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CHARACTERIZATION OF TSK-GEL CM-TOYOPEARL 650 ION EXCHANGER

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SUMMARY

The basic properties and applications to chromatographic separations of the weak cation exchanger TSK-GEL CM-Toyopearl 650 were investigated. It was found to be an ideal support for the medium-performance ion-exchange chromatography of biological substances such as proteins.

INTRODUCTION

Although high-performance liquid chromatography is widely used in many fields, high-performance preparative columns are very expensive and the scale-up of analytical separations is seriously inhibited. For this reason, supports for medium-performance liquid chromatography, which are of large particle size, inexpensive and easy to pack, have been required[1].

TSK-GEL Toyopearl (Toyo Soda, Tokyo, Japan), introduced several years ago, is a support of this kind and has been demonstrated to be an excellent support for medium-performance gel filtration[2]. DEAE-Toyopearl 650, a weak anion exchanger derived from Toyopearl HW65, also has been found to fulfill the above requirements for medium-performance liquid chromatography[3]. In addition, CM-Toyopearl 650, a weak cation exchanger, has become commercially available recently. According to the manufacturer, it was derived by introducing carboxymethyl groups into Toyopearl HW65. The basic properties and applications to chromatographic separations of this cation exchanger are described in this paper.

EXPERIMENTAL

All measurements were performed on CM-Toyopearl 650S (superfine grade) and 650M (medium grade) of lot No. CM65002M. The same materials are available from E. Merck (Darmstadt, F.R.G.).

The titration curve was measured with a Model RAT-11 autotitrator (Hiranuma, Ibaragi, Japan). CM-Toyopearl 650M (10 ml in the swollen state) was titrated in 100 ml of 0.5 *M* sodium chloride solution with 0.5 *M* sodium hydroxide solution.

The adsorption capacity for protein was determined by the static method.

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CM-Toyopearl 650M (3 ml) was mixed with 10 ml of a 2% solution of bovine serum albumin and 0.01 *M* acetate buffer of pH 5.0 (adsorption buffer) was added to make the total volume 50 ml. After the mixture had been left at 25°C for 30 min with occasional swirling, it was filtered through filter-paper, washed with adsorption buffer and then washed repeatedly with *ca.* 15 ml of 0.01 *M* acetate buffer of pH 5.0 containing 0.5 *M* sodium chloride (desorption buffer). The filtrate containing desorption buffer was pooled until it became 100 ml in total and the protein in the filtrate was determined spectrophotometrically at 280 nm.

The recovery of proteins was evaluated on a CM-Toyopearl 650M column (15 × 1.0 cm I.D.). Proteins of 12 mg each were applied to the column equilibrated with the adsorption buffer at a flow-rate of 1 ml/min. After elution for 3 min with the adsorption buffer, the adsorbed proteins were washed out in the desorption buffer. The column effluents were collected for 50 min after sample application. The proteins in the effluents were determined spectrophotometrically at 280 nm.

The pore size was evaluated by measuring the relationship between molecular weight and elution volume, *i.e.*, the molecular weight calibration graph in gel filtration, for protein and polyethylene glycol. Proteins were measured on a CM-Toyopearl 650M column (15 × 1.6 cm I.D.) in 0.1 *M* Tris–HCl buffer of pH 8.0 containing 0.75 *M* sodium chloride with UV detection at 280 nm. The buffer of high pH and with a high salt concentration was employed in order to minimize ionic interactions between the proteins and the ion exchanger. Polyethylene glycols with narrow molecular weight distributions were measured on the same column in 0.1 *M* sodium chloride solution with refractive index detection. The flow-rate was 1 ml/min.

The mechanical stability was evaluated by measuring the relationship between flow-rate and pressure drop on CM-Toyopearl 650S and 650M columns (15 × 1.6 cm I.D.) in 0.1 *M* sodium chloride solution at 25°C.

The swelling properties were evaluated by measuring the bed volume in 0.05 *M* phosphate buffers of pH 4–9 and in 0.02 *M* acetate buffers of pH 5.0 containing

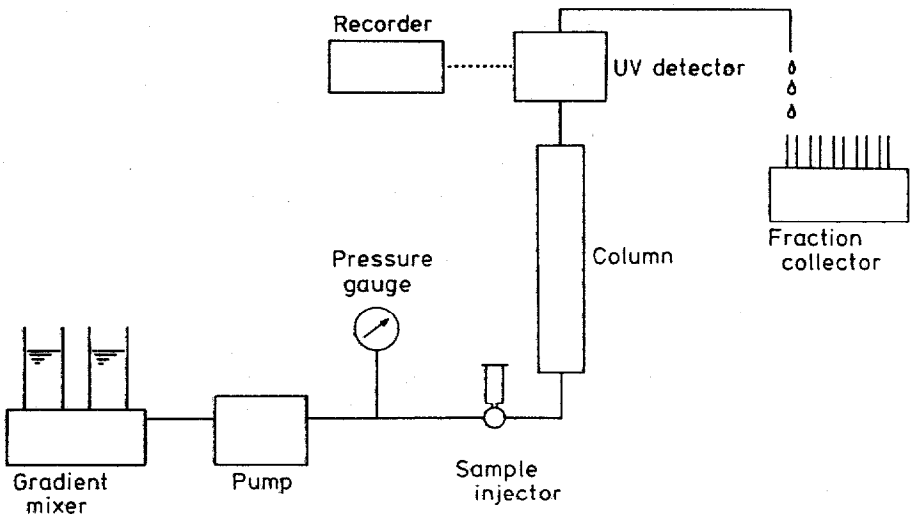

Fig. 1. Schematic diagram of the set-up for ion-exchange chromatography on CM-Toyopearl 650.

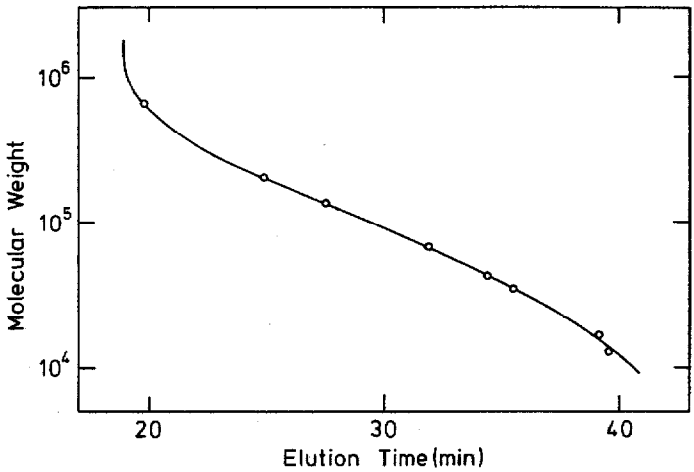

Fig. 2. Calibration graph of TSK-GEL G3000SW two-column system for globular protein.

0–0.5 *M* sodium chloride. CM-Toyopearl 650M was filled into a 60 × 1.6 cm I.D. column to a height of *ca.* 40 cm. The exact bed height in a buffer was determined after elution of the buffer had been continued for 1 day at a flow-rate of *ca.* 1 ml/min. This was repeated for all buffers with a single column.

The chemical stability was evaluated by measuring the change in ion-exchange capacity on treatment of CM-Toyopearl 650M with 0.5 *M* sodium hydroxide solution and 0.5 *M* hydrochloric acid at 25°C. The ion-exchange capacity was determined by measuring the titration curve.

The thermal stability was evaluated by measuring the change in ion-exchange capacity on treatment of CM-Toyopearl 650M with 0.1 *M* phosphate buffer of pH 7.0 at 120°C.

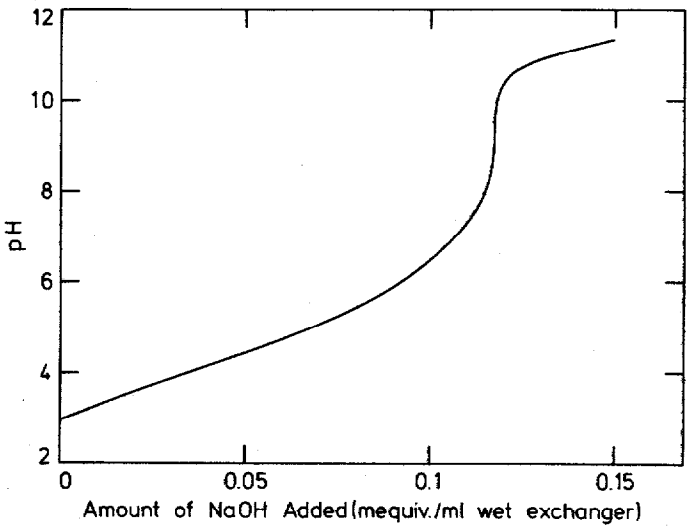

Fig. 3. Titration curve of CM-Toyopearl 650M.

TABLE I

RECOVERY OF PROTEINS FROM CM-TOYOPEARL 650M

Protein	*Recovery (%)*	*Protein*	*Recovery (%)*
Thyroglobulin	95	β-Lactoglobulin	101
γ-Globulin	100	Myoglobin	95
Bovine serum albumin	101	Lysozyme	96
Haemoglobin	75	Ribonuclease	98
Ovalbumin	83	Cytochrome *c*	97

The application to chromatographic separations was investigated with the set-up shown in Fig. 1. Egg white, calf serum (Wako, Osaka, Japan), bovine pancrease ribonuclease (Type I-AS; Sigma, St. Louis, MO, U.S.A.) and α-chymotrypsin (Sigma) were separated by linear gradient elution of two buffers at a flow-rate of 2 ml/min at 25°C. A glass column (15 × 1.6 cm I.D.) with end-fittings at both ends was employed. The packing of the column was carried out by the semi-constant pressure method[4] with a peristaltic pump. The column effluent was monitored continuously with a UV detector at 280 nm. Then the portion of interest was collected manually and subjected to analytical gel filtration and ion-exchange chromatography to examine the degree of purification. In the separation of α-chymotrypsin, the collected fraction was subjected to the determination of enzymatic activity, which was performed using N-benzoyl-L-tyrosine ethyl ester as substrate[5].

The analytical gel filtration was performed on a Toyo Soda TSK-GEL G3000SW (60 × 0.75 cm I.D.) two-column system in 0.1 *M* phosphate buffer of pH 6.8 containing 0.1 *M* sodium sulphate at a flow-rate of 1 ml/min at 25°C. Under these

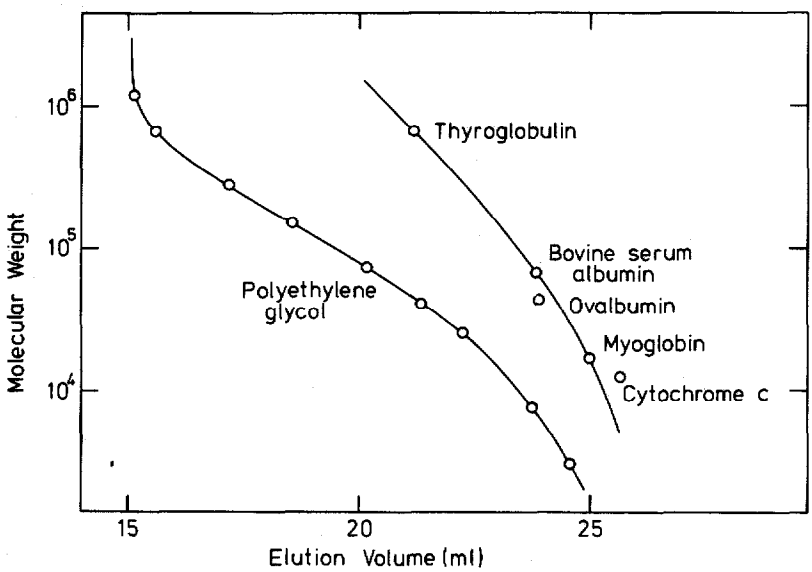

Fig. 4. Plots of molecular weights against elution volumes for proteins and polyethylene glycols on CM-Toyopearl 650M.

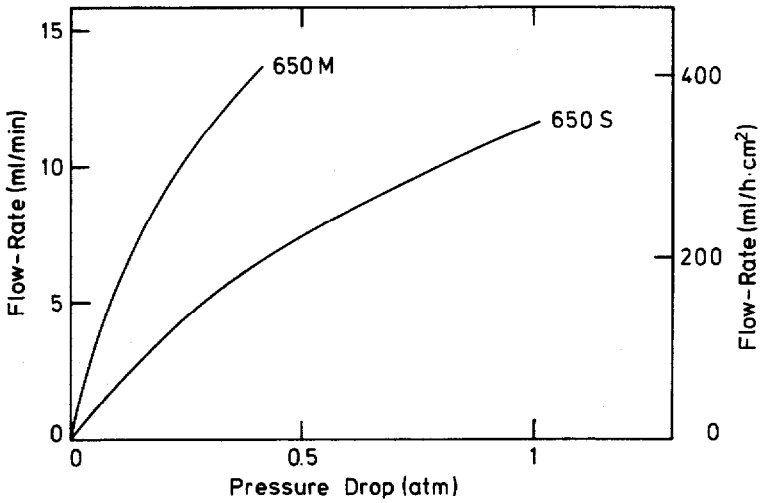

Fig. 5. Flow-rate as a function of pressure drop on CM-Toyopearl 650S and 650M columns (15 × 1.6 cm I.D.).

conditions, proteins in the molecular weight range 10,000–500,000 could be separated in *ca.* 40 min, as can be seen from the molecular weight calibration graph in Fig. 2. Analytical ion-exchange chromatography was performed on TSK-GEL IEX-545 DEAE SIL, IEX-535 CM SIL or IEX-645 DEAE with linear gradient elution of two buffers at a flow-rate of 1 ml/min at 25°C. Each column was 15 × 0.6 cm I.D. A UV detector at 280 nm was also used in analytical separations.

A mixture of ribonuclease (Type I-A; Sigma) and cytochrome *c* (Sigma) was also separated in order to compare CM-Toyopearl 650 with conventional and medium-performance cation exchangers that have been employed in the preparative separation of proteins. CM-Sepharose CL-6B (Pharmacia, Uppsala, Sweden), CM-cellulose CM-52 (Whatman, Maidstone, U.K.), CM-Bio-Gel A (Bio-Rad Labs.,

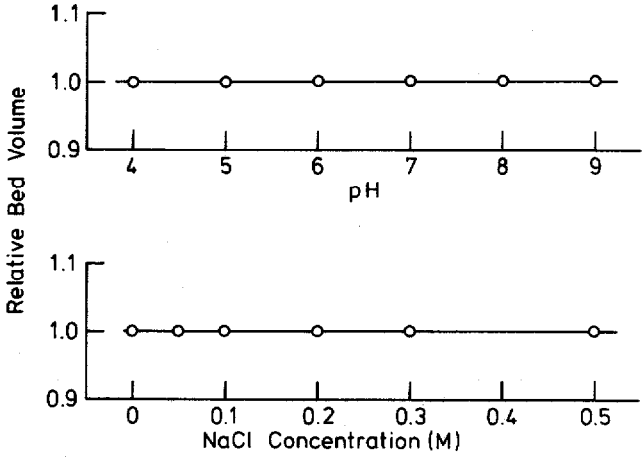

Fig. 6. Bed volume of CM-Toyopearl 650M in 0.05 *M* phosphate buffers of pH 4–9 and in 0.02 *M* acetate buffers of pH 5.0 containing 0–0.5 *M* sodium chloride.

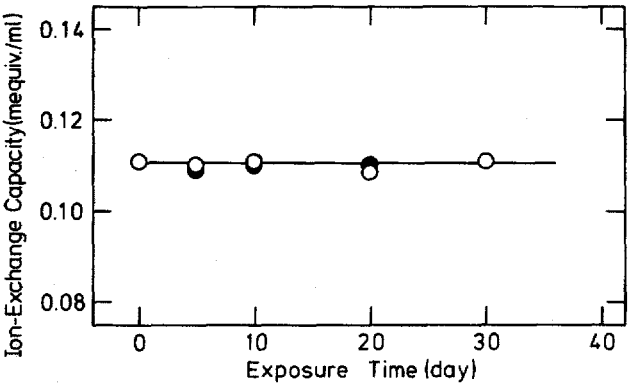

Fig. 7. Chemical stability of CM-Toyopearl 650M in alkaline and acidic aqueous solutions at 25°C. ●, 0.5 *M* sodium hydroxide solution; ○, 0.5 *M* hydrochloric acid.

Richmond, CA, U.S.A.) and CM-Trisacryl M (LKB, Bromma, Sweden) were employed for the comparison.

RESULTS AND DISCUSSION

Basic properties

Fig. 3 shows the titration curve of CM-Toyopearl 650M. The ion-exchange capacity is *ca.* 0.12 mequiv./ml, which is comparable to those of many commercial CM-type ion exchangers. The pK_a is *ca.* 4.7, which is slightly higher than those of many other CM-type ion exchangers.

The adsorption capacity for bovine serum albumin was 45 mg/ml. Some commercial CM-type ion exchangers have adsorption capacities of more than 100 mg/ml. The low adsorption capacity of CM-Toyopearl 650 is probably due to its large pore size. In contrast, it is assumed that CM-Toyopearl 650 has a higher adsorption capacity for proteins with very high molecular weights than other CM-type ion exchangers, as observed for DEAE-Toyopearl 650 (ref. 6).

The recovery of proteins is summarized in Table I. The proteins were recovered

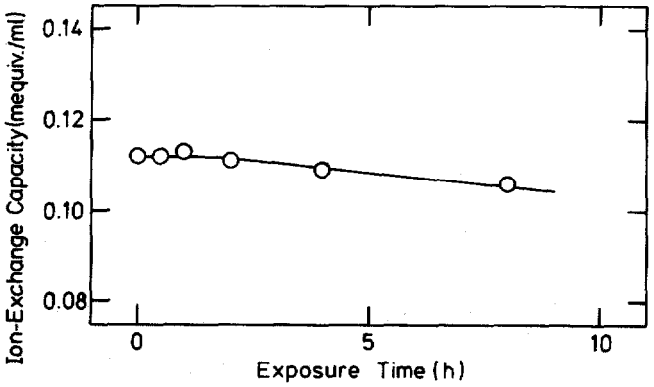

Fig. 8. Thermal stability of CM-Toyopearl 650M in 0.1 *M* phosphate buffer of pH 7.0 at 120°C.

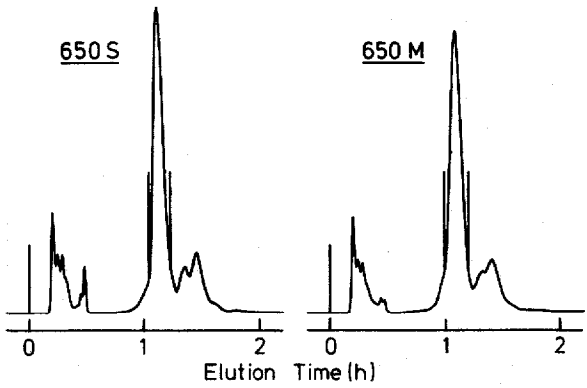

Fig. 9. Separation of egg white proteins by ion-exchange chromatography on CM-Toyopearl 650S and 650M. Sample load, 1 ml of 1:4 diluted solution; column size, 15 × 1.6 cm I.D.; elution, linear gradient from 0.02 *M* acetate buffer of pH 4.5 (200 ml) to 0.02 *M* acetate buffer of pH 4.5 containing 0.2 *M* sodium chloride (200 ml); flow-rate, 2 ml/min; temperature, 25°C; detection, UV absorbance at 280 nm.

almost quantitatively, except for haemoglobin and ovalbumin, for which slightly low recoveries were observed. Haemoglobin seems to be irreversibly adsorbed on many types of supports.

Fig. 4 shows the molecular weight calibration graphs in the gel filtration of proteins and polyethylene glycols. As the total exclusion volume was *ca.* 15 ml, the exclusion limit for proteins is presumed to be tens of millions of daltons. Therefore, CM-Toyopearl 650 should be applicable to proteins with very high molecular weights. The exclusion limit for polyethylene glycol is *ca.* 1,000,000.

Fig. 5 shows the relationship between flow-rate and pressure drop. CM-Toyopearl 650 was found to be mechanically so stable that flow-rates higher than 300

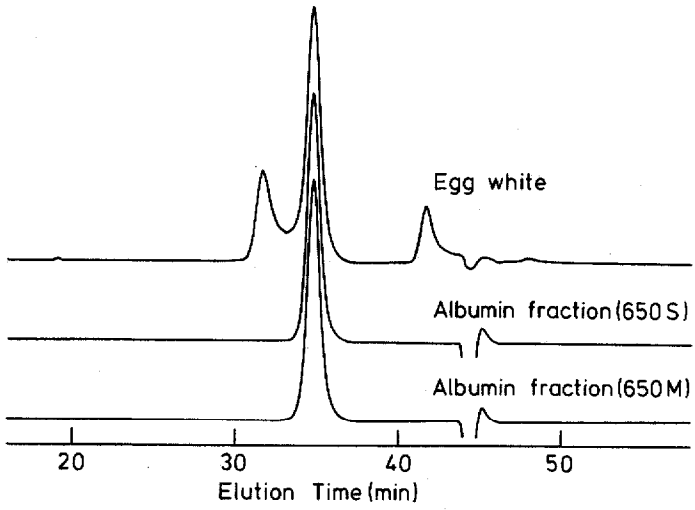

Fig. 10. Analytical gel filtration chromatograms of egg white and albumin fractions on TSK-GEL G3000SW two-column system.

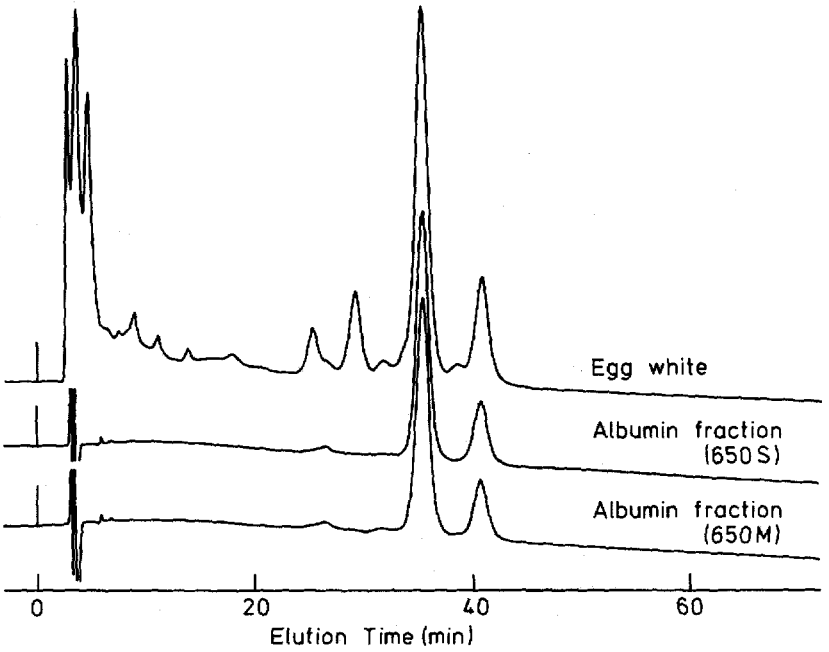

Fig. 11. Analytical ion-exchange chromatograms of egg white and albumin fractions on TSK-GEL IEX-545 DEAE SIL with 180-min linear gradient elution from 0.1 *M* Tris–HCl buffer of pH 7.5 to 0.1 *M* Tris–HCl buffer of pH 7.5 containing 0.2 *M* sodium chloride.

ml/h · cm² were attainable on a 15 × 1.6 cm I.D. column. The upper limit of flow-rate or pressure drop was not established.

Fig. 6 shows the dependence of the swollen volume of CM-Toyopearl 650M on the pH and salt concentration of the buffer. No change in the swollen volume was observed in the pH range 4–9 and at sodium chloride concentrations in the range 0–0.5 *M*. Therefore, it should be possible to repeat separations and re-equilibrations on a column without re-packing.

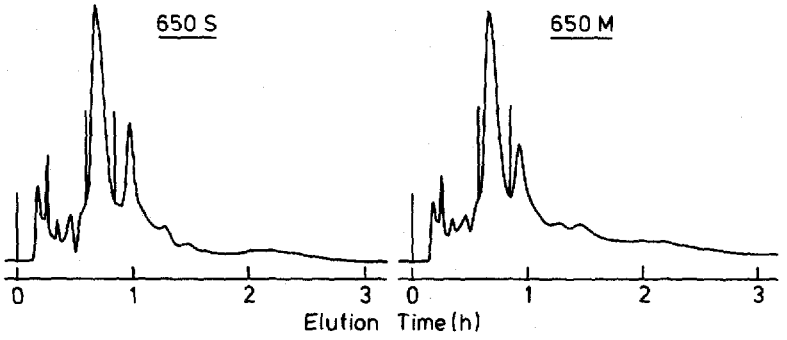

Fig. 12. Separation of calf serum proteins by ion-exchange chromatography on CM-Toyopearl 650S and 650M. Sample load, 0.5 ml of 1:2 diluted solution; column size, 15 × 1.6 cm I.D.; elution, linear gradient from 0.05 *M* acetate buffer of pH 5.0 (200 ml) to 0.05 *M* acetate buffer of pH 5.0 containing 0.5 *M* sodium chloride (200 ml); flow-rate, 2 ml/min; temperature, 25°C; detection, UV absorbance at 280 nm.

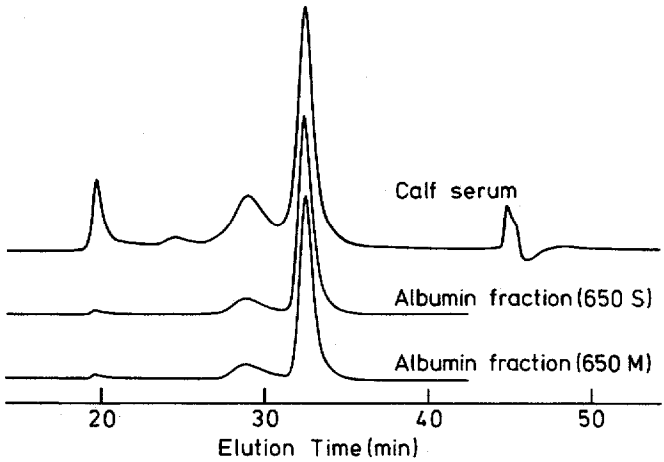

Fig. 13. Analytical gel filtration chromatograms of calf serum and albumin fractions on TSK-GEL G3000SW two-column system.

Fig. 7 shows the change in the ion-exchange capacity of CM-Toyopearl 650M on treatment with 0.5 *M* sodium hydroxide solution and 0.5 *M* hydrochloric acid at 25°C. The ion-exchange capacity remained unchanged for 30 days. Consequently, there should be no problem in washing CM-Toyopearl 650 with alkaline and acidic aqueous solutions for regeneration.

Fig. 8 shows the change in ion-exchange capacity of CM-Toyopearl 650M on treatment with 0.1 *M* phosphate buffer of pH 7.0 at 120°C. Only a slight decrease in ion-exchange capacity was observed during 8 h. Accordingly, CM-Toyopearl 650 can be sterilized by autoclaving at 120°C.

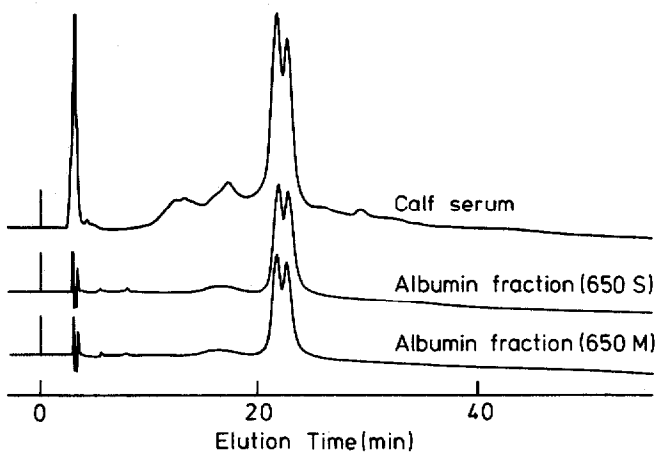

Fig. 14. Analytical ion-exchange chromatograms of calf serum and albumin fractions on TSK-GEL IEX-645 DEAE with 60-min linear gradient elution from 0.05 *M* Tris–HCl buffer of pH 7.5 to 0.05 *M* Tris–HCl buffer of pH 7.5 containing 0.5 *M* sodium chloride.

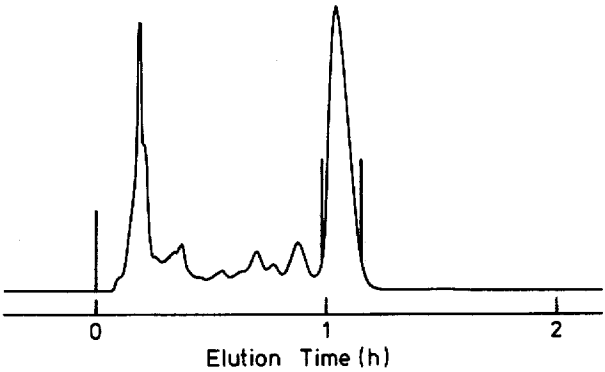

Fig. 15. Separation of commercial ribonuclease by ion-exchange chromatography on CM-Toyopearl 650S. Sample load, 30 mg in 1 ml of initial buffer; column size, 15 × 1.6 cm I.D.; elution, linear gradient from 0.01 *M* Tris–HCl buffer of pH 8.0 (200 ml) to 0.01 *M* Tris–HCl buffer of pH 8.0 containing 0.15 *M* sodium chloride (200 ml); flow-rate, 2 ml/min; temperature, 25°C; detection, UV absorbance at 280 nm.

Application to chromatographic separations

Fig. 9 shows the results of the separation of egg white. The peaks corresponding to albumin between the two vertical lines were fractioned and subjected to analytical gel filtration and ion-exchange chromatography. The results are shown in Figs. 10 and 11, indicating that fairly pure albumin was obtained with only a single process by ion-exchange chromatography on CM-Toyopearl 650. The peaks at an elution time of *ca.* 45 min in the gel filtration chromatograms of the fractions and the peaks just after the sample injection in the ion-exchange chromatograms of the fractions are solvent peaks.

Fig. 12 shows the results of the separation of calf serum. The peaks corresponding to albumin between the two vertical lines were fractioned and subjected to analytical gel filtration and ion-exchange chromatography. The results are shown in Figs. 13 and 14. Albumin was purified to a considerable extent also in this instance.

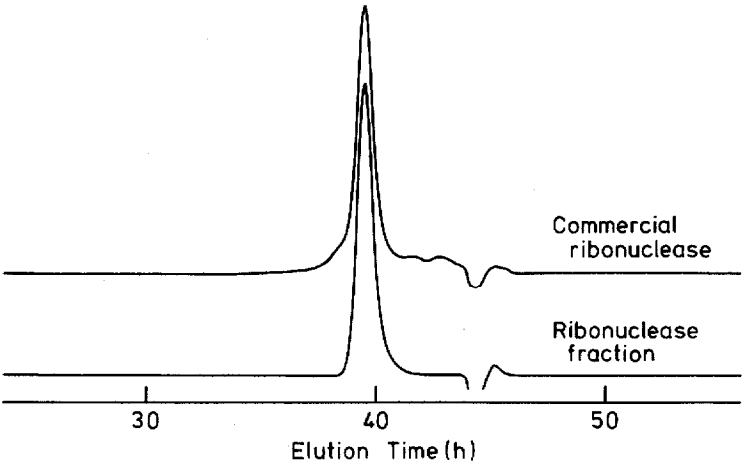

Fig. 16. Analytical gel filtration chromatograms of commercial ribonuclease and ribonuclease fraction on TSK-GEL G3000SW two-column system.

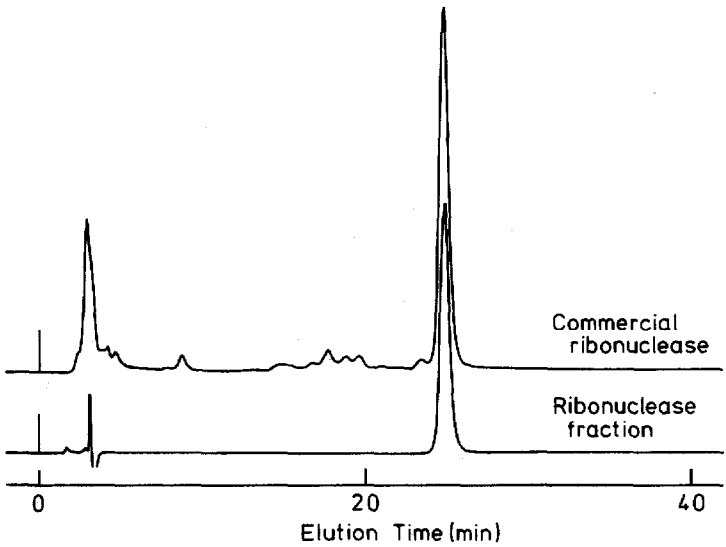

Fig. 17. Analytical ion-exchange chromatograms of commercial ribonuclease and ribonuclease fraction on TSK-GEL IEX-535 CM SIL with 120-min linear gradient elution from 0.05 *M* Tris–HCl buffer of pH 7.5 to 0.05 *M* Tris–HCl buffer of pH 7.5 containing 0.2 *M* sodium chloride.

Superfine grade material with a smaller particle size provided slightly better resolution than medium grade material.

Fig. 15 shows the result of the separation of commercial ribonuclease. The peak corresponding to ribonuclease between the two vertical lines was fractionated and subjected to analytical gel filtration and ion-exchange chromatography. The results are shown in Figs. 16 and 17. Impurities are seen only in ion-exchange chromatogram and only in small amounts.

Fig. 18 shows the separation of α-chymotrypsin. The peak corresponding to α-chymotrypsin between two vertical lines was fractionated and subjected to the determination of enzymatic activity. The recovery of enzymatic activity in the fraction was 96%. Therefore, high recoveries of both protein mass and enzymatic activity can be expected in ion-exchange chromatography on CM-Toyopearl 650.

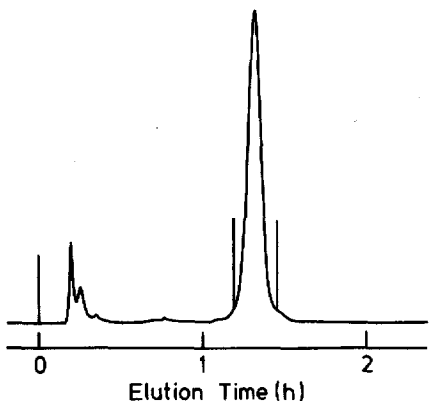

Fig. 18. Separation of commercial α-chymotrypsin by ion-exchange chromatography on CM-Toyopearl 650M. Sample load, 5 mg in 1 ml of initial buffer; column size, 15 × 1.6 cm I.D.; elution. linear gradient from 0.02 *M* Tris–HCl buffer of pH 7.0 (250 ml) to 0.02 *M* Tris–HCl buffer of pH 7.0 containing 0.5 *M* sodium chloride (250 ml); flow-rate, 2 ml/min; temperature, 25°C; detection, UV absorbance at 280 nm.

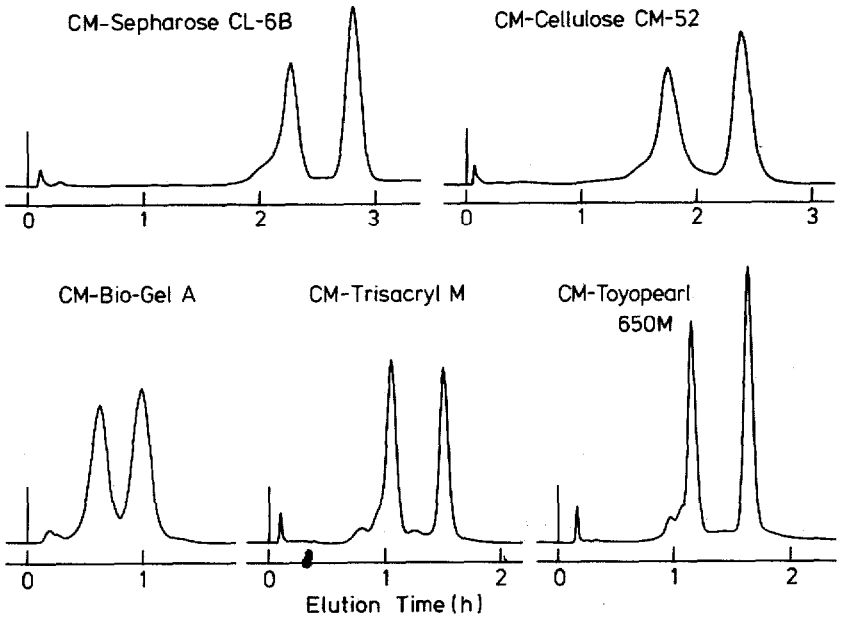

Fig. 19. Separation of a mixture of ribonuclease (9 mg, first peak) and cytochrome *c* (3 mg, second peak) by ion-exchange chromatography on CM-Sepharose CL-6B, CM- cellulose CM-52, CM-Bio-Gel A, CM-Trisacryl M and CM-Toyopearl 650M. Column size, 15 × 1.6 cm I.D.; elution, linear gradient from 0.04 *M* citrate buffer of pH 5.0 (200 ml) to 0.04 *M* citrate buffer of pH 5.0 containing 0.4 *M* sodium chloride (200 ml); flow-rate, 2 ml/min; temperature, 25°C; detection, UV absorbance at 280 nm.

Fig. 19 shows the separations of a mixture of ribonuclease and cytochrome *c* obtained on five commercial ion exchangers. The best separation was attained on CM-Toyopearl 650M. CM-Toyopearl 650M also provided the narrowest peak, which means that the dilution of the sample during chromatography is least on CM-Toyopearl 650M. Fig. 19 also indicates that proteins elute at lower salt concentration on CM-Toyopearl 650 than on CM-Sepharose CL-6B and CM-cellulose CM-52.

CONCLUSION

CM-Toyopearl 650 provides high resolution, especially in the separation of proteins at medium speed. CM-Toyopearl 650 is an ideal support for medium-performance ion-exchange chromatography and should be very suitable for scale-up up to industrial-scale separations owing to its high mechanical stability, stable swollen volume independent of pH and salt concentration and high chemical stability.

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