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PREPARATIVE HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

Preparative columns (150 × 21.5 and 200 × 55 mm I.D.) of TSKgel DEAE-5PW and SP-5PW were evaluated with respect to resolution, sample loading capacity and applications to the purification of proteins. The separation obtained on an analytical column (75 × 7.5 mm I.D.) could be scaled up without loss of resolution on these preparative columns. The sample loading capacities of the 150 × 21.5 and 200 × 55 mm I.D. columns were 40–200 and 240–1000 mg, respectively. Lipoxidase, superoxide dismutase and human growth hormone were purified almost to homogeneity.

INTRODUCTION

Although the high-performance ion-exchange chromatography (HPIEC) of proteins has been well accepted in the analytical field[1–6], it has not yet been extended to large-scale separations, where classical supports are still mainly used. Preparative HPIEC would be of great value when the proteins to be purified are unstable and rapid isolation is required.

Recently, preparative high-performance ion-exchange columns (150 × 21.5 and 200 × 55 mm I.D.) became commercially available and we have evaluated them. In this paper we report the results of resolution, sample loading capacity and applications to the purification of proteins.

EXPERIMENTAL

Chromatographic procedure

TSKgel DEAE-5PW and SP-5PW columns, 75 × 7.5 mm I.D. (particle size 10 μm), 150 × 21.5 mm I.D. (particle size 13 μm) and 200 × 55 mm I.D. (particle size 20 μm), were employed as ion exchangers. All chromatographic separations were performed at 25°C with a Model SP 8700 high-speed liquid chromatograph (Spectra-Physics) or a Model HLC-837 preparative liquid chromatograph (Toyo Soda) equipped with a Model UV-8 variable-wavelength UV detector (Toyo Soda) operated at 280 nm.

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Resolution

A mixture of equal amounts of ovalbumin (Seikagaku, Tokyo, Japan) and trypsin inhibitor (Sigma, St. Louis, MO, U.S.A.) was separted on a DEAE-5PW 75 × 7.5, 150 × 21.5 or 200 × 55 mm I.D. column with a linear gradient of sodium chloride from 0 to 0.5 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) to investigate the resolution. Ovalbumin and trypsin inhibitor (0.2 mg of each) were injected on to a 75 × 7.5 mm I.D. column and the loading was varied in proportion to the column cross-sectional area for columns with different inner diameters. The flow-rate and gradient time were also varied.

Sample loading capacity

The sample loading capacity was studied by separating lipoxidase (P-L Biochemicals, Milwaukee, WI, U.S.A.), superoxide dismutase and human growth hormone with varying loadings. Crude superoxide dismutase, which was prepared from bovine erythrocytes by acetone precipitation[7], was kindly provided by Mr. Mitoma. Crude human growth hormone produced in *E. coli*, which was prepared according to a published procedure[8], was kindly provided by Mr. Okamura.

Lipoxidase was separated on DEAE-5PW 150 × 21.5 and 200 × 55 mm I.D. columns with a linear gradient of sodium chloride from 0 to 0.5 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) at flow-rates of 4 and 30 ml/min, respectively. With the SP-5PW 150 × 21.5 mm I.D. column, lipoxidase was separated with a linear gradient of sodium sulphate from 0 to 0.5 *M* in 0.02 *M* acetate buffer (pH 4.5) at a flow-rate of 4 ml/min.

Crude superoxide dismutase was separated on a DEAE-5PW 150 × 21.5 mm I.D. column with a linear gradient of sodium chloride from 0 to 0.3 *M* in 0.02 *M* Tris–HCl buffer (pH 7.5) at a flow-rate of 4 ml/min.

Crude human growth hormone was separated on a DEAE-5PW 150 × 21.5 mm I.D. column with a linear gradient of sodium chloride from 0.075 to 0.5 *M* in 0.02 *M* glycine buffer (pH 8.0) at a flow-rate of 4 ml/min.

Applications to protein purification

Lipoxidase was purified on DEAE-5PW and SP-5PW columns under the same conditions as described under *Sample loading capacity*.

Crude superoxide dismutase was purified on a DEAE-5PW 200 × 55 mm I.D. column with a linear gradient of sodium chloride from 0 to 0.3 *M* in 0.02 *M* Tris–HCl buffer (pH 7.5) at a flow-rate of 30 ml/min.

Crude human growth hormone was purified on a DEAE-5PW 200 × 55 mm I.D. column with a linear gradient of sodium chloride from 0.075 to 0.5 *M* in 0.02 *M* glycine buffer (pH 8.0) at a flow-rate of 30 ml/min.

The purity of the fractions obtained was checked by analytical ion-exchange chromatography on TSKgel DEAE-5PW (75 × 7.5 mm I.D. column), analytical gel filtration on TSKgel G3000SW (600 × 7.5 mm I.D. column), reversed-phase chromatography on TSKgel Phenyl-5PW RP (75 × 4.6 mm I.D. column) and polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Resolution

Figs. 1 and 2 show the dependence of resolution on gradient time on DEAE-5PW 150 × 21.5 and 200 × 55 mm I.D. columns, respectively, at various flow-rates. The resolution, R(OA,TI), was calculated according the equation

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

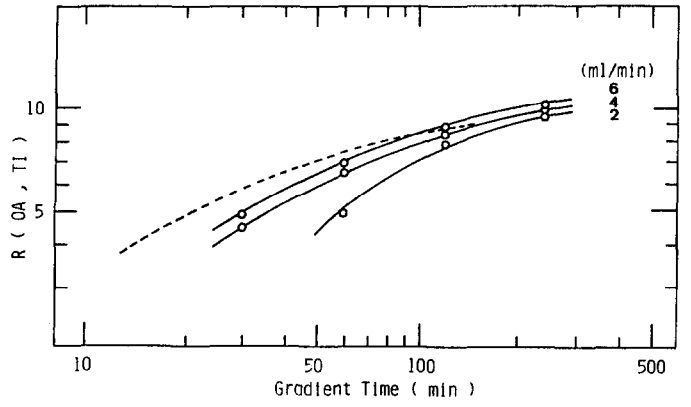

Fig. 1. Dependence of resolution on gradient time on a DEAE-5PW 150 × 21.5 mm I.D. column at various flow-rates. The dashed line indicates the results on the DEAE-5PW analytical column (75 × 7.5 mm I.D.) at a flow-rate of 1 ml/min. Almost the same results were obtained at flow-rates of 6 and 8 ml/min.

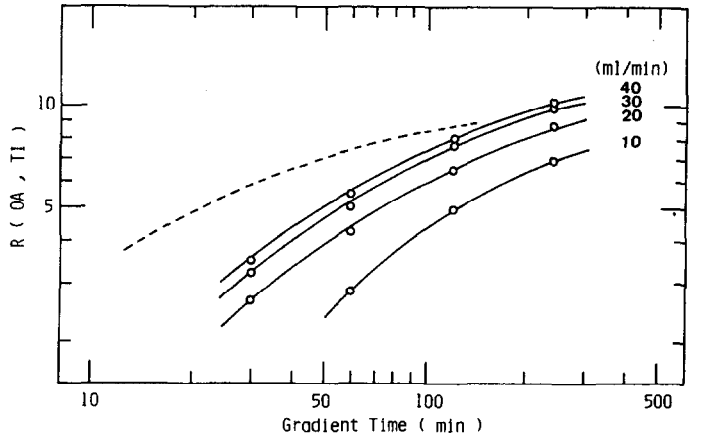

Fig. 2. Dependence of resolution on gradient time on a DEAE-5PW 200 × 55 mm I.D. column at various flow-rates. The dashed line indicates the results on the DEAE-5PW analytical column (75 × 7.5 mm I.D.) at a flow-rate of 1 ml/min.

where V_{TI}, V_{OA}, W_{TI} and W_{OA} are the elution volumes and baseline peak widths of trypsin inhibitor and ovalbumin, respectively.

Figs. 1 and 2 indicate that the resolution was considerably improved by increasing the flow-rate. However, the effect of flow-rate was small above 4 and 30 ml/min on the 150 × 21.5 and 200 × 55 mm I.D. columns, respectively. Therefore, flow-rates of around 4 and 30 ml/min seem to be suitable for these columns.

Fig. 1 shows that the resolution given by the 150 × 21.5 mm I.D. column at flow-rates of 4–6 ml/min is equal or superior to that of the analytical column when the gradient time on the 150 × 21.5 mm I.D. column is double that on the analytical column.

Fig. 2 shows that the resolution given by the 200 × 55 mm I.D. column at flow-rates of 30–40 ml/min is equal or superior to that of the analytical column when the gradient time on the 200 × 55 mm I.D. column is three times than that on the analytical column.

Sample loading capacity

Figs. 3, 4 and 5 show the chromatograms obtained on DEAE-5PW 150 × 21.5, 200 × 55 and SP-5PW 150 × 21.5 mm I.D. columns with increasing sample loadings of commercial lipoxidase, where lipoxidase eluted at 50, 60 and 60 min, respectively.

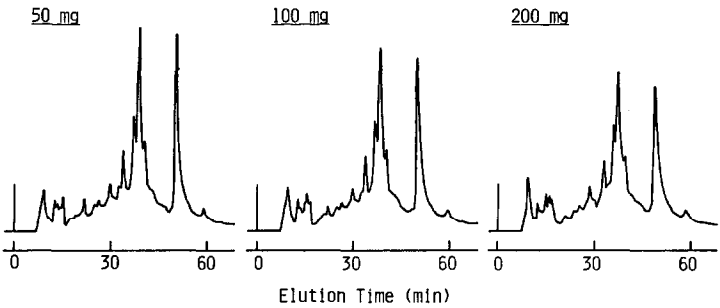

Fig. 3. Chromatograms of lipoxidase (50, 100 or 200 mg) obtained on a DEAE-5PW 150 × 21.5 mm I.D. column with a 120 min linear gradient of NaCl from 0 to 0.5 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) at a flow-rate of 4 ml/min.

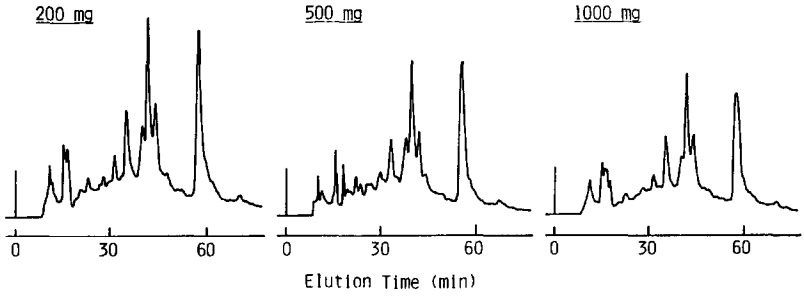

Fig. 4. Chromatograms of lipoxidase (200, 500 or 1000 mg) obtained on a DEAE-5PW 200 × 55 mm I.D. column with a 180 min linear gradient of NaCl from 0 to 0.5 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) at a flow-rate of 30 ml/min.

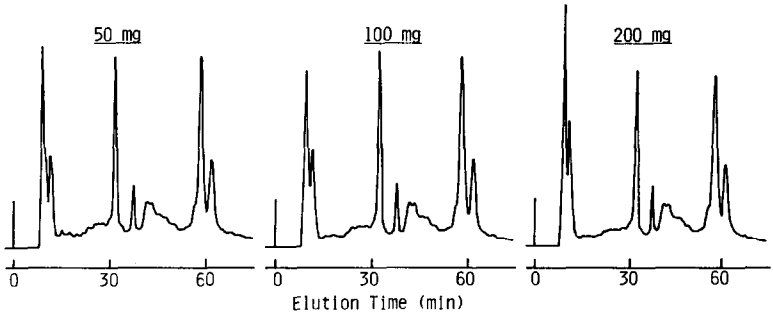

Fig. 5. Chromatograms of lipoxidase (50, 100 or 200 mg) obtained on an SP-5PW 150 × 21.5 mm I.D. column with a 120 min linear gradient of Na_2SO_4 from 0 to 0.5 *M* in 0.02 *M* acetate buffer (pH 4.5) at a flow-rate of 4 ml/min.

Fig. 6 shows the chromatograms obtained on a DEAE-5PW 150 × 21.5 mm I.D. column with increasing sample loadings of crude superoxide dismutase, where superoxide dismutase eluted at 25 min.

Fig. 7 shows the chromatograms obtained on a DEAE-5PW 150 × 21.5 mm I.D. column with increasing sample loadings of crude human growth hormone, where human growth hormone eluted at 33 min.

Figs. 3 and 5 indicate that DEAE-5PW and SP-5PW 150 × 21.5 mm I.D. columns have same sample loading capacity. Figs. 3 and 4 indicate that the sample

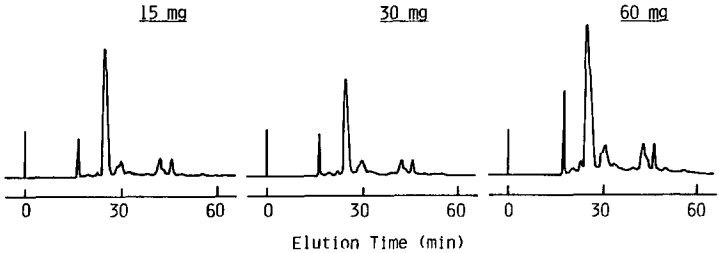

Fig. 6. Chromatograms of crude superoxide dismutase (15, 30 or 60 mg) obtained on a DEAE-5PW 150 × 21.5 mm I.D. column with a 120 min linear gradient of NaCl from 0 to 0.3 *M* in 0.02 *M* Tris–HCl buffer (pH 7.5) at a flow-rate of 4 ml/min.

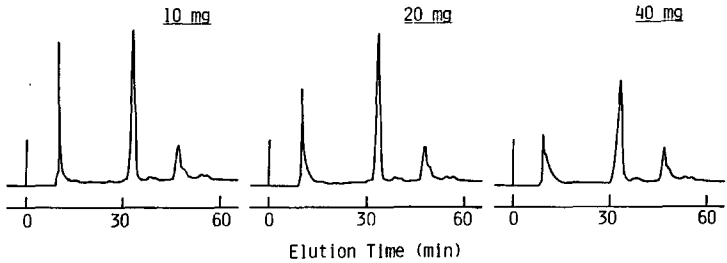

Fig. 7. Chromatograms of crude human growth hormone (10, 20 or 40 mg) obtained on a DEAE-5PW 150 × 21.5 mm I.D. column with a 120 min linear gradient of NaCl from 0.075 to 0.5 *M* in 0.02 *M* glycine buffer (pH 8.0) at a flow-rate of 4 ml/min.

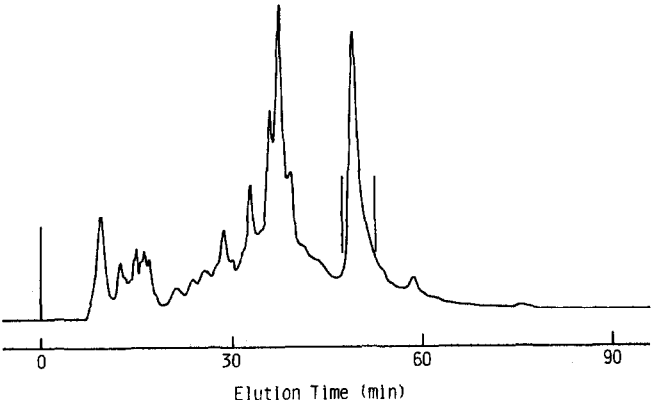

Fig. 8. Chromatogram of lipoxidase (200 mg) obtained on a DEAE-5PW 150 × 21.5 mm I.D. column. Conditions as in Fig. 3.

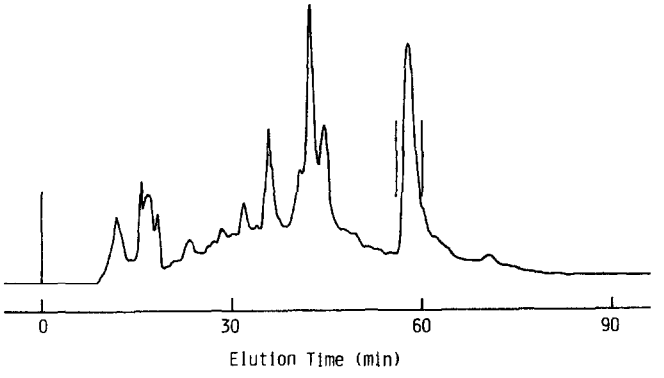

Fig. 9. Chromatogram of lipoxidase (1000 mg) obtained on a DEAE-5PW 200 × 55 mm I.D. column. Conditions as in Fig. 4.

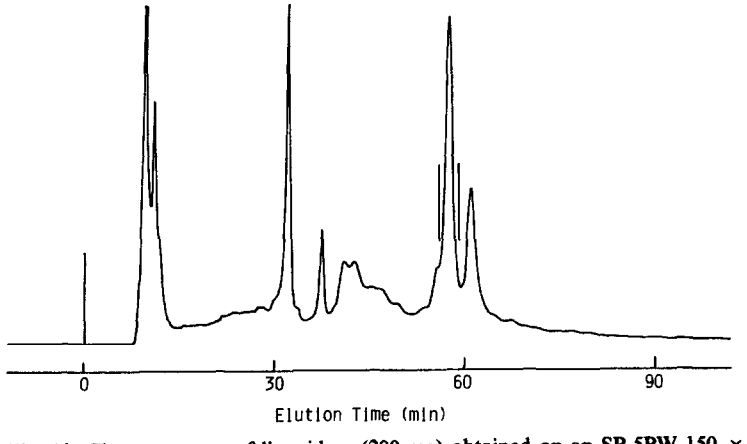

Fig. 10. Chromatogram of lipoxidase (200 mg) obtained on an SP-5PW 150 × 21.5 mm I.D. column. Conditions as in Fig. 5.

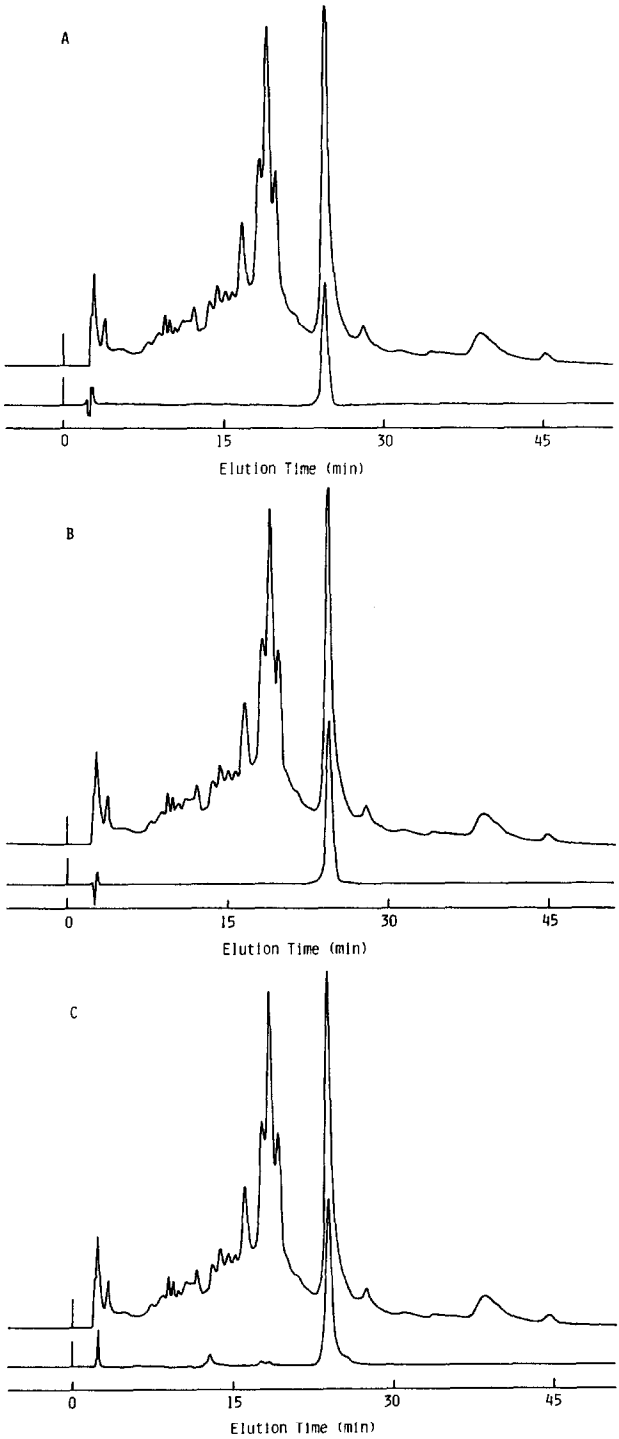

Fig. 11. Analytical ion-exchange chromatograms of original sample and lipoxidase fraction on a DEAE-5PW 75 × 7.5 mm I.D. column with a 60 min linear gradient of NaCl from 0 to 0.5 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) at a flow-rate of 1 ml/min. (A) Fraction from Fig. 8; (B) fraction from Fig. 9; (C) fraction from Fig. 10.

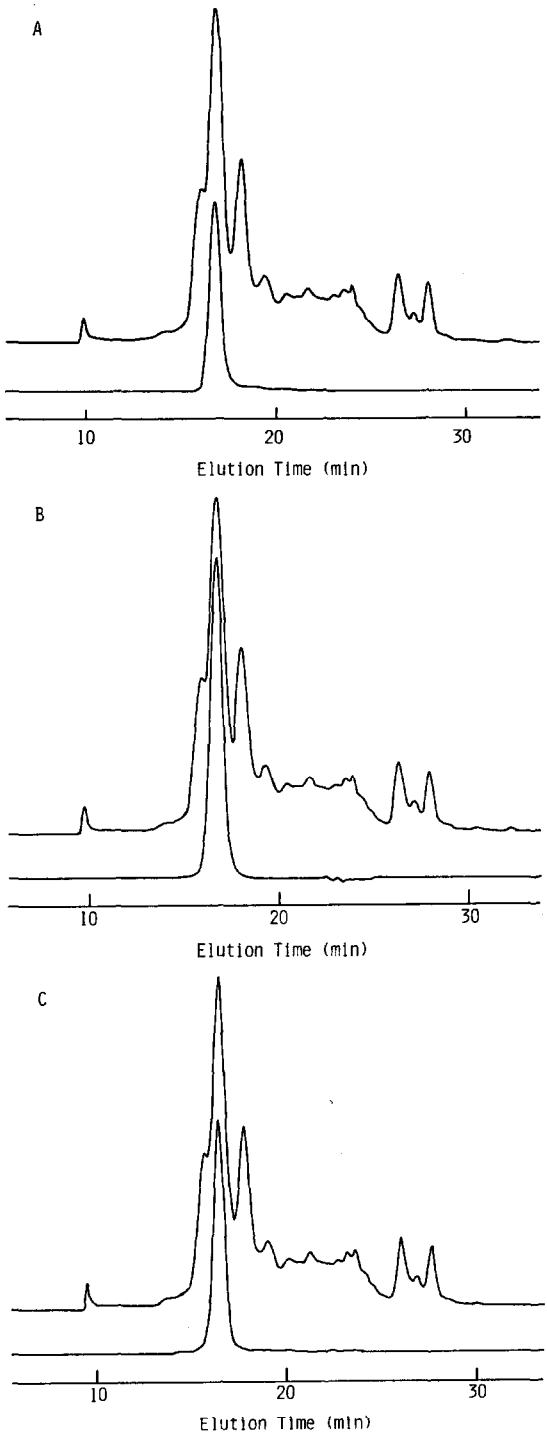

Fig. 12. Analytical gel filtration chromatograms of original sample and lipoxidase fraction on TSKgel G3000SW. Eluent: 0.05 *M* phosphate buffer containing 0.2 *M* NaCl (pH 7.0). Flow-rate: 1.0 ml/min. (A) Fraction from Fig. 8; (B) fraction from Fig. 9; (C) fraction from Fig. 10.

loading capacity seems to be approximately proportional to the column cross-sectional area. Actually, the sample loading capacities of the DEAE-5PW 200 × 55 mm I.D. column for crude superoxide dismutase and crude human growth hormone are 300 and 240 mg, respectively.

We have already studied the sample loading capacity in analytical ion-exchange chromatography and found that the maximum sample loading increased as the number of components in the sample increased[9]. In these experiments we obtained the same results (Figs. 3, 6 and 7).

Applications to protein purification

Figs. 8, 9 and 10 show the results of the separation of commercial lipoxidase on DEAE-5PW 150 × 21.5, 200 × 55 and SP-5PW 150 × 21.5 mm I.D. columns, respectively. When the column effluents between the two vertical lines in Figs. 8, 9 and 10 were fractionated, enzymatic activities of 90, 90 and 80%, respectively, were recovered. The fractions were also subjected to analytical ion-exchange chromato-

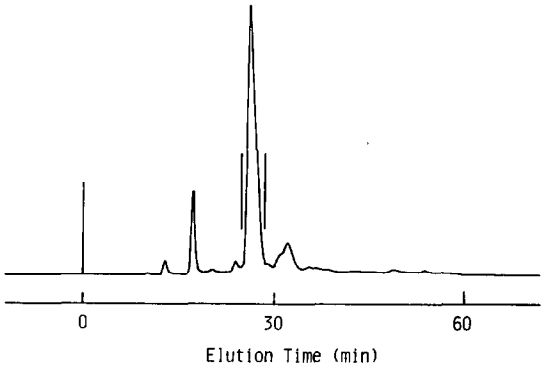

Fig. 13. Chromatogram of crude superoxide dismutase (300 mg) obtained on a DEAE-5PW 200 × 55 mm I.D. column with a 180 min linear gradient of NaCl from 0 to 0.3 *M* in 0.02 *M* Tris–HCl buffer (pH 7.5) at a flow-rate of 30 ml/min.

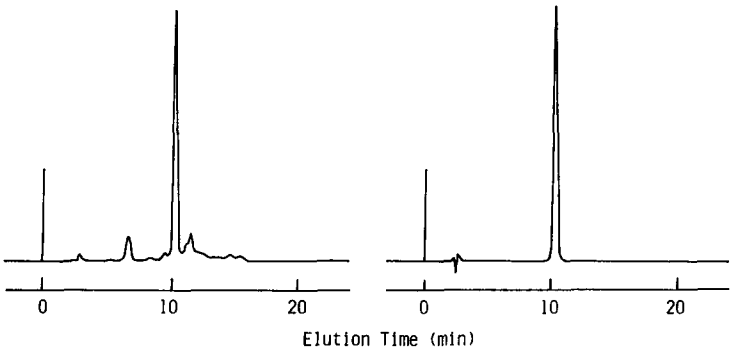

Fig. 14. Analytical ion-exchange chromatograms of original sample and superoxide dismutase fraction on a DEAE-5PW 75 × 7.5 mm I.D. column with a 30 min linear gradient of NaCl from 0 to 0.3 *M* in 0.02 *M* Tris–HCl buffer (pH 7.5) at a flow-rate of 1 ml/min.

graphy and analytical gel filtration. Fig. 11 shows chromatograms of the fraction and the original lipoxidase obtained by analytical ion-exchange chromatography. Fig. 12 shows chromatograms of the fraction and the original lipoxidase obtained by analytical gel filtration.

Fig. 13 shows the separation of crude superoxide dismutase on a DEAE-5PW 200 × 55 mm I.D. column. When the column effluent between the two vertical lines

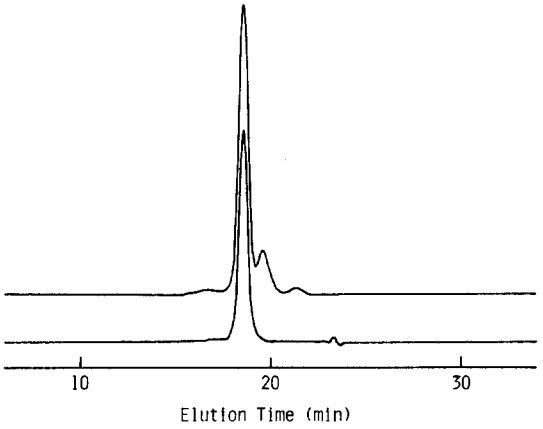

Fig. 15. Analytical gel filtration chromatograms of original sample and superoxide dismutase fraction on TSKgel G3000SW. Conditions as in Fig. 12.

Fig. 16. SDS polyacrylamide gel electrophoresis patterns of (a) original sample and (b) fraction.

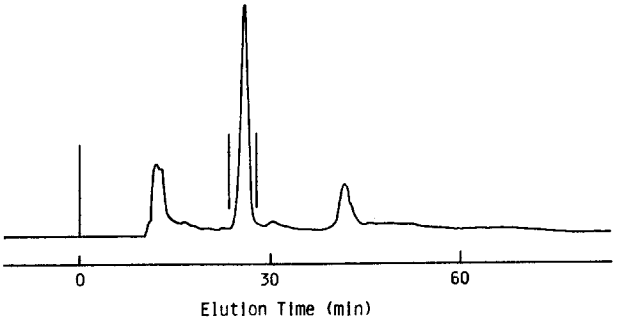

Fig. 17. Chromatogram of crude human growth hormone (240 mg) obtained on a DEAE-5PW 200 × 55 mm I.D. column with a 180 min linear gradient of NaCl from 0.075 to 0.5 *M* in 0.02 *M* glycine buffer (pH 8.0) at a flow-rate of 30 ml/min.

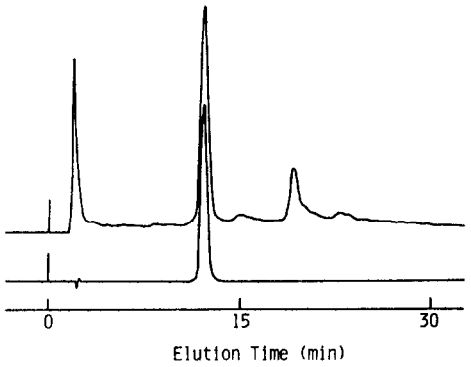

Fig. 18. Analytical ion-exchange chromatograms of original sample and human growth hormone fraction on a DEAE-5PW 75 × 7.5 mm I.D. column with a 60 min linear gradient of NaCl from 0.075 to 0.5 *M* in 0.02 *M* glycine buffer (pH 8.0) at a flow-rate of 1 ml/min.

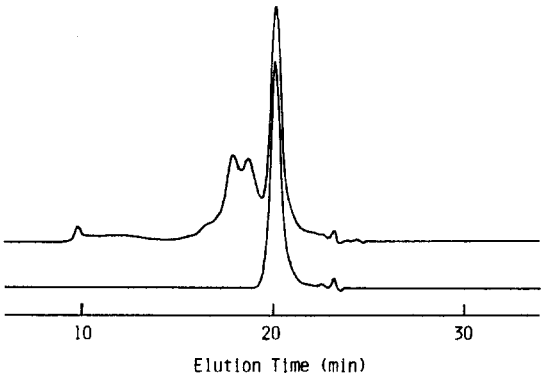

Fig. 19. Analytical ion-exchange chromatograms of original sample and human growth hormone fraction on a DEAE-5PW 75 × 7.5 mm I.D. column with a 60 min linear gradient of NaCl from 0.075 to 0.5 *M* in 0.02 *M* glycine buffer (pH 8.0) at a flow-rate of 1 ml/min.

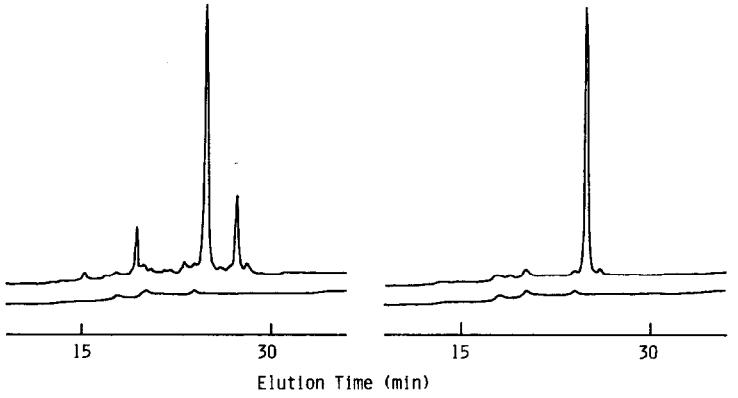

Fig. 20. Reversed-phase chromatograms of original sample and human growth hormone fraction on TSKgel Phenyl-5PW RP with a 2 min linear gradient of acetonitrile from 5 to 20% followed by a 48 min linear gradient of acetonitrile from 20 to 80% at a flow-rate of 1 ml/min. All eluents contained 0.05% of trifluoroacetic acid. Lower curves indicate blank gradient.

was fractionated, an enzymatic activity of 67% was recovered. The reason for the low recovery of activity is the existence of isozymes, which eluted at *ca.* 30 min in Fig. 13. Figs. 14 and 15 show chromatograms of the fraction and the original sample obtained by analytical ion-exchange chromatography and analytical gel filtration, respectively. Fig. 16 shows SDS-PAGE patterns of the fraction and the original sample, Figs. 14–16 indicate that superoxide dismutase was purified almost to homogeneity.

Fig. 17 shows the result of the separation of crude human growth hormone on a DEAE-5PW 200 × 55 mm I.D. column. The peak corresponding to human growth hormone between the two vertical lines was fractionated and subjected to analytical ion-exchange chromatography, analytical gel filtration, reversed-phase chromatography and polyacrylamide gel electrophoresis. The results are shown in Figs. 18–21, and indicate that the fraction contains only very small amounts of impurities in addition to human growth hormone.

Fig. 21. Polyacrylamide gel electrophoresis patterns of (a) original sample and (b) human growth hormone fraction.

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