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Preparative chromatography of proteins

Design calculation procedure for gradient and stepwise elution

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ABSTRACT

A method is presented for determining stepwise elution chromatographic conditions on the basis of the data obtained from linear gradient elution experiments. The separation of crude β-galactosidase by medium-performance (an)ion-exchange chromatography (MPIEC) was chosen as a model separation system. Some factors affecting the purification such as the column dimensions and the residence time were investigated. Large-scale MPIEC was carried out and its performance was compared with that of a small scale-column. The effect of sample loading was also examined.

INTRODUCTION

Stepwise elution, in which a discontinuous change in the salt concentration of the elution buffer is introduced into a column, is one of the elution (separation) methods for proteins in ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and affinity chromatography. The attraction of this method is that the apparatus and the operating procedure are simple. However, there are several disadvantages with stepwise elution, as pointed out previously[1–3]. One of the most serious disadvantages is an artificial peak due to a discontinuous change in the elution buffer. For example, when all the proteins contained in the sample are desorbed completely by the elution buffer, they will be eluted as a single peak[1]. On the other hand, when a protein is not eluted completely by the first elution buffer, the subsequent second elution buffer may cause an artificial, peak frequently called a 'false peak'[1]. These phenomena make it difficult to interpret the experimental results and may lead to a misunderstanding regarding the homogeneity of the eluted fraction.

In ideal-affinity chromatography, only the desired protein is adsorbed on the

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column biospecifically and subsequent desorption yields the purified product. In contrast, a number of contaminant proteins present in the sample are adsorbed on IEC or HIC columns and the desired protein must be resolved during the desorption (elution) process. For example, the desired protein is desorbed while the other contaminants are retained or the contaminants are washed out while the desired protein is retained. For this purpose, a knowledge of the salt concentration dependence of the distribution coefficients, $K(I)$, of the contaminant proteins and of the desired protein is required[1,4,5]. However, the measurement of $K(I)$ for the contaminant proteins by a batch experiment is usually impossible unless a specific detection method for individual contaminant proteins is available. Moreover, the measurement of $K(I)$ is time consuming and laborious.

We previously reported a method for determining $K(I)$ from linear gradient elution experiments and verified the method experimentally[1,6]. In this study, this method was applied to the design of the stepwise elution of crude β-galactosidase with medium-performance IEC (MPIEC) columns. Some factors affecting the purification were investigated. Large-scale MPIEC was carried out and its performance was compared with that of a small scale-column. The effect of sample loading was also examined.

EXPERIMENTAL

The enzyme used was crude β-galactosidase from *Aspergillus oryzae*, obtained from Amano Pharmaceutical (Japan) as Lactase F. The enzyme activity of β-galactosidase was determined spectrophotometerically by measuring the change in the absorbance at 420 nm of the substrate (*o*-nitrophenylgalactoside) with time[7].

The MPIEC packings used are anion-exchange gels having diethylaminoethyl (DEAE) groups, distributed by Tosoh (Tokyo, Japan): DEAE Toyopearl 650S ($d_p = 40$ μm) for columns of 15 × 1.6 cm I.D., 30 × 1.6 cm I.D., 2 × 2.2 cm I.D. and 2 × 10.8 cm I.D.; DEAE Toyopearl 650M ($d_p = 65$ μm) for columns of 15 × 1.4 cm I.D. and 40 × 31 cm I.D.; and DEAE Toyopearl 650C ($d_p = 87$ μm) for columns of 15 × 1.4 cm I.D. All were employed as closed columns. Details of the design of the large column are described elsewhere[8]. The apparatus and the operating method are similar to those in our previous studies[6,9,10]: for small columns, peristaltic pumps, an Altex 153 UV detector and a Horiba (Kyoto, Japan) DS-8M conductivity meter for the measurement of sodium chloride concentration; for large columns, a CCP-8070 pump (Tosoh) and a UV-8070 UV detector (Tosoh).

The elution was performed by an increase in sodium chloride concentration from 0.03 *M* either continuously (linear gradient elution) or discontinuously (stepwise elution) at a fixed pH (7.7) and 25°C. The buffer solution was 14 m*M* Tris–HCl (pH 7.7). The initial buffer contained 0.03 *M* sodium chloride. As Blue Dextran 2000, commonly used for the determination of the column void volume, V_o, in gel filtration chromatography, is adsorbed on DEAE IEC columns, we employed Dextran T-2000 (Pharmacia) pulses for determining V_o. The eluted fractions were collected with a fraction collector and the protein concentration and the enzyme activity were determined.

As a measure of the degree of purification, the purification factor, defined as $(\text{enzyme activity/protein amount})_{\text{recovered}}/(\text{enzyme activity/protein amount})_{\text{sample}}$, was used.

The protein concentration in the recovered fraction and in the sample were determined from the absorbance at 280 nm or by the biuret method. HPIEC was employed to test the homogeneity of the fractionated sample by the MPIEC columns. The sample (the peak fraction from the MPIEC columns) was dialysed against the initial buffer (the same as that for the MPIEC) before it was applied to the HPIEC column (TSK-gel DEAE 5PW, 7.5 × 0.75 cm I.D.).

RESULTS

Determination of K(I)

The elution curve for the sample used (crude β-galactosidase), obtained by linear gradient elution on an MPIEC column, is shown in Fig. 1. Although a number of peaks including a peak having the enzyme activity (peak B) are observed, we focus our attention on the three large peaks A, B and C shown in Fig. 1. Peak A is eluted at very low salt concentrations, so, this peak can be washed out with the starting buffer in the stepwise elution. Therefore, the chromatographic conditions should be such that peak B, the desired enzyme, is eluted whereas peak C is retained. The linear gradient elution experiments were carried out with different slopes of the gradient, g, then the salt concentration at the peak position, I_R, was measured (the experimental results are summarized in Table I). The GH *vs.* I_R plots for peaks B and C are shown in Fig. 2 [GH is the slope of the gradient normalized with respect to the column gel volume; $GH = g(V_t - V_o)$]. The GH–I_R relationship is not dependent on the flow-rate, the column dimensions, the sample volume or the particle diameter. The elution position of peaks B and C in the linear gradient elution experiments can be predicted from these GH–I_R curves[1,4,5,8]. It should also be noted that the slope of the GH–I_R curve corresponds to the number of charges involved in the adsorption equilibria[1,4–6].

In the design of the stepwise elution, the K–I relationship is needed[1,4,5]. We have also shown that the K–I relationship can be obtained from the GH–I_R curve on the basis of the following equation[1,6]:

$$d(GH)/dI = 1/[K(I) - K']$$

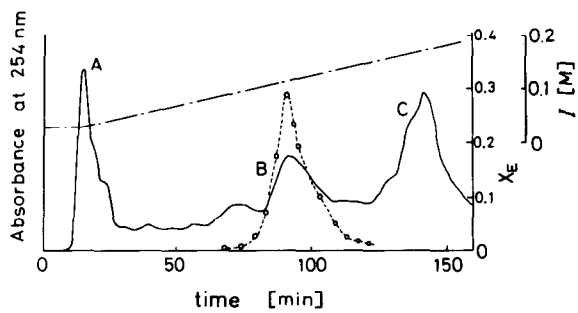

Fig. 1. Elution curve of crude β-galactosidase obtained by linear gradient elution on a MPIEC column. The linear gradient elution experiment was performed with a linear increase in NaCl concentration in the buffer (14 mM Tris–HCl, pH 7.7) from 0.03 M at 25°C. Column DEAE Toyopearl 650S (d_p = 40 μm), 15 × 1.6 cm I.D. Slope of NaCl gradient, $g = 6 \cdot 10^{-4}$ M/ml; F = 2 ml/min. Sample (crude β-galactosidase), 10 ml of 1% solution. (O) X_E = ratio of the enzyme activity to that of the sample. (— · —) I = NaCl concentration.

TABLE I

EXPERIMENTAL DATA PLOTTED IN FIG. 2.

Sample (crude β-galactosidase) concentration 1%; packing DEAE Toyopearl 650; pH, 7.7; initial salt (NaCl) concentration, 0.03 *M*.

d_p (μm)	d_c (cm)	Z (cm)	F (ml/min)	$g \times 10^3$ (M/ml)	$GH \times 10^2$ (M)	I_R for peak B (M)	I_R for peak C (M)	Sample volume (ml)
40	1.6	15	2.0	2.3	4.0	0.123	0.181	5
				1.1	2.0	0.117	0.177	2
				0.56	1.0	0.101	0.159	10
				0.40	0.71	0.100	ND[a]	2
	1.6	30	1.4	0.87	2.9	0.128	0.195	5
65	1.4	15	1.1	1.5	2.1	0.112	0.166	8
			2.0	0.66	0.91	0.108	0.159	2
				2.9	4.0	0.125	0.182	2
87	1.4	15.	0.9	2.3	3.0	0.131	0.195	8
			2.0	1.6	2.1	0.123	0.188	2
				0.72	0.95	0.115	0.174	2

[a] ND = Not determined.

where K' is the distribution coefficient of the gradient substance (in this instance sodium chloride). The $K(I)$ values for peaks B and C thus obtained are shown in Fig. 3. The K values obtained by isocratic elution experiments with the purified fraction of peaks B and C as a sample (symbols in Fig. 3) are in good agreement with the $K(I)$ values determined from the GH–I_R curve.

Effect of the salt concentration of the elution buffer, I_E

The elution behaviour in the stepwise elution can be grouped into two types, as explained previously[1,4,5]. One is the case where a protein is desorbed completely in

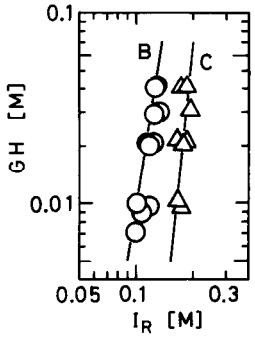

Fig. 2. Relationship between GH and salt concentration at the peak position I_R for peaks B and C in Fig. 1. Linear gradient elution experiments were carried out with various slopes of the gradient g under the experimental conditions shown in Fig. 1. Then the salt (NaCl) concentration at the peak position I_R was measured. $GH = g(V_t - V_0)$; V_t = the total column volume; V_0 = the column void volume. Peak B has the enzyme activity and peak C is the contaminant (see Fig. 1). See Table I for the details of the experimental conditions for the points.

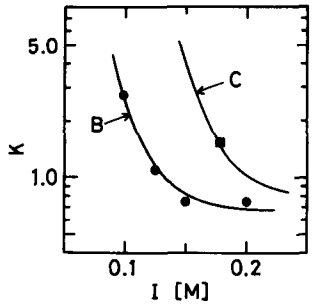

Fig. 3. Relationship between distribution coefficient K and salt (NaCl) concentration, I. The solid curves were obtained from differentiation of the corresponding GH *vs.* I_R curve in Fig. 2[1,6]. The symbols are the experimental results from the isocratic elution experiments with the peak fraction of the linear gradient elution as the sample.

the elution buffer and therefore is eluted in (or near) the spreading front boundary of the elution buffer as a very sharp peak (type I elution). In this case, the K value at $I = I_E$, K_E is less than or nearly equal to K'. The shape of the front boundary of the elution buffer from I_0 to I_E plays a similar role to the steep slope of the gradient. In another case, the protein peak appears after the concentration of the composition of the elution buffer at the exit of the column reaches its initial value (type II elution).

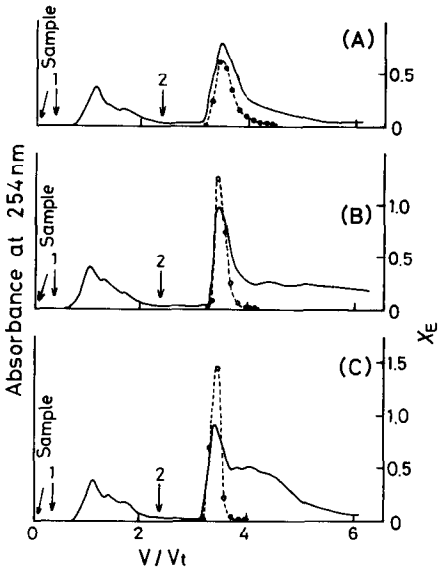

Fig. 4. Effect of the salt concentration of the elution buffer I_E in the stepwise elution. V = the volume from the start of the experiment and V_t is the total column volume. (o) X_E = the ratio of the enzyme activity to that of the sample. Sample = 1% crude β-galactosidase dissolved in the starting buffer [14 mM Tris–HCl (pH 7.7) containing 0.03 M NaCl], 10 ml. Column, DEAE Toyopearl 650S (d_p = 40 μm), 15 × 1.6 cm I.D. The arrows indicate the change in the elution buffer: 1, starting buffer (washing); 2, elution buffer, 14 mM Tris–HCl (pH 7.7) containing (A) 0.13, (B) 0.15 and (C) 0.17 M NaCl. F = 1.3 ml/min; 25°C. The purification factor is (A) 2.6, (B) 2.3 and (C) 2.0. The recovery was 90–93%.

The peak is diluted considerably compared with those in type I elution. K_E in this case is greater than K'. Obviously, it is desirable that the required protein is eluted by type I elution. From Fig. 3, I_E was determined as 0.15, where peak B is eluted by type I elution and the K of peak C is large enough for peak C to be retained in the column. After the sample (1% β-galactosidase, 10 ml) was applied, the column was washed with the initial starting buffer until the UV detector response fell almost to zero. Subsequently, the elution buffer was applied. The elution curves obtained with different I_E values are shown in Fig. 4.

The shape and peak position of the elution curves are markedly influenced by small changes in I_E. The peak elution volume at $I_E = 0.13$ is larger than that at $I_E = 0.15$. The peak height at $I_E = 0.13$ is much lower than that at $I_E = 0.15$ and $I_E = 0.17$. This is typical of type II elution behaviour. Although the peak height at $I_E = 0.17$ is larger than that at $I_E = 0.15$, the purification factor is low. In order to check the homogeneity of the fractions, linear gradient elution of the fractions from the MPIEC column was carried out with an HPIEC column. The resulting chromatograms (Fig. 5) indicate that peak A is eliminated in any of the three stepwise elution experiments but the removal of peak C is dependent on I_E. At $I_E = 0.17$, considerable amounts of peak C still remain although the ratio of the area of peak B to the total area increased from that in the starting sample. Peak C was removed to a great extent at $I_E = 0.13$ and 0.15. The choice of $I_E = 0.15$ from Fig. 3 is therefore suitable for the purification of the present sample.

Effect of residence time

The column productivity, *e.g.*, amount of protein recovered per unit column volume in unit separation time, can be increased by decreasing the residence time.

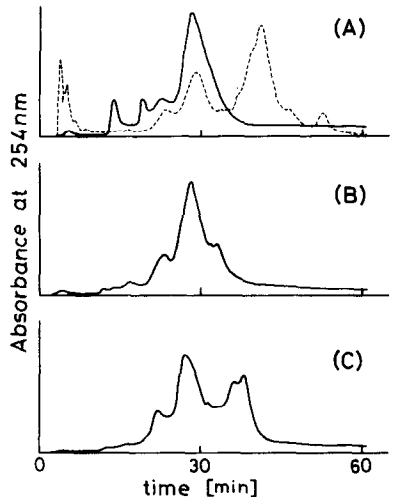

Fig. 5. HPIEC of the purified fraction of β-galactosidase shown in Fig. 4. The linear gradient elution experiment was performed with a linear increase in NaCl concentration in the buffer [14 mM Tris–HCl (pH 7.7)] from 0.03M at 25°C. Column, TSK-gel DEAE 5PW (7.5 × 0.75 cm I.D.); $g = 4.7 \cdot 10^{-3}$ M/ml; $F = 1$ ml/min. The sample used is (A) the fraction in Fig. 4A, (B) the fraction in Fig. 4B and (C) the fraction in Fig. 4C. The dashed curve (A) is the elution curve of the crude sample.

This can be done by increasing the flow-rate or by decreasing the column length. Two experiments were carried out in one instance the superficial velocity was increased by a factor of 2.3 and in the other case the column length was decreased by a factor of 7.5 (Fig. 6). Although the X_E values were lowered on decreasing the residence time, the purification factor values and the HPIEC results (Fig. 7) are similar. This suggests that the column productivity can be increased by using a short column and/or high flow-rates if we ignore the concentration factor.

Effect of sample loading

The next task was to determine the sample loading. Unfortunately, this had to be done by preliminary column experiments as it is difficult to measure the $K(I_o)$ values for the contaminants. The elution curves at $I_E = 0.15$ with different sample volumes and sample concentrations are shown in Fig. 8. Although the elution pattern during the wash out is different, the enzyme peak shapes are similar. The peak for the sample of 1% and 75 ml is markedly concentrated. However, the HPIEC results (Fig. 9) indicate that the ratio of the area of peak C in the fraction at a sample volume of 75 ml (1%) is higher than that at a sample volume of 10 ml (1%). This corresponds to a decrease in the purification factor. It is interesting that both the purification factor values and the chromatograms are similar for equal amounts of the sample, *i.e.*, 10 ml of 1% and 100 ml of 0.1% solution. The sample loading for 75 ml of 1% solution is 25 mg of crude enzyme per ml of column. This value is high for protein separation by liquid chromatography[1,11].

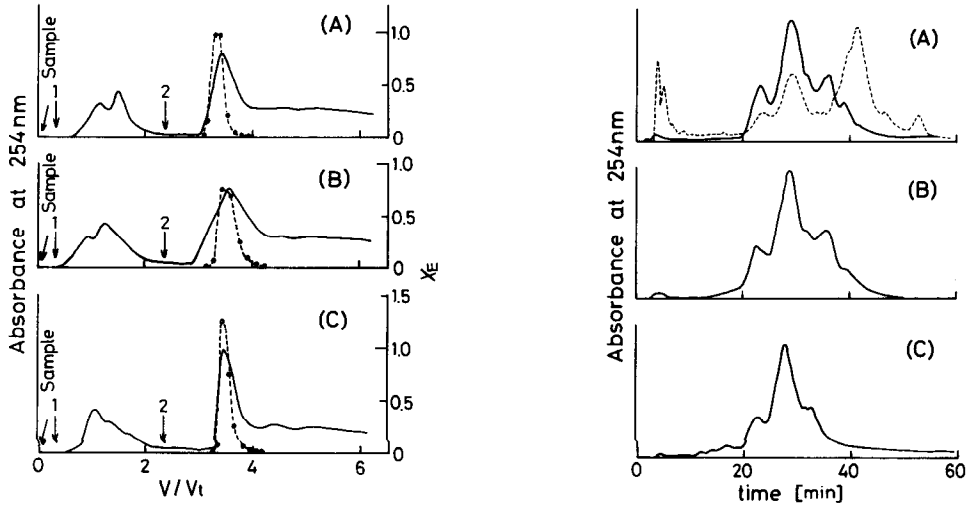

Fig. 6. Effect of residence time. Arrows and symbols as in Fig. 4. Columns DEAE Toyopearl 650 MPIEC ($d_p = 40\mu m$), (A) 15 × 1.6 cm I.D., $F = 3$ ml/min, sample 10 ml of 1% solution, residence time $V_t/F = 10$ min; (B) 2.0 × 2.2 cm I.D., $F = 2.4$ ml/min, sample 2.5 ml of 1% solution, $V_t/F = 3.2$ min; (C) 15 × 1.6 cm I.D., $F = 1.3$ ml/min, sample 10 ml of 1% solution, $V_t/F = 23$ min. Elution buffer, 14 m*M* Tris–HCl (pH 7.7) containing 0.15 *M* NaCl. Other conditions as in Fig. 4. The purification factor is (A) 2.1, (B) 2.0 and (C) 2.3. The recovery was 90–93%.

Fig. 7. HPIEC of the purified fraction of β-galactosidase shown in Fig. 6. Conditions as in Fig. 5. The sample used is (A) the fraction in Fig. 6A, (B) the fraction in Fig. 6B, (C) the fraction in Fig. 6C. The dashed curve is the elution curve of the crude sample.

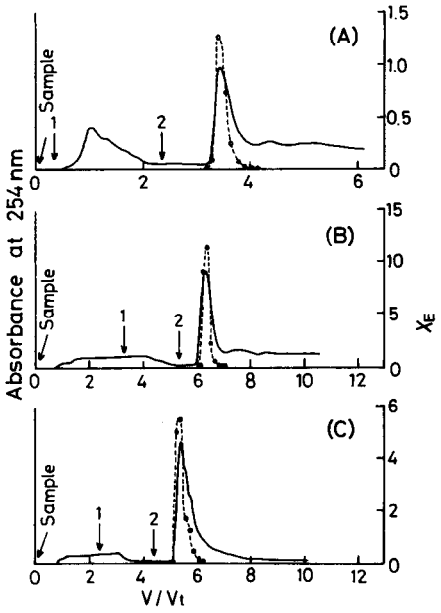

Fig. 8. Effect of the sample loading. Arrows and symbols as in Fig. 4. Column DEAE Toyopearl 650 S (d_p = 40 μm), 15 × 1.6 cm I.D.; elution buffer, 14 m*M* Tris–HCl (pH 7.7) containing 0.15 *M* NaCl; F = 1.3 ml/min; 25°C; sample, (A) 10 ml of 1% solution, (B) 100 ml of 0.1% solution and (C) 75 ml of 1% solution. Other conditions as in Fig. 4. The purification factor is (A) 2.2, (B) 2.5 and (C) 1.7. The recovery was 90–93%.

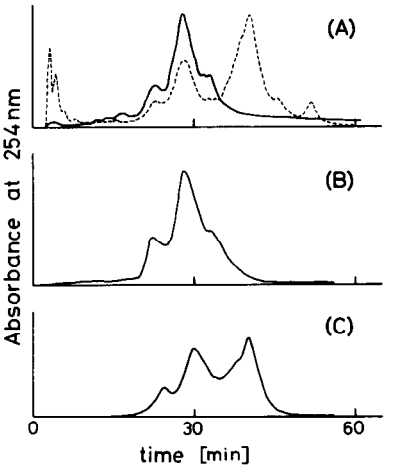

Fig. 9. HPIEC of the purified fraction of β-galactosidase shown in Fig. 8. Conditions as in Fig. 5. The sample used is (A) the fraction in Fig. 8A, (B) the fraction in Fig. 8B and (C) the fraction in Fig. 8C. The dashed curve is the elution curve of the crude sample.

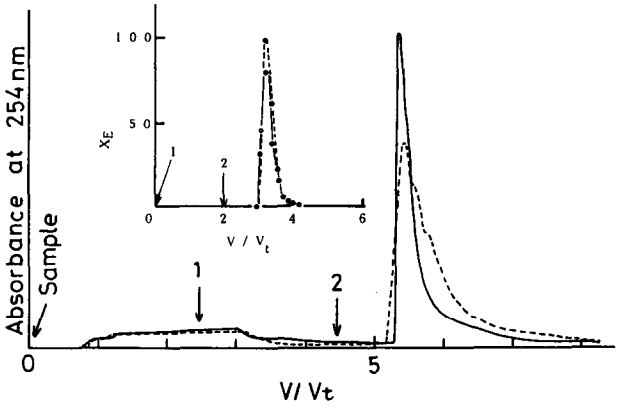

Fig. 10. Comparison of the elution curves of β-galactosidase obtained by stepwise elution with small and large MPIEC columns. Arrows and symbols as in Fig. 4. Columns DEAE Toyopearl 650; elution buffer, 14 m*M* Tris–HCl (pH 7.7) containing 0.15 *M* NaCl. Solid curve, $d_p = 65$ μm, column 40 × 31 cm I.D., $F = 528$ ml/min (superficial velocity $u_0 = 0.7$ cm/min), sample 73.5 l of 1% solution; dashed curve, $d_p = 40$ μm, column 15 × 1.6 cm I.D., $F = 1.5$ ml/min ($u_0 = 0.75$ cm/min), sample 75 ml of 1% solution. Other conditions as in Fig. 4. Inset: sample 30 column volumes of 0.1% solution; superficial velocity 1.8 cm/min for the sample application and washing and 0.32 cm/min for the elution; (○) $d_p = 40$ μm, column 2 × 10.8 cm I.D.; (●) $d_p = 40$ μm, column 2 × 2.2 cm I.D. Other conditions as in Fig. 4.

Large-scale application

Fig. 10 shows a comparison of the elution curve at $I_E = 0.15$ for analytical and large-scale columns. The superficial velocity and the ratio of the sample volume to the total column volume were set equal for the two columns, but the particle diameter in the large column is larger and the column is longer. As can be seen, the elution profiles for the two columns are similar. The peak height for the large column is higher, owing to the longer column. As the superficial velocity is the same, the number of the theoretical plates N increases with increasing column length Z at the same particle diameter d_p. On the other hand, N is proportional to the $1/d_p$ at low flow-rates and to $1/d_p^2$ at high flow-rates[1]. Consequently, an increase in Z from 15 to 40 cm and in d_p from 40 to 65 μm will result in a 1.3-fold increase in N, if we assume that N is proportional to $d_p^{-1.5}$. This increase in N is responsible for the higher peak concentration of the large column. However, the separation time for the large column was 2.67 times longer than that for the small one. It should be noted that when the N value of the two columns are set equal, the resulting peak heights are the same.

The inset in Fig. 10 shows the results obtained with a very short column (length 2 cm) with small (2 cm) and large (10.8 cm) diameters. The enzyme activity profiles are similar. In this case, a large volume of sample containing the enzyme at a relatively low concentration was applied. As a large volume must be processed, the flow-rate during the sample application was high, but was reduced at the elution stage because the concentration factor of the target enzyme in this type of stepwise elution is strongly dependent on the flow-rate[12].

DISCUSSION

Several strategies are possible for increasing the column productivity[1,13,14]. Displacement and/or frontal chromatography may be alternative methods for preparative separations of proteins. Liao and Horváth[15] examined the frontal and displacement IEC of an enzyme similar to that used in this study and found that the contaminant itself can be used as a displacer. They also claimed that the productivity is much higher than that shown in linear gradient elution experiments in our previous study[9].

In fact, the frontal and displacement mode can be applied to the present system. Fig. 11 shows the breakthrough curve (the profiles in frontal chromatography). It is seen that the concentrated and purified enzyme can be recovered by this method. However, during this experiment clogging of the column and a resulting increase in pressure drop were observed. Moreover, it is difficult to monitor the target protein with a UV detector. In the work of Liao and Horváth[15], a large amount of the target enzyme was discarded and the recovery was relatively low (the final recovery was 33%).

Although several theoretical and experimental studies have been reported recently[14–17], it seems that more studies are needed to perform frontal and displace-

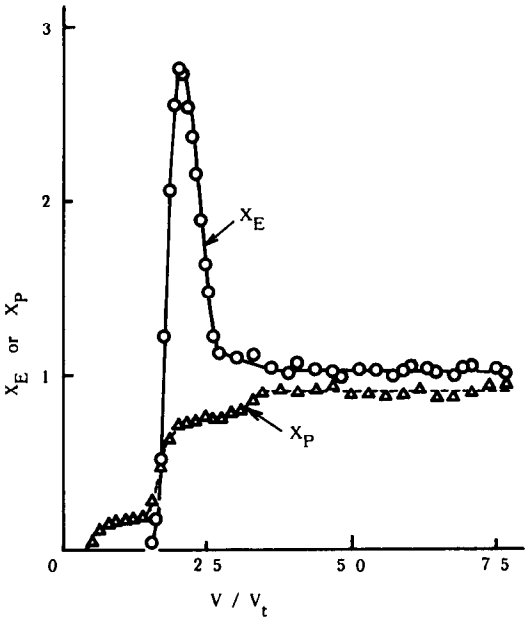

Fig. 11. Breakthrough curve (frontal chromatography). The sample (1% β-galactosidase) dissolved in the buffer [14 m*M* Tris–HCl (pH 7.7) containing 0.03 *M* NaCl] was applied to the column (DEAE Toyopearl 650, 7.5 × 0.75 cm I.D., $d_p = 40$ μm) equilibrated with that buffer at $F = 1$ ml/min and 25°C until $V/V_t = 77$ ($V = 250$ ml). The eluted fraction was collected by a fraction collector and the protein concentration and the enzyme activity were assayed. When the fractions between $V/V_t = 16$ and 27 are recovered, the purification factor is 2.0 and the recovery is 70%. (○) X_E = ratio of the enzyme activity to that of the sample; (△) Xp = ratio of the protien concentration to that of the sample.

ment chromatography as an efficient method for the preparative separation of proteins.

The purification factor values obtained with the stepwise elution in this study are lower than those in linear gradient elution reported previously[9]. The elution volume used (about four column volumes as shown in Fig. 4) was almost the same for linear gradient elution at $GH = 0.029$ M and $u = 1.7$ cm/min (see Fig. 7 in ref. 9). Moreover, the determination of the chromatographic conditions is not easy, as shown in this study, while the scaling-up of linear gradient elution is easily designed on the basis of the GH–I_R relationship shown in Fig. 2[1,6,9] and the dimensionless variable $[(D_m I_a Z)/(GHud_p^2)]^{1/2}$ (ref. 10).

The most attractive feature of the stepwise elution is the simple operation and the highly concentrated fraction. It is not advantageous to search for chromatographic conditions such that the desired protein is purified to homogeneity. Rather it is advisable to use stepwise elution as a fast purification and concentration method for proteins.

Usually, a very large volume of liquids containing a desired protein must be processed at an early stage of the purification (downstream) process. This might be done by, for example, ultrafiltration. However, the use of liquid chromatography is advantageous as it not only concentrates but also purifies the desired protein. For this purpose, a column of large diameter and short length packed with small particles such as that shown in Fig. 10 is suitable as it can permit high volumetric flow-rates and process a sample of large volume in a short cycle time. The productivity, defined as (amount of the recovered enzyme)/[(column volume)(separation time)], is 0.05 mg enzyme/(ml column)·min and the concentration factor is about 50-fold for the experimental results shown in the inset in Fig. 10. This means that, for example, a 5-l IEC column (10 cm × 25 cm I.D.) can process 150 l of solution of 0.01% enzyme used in this study within 60 min.

SYMBOLS

D_m	molecular diffusion coefficient (cm^2/s)
d_c	column diameter (cm)
d_p	particle diameter (μm)
F	volumetric flow-rate (ml/min)
GH	$g(V_t - V_o)$, slope of the gradient normalized with respect to the column gel volume (M)
g	slope of the linear gradient (M/ml)
I_a	dimensional constant having a numerical value of 1 (M)
I	salt concentration (M)
I_E	salt concentration of the elution buffer (M)
I_R	salt concentration at peak position (M)
I_0	salt concentration of the starting buffer (M)
K	distribution coefficient of proteins as a function of I, $K(I)$
K'	distribution coefficient of a salt
K_E	K at $I = I_E$
u	u_0/ε, linear mobile phase velocity (cm/min)
u_0	$F/(\pi d_c^2/4)$, superficial velocity (cm/min)

V elution volume from the start of elution (ml)
V_o εV_t, column void volume (ml)
V_t total column volume (ml)
X_E ratio of the enzyme activity to that of the sample
X_P ratio of the protein concentration to that of the sample
Z column length (cm)
ε void fraction of the column

REFERENCES

1 S. Yamamoto, K. Nakanishi and R. Matsuno, *Ion Exchange Chromatography of Proteins*, Marcel Dekker, New York, 1988.
2 C. J. O. R. Morris and P. Morris, *Separation Methods in Biochemistry*, Pitman, London, 2nd ed., 1964, p. 93.
3 D. L. Saunders, in E. Heftmann (Editor), *Chromatography*, Van Nostrand-Reinhold, New York, 3rd ed., 1975, p. 84.
4 S. Yamamoto, K. Nakanishi, R. Matsuno and T. Kamikubo, *Biotechnol. Bioeng., 25* (1983) 1465.
5 S. Yamamoto, K. Nakanishi, R. Matsuno and T. Kamikubo, *Biotechnol. Bioeng., 25* (1983) 1373.
6 S. Yamamoto, M. Nomura and Y. Sano, *AIChE J.*, 33 (1987) 1426.
7 E. Steers, P. Cuatrecasas and H. Pollard, *J. Biol. Chem.*, 246 (1971) 196.
8 K. Nishimoto, Y. Nakahara, K. Matsubara and K. Sakamoto, *Toyo Soda Kenkyu Hokoku*, 31 (1987) 45.
9 S. Yamamoto, M. Nomura and Y. Sano, *J. Chromatogr.*, 396 (1987) 355.
10 S. Yamamoto, M. Nomura and Y. Sano, *J. Chromatogr.*, 409 (1987) 101.
11 J.-Ch. Janson and P. Hedman, *Adv. Biochem. Eng.*, 25 (1982) 43.
12 S. Yamamoto, T. Suehisa and Y. Sano, *AIChE Annual Meeting, San Francisco 1989*, paper No. 180f.
13 I. Mazsaroff, R. Bischoff, P. Tice and F. E. Regnier, *J. Chromatogr.*, 437 (1988) 429.
14 L. Velayudhan and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 13.
15 A. Liao and Cs. Horváth, *Ann. N.Y. Acad. Sci., Biochemical Engineering VI*, 589 (1990).
16 M. W. Philips, G. Subramanian and S. M. Cramer, *J. Chromatogr.*, 454 (1988) 1.
17 V. Svoboda, *J. Chromatogr.*, 464 (1989) 1.