Adsorption Chromatography of Proteins: Determination of Optimum Conditions

A simple and convenient method for predicting optimum chromatographic conditions from linear gradient elution experiments with a small column is presented and verified experimentally. The method was found to be very useful for a rapid survey of the nature of unknown proteins, for the estimation of optimum chromatographic conditions, and for scaling up the separation of proteins in ion exchange and hydrophobic interaction chromatography.

Two applications were successfully carried out on the basis of the proposed method: (1) Separation of a protein mixture of ovalbumin and β-lactoglobulins A and B with both anion and cation ion exchange columns by means of column switching technique. In this method, the sample is always subjected to the elution process until it is collected as a purified fraction through the two columns. A special valving system is devised for this method. (2) Large-scale ion exchange chromatography separation of β-galactosidase. Linear gradient elution of crude β-galactosidase was carried out with a 30 L ion exchange gel column on the basis of data obtained with a small (23 mL) column.

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Introduction

The development of biotechnology requires excellent separation methods such as various types of liquid chromatography for processing large amounts of proteins and enzymes.

Linear salt gradient elution is the most common and useful method for separation of proteins by adsorption chromatography procedures such as ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and several types of group-specific affinity chromatography. In isocratic elution the composition of the elution buffer is the same as that of the starting buffer, and in stepwise elution the composition of the elution buffer is changed discontinuously from that of the starting buffer. In comparison with these methods, linear gradient elution has the following advantages (Morris and Morris, 1964; Matsuno et al., 1987):

1. An artificial peak caused by discontinuous changes of the elution buffer is avoided
2. A number of components can be eluted in a single elution experiment without using excessively large effluent volumes
3. The resolution is increased simply by reducing the slope of the gradient (Kato et al., 1982, 1983, 1985; Matsuno et al., 1987; Yamoto et al., 1987b)

The movement of protein zones in linear gradient elution is drawn schematically in Figure 1 in comparison with that in isocratic elution. Initially, the protein zones including a protein of interest are retained at the top of an IEC or HIC column. As the linear increase (decrease) of the salt concentration is introduced to the IEC or HIC column, the zones start to move. Then their velocities increase drastically because the adsorption of proteins on the stationary phase depends strongly on the salt concentration. Finally, they move with a certain salt concentration. After this period, the distance between the two zones hardly varies with time, while in isocratic elution it increases with time, as shown in Figure 1.

The movement of the protein zone in linear gradient elution can be described by equilibrium theory (Drake, 1955; Freiling, 1955) as:

$$dz_p/dt = u/[1 + HK(I)] \tag{1}$$

When the linear gradient of a salt concentration is assumed to be established ideally in the column, the salt concentration or ionic strength I as a function of z_p and t is given as

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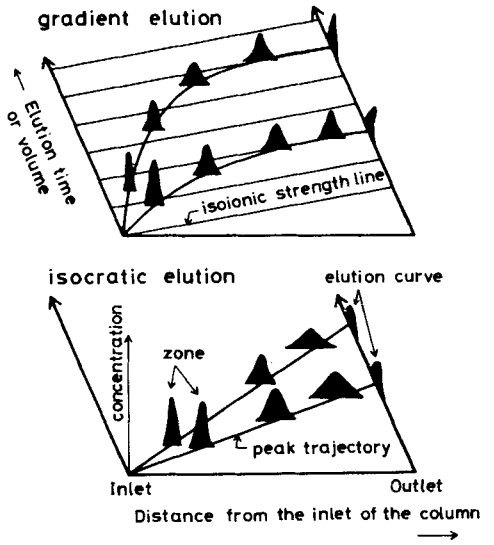

Figure 1. Movement of zones during linear gradient elution and isocratic elution.

$$I = I_o + G[(ut/Z) - (1 + HK')(z_p/Z)] \qquad (2)$$

where $G = gV_o$ is the slope of the gradient normalized with respect to the column void volume and K' is the distribution coefficient of the salt. When the distribution coefficient of a protein K is known as a function of I, integration of Eq. 1 together with Eq. 2 with respect to time yields the peak retention time t_R. This calculation procedure was first presented by Drake (1955) and Freiling (1955). Pitt (1976) has shown by numerical calculation that the t_R value obtained by this model is coincident with that obtained from a mass balance model taking into account the zone spreading. However, since the analytical solution of Eqs. 1 and 2 cannot be obtained unless several simplified assumptions are made (Drake, 1955), the numerical integration must be carried out for each set of operating conditions, which is rather laborious.

Yamamoto et al. (1983) obtained Eq. 3 from Eqs. 1 and 2, and presented a simple graphical method for obtaining the ionic strength at t_R, I_R based on Eq. 3.

$$\int_{I_o}^{I_R} dI/[K(I) - K'] = GH \qquad (3)$$

When $K(I) - K'$ is known, we integrate the lefthand side of Eq. 3 as a function of I and plot it against I. With the aid of this curve, we can predict I_R with a single parameter GH, which is composed of the column volume and the slope of the gradient.

In our previous study (1983), linear gradient elution experiments of proteins were carried out with various ion exchange gel columns for a wide range of experimental conditions. The I_R values obtained were in good agreement with the values calculated by Eq. 3 with $K(I)$ measured by separate batch experiments (Yamamoto et al., 1983). However, the determination of $K(I)$ by the batch experiment over a wide range of I was found to be tedious and time-consuming.

On the other hand, our previous study also suggests that in the case of $K(I)$ unknown, once the GH vs. I_R plot is prepared with a certain column, it can be used for predicting I_R and hence t_R in linear gradient elution of various column dimensions and the slope of the gradient. The purpose of this paper is first to verify this experimentally.

Our second purpose is to show that $K(I)$ can be obtained from the GH vs. I_R plot. The derivative of Eq. 3 with respect to I becomes

$$d(GH)/dI = 1/[K(I) - K'] \qquad (4)$$

Therefore, the differentiation of the GH vs. I_R curve gives $1/[K(I) - K']$ irrespective of the type of the salt concentration dependence of K. Since K' is constant under the usual experimental conditions, we can obtain K' from a pulse response experiment or from frontal analysis. Using the above procedure, we have determined $K(I)$ of several standard proteins in IEC and HIC in this study. Good agreements are observed between K values obtained by the proposed method and those by the batch or isocratic elution method. Stepwise elution designed on the basis of this method also has shown the utility of the method.

Our third purpose is to show that the GH vs. I_R plot can be also used as a method for determining not only the optimum chromatographic conditions but also the characteristics of proteins.

When $K(I) - K'$ is approximated by Eq. 5, Eq. 6 is obtained from Eq. 3 [$I_o^{(B+1)}$ is negligibly small compared with $I_R^{(B+1)}$ in the usual liquid chromatography conditions].

$$K - K' = AI^{-B} \qquad (5)$$

$$GH = I_R^{(B+1)}/[A(B + 1)] \qquad (6)$$

B implies the number of points involved in the adsorption equilibria, as shown later. Therefore, we expect that the GH vs. I_R curve in IEC becomes steep and shifts to higher I_R values with an increase in the net charge of a given protein. This corresponds to an increase of pH in the case of anion exchange columns and a decrease of pH in the case of cation exchange columns.

In the case of HIC, it is difficult to predict the degree of affinity of a given protein to the HIC column. However, the above consideration still holds. Namely, the GH vs. I_R curve becomes steep and shifts to lower I_R values with an increase in the number of points involved in the adsorption equilibria. Therefore, if we prepare GH vs. I_R curves for various pH, we can use them not only for determining the optimum chromatographic conditions of proteins but also for obtaining important information on the protein characteristics. The present experimental results for IEC and HIC have shown the utility of the proposed method.

Furthermore, the following two successful applications of the proposed method are demonstrated:

1. The separation of a protein mixture of ovalbumin and β-lactoglobulins A and B with both anion and cation ion exchange columns by means of column switching techniques. In this method, the sample is always subjected to the elution process until it is collected as a purified fraction through the two columns. A special valving system is devised for this method.

2. The large-scale IEC separation of β-galactosidase. The linear gradient elution of crude β-galactosidase was carried out with a 30 L IEC column on the basis of the data obtained with a small (23 mL) column.

Experimental Method

The proteins employed are listed in Table 1. The following buffer systems were used: 60 mM sodium acetate-acetic acid (pH 4.0), 10 mM sodium acetate-acetic acid (pH 5.2), 10 mM sodium acetate-acetic acid (pH 5.6), 14 mM tris-HCl (pH 7.7), and 4 mM tris-HCl (pH 8.5). The ionic strength I of these buffers was around 0.01. Chromatographic media (gels) from Toyo Soda used in this study included DEAE Toyopearl 650M (d_p = 65 μm), DEAE Toyopearl 650S (d_p = 40 μm), CM Toyopearl 650S (d_p = 36 μm), Buthyl Toyopearl 650S (d_p = 38 μm), and Buthyl Toyopearl 650C (d_p = 92 μm). These gels were packed into the column according to a procedure similar to that of Kato et al. (1981) or that of Yamamoto et al. (1986).

Experimental apparatus was essentially the same as that employed in our previous studies (Yamamoto et al., 1983, 1986) except that closed columns were employed. Therefore, sample injection into the column was carried out by means of a Rheodyne 7120 injection valve in the case of isocratic elution. The sample solution was directly introduced to the column with the aid of the pump in the case of both linear gradient elution and stepwise elution. A block diagram of the apparatus for the column experiment in linear gradient elution and stepwise elution is given by Figure 14 when valves V3 and V4 are set to positions *A* and *B*, respectively. Experiments were carried out in a constant-temperature box maintained at 25°C. The elution was performed by the increase of NaCl concentration from 0.03 M in IEC and by the decrease of $(NH_4)_2SO_4$ concentration from 2 M in HIC.

The distribution coefficient K was determined from the elution volume $V_e = t_R v$ in isocratic elution experiments according to the relation

$$K = (V_e - V_o)/(V_t - V_o) \qquad (7)$$

V_o was determined from a Dextran T-2000 (Pharmacia) pulse.

Table 1. Proteins Employed in Study

Protein*	MW**	pI**	Supplier
Cytochrome C from horse heart	13,000	9.0–9.4	Sigma
Ribonuclease A (RNAse) from bovine pancreas	14,000	8.7–8.9	Sigma
Myoglobin from equine skeletal muscle	18,000	7.3–7.6	Sigma
β-lactoglobulin A (LG-A)	35,000	5.1	Contained in bovine β-lactoglobulin, ICN Pharmaceuticals
β-lactoglobulin B (LG-B)	35,000	5.2–5.3	
Ovalbumin (OA) from egg white	44,000	4.7	Seikagaku Kogyo
β-galactosidase from *Aspergillus oryzae*	130,000†	4.2	Amano Pharmaceutical

*Abbreviations in parentheses are used in figures and text.

**Molecular weight (MW) and isoelectric point (pI) values from Righetti and Caravaggio (1976), Righetti et al. (1981), Smith (1968).

†Determined by high-performance gel filtration chromatography (TSK G-3000SW) with the aid of a calibration curve prepared with standard proteins having known MW values

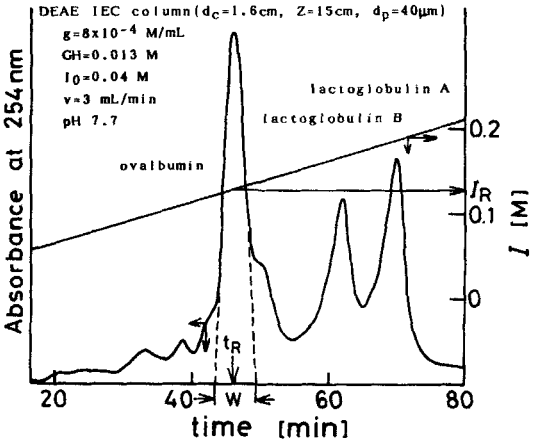

Figure 2. Typical elution curves in linear gradient elution with a DEAE IEC column.
Sample: 0.5% OA + 0.5% LG-A and LG-B, 1 mL

When K was larger than about 5, a batch experiment and/or frontal analysis was carried out according to the same procedure as that in our previous study (Yamamoto et al., 1983).

Results

Effect of sample load

Figures 2 and 3 show typical elution curves obtained from linear gradient elution experiments in IEC and HIC, respectively. The peak retention time, t_R, and the peak width at the base line, W, were measured for various sample volumes to examine the effect of sample volume on t_R and W. As seen in Figures 4 and 5, both the elution volume $V_e = t_R v$ and W remain constant at low sample volumes. Although a further increase of sample volume causes a considerable increase of W, t_R still remains constant. The highest sample loads shown in Figures 2 and 3 are 1.1 (mg protein/mL column) in IEC and 1.7 (mg protein/mL column) in HIC. These sample loads are much higher

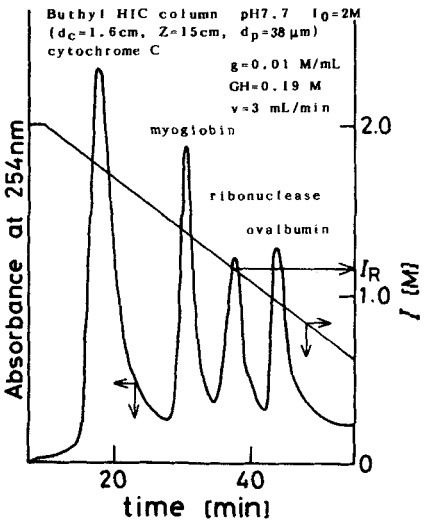

Figure 3. Typical elution curves in linear gradient elution with a Buthyl HIC column.
Sample: 0.2% cytochrome C + 0.2% myoglobin + 0.4% RNAse + 0.4% OA, 2 mL

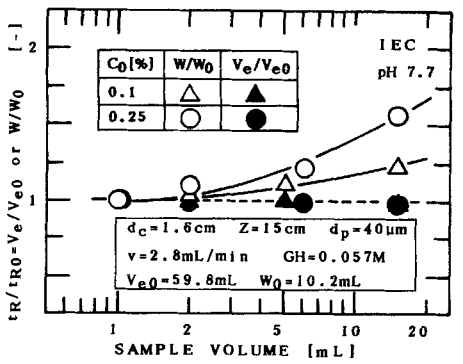

Figure 4. Effect of sample load on peak position (t_R or $V_e = t_R v$) and on peak width W in linear gradient elution IEC.

$V_{eo} = t_{Ro}v$ and W_o values at sample volume = 1 mL

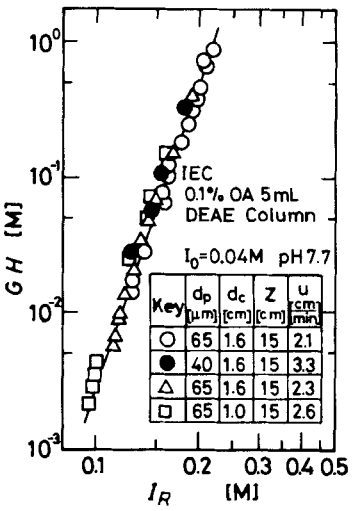

Figure 6. GH vs. I_R for OA with DEAE IEC columns.

I_R, ionic strength = molarity of NaCl + molarity of buffer (0.01)

than those for analytical separations (Kato et al., 1982; Vanecek and Regnier, 1980). Even at such high sample loads the variation of t_R is negligible, although W increases by a factor of 1.5 to 2.0. Similar experimental results were obtained by a number of investigators (Yamamoto et al., 1983; Kato et al., 1982; 1985; Vanecek and Regnier, 1980).

This is because the linear part of the isotherm of proteins very rapidly becomes wider with increasing I in IEC and decreasing I in HIC (Yamamoto et al., 1983). Consequently, the protein zone moves with the velocity governed only by I soon after elution although the protein follows a nonlinear isotherm at the beginning of the elution (Morris and Morris, 1964; Yamamoto et al., 1983).

These experimental results permit the assumption involved in the proposed method that K is dependent only on I and not much affected by the initial protein concentration, and thus permit use of the proposed method for preparative protein separation.

Relation between GH and I_R

The linear gradient elution experiment whose results are shown in Figures 2 and 3 were carried out for a wide range of the slope of the gradient g, the flow rate, and the column dimension. Then, the values of I_R obtained were plotted against GH to give the curves shown in Figures 6 and 7. The curves are not dependent on the column size, the flow rate, or d_p. Accordingly, once the GH vs. I_R curve is obtained with a certain small IEC or HIC column, the I_R and hence t_R value in linear gradient elution can be predicted from the curve for a given set of g, d_c, and Z. This is very useful since the height to diameter ratio ($=Z/d_c$) and the flow rate, as well as the slope of the gradient, must often be changed in the scaling-up due to the mechanical properties of the packing materials (gels) (Janson and Hedman, 1982).

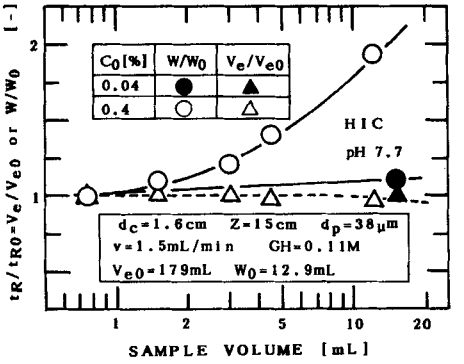

Figure 5. Effect of sample load on peak position (t_R or $V_e = t_R v$) and on peak width W in linear gradient elution HIC.

$V_{eo} = t_{Ro}v$ and W_o values at sample volume = 0.75 mL

Determination of K as a function of I from the GH vs. I_R curve

Knowledge of $K(I)$ is required for the design of the stepwise elution of proteins, since we must choose the condition (salt concentration) where a desired protein is retained (or eluted) while the other proteins or contaminants are eluted (or retained). It is also needed in models of linear gradient elution that consider zone spreading (Pitt, 1976; Yamamoto et al., 1983; Gibbs and Lightfoot, 1986).

On the basis of Eq. 4, the values of $K(I)$ are obtained by the slope of the curve of GH vs. I_R. K' values were determined by the

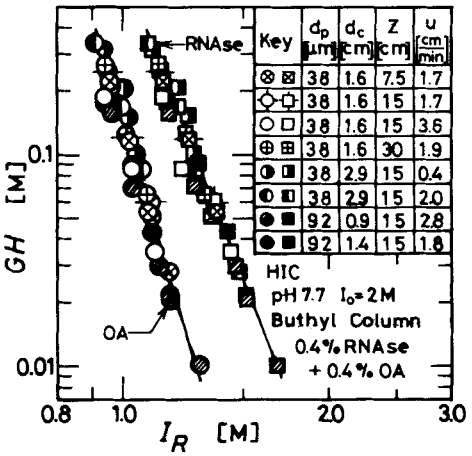

Figure 7. GH vs. I_R for OA and RNAse with Buthyl HIC columns.

$I_R = (NH_4)_2SO_4$ concentration

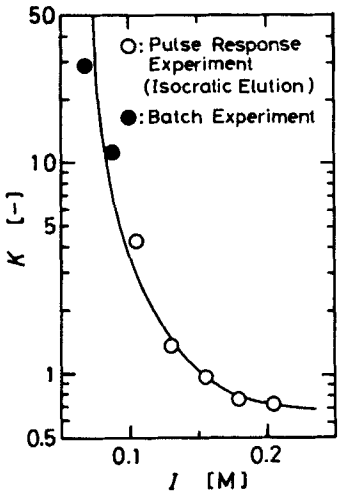

Figure 8. Relation between K and I for OA in DEAE IEC.
Curve obtained from differentiation of curve in Figure 6

pulse response experiment [$K' = 0.66$ for NaCl in IEC and $K' = 0.76$ for $(NH_4)_2SO_4$ in HIC]. As shown in Figures 8 and 9, good agreement is observed between K values obtained by this method and those by the isocratic elution and batch experiments. We have shown that the peak position t_R in stepwise elution can be predicted on the basis of K value at the salt concentration (ionic strength) of the elution buffer (Yamamoto et al., 1983). Therefore, once the curve of GH vs. I_R is obtained, we can predict t_R in both linear gradient elution and stepwise elution for a given combination of the operating variables such as the slope of the gradient, the salt concentration of the elution buffer, and the column dimension.

The elution curves in stepwise elution designed on the basis of Figure 9 are shown in Figure 10. It is seen that four proteins are well separated and the predicted t_R values (arrows in the figure) are in good agreement with the experimental values.

GH vs. I_R plots for various pH

When the ion exchange gel particle is considered to consist of matrices having charged groups (solid phase) and interstices

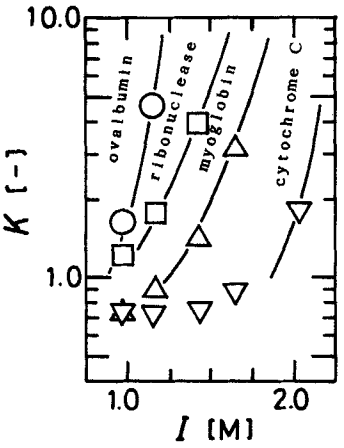

Figure 9. Relation between K and I in Buthyl HIC.
Curves obtained from differentiation of curves in Figure 7 (data of GH vs. I_R for myoglobin and cytochrome C are not shown in Figure 7)

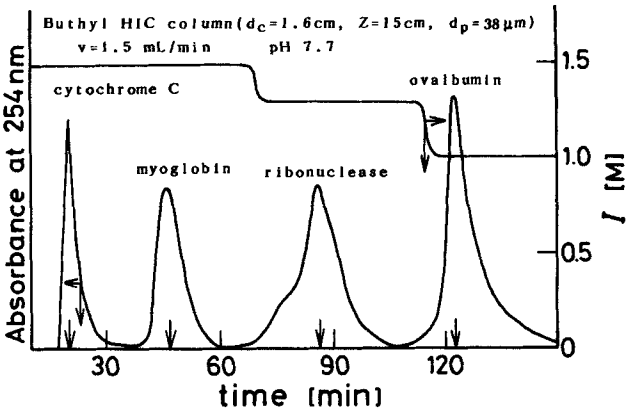

Figure 10. Elution curves in stepwise elution with a Buthyl HIC column.
Sample: 0.25% cytochrome C + 0.25% myoglobin + 0.5% RNAse + 0.5% OA, 0.5 mL

among them (pores), the distribution coefficient K is expressed by

$$K = K_{crt} + K_p \tag{8}$$

Here, K_{crt} is the distribution coefficient under the condition where the interaction between a given protein and the charged group is negligible—i.e., gel filtration (size exclusion) mode—and K_p is that between the protein concentration in the pore and that in the solid phase (Yamamoto et al., 1983; Arnold et al., 1985; Matsuno et al., 1987). Furthermore, when the law of mass action is assumed to be valid, K_p is given as (Boardman and Partridge, 1955; Kopaciewicz et al., 1983):

$$K_p = AI^{-B} \tag{9}$$

Then,

$$K = K_{crt} + AI^{-B} \tag{10}$$

We found that Eq. 10 can describe the experimental relation between K and I in IEC fairly well (Yamamoto et al., 1983). Therefore, when K' is close to K_{crt}, Eq. 5 is also a good approximation for K in IEC.

From Eq. 6, we expect that when a GH vs. I_R relation in IEC is plotted on a log-log scale, a straight curve is obtained; the curve shifts to larger I_R values and its slope becomes steep with an increase of B, that is, the number of charges involved in the adsorption equilibria.

In order to verity the above relations, we carried out linear gradient elution experiments at various pH using a DEAE and CM ion exchange gel columns (1.6 × 15 cm). A synthetic mixture of proteins including ovalbumin, β-lactoglobulin A, and β-lactoglobulin B was employed as a sample. pH was changed from 4.0 to 8.5. As is seen from Figure 11, the curve shifts to larger I_R values and its slope becomes steep with an increase in the net charge, that is, increasing pH in the case of the DEAE column and decreasing pH in the case of the CM column. These findings are in good agreement with the expectations drawn from Eq. 6. However, near the respective pI, the retention behavior of the proteins is very different. For example, at pH 5.2 both β-lactoglobulins A and B were adsorbed to either of the

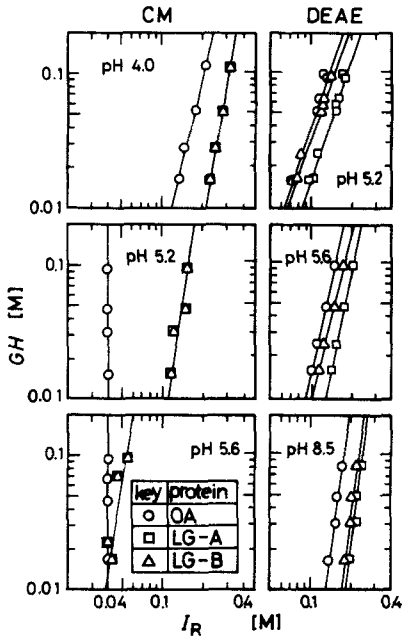

Figure 11. *GH* vs. I_R for DEAE and CM IEC columns at various pH.
I_o = 0.04.

anion and cation columns while ovalbumin was not retained on the cation column. These experimental results suggest that the retention mechanism of proteins in IEC cannot be explained on the basis of the simple net charge concept (Kopaciewicz et al., 1983; Haff et al., 1983). So, we must rely on the experimental results for the prediction of the retention behavior of proteins in IEC.

Figure 12 shows the *GH* vs. I_R curves at various pH in HIC. The slope of the curves is steep, which implies that a number of points are involved in the adsorption equilibria. The variation of the *GH* vs. I_R curve with pH is strongly dependent on respective proteins. In the case of ribonuclease, which is known to maintain its native conformation over a wide range of pH (Buzzell and Tanford, 1956), the curve did not much vary with pH. On the contrary, myoglobin was not desorbed from the HIC column at pH 5.2 and was eluted only when a buffer solution of pH 7.7 was used. The curve for cytochrome C shifted to lower I_R values with a decrease in pH, which means that the retention volume

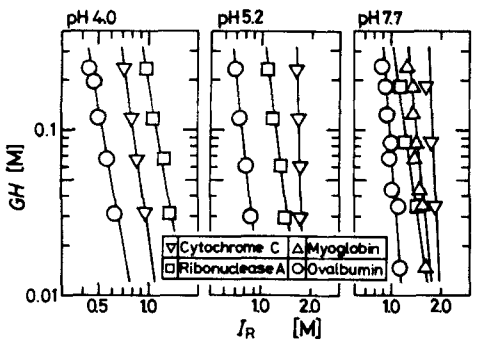

Figure 12. *GH* vs. I_R for Buthyl HIC columns at various pH.
I_o = 2.0.

becomes larger with decreasing pH. Consequently, ribonuclease was eluted before cytochrome C at pH 4.0 while the order is reversed at pH 5.2. It seems that the retention behavior of proteins in HIC is hard to expect, and therefore the utility of the proposed method is again stressed.

It should be mentioned that the assumption of Eq. 5 is not the necessary condition for the above discussion. It is employed only to show that the *GH* vs. I_R plot reflects the adsorption equilibria between proteins and IEC or HIC columns.

Application of present method to actual protein separation

To achieve a fine separation, the values of I_R at a certain *GH* should differ considerably. Therefore, we can determine from Figure 11 that the optimum condition for the separation of the three proteins with a single column is pH 5.6 with the DEAE gel column. As shown in Figure 13, the resolution of the proteins is fairly good and the t_R values estimated from Figure 11 (arrows in the figure) are in good agreement with the experimental results.

However, a more efficient separation process consisting of three separation steps was devised from Figure 11. The first step is the fractionation of ovalbumin, which is not adsorbed to the CM column at pH 5.2. The second step is the desorption of lactoglobulins A and B from the CM column. In this step, they are not separated but are eluted as a single peak. The third step is the separation of β-lactoglobulins A and B on the DEAE column at pH 5.2. Since the elution position in each step can be predicted by the proposed method before chromatography, it is possible to couple columns in a series and skip the process of collecting fractions. This type of process is called column switching technique, multidimensional column chromatography, or coupled multiple-column chromatography (Kopaciewicz and Regnier, 1983). We designed such an on-line separation process using the column switching technique; it consists of a very small CM gel column (0.75 × 7.5 cm), a small DEAE gel column (1.6 × 15 cm), and a valving system, as shown in Figure 14. We chose a low *GH* value so that the I_R value for β-lactoglobulins A and B from the CM column is low. Consequently, they can be readsorbed to the DEAE column without desalting the fraction. Therefore, we can skip the desalting process and the sample is always subjected to the elution process until it is collected as a purified fraction. The experimental elution profiles are shown in

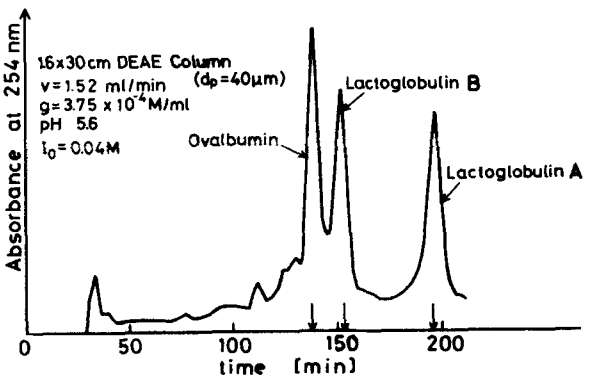

Figure 13. Elution curves in linear gradient elution with a DEAE IEC column.
Sample: 0.5% OA + 0.5% LG-A and LG-B, 2 mL.

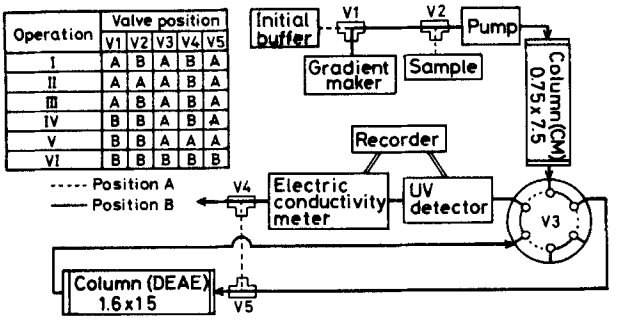

Figure 14. Block diagram of column switching technique.

Figure 15. The resolution of the three proteins separated by means of this process was far better than that with the single-step process shown in Figure 13. In addition, the separation time was 140 min, which was 70% of the single-step separation. These results demonstrate not only the utility of the proposed method but also the versatility of the column switching technique.

It is desirable that large-scale liquid chromatography can be designed and operated on the basis of data obtained with a small column. As an example, the large-scale linear gradient elution IEC of crude β-galactosidase was carried out according to the GH vs. I_R plot prepared with a small, 23 mL, column. Note that the large (30 L) column employed is not geometrically similar to the small one, as is often the case with the large-scale chromatography of proteins (Janson and Hedman, 1982). The slope of the gradient g was adjusted so that the GH values of the small and large IEC columns became equal. As seen in Figure 16, the peak positions as well as the peak shapes for the small and large IEC columns expressed in V/V_o are quite similar. The deviations observed in some peaks are probably due to experimental difficulties in preparing the large volume of sample and buffer solutions, in packing the large column, and so on. We further

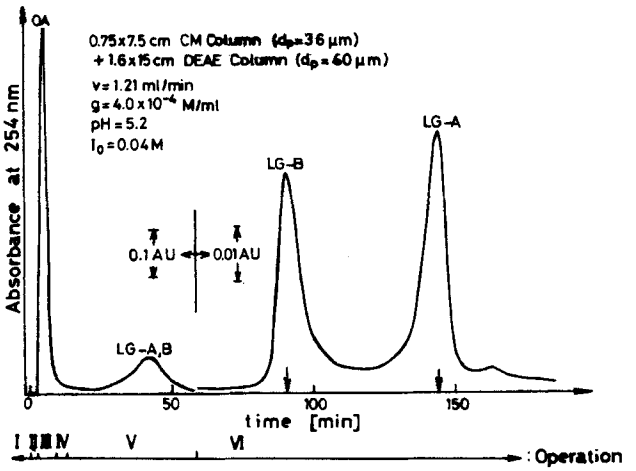

Figure 15. Elution curves in separation experiment using column switching technique.

Sample: 0.25% OA + 0.25% LG-A and LG-B, 2.5 mL
Roman numerals I–VI correspond to Figure 14; arrows represent peak positions estimated from Figure 11
GH value = 0.00069 M for CM column, = 0.0073 M for DEAE column

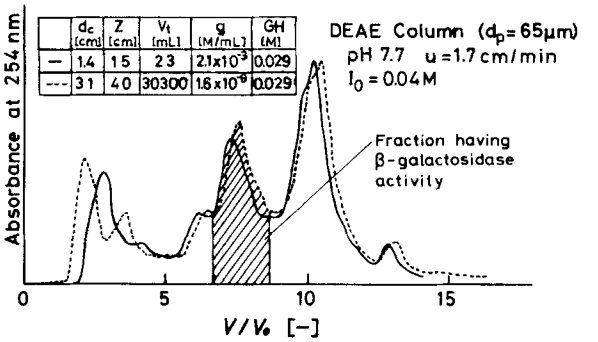

Figure 16. Elution curves of small- and large-scale linear gradient elution IEC.

Sample (1% β-galactosidase) was applied so that sample volume was $\frac{1}{3}V_t$; the sample load was 3.3 mg crude enzyme/mL column

scaled this up to a 113 L column. The results will be reported elsewhere, and variables affecting the purification factor also will be examined (Yamamoto et al., 1987a).

It should be mentioned that the packing materials (gels) employed in this study are more rigid and smaller than conventional soft gels for protein separation (Yamamoto et al., 1986; Kato et al., 1981, 1983; Janson and Hedman, 1982). These properties of the gels not only make scale-up easy but also give the high resolution at high flow rates shown in this study.

Discussion

Separation by liquid chromatography is based on the differential migration velocity caused by the difference of the affinity of substances to the packing material (stationary phase) (Giddings, 1965; Morris and Morris, 1964). Therefore, biospecific affinity chromatography with immobilized monoclonal antibodies is probably the most efficient method of liquid chromatography with proteins at present (Chase, 1984a, b); only a protein of interest is adsorbed to the stationary phase selectively. Therefore, once a column is saturated with the protein, a subsequent one-step desorption yields the highly purified fraction. However, since the affinity is so strong, the desorption must be done with extremely low pH solutions and/or with protein denaturants such as urea and guanidine hydrochloride; these may denature proteins (Chase, 1984a). Furthermore, the preparation of monoclonal antibodies immobilized packing materials is laborious and time-consuming.

On the other hand, various packing materials (gels) are commercially available for IEC and HIC. Moreover, proteins can be eluted (desorbed) simply by the change of the salt concentration. However, a number of overlapped elution curves appear in the linear gradient elution IEC or HIC of crude bioproducts, as shown in Figure 16, since biospecific adsorption of a desired protein is not involved. Let us focus our attention on the resolution of two adjacent elution curves involved in such experiments. The resolution is determined by the distance between the two peak positions and by the peak widths. The optimum chromatographic conditions are therefore such that the distance between the two peak positions is long.

In gel filtration chromatography (GFC), the distribution coefficient K is usually a unique function of molecular size (Whitaker, 1963). So, once K of two given proteins is determined for a certain GFC column, their peak positions and hence

the distance between the two peaks for various column and operating variables can easily be calculated by Eq. 7.

On the other hand, the K of proteins in IEC and HIC depends on the salt concentration I, pH, and protein concentration. Moreover, the salt concentration dependence of K in IEC and HIC is very strong. Therefore, it is very cumbersome to measure K over such a wide range of experimental conditions by a batch experiment. Moreover, when more than one component is included in the sample, the K value of each component cannot be measured by the batch experiment unless a specific measurement method for each component is available. In contrast, the proposed method is applicable to such a case when each peak is resolved. So, it is useful for determining rapidly the optimum chromatographic conditions such as the initial pH and the salt concentration of the elution buffer in stepwise elution and the slope of the gradient in linear gradient elution.

Once such chromatographic conditions are found, the next task is to choose the variables increasing the peak width, such as the column and operating variables (flow rate, column dimension, particle diameter, and so on) and the sample load. We derived an equation that relates the resolution in linear gradient elution to the column and operating variables such as the flow rate, the particle diameter, the column length and the slope of the gradient, and verified it experimentally (Matsuno et al., 1987; Yamamoto et al., 1987b).

In the case of IEC, the sample load in mg protein/mL column is 0.1–0.5 for analytical purposes (Kato et al., 1982; Vanecek and Regnier, 1980). A further increase of the sample load broadens the peak width as shown in Figures 4 and 5. Consequently, the separation efficiency decreases considerably. So, the sample load as well as the variables increasing the peak width mentioned above in IEC and HIC must be carefully chosen so that the desired separation efficiency can be obtained.

It should be noted that the increase of the sample load up to 1–1.6 mg protein/mL column hardly affected the t_R (or I_R) value in this study. This sample load is not low as compared with that in biospecific affinity chromatography (Arnold and Blanch, 1986; Chase, 1984b; Clonis et al., 1986; Janson and Hedman, 1982). When the sample load is further increased, for example to 30 mg protein/mL column as shown in the plasma protein fractionation by Janson and Hedman, the t_R value may deviate from that predicted by the present method. However, even when the aim is to maximize throughput by such overloads, knowledge of t_R at low sample loads is quite important (Knox and Pyper, 1986). For this reason, the present method is useful for the large-scale separation of proteins by IEC and HIC.

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Notation

A = parameter, Eq. 5
AU = absorbance unit
B = parameter, Eq. 5
CM = carboxymethyl
C_o = initial protein concentration, %
DEAE = diethylaminoethyl
d_c = column diameter, cm
d_p = particle diameter, μm
G = gV_o, M
g = absolute value of slope of linear gradient, M/mL
H = $(V_t - V_o)/V_o$
I = salt concentration in HIC, M; ionic strength in IEC, M
I_R = I at t_R, M
I_O = I at start, M
K = distribution coefficient for a protein
K' = K for a salt
K_{crt} = K in gel filtration mode
K_p = distribution coefficient, defined as protein concentration in solid phase of gel divided by that in pore
LG-A = β-lactoglobulin A
LG-B = β-lactoglobulin B
MW = molecular weight
OA = ovalbumin
pI = isoelectric point
RNAse = ribonuclease A
t = time, min
t_R = peak position (retention time), min
t_{RO} = t_R at low sample loads, min
u = vZ/V_o, linear mobile phase velocity, cm/min
V = tv, elution volume, mL
V_e = $t_R v$, elution volume at t_R, mL
V_{eo} = V_e at low sample loads, mL
V_o = column void volume, mL
V_t = total column volume, mL
W = peak width measured at base line, Figure 2, mL
W_o = W at low sample loads, mL
v = volumetric flow rate, mL/min
Z = column length, cm
z_p = distance of peak position of protein zone from inlet of column, cm

Literature Cited

Arnold, F. H., and H. W. Blanch, "Analytical Affinity Chromatography. II: Rate Theory and the Measurement of Biological Binding Kinetics," *J. Chromatogr.*, **355**, 13 (1986).

Arnold, F. H., H. W. Blanch, and C. R. Wilke, "Liquid Chromatography Plate Height Equations," *J. Chromatogr.*, **330**, 159 (1985).

Boardman, N. K., and S. M. Partridge, "Separation of Neutral Proteins on Ion-Exchange Resins," *Biochem. J.*, **59**, 543 (1955).

Buzzel, J. G., and C. Tanford, "The Effect of Charge and Ionic Strength on the Viscosity of Ribonuclease," *J. Phys. Chem.*, **60**, 1204 (1956).

Chase, H. A., "Affinity Separations Utilizing Immobilized Monoclonal Antibodies—A New Tool for the Biochemical Engineer," *Chem. Eng. Sci.*, **39**, 1099 (1984a).

———, "Prediction of the Performance of Preparative Affinity Chromatography," *J. Chromatogr.*, **297**, 179 (1984b).

Clonis, Y. D., K. Jones, and C. R. Lowe, "Process-scale High-performance Liquid Affinity Chromatography," *J. Chromatogr.*, **363**, 31 (1986).

Drake, B., "Contributions to the Theory of Gradient Elution Analysis," *Arkiv. Kemi.*, **8**, 1 (1955).

Freiling, E. C., "Ion Exchange as a Separation Method. IX: Gradient Elution Theory," *J. Am. Chem. Soc.*, **77**, 2067 (1955).

Gibbs, S. J., and E. N. Lightfoot, "Scaling Up Gradient Elution Chromatography," *Ind. Eng. Chem. Fundam.*, **25**, 490 (1986).

Giddings, J. C., *Dynamics of Chromatography*, Dekker, New York, 1965.

Haff, L. A., L. G. Fagerstam, and A. R. Barry, "Use of Electrophoretic Titration Curves for Predicting Optimal Chromatographic Conditions for Fast Ion-Exchange Chromatography of Proteins," *J. Chromatogr.*, **266**, 409 (1983).

Janson, J.-Ch., and P. Hedman, "Large-scale Chromatography of Proteins," *Advances in Biochem. Eng.*, A. Fiechter, ed., Springer-Verlag, Berlin, **25**, 43 (1982).

Kato, Y., K. Komiya, T. Iwaeda, H. Sasaki, and T. Hashimoto, "Packing of Toyopearl Column for Gel Filtration. I: Influence of Packing Velocity on Column Performance; II: Dependence of Optimal Packing Velocity on Column Size; III: Semiconstant-Pressure Packing," *J. Chromatogr.*, **205**, 185; **206**, 135; **208**, 71 (1981).

Kato, Y., K. Komiya, and T. Hashimoto, "Study of Experimental Conditions in High-Performance Ion-Exchange Chromatography of Proteins," *J. Chromatogr.*, **246,** 13 (1982).
———, "Operational Variables in Medium-Performance Ion-Exchange Chromatography of Proteins on DEAE-Toyopearl 650," *J. Chromatogr.*, **256,** 143 (1983).
———, "Preparative High-Performance Hydrophobic Interaction Chromatography of Proteins on TSKgel Phenyl-5PW," *J. Chromatogr.*, **333,** 202 (1985).
Knox, J. H., and H. M. Pyper, "Framework for Maximizing Throughput in Preparative Liquid Chromatography," *J. Chromatogr.*, **363,** 1 (1986).
Kopaciewicz, W., and F. E. Regnier, "A System for Coupled Multiple-Column Separation of Proteins," *Anal. Biochem.*, **129,** 472 (1983).
Kopaciewicz, W., M. A. Rounds, J. Fausnaugh, and F. E. Regnier, "Retention Model for High-Performance Ion-Exchange Chromatography," *J. Chromatogr.*, **266,** 3 (1983).
Matsuno, R., K. Nakanishi, and S. Yamamoto, *Ion Exchange Chromatography of Proteins,* Dekker, New York, in press (1987).
Morris, C. J. O. R., and P. Morris, *Separation Methods in Biochemistry,* 2nd ed., Pitman, London (1964).
Pitt, W. W., "Gradient-Elution Ion-Exchange Chromatography: A Digital Computer Solution of the Mathematical Model," *J. Chromatogr. Sci.*, **14,** 396 (1976).
Righetti, P. G., and T. Caravaggio, "Isoelectric Points and Molecular Weights of Proteins. A Table," *J. Chromatogr.*, **127,** 1 (1976).
Righetti, P. G., G. Tudor, and K. Ek, "Isoelectric Points and Molecular Weights of Proteins. A New Table," *J. Chromatogr.*, **220,** 115 (1981).
Smith, M. H., "Molecular Weights of Proteins and Some Other Materials Including Sedimentation, Diffusion and Frictional Coefficients, and Partial Specific Volumes," *Handbook of Biochemistry,* H. A. Sober, ed. Chem. Rubber Co., C-3 (1968).
Vanecek, G., and F. E. Regnier, "Variables in the High-Performance Anion-Exchange Chromatography of Proteins," *Anal. Biochem.*, **109,** 345 (1980).
Whitaker, J. R., "Determination of Molecular Weights of Proteins by Gel Filtration on Sephadex," *Anal. Chem.*, **35,** 1950 (1963).
Yamamoto, S., K. Nakanishi, R. Matsuno, and T. Kamikubo, "Ion-Exchange Chromatography of Proteins—Prediction of Elution Curves and Operating Conditions. I: Theoretical Considerations; II: Experimental Verification," *Biotechnol. Bioeng.*, **25,** 1465, 1373 (1983).
Yamamoto, S., M. Nomura, and Y. Sano, "Scaling Up of Medium-Performance Gel Filtration Chromatography of Proteins," *J. Chem. Eng. Japan,* **19,** 227 (1986).
———, "Purification of β-Galacotosidase by Large-Scale Gradient Elution Ion Exchange Chromatography," *J. Chromatogr.*, **395,** 355 (1987a).
———, "The Resolution of Proteins in Linear Gradient Elution Ion Exchange and Hydrophobic Interaction Chromatography," *J. Chromatogr.*, in press (1987b).

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