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STUDY OF EXPERIMENTAL CONDITIONS IN HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY OF PROTEINS

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SUMMARY

Commercial ovalbumin was measured by high-performance ion-exchange chromatography on TSK-GEL IEX-545 DEAE SIL and linear gradient elution with NaCl to investigate the effects of gradient, flow-rate, column length and sample loading on resolution and retention. As the gradient became less steep, both resolution and separation time increased. At constant gradient time, the resolution increased and the separation time decreased with increasing flow-rate, but at constant gradient volume, both resolution and separation time increased with decreasing flow-rate. Columns of 75 mm and 150 mm in length provided almost identical resolution. The maximum sample loading was approximately 0.5 mg for columns of 6 mm I.D. and almost independent of column length and injection volume.

INTRODUCTION

Since Peterson and Sober[1] developed cellulose ion-exchangers in 1956, ion-exchange chromatography has been widely used for the separation and purification of proteins. With the introduction in 1976 of rigid or semi-rigid ion-exchangers having high protein capacity and low irreversible adsorption by Chang *et al.*[2] and Mikes *et al.*[3] high-performance ion-exchange chromatography of proteins has also become possible. As a result, the application of ion-exchange chromatography to proteins has rapidly been extended, especially in the analytical field. However, previous studies concerning chromatographic operational variables do not seem to have resulted in the optimization of separation conditions[4–13].

This paper reports the effects of salt concentration gradient, flow-rate, column length and sample loading on the linear gradient elution of ovalbumin with NaCl on a new chemically bonded ion-exchanger, TSK-GEL IEX-545 DEAE SIL.

EXPERIMENTAL

Ovalbumin purchased from Seikagaku (Tokyo, Japan) was used in all measurements. As shown later, this sample contains several components which can be separated in ion-exchange chromatography, although it is fairly homogeneous in terms of molecular weight.

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Ion-exchange chromatographic measurements were performed with a high-speed liquid chromatograph Model HLC-803C equipped with gradient generator Model GE-2 (Toyo Soda, Tokyo, Japan). The liquid chromatograph consists of a reciprocating single-plunger pump, a valve loop injector and a variable-wavelength UV detector. The UV detector was operated at 280 nm. The gradient generator is a nozzle flapper type and can generate linear, convex or concave gradients of two solvents. A gradient time between 1 and 999 min can be selected. Commercially available prepacked columns of TSK-GEL IEX-545 DEAE SIL were used. The column packings were weak anion exchangers prepared by introducing diethyl-aminoethyl groups into chemically bonded hydrophilic layers of TSK-GEL G3000SW, which is a silica-based support of particle diameter 10 μm and pore diameter 250 Å for high-performance gel filtration. A 150 × 6 mm I.D. column was generally used. A 75 × 6 mm I.D. column was also used to study the effects of column size and sample loading. A linear gradient of NaCl was employed in all measurements. The initial buffer was 0.1 *M* Tris·HCl of pH 7.50 and the final buffer was 0.1 *M* Tris·HCl containing 0.2 *M* NaCl of pH 7.50. Ovalbumin did not move along the column at all in the initial buffer. Gradient elution was started at the same time as sample injection. Usually 0.21 ml of 0.2% solution were injected. To study the effect of sample loading, however, both the injection volume and sample concentration were varied. All measurements were carried out at 25 ± 0.5°C.

RESULTS AND DISCUSSION

Evaluation of resolution

Since several peaks were observed in chromatograms of ovalbumin as exemplified in Fig. 1, the resolution was calculated for two major peaks a and b according to eqn. 1

$$R(a,b) = 2(V_b - V_a)/(W_a + W_b) \quad (1)$$

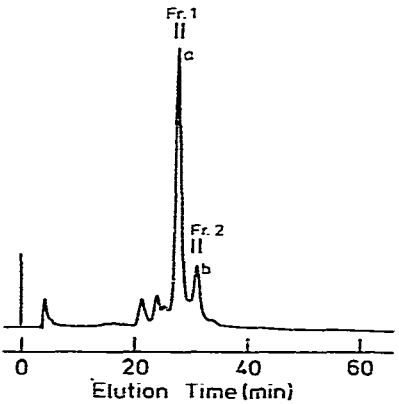

Fig. 1. An example of a chromatogram of ovalbumin obtained by high-performance ion-exchange chromatography. Column: TSK-GEL IEX-545 DEAE SIL (150 × 6 mm I.D.). Elution: 90-min linear gradient from 0.1 *M* Tris·HCl buffer (pH 7.50) to 0.1 *M* Tris·HCl buffer containing 0.2 *M* NaCl (pH 7.50); flow-rate, 1 ml/min. Sample loading: 0.2%, 0.21 ml. Temperature: 25°C. Detector: UV spectrophotometer at 280 nm.

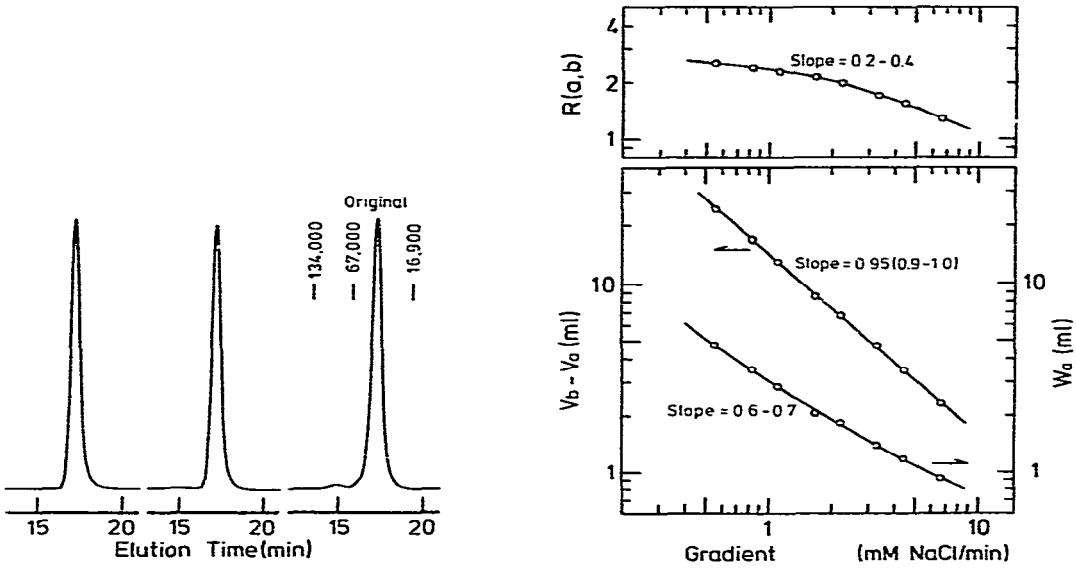

Fig. 2. Chromatograms of fractions 1 and 2 from Fig. 1 and the original ovalbumin sample obtained by high-performance gel filtration. Column: TSK-GEL G3000SW (600 × 7.5 mm I.D.). Eluent: 0.1 *M* phosphate buffer containing 0.1 *M* Na_2SO_4 (pH 6.6); flow-rate, 1 ml/min. Injection volume: 0.1 ml. Temperature: 25°C. Detector: UV spectrophotometer at 210 nm. Vertical bars indicate the elution positions of myoglobin, bovine serum albumin monomer and dimer. Numerical values on the bars are molecular weights of the proteins.

Fig. 3. Dependences of resolution, peak interval and peak width on *b*. Gradient times (min): 30, 45, 60, 90, 120, 180, 240 and 360. Other conditions as in Fig. 1.

where V_a, V_b, W_a and W_b are the elution volumes and baseline peak widths of the components a and b, respectively. The two peaks a and b were separated as in Fig. 1 and subjected to high-performance gel filtration on TSK-GEL G3000SW. The two fractions were eluted at the same position as the original ovalbumin as seen in Fig. 2, which implies that components a and b have almost the same molecular weights and are not separated on the basis of molecular weight in ion-exchange chromatography.

Effect of the salt concentration gradient

The effect of the slope (*b*) of the salt concentration gradient on resolution and

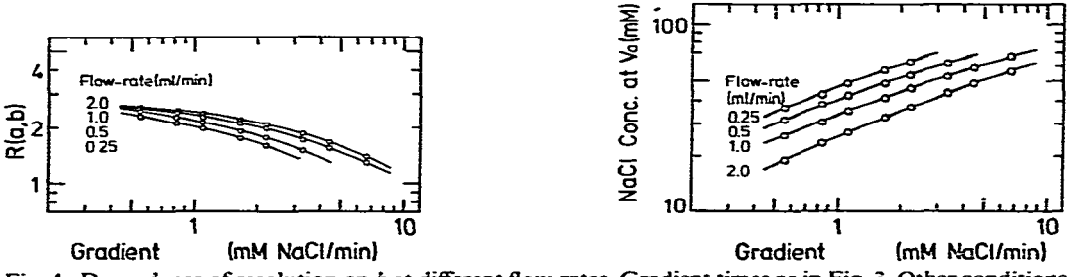

Fig. 4. Dependence of resolution on *b* at different flow-rates. Gradient times as in Fig. 3. Other conditions as in Fig. 1.

Fig. 5. Dependence of effluent salt concentration at which ovalbumin was eluted from the column on *b*. Gradient times as in Fig. 3. Other conditions as in Fig. 1.

retention was investigated by measuring ovalbumin with gradient times of 30–360 min. Fig. 3 shows the dependences of resolution, peak interval and peak width on b obtained at a flow-rate of 1 ml/min. Higher resolution was achieved in inverse proportion to $b^{0.2-0.4}$, *i.e.*, as the gradient time became longer. This is because the peak interval increased approximately in inverse proportion to $b^{0.95}$ while peak width increased approximately in inverse proportion to $b^{0.6-0.7}$. However, since the latter dependence becomes more pronounced with decreasing b, the resolution tends to become constant as the gradient becomes less steep. The same qualitative results were obtained also at flow-rates of 0.25, 0.5 and 2 ml/min as shown in Fig. 4. The dependence of the sodium chloride concentration at the elution volume on b is shown in Fig. 5, indicating that ovalbumin is eluted at lower salt concentrations as the gradient becomes less steep. Detection sensitivity increased in proportion to $b^{0.6-0.7}$ as can be understood from the dependence of the peak width on b in Fig. 3. The separation time became longer as the gradient became less steep as seen in Fig. 6.

Effect of flow-rate

The effect of flow-rate on resolution and retention was investigated by measuring ovalbumin at flow-rates of 0.25, 0.5, 1 and 2 ml/min. Measurements were carried out at either constant gradient time or constant gradient volume (product of flow-rate and gradient time).

Fig. 7 shows the dependences of resolution, peak interval and peak width on flow-rate obtained with constant gradient time. Higher resolution was achieved in proportion to (flow-rate)$^{0.05-0.1}$. This is because the peak interval increased approximately in proportion to (flow-rate)$^{1.0}$ while peak width increased approximately in proportion to (flow-rate)$^{0.9-0.95}$. As shown in Fig. 8, the same qualitative results were obtained also with other b values. Fig. 9 shows flow-rate dependence of the effluent salt concentration at which ovalbumin was eluted. Ovalbumin was eluted at lower salt concentrations as the flow-rate became higher. This means that higher resolution is attainable in a shorter time by increasing the flow-rate at constant gradient time, as seen in Figs. 10 and 11. Detection sensitivity, however, decreased almost in inverse proportion to flow-rate.

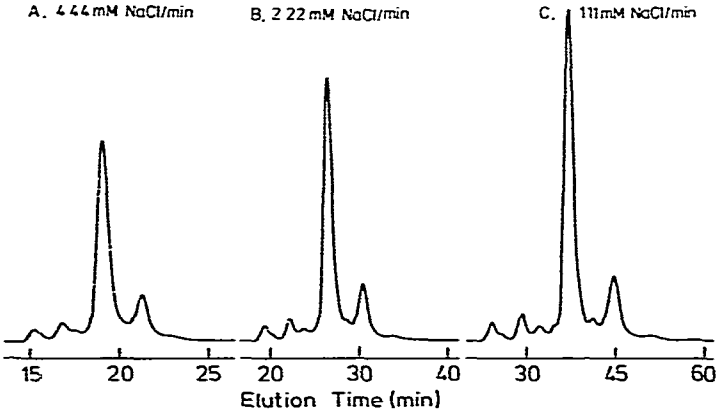

Fig. 6. Chromatograms of ovalbumin obtained with gradients of 4.44 mM NaCl/min (A), 2.22 mM NaCl/min (B) and 1.11 mM NaCl/min (C). Gradient times: 45 min (A), 90 min (B) and 180 min (C). Other conditions as in Fig. 1.

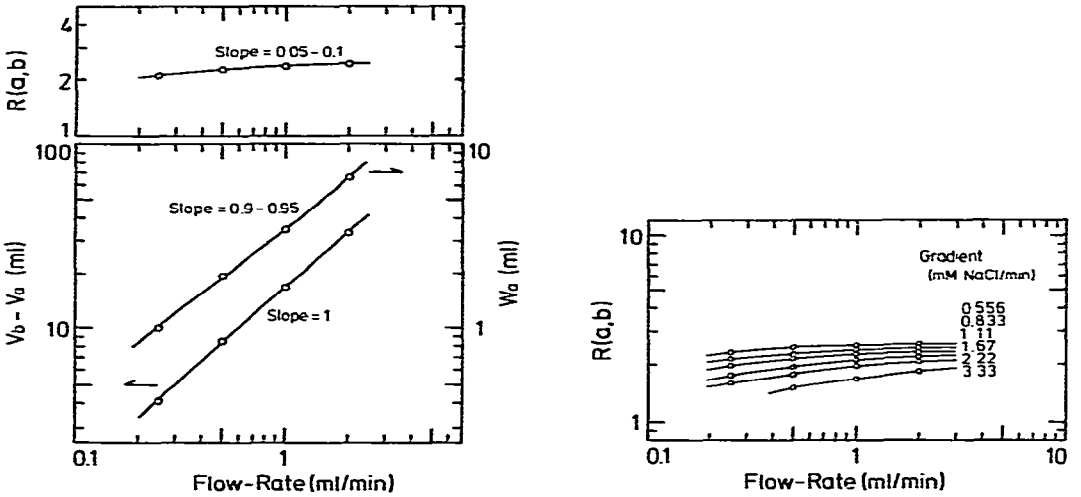

Fig. 7. Dependences of resolution, peak interval and peak width on flow-rate at constant gradient time. Gradient time: 240 min (corresponds to a gradient of 0.833 m*M* NaCl/min). Flow-rates (ml/min): 0.25, 0.5, 1 and 2. Other conditions as in Fig. 1.

Fig. 8. Dependence of resolution on flow-rate at different constant gradient times. Gradient times as in Fig. 3. flow-rates as in Fig. 7; other conditions as in Fig. 1.

Fig. 12 shows the dependences of resolution, peak interval and peak width on flow-rate obtained with constant gradient volume (60 ml). In this case, higher resolution was achieved in inverse proportion to (flow-rate)$^{0.1-0.3}$. This is because the peak width decreased approximately in proportion to (flow-rate)$^{0.1-0.3}$ while peak interval was almost unchanged with decreasing flow-rate. As shown in Fig. 13, the same qualitative results were obtained also with other *b* values, and this tendency has been observed also by Vanecek and Regnier[10]. Fig. 14 shows flow-rate dependence of the effluent salt concentration at which ovalbumin was eluted. Ovalbumin was eluted at lower salt concentrations as the flow-rate decreased. However, the separation time became longer almost in inverse proportion to flow-rate as seen in Fig. 15. On the other hand, the detection sensitivity increased in inverse proportion to (flow-rate)$^{0.1-0.3}$.

As shown above, in the case of a constant gradient volume the flow-rate effect is the same as in the case of isocratic elution. In the case of a constant gradient time, however, the effect of flow-rate on resolution is opposite to that in isocratic elution.

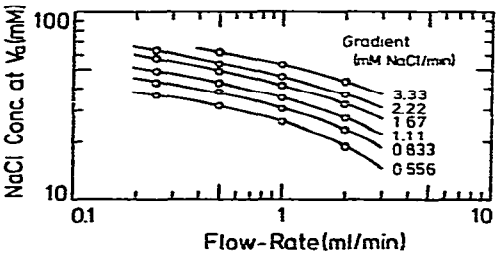

Fig. 9. Dependence of effluent-salt concentration at which ovalbumin was eluted on flow-rate at different constant gradient times. Details as in Fig. 8.

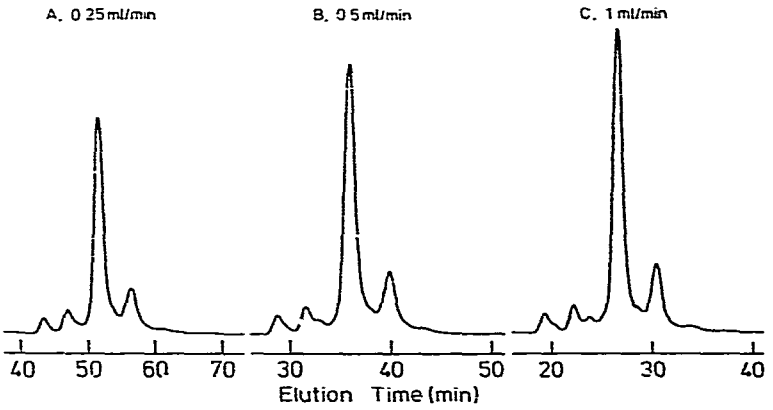

Fig. 10. Chromatograms of ovalbumin obtained at different flow-rates: (A) 0.25 ml/min, (B) 0.5 ml/min and (C) 1 ml/min. Gradient time: 90 min, other conditions as in Fig. 1.

Effect of column length

The effect of column length on resolution and retention was investigated by measuring ovalbumin on 150 × 6 mm I.D. and 75 × 6 mm I.D. columns. The height equivalent to a theoretical plate of both columns evaluated by measuring uric acid isocratically in 1/15 *M* phosphate buffer of pH 6.4 at a flow-rate of 1 ml/min was *ca.* 35 μm. The results are summarized in Table I. Almost identical peak intervals, peak widths and resolutions were obtained on both columns. However, the 150-mm column provided slightly higher resolution than the 75-mm column as the gradient became less steep, although the difference is not so great as reported by Vanecek and Regnier[10]. They observed an approximately 11% increase in resolution with a 5-cm incremental increase in column length. Fig. 16 shows a comparison of chromatograms of ovalbumin obtained on two columns with a gradient of 0.833 m*M* NaCl/min. Ovalbumin was eluted at lower salt concentrations on the 75-mm column than on the 150-mm column.

Effect of sample loading

The effect of sample loading on resolution and retention was investigated by injecting 0.21 ml and 1.64 ml of solutions of various concentrations. Figs. 17 and 18

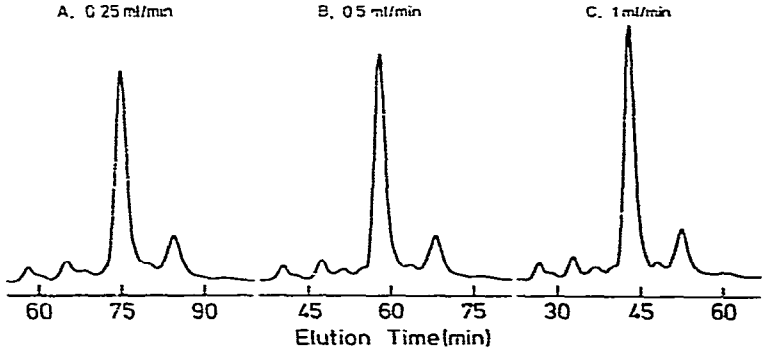

Fig. 11. Chromatograms of ovalbumin obtained at different flow-rates. Gradient time: 240 min. Flow-rates as in Fig. 10. Other conditions as in Fig. 1.

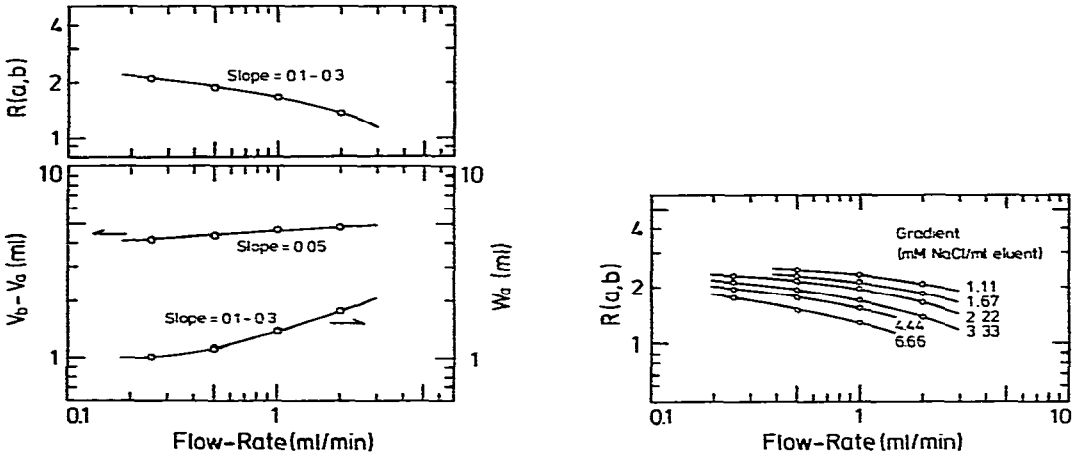

Fig. 12. Dependences of resolution, peak interval and peak width on flow-rate at constant gradient volume. Flow-rate and gradient time were varied simultaneously keeping the gradient volume at 60 ml (corresponds to a gradient of 3.33 m*M* NaCl per ml eluent). Gradient times and flow-rates; 30 min and 2 ml/min; 60 min and 1 ml/min; 120 min and 0.5 ml/min; 240 min and 0.25 ml min. Other conditions as in Fig. 1.

Fig. 13. Dependence of resolution on flow-rate at different constant gradient volumes. Gradient times as in Fig. 3. Flow-rates (ml/min): 0.25, 0.5, 1 and 2. Other conditions as in Fig. 1.

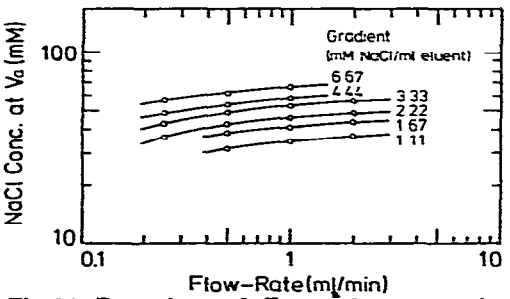

Fig. 14. Dependence of effluent salt concentration at which ovalbumin was eluted on flow-rate at different constant gradient volumes. Gradient times as in Fig. 3, flow-rates as in Fig. 13; other conditions as in Fig. 1.

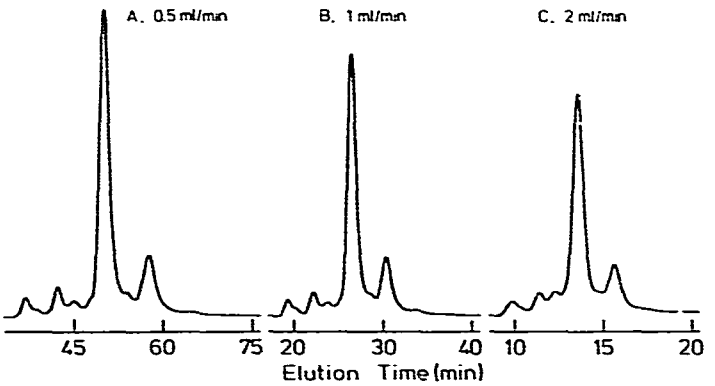

Fig. 15. Chromatograms of ovalbumin obtained at different flow-rates and a gradient volume of 90 ml (corresponds to a gradient of 2.22 m*M* NaCl per ml eluent). Gradient times and flow-rates: (A) 180 min, 0.5 ml/min; (B) 90 min, 1 ml/min; (C) 45 min, 2 ml/min. Other conditions as in Fig. 1.

TABLE I

COMPARISON OF RESOLUTION, PEAK INTERVAL, PEAK WIDTH AND EFFLUENT SALT CONCENTRATION AT WHICH OVALBUMIN WAS ELUTED

Columns: 150 × 6 mm I.D. and 75 × 6 mm I.D. Conditions as in Fig. 1 except column size and gradient time were varied.

Gradient (mM NaCl/min) (b)	$V_b - V_a$ *(ml)*		W_a *(ml)*		*R(a,b)*		*NaCl concn. at* V_a *(mM)*	
	150 mm	*75 mm*	*150 mm*	*75 mm*	*150 mm*	*75 mm*	*150 mm*	*75 mm*
0.556	24.4	23.4	4.74	4.90	2.51	2.42	25.8	20.0
0.833	16.7	16.1	3.47	3.52	2.37	2.23	30.6	24.0
1.11	13.0	12.4	2.86	2.91	2.27	2.10	34.7	27.2
1.67	8.57	8.47	2.04	2.18	2.12	2.05	41.0	32.3
2.22	6.73	6.49	1.84	1.84	1.96	1.87	45.6	36.4
3.33	4.68	4.47	1.39	1.38	1.69	1.69	52.9	42.3
4.44	3.47	3.42	1.17	1.12	1.53	1.57	58.5	46.8
6.67	2.30	2.35	0.92	0.90	1.28	1.31	66.9	54.3

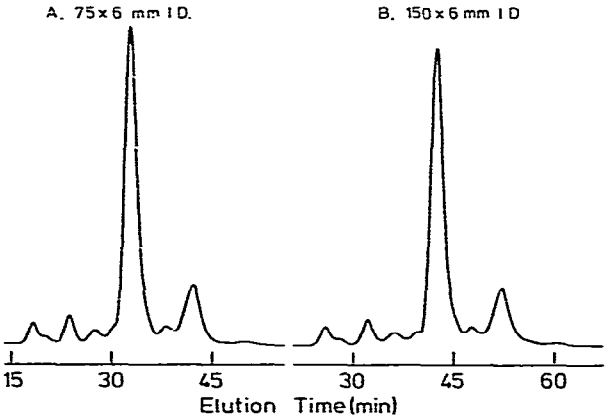

Fig. 16. Chromatograms of ovalbumin obtained on columns of different size: (A) 75 × 6 mm I.D., (B) 150 × 6 mm I.D.; gradient time: 240 min. Other conditions as in Fig. 1.

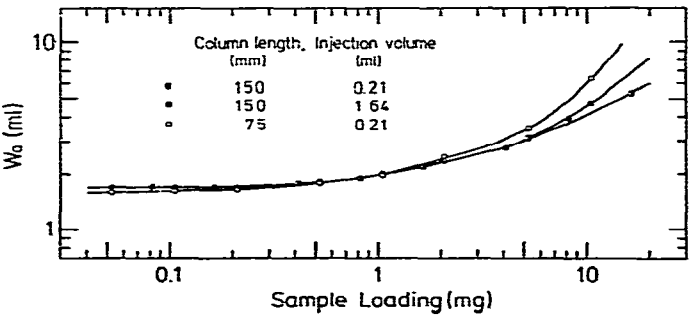

Fig. 17. Dependence of peak width on sample loading. Column sizes: 150 × 6 mm I.D. or 75 × 6 mm I.D. Sample loading: 0.005–5%, 0.21 ml or 1.64 ml. Other conditions as in Fig. 1.

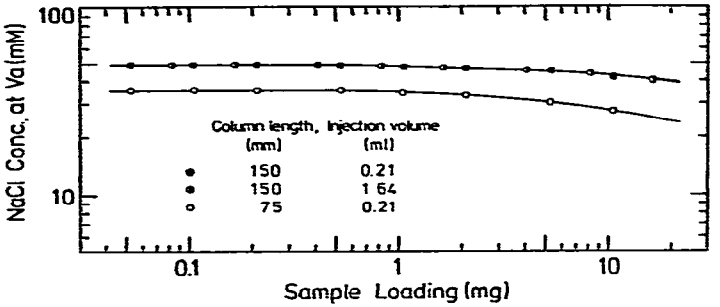

Fig. 18. Dependence of effluent salt concentration at which ovalbumin was eluted on sample loading. Details as in Fig. 17.

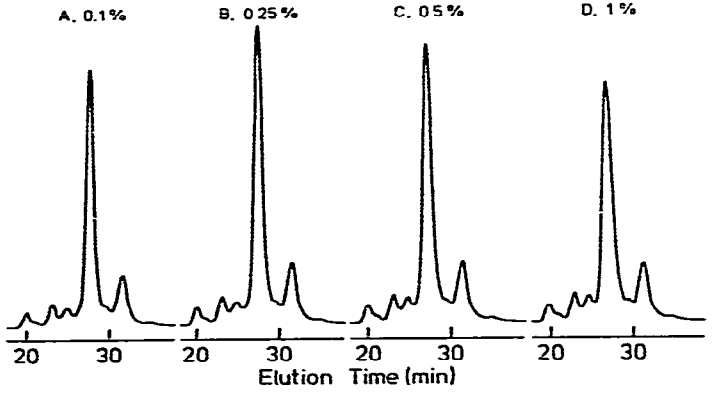

Fig. 19. Chromatograms of ovalbumin obtained with solutions of different concentrations. Sample concentrations: (A) 0.1%, (B) 0.25%, (C) 0.5% and (D) 1%. Other conditions as in Fig. 1.

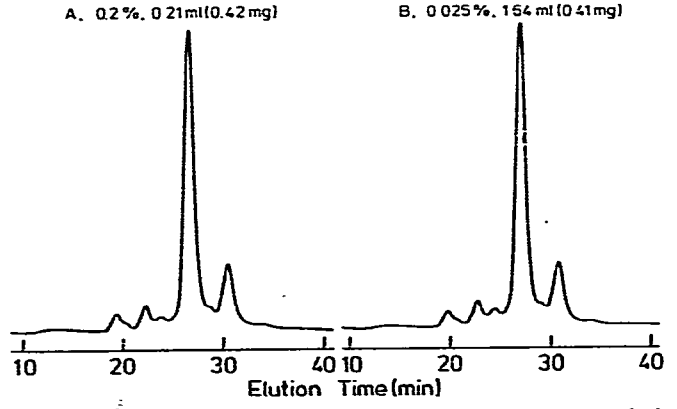

Fig. 20. Chromatograms of ovalbumin obtained with sample loadings of (A) 0.2%, 0.21 ml (0.42 mg protein) and (B) 0.025%, 1.64 ml (0.41 mg protein). Conditions except sample loading as in Fig. 1.

show the dependences of, respectively, peak width and effluent salt concentration at which ovalbumin was eluted. The sample loading at which the peak width begins to increase or elution begins to become faster is almost independent of both column length and injection volume and is *ca.* 0.5 mg. However, the increase in peak width above a sample loading of 0.5 mg is more pronounced on the 75-mm column than on the 150-mm column. Although it has been reported that resolution decreases little up to considerably high sample loadings in ordinary low pressure ion-exchange chromatography of proteins[4], sample loading should be kept less that 1–2 mg per unit column section area (cm^2) in high-performance ion-exchange chromatography of proteins. Chromatograms of ovalbumin obtained at various sample loadings are shown in Fig. 19, which indicates that peak width becomes wider, elution becomes faster and peak shape becomes tailing with increasing sample loading. The effect of the sample loading on each component in the sample seems to depend not on the whole amount injected but on the respective amount of each component. This is why the separation between peaks a and b (see Fig. 1) seems to be almost unchanged in the chromatograms in Fig. 19. Fig. 20 shows chromatograms of ovalbumin obtained with different injection volumes and sample concentrations, but almost identical sample loadings.

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