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Effect of chromatographic conditions on resolution in high-performance ion-exchange chromatography of proteins on macroporous anion-exchange resin

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Abstract

We explored chromatographic conditions to obtain high resolution in protein separations by ion-exchange chromatography (IEC) on a macroporous anion-exchange resin of 10 μm in particle diameter. We studied effects of flow-rate, gradient time (steepness of salt concentration gradient) and column length on resolution in wide ranges. It was found that very high resolutions are attainable at long gradient times with long columns. The resolution continuously became higher as the gradient time and the column length became longer except in some special cases. The dependence of resolution on gradient time was particularly great when the column was long and the gradient time for the change of 0–0.5 M NaCl was longer than 2 h. On the other hand, the effect of flow-rate on resolution was very small. Although the separations at long gradient times with long columns have not been popular in high-performance IEC and it takes several hours for one separation, such separations should be advantageous when very high resolutions are required like in proteomics research.

Keywords: Flow-rate; Salt gradients; Column length; Resolution; Proteins

1. Introduction

Various modes of HPLC have been widely employed for protein analyses since the end of the 1970s. Proteins are usually separated in less than 1 h with high resolution. But, the HPLC is not powerful enough in some cases, e.g., in proteomics research, where extremely high resolutions are required. In proteomics research, two-dimensional gel electrophoresis has mainly been employed and HPLC has not been the method of choice due to its insufficient resolution. However, the two-dimensional gel electrophoresis has some disadvantages and alternative methods have been desired. HPLC seems one of promising candidates if it can be improved in terms of resolution. Thus, much effort is being made to obtain higher resolution, in particular by using two-dimensional HPLC [1–8]. Another possible approach to achieve higher resolution is to adopt strictly optimized chromatographic conditions regardless of a single- or two-dimensional separation. However, sufficient attention has not been paid to it and it does not seem that chromatographic conditions to achieve the highest resolution for specific objectives are fully understood. Consequently, we explored chromatographic conditions to obtain high resolution in protein separations by high-performance ion-exchange chromatography (IEC) on a nonporous anion-exchange resin of 2.5 μm in particle diameter [9]. We studied effects of gradient time (steepness of salt concentration gradient), flow-rate and column length on resolution in much wider ranges than had been studied before [10,11]. As a result, it was found that very high resolutions are attainable under the conditions of a combination of long gradient time, low flow-rate and long column. However, these conditions have never been recommended and have rarely been employed in high-performance IEC, as long as authors know. Then, we wondered if they are effective in general to achieve high resolution on any ion-exchange resins. Accordingly, in this paper we investigate the effects of chromatographic conditions on resolution on other anion-exchange resin as an extension of the previous study.

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2. Experimental

2.1. Chromatographic measurements

Chromatographic measurements were carried out with a system consisting of a model CCPM II dual-piston pump, a model UV-8010 variable-wavelength UV detector operated at 220 nm and a model SC-8020 system controller/data processor (Tosoh, Tokyo, Japan). A sample of trypsin inhibitor was separated at 25 °C at flow-rates of 0.03–2 ml/min by 0.94–960 min linear gradients of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1). A 20-μl volume of solution containing 4–32 μg trypsin inhibitor in the initial eluent was injected. Columns of 0.5, 1, 2, 3.5, 7.5 and 15 cm in length × 4.6 mm i.d. packed with TSKgel Bio-Assist Q (Tosoh), which is a macroporous anion-exchange resin of 10 μm in particle diameter and 400 nm in pore diameter, were used. The operating flow-rates were kept below approximately one-third of the packing flow-rates for the sake of long-term stability of the columns. The flow-rate ranges operated on these columns are summarized in Table 1.

2.2. Materials

Trypsin inhibitor employed was a commercial sample purchased from Sigma (St. Louis, MO, USA; type I-S: from soybean, sample code T-9003, lot 30K7020). According to the manufacturer, the sample was chromatographically prepared and lyophilized. The sample was reasonably pure although it contained some components, as can be seen later.

2.3. Calculation of resolution

The resolution was calculated for the largest and the second largest peaks in chromatograms of trypsin inhibitor (peaks 1 and 2 in Fig. 1). The peak 2 contained some minor components together with the main component and the width was affected by the degree of separation between them. Therefore, the width of peak 2 was not used in the calculation of resolution. The resolution was calculated from retention times of peaks 1 and 2 and the width of peak 1 by considering the widths of peaks 1 and 2 are identical.

Table 1
Flow-rate ranges operated on columns of different lengths

Column size (mm)	Flow-rate range (ml/min)
5 × 4.6	0.5–2
10 × 4.6	0.5–2
20 × 4.6	0.5–2
35 × 4.6	0.03–1
75 × 4.6	0.03–0.5
150 × 4.6	0.03–0.5

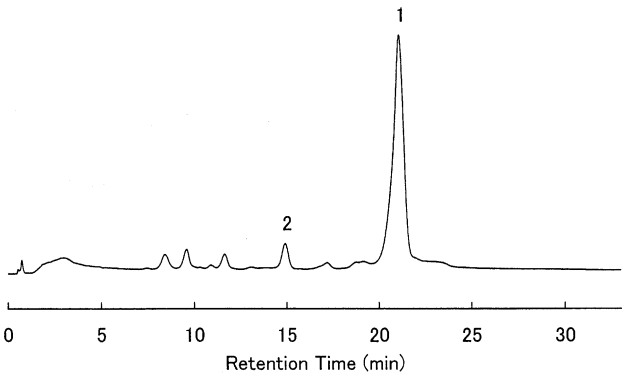

Fig. 1. Example of separation of a commercial sample of trypsin inhibitor, which was obtained on a 35 mm × 4.6 mm i.d. column by a 30-min linear gradient of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at a flow-rate of 1 ml/min.

3. Results and discussion

3.1. Effect of flow-rate on resolution

The dependence of resolution on flow-rate at constant gradient times on 3.5-cm long column is shown in Fig. 2, which indicates that the flow-rate has little effect on resolution. The resolution became lower although very slightly as the flow-rate decreased when the gradient volume (the product of gradient time and flow-rate) was less than 15 ml. But, the resolution did not depend on the flow-rate at gradient volumes of 15 ml or more. The reason for this was as follows. As the flow-rate became higher, retention times of components 1 and 2 decreased in parallel and their difference was constant. The peak width expressed in time was also independent of flow-rate. Consequently, the resolution was unchanged with flow-rate.

These results are quite different from the one observed on a nonporous anion-exchange resin before [9]. The flow-rate significantly affected the resolution in the case of the

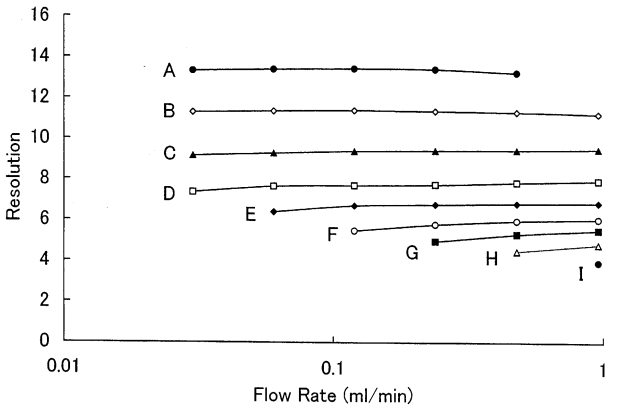

Fig. 2. Dependence of resolution on flow-rate in the separation of trypsin inhibitor on a 35 mm × 4.6 mm i.d. column by (A) 960 min; (B) 480 min; (C) 240 min; (D) 120 min; (E) 60 min; (F) 30 min; (G) 15 min; (H) 7.5 min; and (I) 3.75 min linear gradients of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at flow-rates of 0.03–1 ml/min.

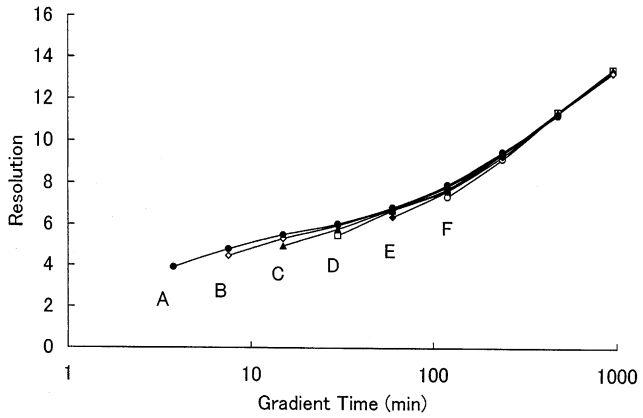

Fig. 3. Dependence of resolution on gradient time in the separation of trypsin inhibitor on a 35 mm × 4.6 mm i.d. column by 3.75–960 min linear gradients of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at flow-rates of (A) 1 ml/min; (B) 0.5 ml/min; (C) 0.25 ml/min; (D) 0.12 ml/min; (E) 0.06 ml/min; and (F) 0.03 ml/min.

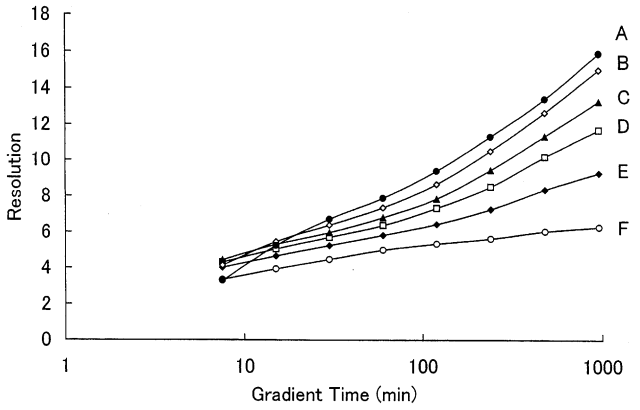

Fig. 4. Dependence of resolution on gradient time in the separation of trypsin inhibitor on (A) 15 cm; (B) 7.5 cm; (C) 3.5 cm; (D) 2 cm; (E) 1 cm; and (F) 0.5 cm long columns by 7.5–960 min linear gradients of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at a flow-rate of 0.5 ml/min.

nonporous resin. Also, the resolutions became maximums at certain flow-rates. It was because the peak width varied with flow-rate and it was reflected to the flow-rate dependence of resolution. The interval between peaks of components 1 and 2 was constant also in the case of the nonporous resin.

3.2. Effect of gradient time on resolution

The dependence of resolution on gradient time at constant flow-rates on 3.5-cm long column is shown in Fig. 3. The resolution continuously became higher as the gradient time became longer, suggesting that there is a possibility of obtaining very high resolutions by employing long gradient times. Interestingly, the resolution increased at different rates at gradient times shorter and longer than 2 h. The increase rate was larger at gradient times of 2 h and longer. Although it is a matter that can be expected from the flow-rate dependence of resolution, all plots of resolution against gradient time for different flow-rates overlapped except in the case of small gradient volumes of less than 15 ml.

The results mentioned above are also quite different from the one observed on a nonporous anion-exchange resin before [9]. On the nonporous resin the plots of resolution against gradient time shifted each other and maximum resolutions were observed when the flow-rates were high. Such maximums did not exist on the macroporous resin.

The reason for the difference in gradient time dependence of resolution on the two anion-exchange resins was as follows. As the gradient time became longer, retention times of components 1 and 2 increased and their difference increased almost in proportion to gradient time on both resins. However, the gradient time dependence of peak width was significantly different on the two resins. On the macroporous resin, the increase rate of peak width was always less than in proportion to gradient time and therefore the resolution continuously became higher as the gradient time became longer. On the nonporous resin, on the other hand, the peak width increased complicatedly and sometimes at the rate higher than in proportion to gradient time. Then, the resolution complicatedly depended on gradient time.

3.3. Effect of column length on resolution

Similar effects of flow-rate and gradient time on resolution as described on 3.5-cm long columns were observed on the columns of other lengths. However, the dependence of resolution on gradient time was more significant when the column was longer, as shown in Fig. 4. Also, longer columns provided the higher resolutions than shorter columns at the same gradient time and flow-rate except in the case of short gradient times, as shown in Fig. 5. In addition, the effect of column length was greater at longer gradient times. When the gradient time was short, the effect of column length was small and at gradient times of 7.5 and 15 min the resolution even became slightly lower with increasing the column

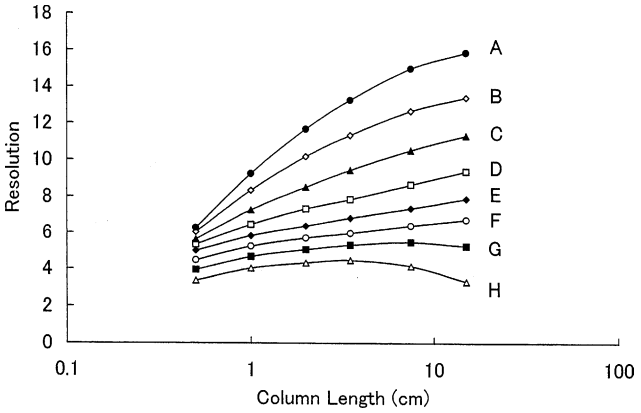

Fig. 5. Dependence of resolution on column length in the separation of trypsin inhibitor on 0.5–15 cm long columns by (A) 960 min; (B) 480 min; (C) 240 min; (D) 120 min; (E) 60 min; (F) 30 min; (G) 15 min; and (H) 7.5 min linear gradients of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at a flow-rate of 0.5 ml/min.

length over 3.5 and 7.5 cm, respectively. The reason for the increase in resolution with column length was as follows. As the column became longer, retention times of components 1 and 2 gradually became longer and their difference slightly increased. On the contrary, the peak width decreased with increasing the column length. Then, higher resolutions were obtained with longer columns. In the case of short gradient times, the decrease in peak width with column length was small and in some domains the peak width even increased with column length. They were reflected to the column length dependence of resolution.

The dependence of resolution on column length was almost the same on the two anion-exchange resins.

3.4. Chromatographic conditions to obtain high resolution

The results described in Sections 3.1–3.3 suggest that employing both long gradient time and long column is the way to attain the highest resolution. The longer the gradient time and the column are, the higher resolution is achievable. The flow-rate can be selected based on other considerations such as pressure drop, solvent consumption and re-equilibration time.

On the other hand, short gradient times must be employed when rapid separations are required. In such cases short columns of 2–3.5 cm long are superior to longer ones. High flow-rates like 2 ml/min are also preferable, as illustrated in Fig. 6. Higher flow-rates provide higher resolutions at short gradient times with short columns although the effect of flow-rate on resolution is not so significant as in the case of nonporous anion-exchange resin.

3.5. Relationship between support properties and the effect of chromatographic conditions on resolution

As explained in previous sections, considerably different effects of flow-rate and gradient time on resolution were found in the separations of trypsin inhibitor on TSKgel DEAE-NPR and BioAssist Q. These two anion-exchange resins differ not only in pore structure (nonporous and macroporous) but also in particle size (2.5 and 10 μm), ion-exchange capacity (0.15 and 0.10 meq./ml resin) and protein adsorption capacity (5 and 90 mg/ml resin for bovine serum albumin). In addition, their retention strengths and selectivity for proteins also differ, as can be seen from Figs. 7 and 8. Therefore, it is difficult to estimate from the results obtained so far alone which properties are responsible for the difference in the effects of chromatographic conditions on resolution. The collection of more data on other supports is necessary to clarify the relationship between the support properties and the effects of chromatographic conditions on resolution. If it becomes clear, the information will be useful for designing superior supports for the separations with very high resolutions.

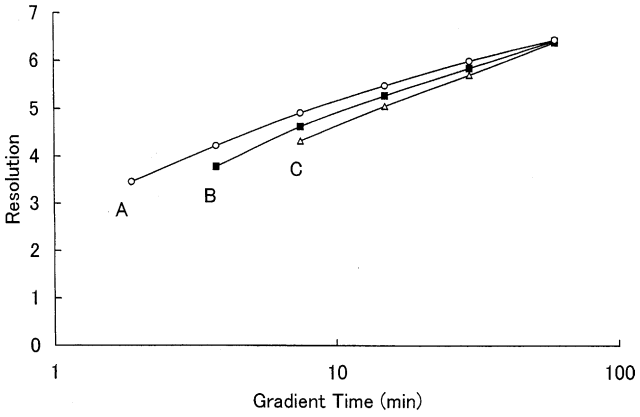

Fig. 6. Dependence of resolution on gradient time in the separation of trypsin inhibitor on a 20 mm × 4.6 mm i.d. column by 1.88–60 min linear gradients of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at flow-rates of (A) 2 ml/min; (B) 1 ml/min; and (C) 0.5 ml/min.

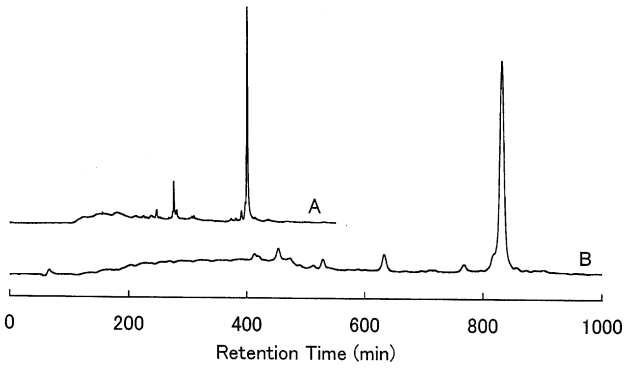

Fig. 7. Comparison of separations of a commercial sample of trypsin inhibitor obtained on (A) DEAE-NPR; and (B) BioAssist Q columns of 150 mm × 4.6 mm i.d. by a 960-min linear gradient of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at a flow-rate of 0.03 ml/min.

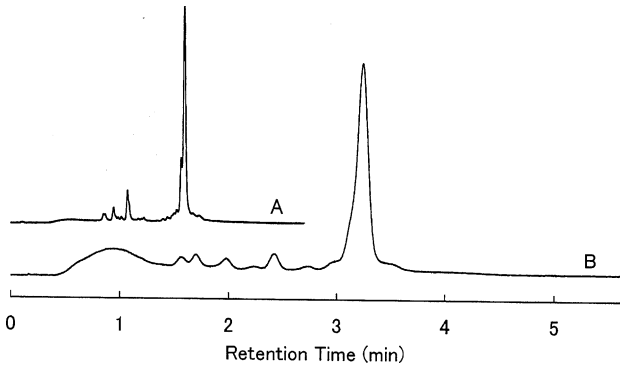

Fig. 8. Comparison of separations of a commercial sample of trypsin inhibitor obtained on (A) DEAE-NPR; and (B) BioAssist Q columns of 20 mm × 4.6 mm i.d. by a 3.75-min linear gradient of sodium chloride from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.1) at a flow-rates of 2 ml/min.

4. Conclusions

Very high resolutions are attainable at long gradient times with long columns in protein separations by ion-exchange

chromatography on the macroporous anion-exchange resin, TSKgel BioAssist Q. The resolution continuously becomes higher as the gradient time and the column length become longer except in some special cases. The dependence of resolution on gradient time is particularly great when the column is long and the gradient time for the change of 0–0.5 M NaCl is longer than 2 h. On the other hand, the effect of flow-rate on resolution is very small. Although the separations at long gradient times with long columns are not popular in current high-performance IEC and it takes several hours for one separation, such separations should be advantageous when very high resolutions are required like in proteomics research.

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