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NEW ION EXCHANGER FOR THE SEPARATION OF PROTEINS AND NUCLEIC ACIDS

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SUMMARY

The basic properties of the new weak anion exchanger TSK-GEL IEX-645 DEAE and applications to the separations of proteins and nucleic acids were investigated. IEX-645 DEAE was found to be very versatile for high-performance ion-exchange chromatography of biopolymers. It was especially superior in applications at high pH and to high-molecular-weight samples.

INTRODUCTION

The high-performance ion-exchange chromatography of proteins as developed by Chang *et al.*[1] is gaining wide acceptance. For this technique, silica-based column materials of pore diameter 250–300 Å are mainly used. Such materials have highly appropriate physical properties, but there are some problems in chemical stability. They are especially inconvenient in anion-exchange chromatography since they are unstable in basic buffers.

Columns packed with a new chemically stable ion exchanger derived from TSK-GEL G5000PW, which is a hydrophilic-polymer-based material of large pore size for high-performance gel filtration, have become commercially available under the trade-name of TSK-GEL IEX-645 DEAE (Toyo Soda, Tokyo, Japan). According to the manufacturer, this new ion exchanger was developed by introducing diethylaminoethyl groups into G5000PW of particle diameter 10 μm.Its basic properties and applications to the separation of proteins and nucleic acids are described in this paper.

EXPERIMENTAL

The titration curve was established with a Model RAT-11 autotitrator (Hiranuma, Ibaragi, Japan). A 10-ml volume of IEX-645 DEAE in the swollen state was titrated in 100 ml of 0.5 *M* sodium chloride with 0.5 *M* hydrochloric acid.

The adsorption capacity for proteins was determined by the static method. A 3-ml volume IEX-645 DEAE was mixed with 10 ml of a 1.5% solution of thyroglobulin, ferritin or bovine serum albumin, and 0.05 *M* Tris–HCl buffer of pH 8.3 (adsorption buffer) was added to give a total volume of 50 ml. After the mixture had been left at 25°C for 30 min with occasional swirling, it was filtered through filter-paper, washed

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with adsorption buffer and then washed repeatedly with *ca.* 15 ml of 0.05 *M* Tris–HCl buffer of pH 8.3 containing 0.5 *M* sodium chloride (desorption buffer). The filtrate containing desorption buffer was pooled until it had a total volume of 100 ml and the protein content was determined spectrophotometrically at 280 nm.

The recovery of proteins from IEX-645 DEAE was evaluated by injecting proteins (0.4 mg) into a column (15 × 0.6 cm I.D.) equilibrated with 0.05 *M* Tris–HCl buffer of pH 8.3 containing 0.5 *M* sodium chloride Chromatography at a flow-rate of 1 ml/min and spectrophotometric determination of the proteins (at 225 nm) in the column effluent pooled for 20 min after protein injection, gave the recovery.

The pore size of IEX-645 DEAE was evaluated by measuring the relationship between molecular weight and elution volume, *i.e.*, the molecular weight calibration curve in gel filtration, for protein and polyethylene glycol. Proteins were measured on a column (15 × 1.6 cm I.D.) with 0.1 *M* phosphate buffer of pH 5.5 containing 0.5 *M* sodium chloride by UV detection at 280 nm. Polyethylene glycols with narrow molecular-weight distributions were measured on the same column in distilled water by refractive index detection. The flow-rate was 1 ml/min.

Ion-exchange chromatographic measurements were carried out on a column (15 × 0.6 cm I.D.) with a high-speed liquid chromatograph Model HLC-803C equipped with a gradient generator Model GE-2 and variable wavelength UV detector Model UV-8 (Toyo Soda). Proteins and nucleic acids were separated by linear gradient elution with two buffers at a flow-rate of 1 ml/min at 25°C and detected at 280 nm and 260 nm, respectively. In the separation of some enzymes the column effluent was collected and the enzymatic activity was determined.

All proteins and total *Escherichia coli* RNA were commercial products. Total *E. coli* RNA contains 4S tRNA (MW 23,000 – 27,000), 5S rRNA (39,000), 16S rRNA (560,000) and 23S rRNA (1,100,000). HaeIII-cleaved pBR322 DNA was a gift from Dr. Murotsu (Osaka University). This sample contains 22 double stranded restriction fragments of 7, 11, 18, 21, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540 and 587 base pairs.

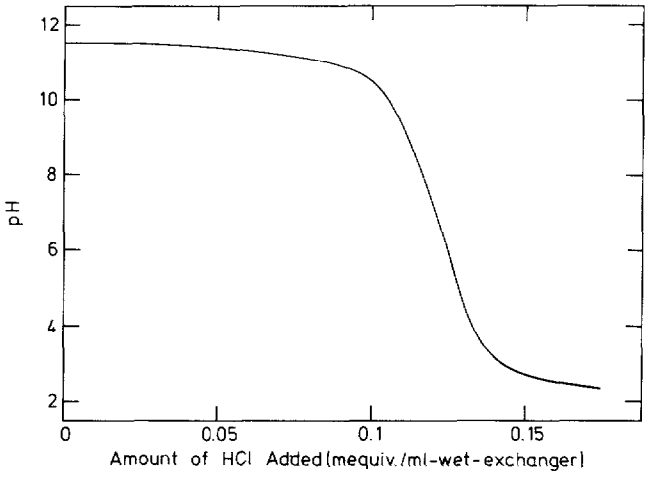

Fig. 1. Titration curve of IEX-645 DEAE.

TABLE I

ADSORPTION CAPACITY OF IEX-645 DEAE FOR PROTEINS IN 0.05 *M* TRISH–HCl BUFFER OF pH 8.3 AT 25°C

Protein	*Adsorption capacity (mg/ml)*
Thyroglobulin	35
Ferritin	32
Bovine serum albumin	43

RESULTS AND DISCUSSION

Basic properties

Fig. 1 shows the titration curve, indicating that IEX-645 DEAE contains only one type of ionic group with $pK_a \approx 11.5$ and shows little buffering activity at pH < 10. Therefore, little pH fluctuation of the eluent in the column, little change in adsorption strength of the ion exchanger and rapid re-equilibration of the column can be expected in ion-exchange chromatorgraphy on IEX-645 DEAE with pH gradient elution. Furthermore, owing to the high pK_a value, IEX-645 DEAE may be applicable to the separation of some basic proteins. The ion-exchange capacity is *ca.* 0.12 mequiv./ml.

The adsorption capacity for proteins is summarized in Table I. IEX-645 DEAE has a fairly high adsorption capacity even for ferritin and thyroglobulin, with molecular weights of 440,000 and 660,000, respectively. Therefore, it should be applicable to proteins with very high molecular weights.

The recovery of proteins was almost quantitative for all proteins investigated (Table II). The recovery of enzymatic activity was also high (> 80%) as shown later.

Fig. 2 shows the molecular-weight calibration obtained by gel filtration of proteins and polyethylene glycols. Proteins as well as polyethylene glycols were separated according to their molecular size without ionic interactions because a buffer of low pH and with a high salt concentration was employed as the eluent. The exclusion limit for

TABLE II

RECOVERY OF PROTEINS FROM IEX-645 DEAE IN 0.05 *M* TRIS–HCl BUFFER OF pH 8.3 CONTAINING 0.5 *M* SODIUM CHLORIDE AT 25°C

Sample loading: 0.4 mg.

Protein	*Recovery (%)*
Thyroglobulin	91
Ferritin	98
γ-Globulin	98
Bovine serum albumin	99
Hemoglobin	94
Ovalbumin	99
β-Lactoglobulin	98
Trypsin inhibitor	101
Myoglobin	97

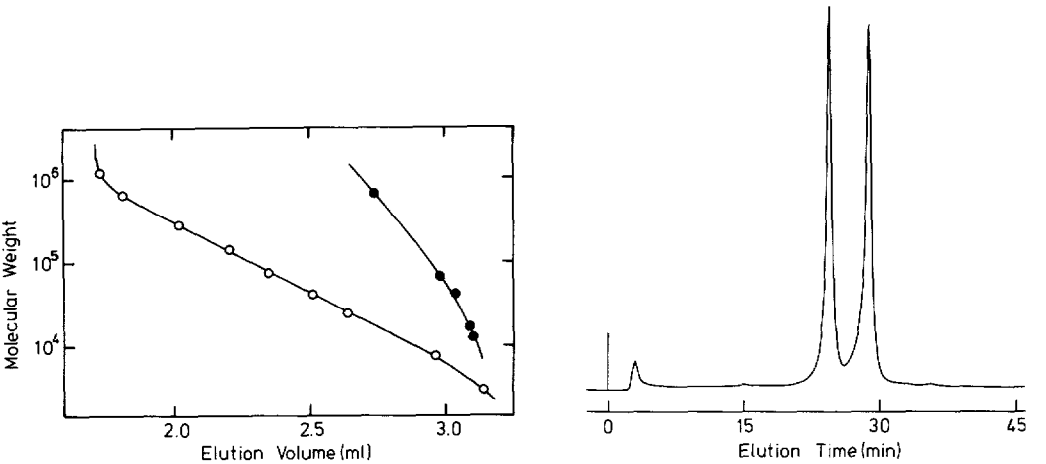

Fig. 2. Plots of molecular weights against elution volumes for proteins (○) and polyethylene glycols (●), chromatographed on an IEX-645 DEAE column (15 × 0.6 cm I.D.).

Fig. 3. Chromatogram of β-lactoglobulin (0.4 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-645 DEAE with a 60-min linear elution gradient from 0.02 *M* piperazine–HCl buffer of pH 6.0 to 0.02 *M* piperazine–HCl buffer of pH 6.0 containing 0.5 *M* sodium chloride, at a flow-rate of 1 ml/min. The piperazine–HCl buffer was prepared by dissolving 3.88 g piperazine · hexahydrate in 950 ml distilled water, adjusting to pH 6.0 with 1 *M* hydrochloric acid and diluting to 1000 ml with distilled water.

proteins is much higher than one million, suggesting that IEX-645 DEAE has a very large pore size. Therefore, high resolution is expected in protein separations[2]. The exclusion limit for polyethylene glycol is about one million.

Applications to protein separation

Figs. 3 and 4 show chromatrograms of bovine β-lactoglobulin (ICN Pharmaceuticals, Cleveland, OH, U.S.A.) and standard human serum (Boehringer, Mann-

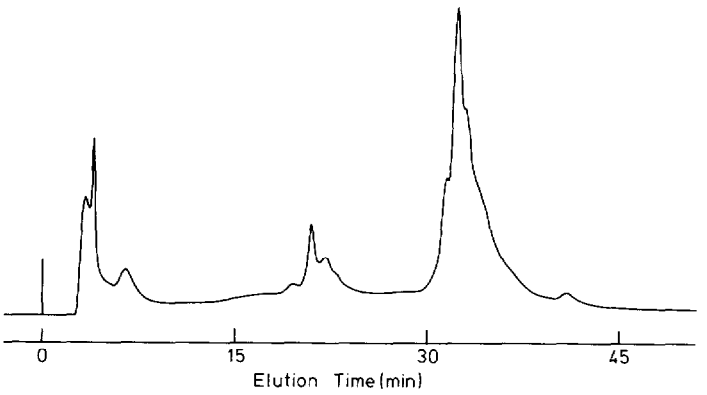

Fig. 4. Chromatogram of standard human serum (0.1 ml of 1:20 diluted serum) obtained by high performance ion-exchange chromatography on IEX-645 DEAE with a 60-min linear elution gradient from 0.05 *M* Tris–HCl buffer of pH 7.5 to 0.05 *M* Tris–HCl buffer of pH 7.5 containing 0.5 *M* sodium chloride, at a flow-rate of 1 ml/min.

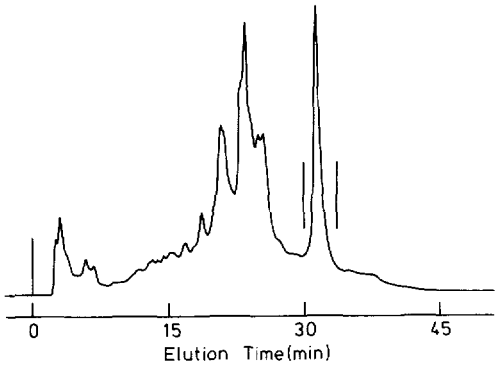

Fig. 5. Chromatogram of lipoxidase (1 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-645 DEAE with a 60-min linear gradient from 0.02 *M* ethanolamine–HCl buffer of pH 9.0 to 0.02 *M* ethanolamine–HCl buffer of pH 9.0 containing 0.5 *M* sodium chloride, at a flow-rate of 1 ml/min. The ethanolamine–HCl buffer was prepared by dissolving 1.22 g ethanolamine in 950 ml distilled water, adjusting to pH 9.0 with 1 *M* hydrochloric acid and diluting to 1000 ml with distilled water.

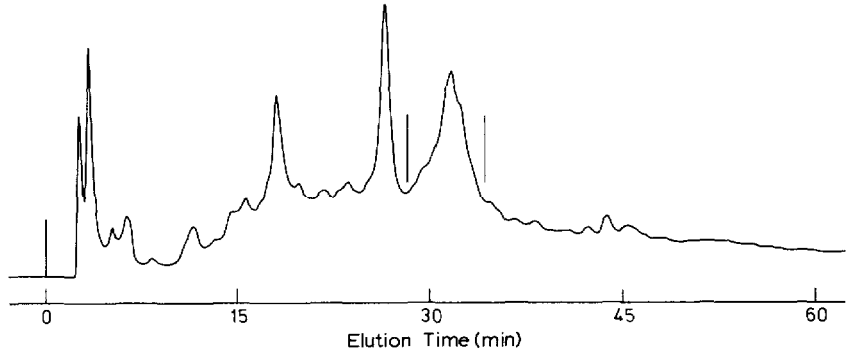

Fig. 6. Chromatogram of catalase (1 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-645 DEAE. Conditions as in Fig. 5.

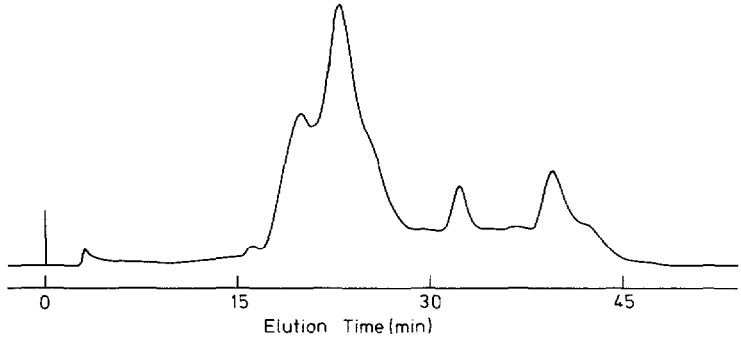

Fig. 7. Chromatogram of fibrinogen (0.5 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-645 DEAE with a 120-min linear gradient from 0.1 *M* Tris–HCl buffer of pH 7.5 to 0.1 *M* Tris–HCl buffer of pH 7.5 containing 1.0 *M* sodium chloride, at a flow-rate of 1 ml/min.

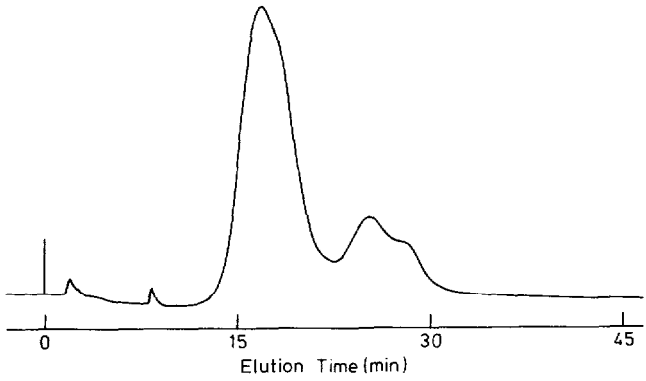

Fig. 8. Chromatogram of fibrinogen (0.5 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-545 DEAE SIL. Conditions as in Fig. 7.

heim, G.F.R.) as examples of separations of proteins with typical molecular weights. β-Lactoglobulin A chain (second peak) and B chain (first peak) are separated in Fig. 3. In Fig. 4, the peak eluted in *ca.* 33 min is albumin and the sharp peak eluted in *ca.* 19 min is transferrin. The analysis of these two samples under the same conditions on TSK-GEL IEX-545 DEAE SIL (Toyo Soda)[3], which is a silica-based weak anion exhanger of particle diameter 10 μm and pore diameter *ca.* 250 Å, provided almost identical chromatograms.

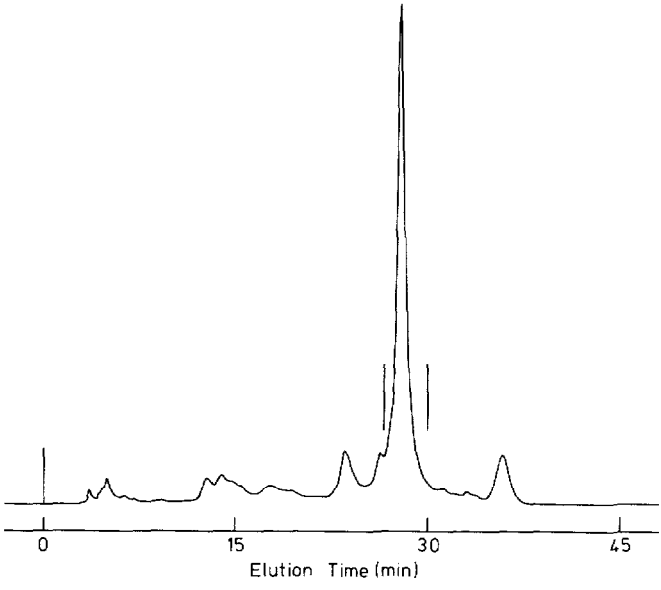

Fig. 9. Chromatogram of α-chymotrypsin (0.2 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-645 DEAE with a 60 min linear elution gradient from 0.02 *M* 1,3-diaminopropane–HCl buffer of pH 9.8 to 0.02 *M* 1,3-diaminopropane–HCl buffer of pH 9.8 containing 0.5 *M* sodium chloride, at a flow-rate of 1 ml/min. The 1,3-diaminopropane–HCl buffer was prepared by dissolving 1.48 g 1,3-diaminopropane in 950 ml distilled water, adjusting to pH 9.8 with 1 *M* hydrochloric acid and diluting to 1000 ml with distilled water.

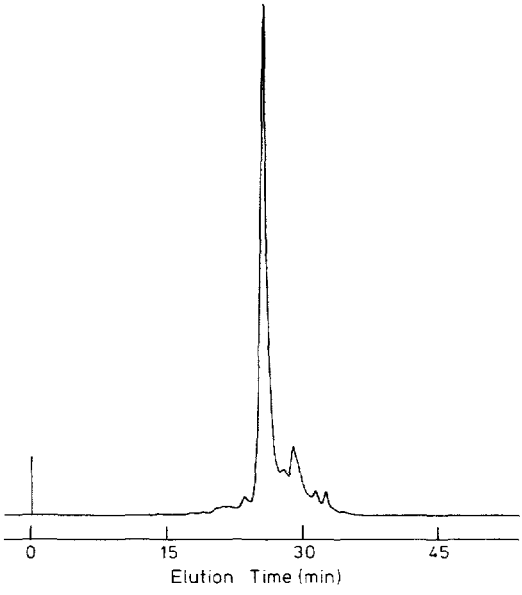

Fig. 10. Chromatogram of trypsinogen (0.3 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-645 DEAE. Conditions as in Fig. 9.

Figs. 5–7 show the chromatograms of lipoxidase (P-L Biochemicals, Milwaukee, WI, U.S.A.), catalase (Sigma, St. Louis, MO, U.S.A.) and fibrinogen (Wako, Osaka, Japan) of molecular weights of 100,000, 240,000 and 330,000, respectively, as examples of separations of high-molecular-weight proteins. In Fig. 5, the peak eluted in *ca* 32 min is lipoxidase and all other peaks are impurities in the sample. The buffer of pH 9.0 was employed for this separation because after separation, the enzymatic activity was measured at pH 9.0. When this sample was chromatographed in buffer of pH 7.5

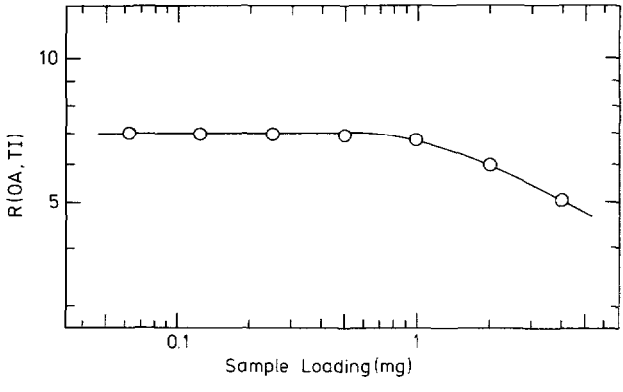

Fig. 11. Dependence of resolution on sample load in the separation of a mixture of equal amounts of ovalbumin and trypsin inhibitor on IEX-645 DEAE with a 60-min linear elution gradient from 0,05 *M* Tris–HCl buffer of pH 7.5 to 0.05 *M* Tris–HCl buffer of pH 7.5 containing 0.5 *M* sodium chloride, at a flow-rate of 1 ml/min.

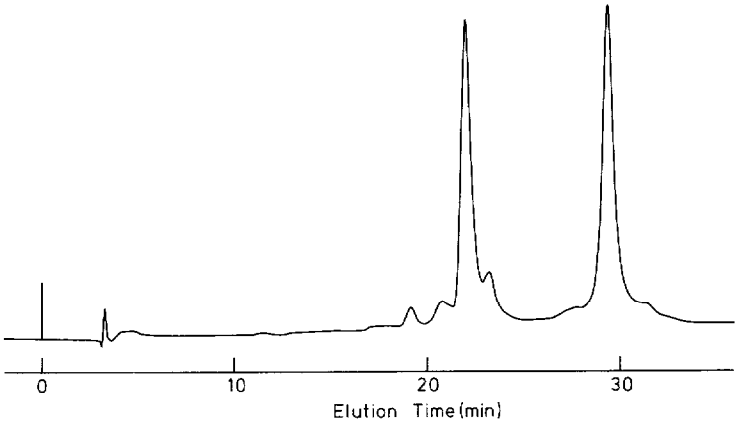

Fig. 12. Chromatogram of a mixture of equal amounts of ovalbumin and trypsin inhibitor. Conditions as in Fig. 11.

on IEX-645 DEAE and IEX-545 DEAE SIL, the peak of lipoxidase was slightly narrower on the former although similar elution patterns were obtained with both columns. In Fig. 6 catalase is present in the broad peak eluted in *ca.* 30 min. This sample also contains many impurities and catalase could not be separated as an discrete peak. Several peaks also appear in Fig. 7 although they have not been assigned. This sample was eluted earlier and was not well separated on IEX-545 DEAE SIL as seen in Fig. 8. Consequently, it may be said that IEX-645 DEAE provides a better separation than IEX-545 DEAE SIL for proteins of high molecular weight, roughly above 10^5 daltons.

Figs. 9 and 10 show chromatograms of α-chymotrypsin(Sigma) and trypsinogen (Sigma) of p*I* 8.5 and 9.3, respectively, as examples of separations of basic proteins. These separations performed with buffer of pH 9.8 were only possible because IEX-645 DEAE can be used at this high pH, they are impossible on silica-based IEX-545 DEAE SIL.

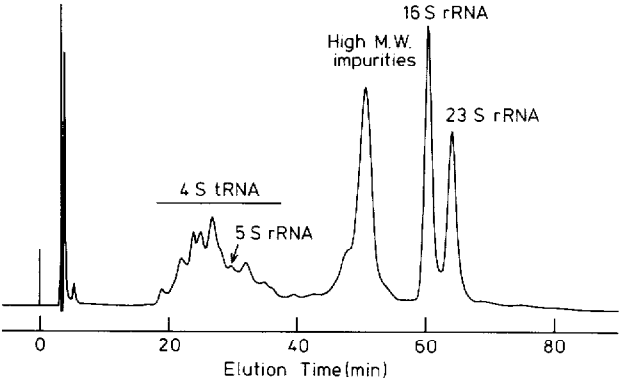

Fig. 13. Chromatogram of Total *E. coli* RNA obtained by high-performance ion-exchange chromatography on IEX-645 DEAE with a 300-min linear elution gradient from 0.1 *M* Tris–HCl buffer of pH 7.6 containing 0.3 *M* sodium chloride to 0.1 *M* Tris–HCl buffer of pH 7.6 containing 1 *M* sodium chloride, at a flow-rate of 1 ml/min.

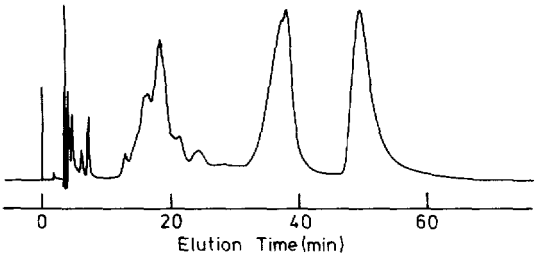

Fig. 14. Chromatogram of Total *E. coli* RNA obtained by high-performance ion-exchange chromatography on IEX-545 DEAE SIL. Conditions as in Fig. 13.

The recovery of enzymatic activity was investigated in the separation of lipoxidase, catalase and α-chymotrypsin. Column effluents between the two vertical lines in Figs. 5, 6 and 9 were fractionated, and the enzymatic activity was determined by the methods recommended by the manufacturers with linoleic acid, hydrogen peroxide and benzoyl-L-tyrosine ethyl ester, respectively, as substrates. Enzymatic activities as high as 95%, 80% and 93% were recovered for lipoxidase, catalase and α-chymotrypsin, respectively.

Fig. 11 shows the dependence of resolution on sample loading for a mixture of equal amounts of ovalbumin (Seikagaku, Tokyo, Japan) and trypsin inhibitor(Sigma). Resolution, R(OA, TI), was calculated from chromatograms exemplified by Fig. 12 according to:

$$R(\mathrm{OA, TI}) = 2\,(V_{\mathrm{TI}} - V_{\mathrm{OA}})/(W_{\mathrm{TI}} + W_{\mathrm{OA}}) \quad (1)$$

where V_{TI}, V_{OA}, W_{TI} and W_{OA} are the elution volumes and baseline widths of the peaks of ovalbumin and trypsin inhibitor, respectively. It was constant up to sample loads of *ca.* 1 mg and then decreased with increasing sample loads. Since the sample

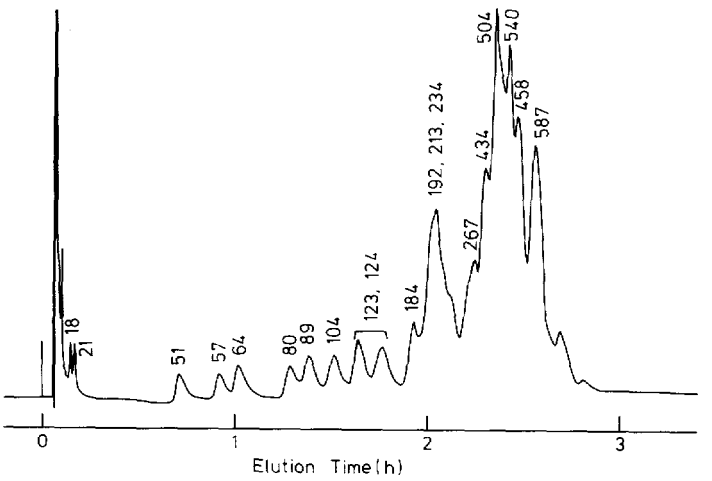

Fig. 15. Chromatogram of HaeIII-cleaved pBR322 DNA (0.027 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on IEX-645 DEAE with a 300-min linear elution gradient from 0.1 *M* Tris–HCl buffer of pH 7.6 containing 0.35 *M* sodium chloride to 0.1 *M* Tris–HCl buffer of pH 7.6 containing 0.5 *M* sodium chloride, at a flow-rate of 1 ml/min.

load in Fig. 11 is the total of two proteins, the maximum sample load is estimated to be *ca.* 0.5 mg for each component, which is equivalent to that attainable on IEX-545 DEAE SIL[4].

Applications to nucleic acid

A chromatogram of Total *E. coli* RNA is shown in Fig. 13. Peaks were collected and assigned by high-performance gel filtration on TSK-GEL G4000SW[5]. Since tRNA is a mixture of components which are specific for various animo acids, many peaks are seen at elution times of 18 – 40 min. Two high-molecular-weight rRNAs (16S and 23S) are well separated. Fig. 14 shows that these components could not be separated on IEX-545 DEAE SIL under the same conditions, probably due to its smaller pore size. Although the total *E. coli* RNA sample also contains high-molecular-weight impurities which are not separated from 16S and 23S rRNA in gel filtration on G4000SW, they are eluted as a discrete peak from the ion-exchange column.

Fig. 15 shows a chromatogram of HaeIII-cleaved pBR322 DNA. Peaks were assigned by polyacrylamide slab gel electrophoresis. Two fragments of 123 and 124 base pairs could not be identified individually. Small fragments of 8 and 11 base pairs should have been eluted near the void volume of the column and could not be confirmed. The components are mostly eluted in the order of increasing base pair number. Therefore, it can be said that double-stranded DNA fragments were mainly separated according to the chain length.

Although large fragments were not separated so well, all components up to the 587 base-pair fragment were eluted.

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