Sephadex C-50[9] were used as a carrier for purification of urokinase. Therefore, it is considered that the κ-carrageenan beads are a useful carrier for purification of urokinase and other proteins.

Application to gel filtration of cross-linked and desulphated carrageenan beads

We investigated the application of carrageenan beads for molecular sieving of proteins. However, κ-carrageenan, like agarose or agar, is a kind of strong ion exchanger. Therefore, the charged groups are partly responsible for zone spreading in the gel filtration of proteins, and decreased recovery of proteins due to ion–ion interaction is often observed, especially at low ionic strength. In order to reduce this ion–ion interaction, sulphate groups of κ-carrageenan beads were removed by alkaline hydrolysis in the presence of the sodium borohydride, according to the method of Porath *et al.*[6] used for agarose beads. Using these cross-linked and desulphated κ-carrageenan beads, experiments on the gel filtration were carried out as follows. A solution containing urease (mol. wt. 480,000), asparaginase (mol. wt. 120,000) and

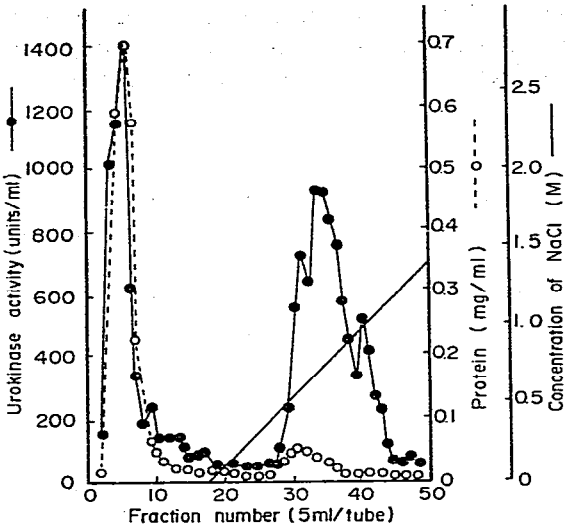

Fig. 1. Chromatography of urokinase on a column of κ-carrageenan beads. Crude urokinase (9.32 mg, specific activity 5395 units per milligram of protein) was dissolved in 10 ml of 0.01 *M* phosphate buffer (pH 8.0) containing 0.1 *M* KCl. The enzyme solution was applied to a carrageenan bead column (7.3 × 1.3 cm I.D.) equilibrated with the same buffer. After the column was thoroughly washed with the same buffer, the enzyme was eluted by linear gradients from 0 to 2.5 *M* NaCl in the same buffer at 4°C. The flow-rate was *ca.* 10 ml/h, and fractions of 5 ml were collected.

bovine serum albumin (mol. wt. 68,500) was chromatographed on a column (22 × 2.5 cm I.D.) packed with the cross-linked and desulphated κ-carrageenan beads. The elution pattern is shown in Fig. 2. Each protein was molecular-sieved according to its molecular weight, and K_{av} (distribution coefficient) was calculated to be 0.37 for

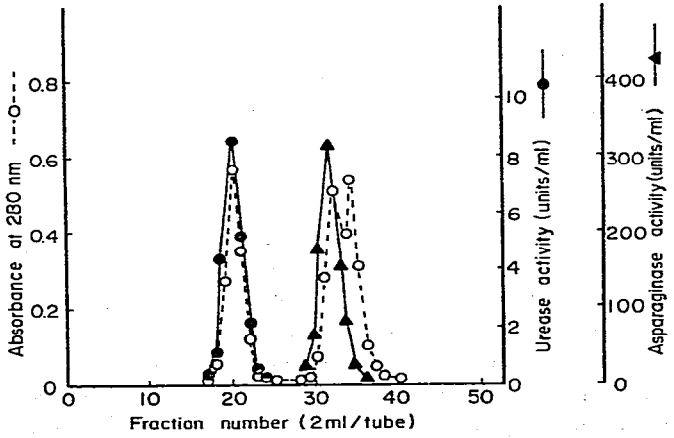

Fig. 2. Gel filtration of proteins on a column of cross-linked and desulphated κ-carrageenan beads. Urease (10 mg), asparaginase (5 mg) and bovine serum albumin (5 mg) were dissolved in 1 ml of 0.01 *M* phosphate buffer (pH 7.0) containing 0.05 *M* KCl. The protein solution was applied to a column (22 × 2.5 cm I.D.) of cross-linked and desulphated κ-carrageenan beads. Elution was carried out with the same buffer at 4°C. The flow-rate was kept at 10 ml/h, and fractions of 2 ml were collected.

urease, 0.59 for asparaginase and 0.63 for bovine serum albumin. This behaviour of gel filtration is similar to that observed using agarose beads. These results show that the cross-linked and desulphated κ-carrageenan beads are a useful carrier for molecular sieving of proteins.

Protein adsorption on tannin–carrageenan beads

In previous papers[7,10], we reported that chinese gallotannin used as a protein-precipitating agent can be covalently bound to aminohexyl-cellulose, and the immobilized tannin is useful as a specific adsorbent for proteins. In order to discover a more advantageous protein adsorbent, tannin–carrageenan beads and various tannin–matrix complexes were prepared, and their adsorption capacities for glucoamylase compared. The results are shown in Table I. The tannin–κ-carrageenan beads adsorbed glucoamylase to a high level in comparison with other tannin–matrix complexes containing sulphate groups, and the adsorption capacity of tannin–κ-carrageenan beads for the enzyme was similar to that of tannin immobilized with aminohexyl-cellulose. Thus it is considered that κ-carrageenan beads are a suitable matrix for binding tannin, and the tannin–κ-carrageenan beads are advantageous as a protein adsorbent.

TABLE I

ADSORPTION CAPACITY OF VARIOUS TANNIN–MATRIX COMPLEXES FOR GLUCOAMYLASE

Tannin–matrix complex (1 g wet weight) was suspended in 40 ml of glucoamylase solution (10 mg protein/ml of 0.1 *M* acetate buffer, pH 4.3) and the mixture was shaken at 25°C for 2 h. The mixture was then filtered and the complex was washed with water. The amount of protein adsorbed on the complex was calculated from the difference between the protein concentration in the initial solution and that in the filtrate.

Tannin–matrix complex	*Adsorption capacity for glucoamylase (mg/ml of adsorbent)*
Tannin–Fe–carrageenan beads	48.0
Tannin–Fe–carrageenan powder	8.2
Tannin–Fe–agar beads	14.4
Tannin–Fe–agar powder	8.0
Tannin–Fe–Amberlite IR-120	8.0
Tannin–Fe–SP-Sephadex C-25	30.0
Tannin–aminohexyl-cellulose	57.1

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