Journal of Chromatography, 396 (1987) 355–362
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 480

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Purification of β-galactosidase by large-scale gradient elution ion-exchange chromatography

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(Received February 2nd, 1987)

The purification and separation of proteins includes separation steps involving various types of liquid chromatography (LC). Although the optimization of such a separation process is essential for industrial purposes, it is still difficult to determine what type of LC should preferably be employed. If two or more LC separation modes are needed, the sequence must be determined, which is also a difficult problem at present.

Such difficulties arise from the fact that each LC mode has a large number of operating and column variables that must be carefully chosen in order to obtain a desired resolution of a given protein. Another problem is the low speed of LC of proteins. The LC of proteins has been operated at low pressure and low flow-rates as the packing materials (gels) are soft and compressible. In addition, these properties of soft gels make large-scale LC difficult[1].

In order to circumvent such difficulties, packing materials much smaller and more rigid than the soft gels have been developed. LC with such packing materials, known as high- or medium-performance LC, will be employed more frequently as a tool for the large (industrial) scale purification of biological products such as proteins and enzymes.

In this paper, we present a case study of the large-scale medium-performance LC purification of crude β-galactosidase by the following strategy. We first determined the suitable mode of LC for the purification of the above-mentioned enzyme by using high-performance liquid chromatography (HPLC) with 10-μm particles and medium-performance LC with 40-μm particles. The following three modes, in which no biospecific affinity interaction is involved, were employed: gel filtration chromatography, ion-exchange chromatography and hydrophobic interaction chromatography.

The degree of purification and the recovery of the enzyme were also measured. It was determined from these results that the most suitable LC mode for the purification of the enzyme was ion exchange.

The effects of the slope of the salt gradient on the degree of purification and on the elution position were then investigated with a small medium-performance ion-exchange column according to our method reported elsewhere[2–4]. On the basis of these data, large-scale gradient elution medium-performance ion-exchange chromatography was carried out with 65-μm gel. The elution curves obtained with 113-, 30- and 2.7-l columns were similar to the curve with a small column of 23 ml.

0021-9673/87/$03.50

TABLE I

CHROMATOGRAPHIC COLUMNS EMPLOYED

MPGFC = medium-performance gel filtration chromatography; HPGFC = high-performance gel filtration chromatography; MPIEC = medium-performance ion-exchange chromatography; HPIEC = high-performance ion-exchange chromatography; MPHIC = medium-performance hydrophobic interaction chromatography; HPHIC = high-performance hydrophobic interaction chromatography.

Type	*Gel**	$d_p(\mu m)$	$d_c(cm)$	$Z(cm)$	$V_t(ml)$	*Elution method*
MPGFC	HW55F	44	2.2	60	228	Isocratic (containing
HPGFC	G3000SW	11	0.75	30	13.3	0.1 *M* NaCl) at pH 7.7
MPIEC	DEAE 650S	40	1.6	15	30	Linear increase of NaCl
MPIEC	DEAE 650M**	65	1.4	15	23	from 0.03 *M* at pH 7.7***
HPIEC	DEAE 5PW	10	0.75	7.5	3.3	
MPHIC	Butyl 650S	37	1.6	15	30	Linear decrease of ammonium
HPHIC	Phenyl 5PW	10	0.75	7.5	3.3	sulphate from 2 *M* at pH 7.7

* All gels employed are commercial products from Toyo Soda (Tokyo, Japan).
** For the large-scale ion-exchange chromatograph shown in Fig. 7, DEAE 650M was employed.
*** The experiments were also carried out at pH 5.2 for ion-exchange chromatography.

EXPERIMENTAL

The enzyme used was crude β-galactosidase from *Aspergillus oryzae*, commercially available from Amano Pharmaceutical as Lactase F. The HPLC columns and small medium-performance LC columns (gels) employed are listed in Table I. The apparatus and the operating method were the same as those in our previous studies[3–5]. The details of the design of the large columns were described in ref. 6.

The enzyme activity of β-galactosidase was determined spectrophotometrically by measuring the change in the absorbance at 420 nm of the substrate (*o*-nitrophenylgalactoside) with time, ΔA_{420}. The buffer solutions used were 14 m*M* Tris–HCl (pH 7.7, ionic strength 0.01) and 15 m*M* acetate (pH 5.2, ionic strength 0.01). The elution methods are summarized in Table I. The experiments were carried out at 25°C.

The elution volume, V_e, of a solute in gel filtration chromatography can be described by the equation[7].

$$V_e = V_0 + K(V_t - V_0) \quad (1)$$

where V_0 is the column void volume, V_t is the total column volume and K is the distribution coefficient. This equation can also be employed for solutes that are not adsorbed on ion-exchange columns. As Blue Dextran 2000, commonly used for the determination of V_0 in gel filtration chromatography[7] is adsorbed on DEAE ion-exchange columns, we employed Dextran T-2000 pulses (1%, 0.4 ml) for determining V_0 of small DEAE ion-exchange columns. For large DEAE ion-exchange columns (2.7, 30 and 113 l), V_0 was determined from V_e using acetone pulses (concentration 0.5%; volume 0.5–0.6% of V_t) according to the equation

$$V_0 = (V_e - K_{acetone}V_t)/(1 - K_{acetone}) \quad (2)$$

where $K_{acetone}$ (=0.73) is the K value for acetone pulses (0.5%, 0.4 ml) determined with small DEAE ion-exchange columns according to eqn. 1.

RESULTS AND DISCUSSION

Purification of β-galactosidase by HPLC and medium-performance LC in various separation modes

Fig. 1 shows the elution curves obtained with high- and medium-performance gel filtration chromatography. Basically, substances are eluted in order of decreasing molecular weight (MW) in gel filtration chromatography[7]. Hence it is easy to predict the elution behaviour in medium-performance gel filtration chromatography from that in high-performance gel filtration chromatography. As seen in Fig. 1, the resolution of high-performance gel filtration chromatography is much better than that of medium-performance gel filtration chromatography. Although we can increase the resolution by decreasing the flow-rate and/or increasing the column length[7,8], the separation time also increases.

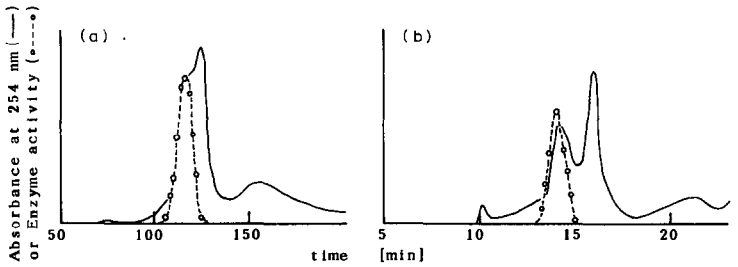

Fig. 1. Elution curves of β-galactosidase with (a) medium- and (b) high-performance gel filtration chromatography. The dotted and solid curves in Figs. 1–3 represent the activity of β-galactosidase (ΔA_{420}, arbitrary units) and the absorbance at 254 nm (UV detector output, arbitrary units), respectively. Sample: 1%, 1 ml (medium performance); 1%, 0.1 ml (high performance). Flow-rate (v), 1 and 0.5 ml/min, respectively.

Gel filtration chromatography is also useful for determining MW[7]. The MW of β-galactosidase was determined to be 130000 from its elution volume in high-performance gel filtration chromatography with the aid of the calibration graph prepared with standard proteins of known MW.

In ion-exchange chromatography, the resolution can be increased simply by decreasing the slope of the gradient, g[2,9,10]. Hence the g value for the medium-performance column was set lower than that for the high-performance column. As shown in Fig. 2, the elution behaviour of high- and medium-performance ion-exchange chromatography is similar, although many small peaks are found in high-performance ion-exchange chromatography which cannot be resolved well in medium-performance ion-exchange chromatography. Similar findings are drawn from the results of hydrophobic interaction chromatography shown in Fig. 3. The enzyme activity *vs.* time curve in hydrophobic interaction chromatography is not as sharp as that in ion-exchange chromatography.

Table II summarizes the recovery and the degree of purification obtained with

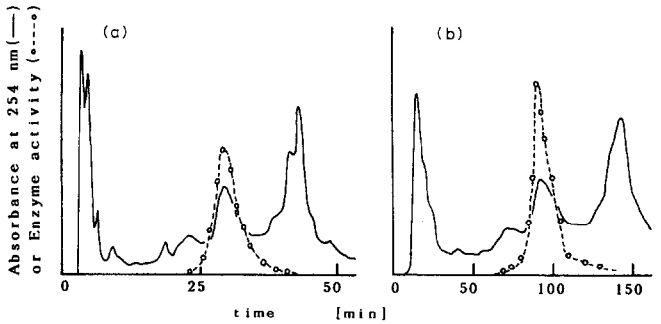

Fig. 2. Elution curves of β-galactosidase with (a) high- and (b) medium-performance ion-exchange chromatography. High performance: sample, 1%, 1 ml; $g = 0.0047$ M/ml; $v = 1$ ml/min. Medium performance: sample, 1%, 10 ml; $g = 6 \cdot 10^{-4}$ M/ml; $v = 2$ ml/min.

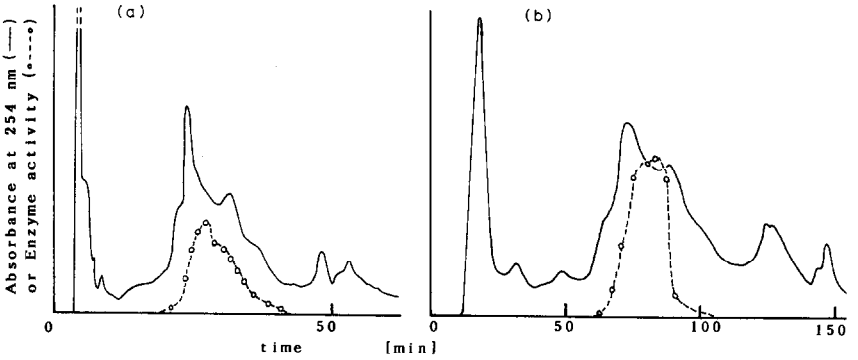

Fig. 3. Elution curves of β-galactosidase with (a) high- and (b) medium-performance hydrophobic interaction chromatography. Sample and v as in Fig. 2. $g = -0.033$ M/ml (high performance); -0.0067 M/ml (medium performance).

medium-performance gel filtration, ion-exchange and hydrophobic interaction chromatography. As a measure of the degree of purificaiton, the purification factor (PF) defined by the following equation was employed:

$$\mathrm{PF} = \frac{(\text{enzyme activity/amount of protein})_{\text{recovered}}}{(\text{enzyme activity/amount of protein})_{\text{sample}}} \tag{3}$$

TABLE II

SUMMARY OF PURIFICATION OF β-GALACTOSIDASE BY MEDIUM PERFORMANCE LC

The purification factors are obtained from the experimental results shown in Figs. 1–3.

Mode of separation	*Purification factor*	*Recovery (%)*
Crude	1.0	100
MPGFC (HW55F)	1.5	100
MPIEC (DEAE 650S)	3.3	95
MPHIC (Butyl 650S)	1.7	100

The protein concentration was determined from the absorbance at 280 nm. The purification factor for ion-exchange chromatography is high, although the recovery is slightly lower than for the other two LC modes. The enzyme activity *vs.* time curve corresponds to a single peak obtained by the UV detector (Fig. 2). This is also favourable for the purification. In ion-exchange chromatography, pH is also an important parameter affecting the resolution[2,4,11]. The purification factor obtained at pH 5.2 was 2.0–2.5, which was lower than that at pH 7.7.

The purity of each peak fraction obtained by medium-performance LC was checked by high-performance ion-exchange chromatography. It is seen from Fig. 4 that the purity of the fraction from medium-performance ion-exchange chromatography is high compared with the other two LC modes. Similar results were also obtained by a disc gel electrophoresis experiment (data not shown).

Large-scale gradient elution medium-performance ion-exchange chromatography

As the enzyme (β-galactosidase) was found to be highly purified by medium-

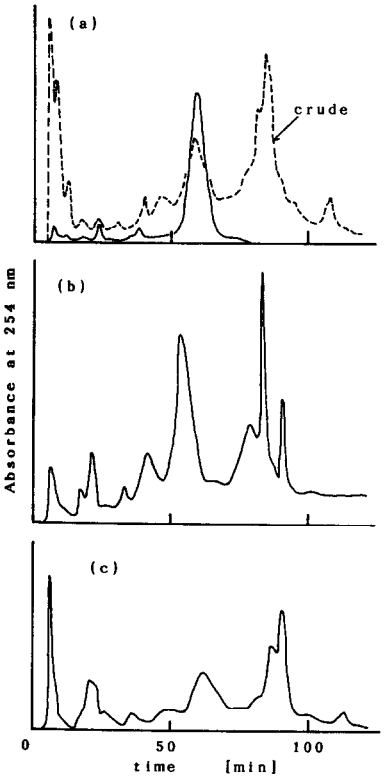

Fig. 4. Elution curves of the purified fraction of β-galactosidase by (a) medium-performance ion-exchange chromatography, (b) medium-performance hydrophobic interaction chromatography and (c) medium-performance gel filtration chromatography with high-performance ion-exchange chromatography. The sample (the peak fraction of medium performance LC shown in Figs. 1–3) was dialysed against the initial buffer before it was applied to the DEAE 5 PW column. The elution curve for crude β-galactosidase is shown by a dotted curve in (a). g = 0.0047 M/ml; v = 0.5 ml/min.

performance ion-exchange chromatography, we further investigated factors that affect the purification factor to carry out large-scale gradient elution medium-performance ion-exchange chromatography.

We have shown[2,4] that the most important variable in linear gradient elution is the parameter $GH = g(V_t - V_0)$. When the salt concentration at the peak position I_R is measured as a function of GH for a given small column, the elution position can be predicted from the GH *vs.* I_R curve for larger columns and/or for different slopes of the gradient g[2,4]. We have also reported that the resolution increases with decreasing GH^2.

Fig. 5 shows the GH *vs.* I_R plot for β-galactosidase. As the pH increases from 5.2 to 7.7, the curve becomes steeper and shifts to larger I_R values. This means that the enzyme is adsorbed on the ion-exchange column more strongly with increase in pH[2,5]. It is also seen that the variation of I_R is negligible for the ranges of flow-rate, sample volume and particle diameter employed.

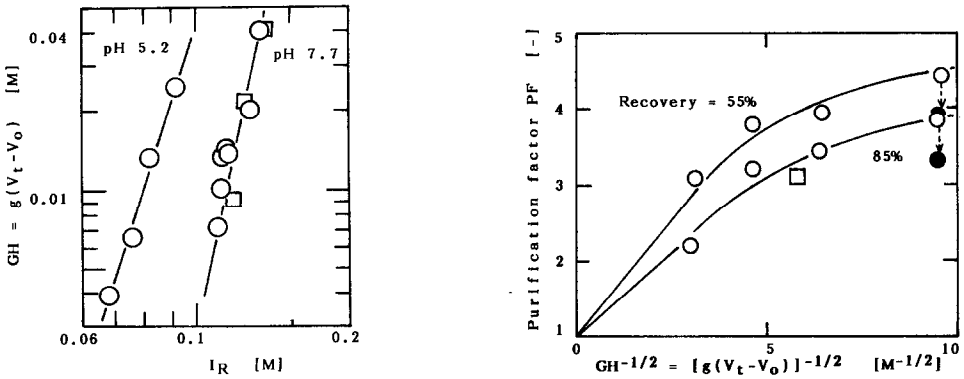

Fig. 5. Effect of the slope of the gradient on the elution position of β-galactosidase. I_R is the ionic strength = NaCl molarity + ionic strength of buffer (= 0.01) at which the β-galactosidase peak appeared at the column outlet. V_0 and V_t are the void volume and the total column volume, respectively. Sample (1% β-galactosidase) volume, 1–10 ml; flow-rate (v), 2–3 ml/min. ○, DEAE 650S; □, DEAE 650M.

Fig. 6. Effect of GH on the purification factor as a function of recovery at pH 7.7. Sample (1% β-galactosidase) volume: ○, 10 ml; ●, 50 ml. Flow-rate, (v), 2 ml/min, ○,●, DEAE 650S; □, DEAE 650M.

Fig. 6 shows the relationship between purification factor and GH as a function of recovery at pH 7.7. The purification factor was calculated from the fractions, the sum of the enzyme activities of which becomes equal to a specified value (in this instance either 55 or 85%) of that initially applied to the column. Therefore, considerable parts of the leading and tailing portions of the enzyme activity *vs.* time curve were ignored for the calculation of the purification factor when the recovery was 55%. There is a linear relationship between purification factor and $GH^{-1/2}$ at low $GH^{-1/2}$ values. Thus, the degree of purification increases with decreasing slope of the gradient g. However, the slope of the purification factor *vs.* $GH^{-1/2}$ curve becomes shallow with further increase in $GH^{-1/2}$.

It is also seen from Fig. 6 that the purification factor decreases with increase in particle diameter, sample volume and recovery at a certain GH and flow-rate. It

should be kept in mind that the increase in $GH^{-1/2}$ increases the separation time. For example, a two-fold increase of $GH^{-1/2}$ causes almost a four-fold increase in separation time. Therefore, we must consider the relationship between purification factor, the separation time, the recovery and the amount of sample when choosing the operating conditions. Here, as model case, we chose a value of $GH = 0.029$.

For the large columns the ratio between the length and the diameter of the column is different from that for the small columns. Therefore, the slope of the gradient g is adjusted to that $GH = g(V_t - V_0)$ becomes equal to 0.029. Fig. 7 shows the elution curves of β-galactosidase with the small and large medium-performance ion-exchange columns ($d_p = 65$ μm). When we consider the experimental difficulties in preparing a large volume of buffer and sample solutions, in making a large-scale linear increase of NaCl, in packing large columns and in determining V_0, we can conclude that the peak positions with respect to V/V_0 and also the peak shapes are similar.

This study has shown that linear salt gradient medium-performance ion-ex-

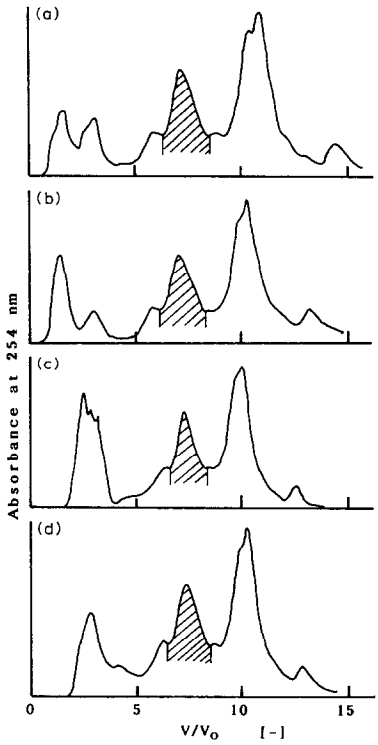

Fig. 7. Comparison of the elution curves of β-galactosidase by linear gradient elution with DEAE columns ($d_p = 65$ μm) of various column dimensions. The sample (1% β-galactosidase) volume is approximately one-third of the total column volume, V_t. V_0 is the column void volume and V is the elution volume from the start of the linear gradient elution. The shaded area indicates the fractions having β-galactosidase activity. (a) $d_c = 60$ cm; $Z = 40$ cm; $V_t = 113$ l; $g = 4.3 \cdot 10^{-7}$ M/ml; $v = 1600$ ml/min; sample, 40 l. (b) $d_c = 31$ cm; $Z = 40$ cm, $V_t = 30$ l; $g = 1.6 \cdot 10^{-6}$ M/ml; $v = 500$ ml/min; sample, 10 l. (c) $d_c = 10.8$ cm; $Z = 30$ cm, $V_t = 2.7$ l; $g = 1.8 \cdot 10^{-5}$ M/ml; $v = 60$ ml/min; sample, 1 l. (d) $d_c = 1.4$ cm; $Z = 15$ cm; $V_t = 23$ ml; $g = 2.1 \cdot 10^{-3}$ M/ml; $v = 1$ ml/min; sample, 8 ml.

change chromatography of proteins with 65-μm gel can be successfully scaled up to the 113-l column from the data obtained with the 23-ml column. HPLC is also appears to be useful for a rapid survey of the optimal chromatographic conditions of a given protein and for the rapid determination of the purity of the fractionated protein.

SYMBOLS

DEAE	Diethylaminoethyl;
d_c	column diameter (cm);
d_p	particle diameter (μm);
GH	$g(V_t - V_0)$ (M);
g	slope of the linear gradient (M/ml);
I_R	ionic strength = sodium chloride molarity + 0.01 (ionic strength of the buffer solution) at peak position (M);
K	distribution coefficient defined by eqn. 1;
MW	molecular weight;
V	elution volume from the start of linear gradient elution (ml);
V_e	peak elution volume (ml);
V_0	column void volume (ml);
V_t	total column volume (ml);
v	volumetric flow-rate (ml/min);
Z	column length (cm).

ACKNOWLEDGEMENTS

We gratefully acknowledge support from the Ministry of Education, Science and Culture of Japan, Grant No. 60750873, and from the Saneyoshi Scholarship Foundation, Grant No. 5820. We are also grateful to Messrs. K. Matsubara, K. Sakamoto and H. Sasaki of Toyo Soda for their assistance in carrying out large-scale ion-exchange chromatography.

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