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Effect of chromatographic conditions on resolution in high-performance ion-exchange chromatography of proteins on nonporous support

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Abstract

We explored chromatographic conditions to obtain high resolution in protein separations by ion-exchange chromatography (IEC) on a nonporous anion-exchange resin of 2.5 μm in particle diameter. We studied the 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. It was found that two distinct conditions exist that provide high resolution. The first is a condition which has widely been employed in current high-performance IEC, namely, a combination of short gradient time, high flow-rate and comparatively short column. Separation times are usually 5–30 min, and even more rapid (1–2 min) separations are possible. The second is the condition which has rarely been employed in high-performance IEC. It is a combination of long gradient time, low flow-rate and long column. Although it takes several hours for one separation, very high resolution is attainable.

Keywords: Gradient elution; Column length; Ion-exchange chromatography; Resolution; Proteins

1. Introduction

Various modes of HPLC of proteins have progressed greatly since the middle of the 1970s by the development of spherical macroporous or nonporous microparticulate supports [1–16] and extensive studies of chromatographic conditions [7,8,10–12,14,15,17,18]. As a result, proteins can be separated in less than 1 h with high resolution and HPLC is now widely used for protein analyses.

On the other hand, proteomics has become popular since the end of 1990s. In proteomics, protein separation is one of key technologies. Very high resolution is required and two-dimensional gel electrophoresis has mainly been employed. HPLC has not been the method of choice because the resolution in currently-used HPLC is not sufficient for proteomics. However, 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 [19–24]. Another way 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 high-

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est resolution for specific objectives are fully understood.

In this paper we explore chromatographic conditions to obtain high resolution in protein separations by high-performance ion-exchange chromatography (IEC). Although such investigations were performed in the past [10,17], we study effects of gradient time (steepness of salt concentration gradient), flow-rate and column length on resolution in much wider ranges than before.

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 0.03–4 ml/min by 0.94–960 min linear gradients of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1). A 20-μl volume of solution containing 4 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 DEAE-NPR (Tosoh), which is a nonporous anion-exchange resin of 2.5 μm in particle diameter, were used. The operating pressure was kept below 100 bar for the sake of long-term stable operation of the HPLC system employed although its maximum operational pressure is 350 bar according to the manufacture. The flow-rate range operated on each column is summarized in Table 1.

Table 1
Flow rate ranges operated on columns of different lengths

Column size (mm)	Flow-rate range (ml/min)
5×4.6	0.5–4
10×4.6	0.06–4
20×4.6	0.03–2
35×4.6	0.03–1
75×4.6	0.03–0.5
150×4.6	0.03–0.25

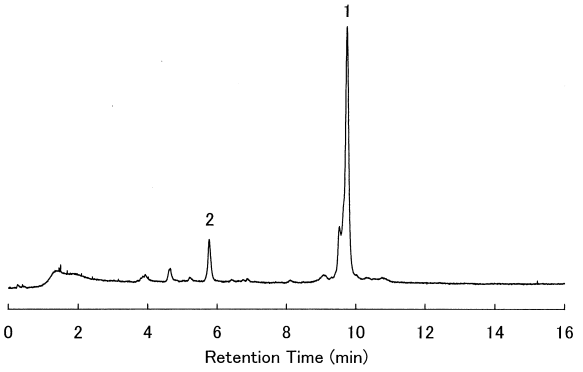

Fig. 1. Example of separation of a commercial sample of trypsin inhibitor, which was obtained on a 35×4.6 mm I.D. column using a 30-min linear gradient of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1) at a flow-rate of 1 ml/min.

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

Resolution was calculated for the largest and the second largest peaks in chromatograms of trypsin inhibitor (peaks 1 and 2 in Fig. 1). 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.

3. Results and discussion

3.1. Effect of gradient time on resolution

The dependence of resolution on gradient time on 3.5-cm long column is shown in Fig. 2. At flow-rates

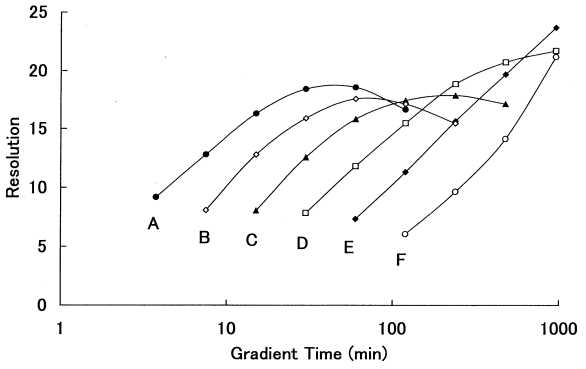

Fig. 2. Dependence of resolution on gradient time in the separation of trypsin inhibitor on a 35×4.6 mm I.D. column by 3.75–960 min linear gradients of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1) at flow-rates of (A) 1 ml/min, (B) 0.5 ml/min, (C) 0.25 ml/min, (D) 0.12 ml/min, (E) 0.06 ml/min and (F) 0.03 ml/min.

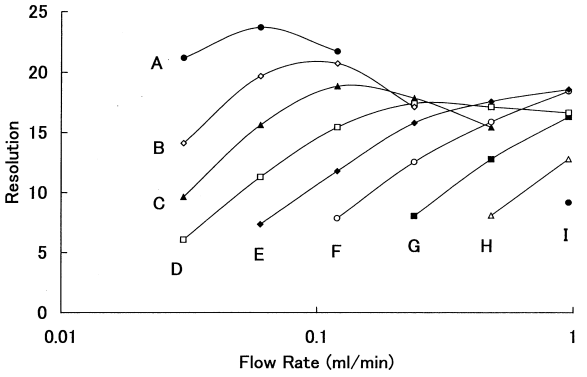

Fig. 3. Dependence of resolution on flow-rate in the separation of trypsin inhibitor on a 35×4.6 mm I.D. column by (A) 960 min, (B) 480 min, (C) 240 min, (D) 120 min, (E) 60 min, (F) 30 min, (G) 15 min, (H) 7.5 min and (I) 3.75 min linear gradients of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1) at flow-rates of 0.03–1 ml/min.

of 0.25–1 ml/min, maximum resolutions were observed at gradient times between 30 and 240 min. The gradient times providing the maximum resolutions were longer at lower flow-rates. On the other hand, the resolution monotonically became higher without reaching the maximum with increasing the gradient time at flow-rates of 0.03–0.12 ml/min. However, it is supposed that the resolution becomes maximum at gradient times longer than 960 min although this has not been confirmed experimentally. Fig. 2 also suggests that the maximum resolutions at constant flow-rates are higher at extremes of high and low flow-rates than at intermediate flow-rates.

3.2. Effect of flow-rate on resolution

The dependence of resolution on flow-rate on 3.5-cm long column is shown in Fig. 3. At gradient times of 120 min and longer, maximum resolutions were observed at certain flow-rates. The flow-rates providing the maximum resolutions were lower at longer gradient times. On the other hand, the resolution monotonically became higher with increasing the flow-rate at gradient times of 60 min and less. However, it is supposed that the resolution reaches the maximum and then decreases at flow-rates higher than 1 ml/min. Indeed, this happened on shorter columns on which separations were conducted at flow-rates up to 2 or 4 ml/min. A flow-rate of 1 ml/min provided the maximum resolutions at gradient times of 30 and 60 min on 0.5-, 1- and 2-cm long columns. In addition, a flow-rate of 2 ml/min provided the maximum resolutions at a gradient time of 15 min on 0.5- and 1-cm long columns. Fig. 3 also suggests that the maximum resolutions at constant gradient times are higher at extremes of long and short gradient times than at intermediate gradient times.

By combining information in Figs. 2 and 3 high resolutions are attainable under the conditions of short gradient time/high flow-rate and long gradient time/low flow-rate.

3.3. Effect of column length on resolution

Longer columns provided higher resolution than shorter columns at the same gradient time and flow-rate although similar effects of gradient time and flow-rate on resolution as described on 3.5-cm long column were observed on the columns of different lengths. In particular, the dependence of resolution on the column length was great in the region of long gradient time and low flow-rate, as shown in Fig. 4. Therefore, if long separation times like several hours are acceptable, the optimum condition to achieve the highest resolution is a combination of long gradient

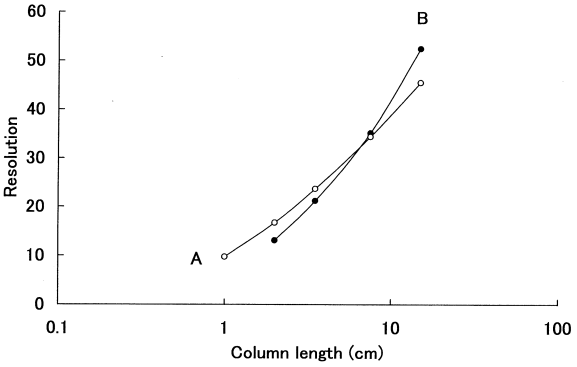

Fig. 4. Dependence of resolution on column length in the separation of trypsin inhibitor on 1–15 cm long columns by a 960-min linear gradient of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1) at flow-rates of (A) 0.06 ml/min and (B) 0.03 ml/min.

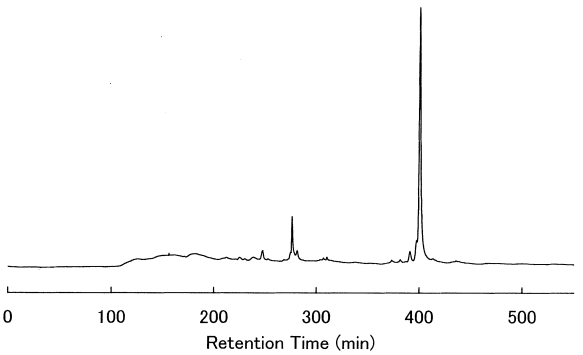

Fig. 5. Example of high-resolution separation of a commercial sample of trypsin inhibitor, which was obtained on a 150×4.6 mm I.D. column by a 960-min linear gradient of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1) at a flow-rate of 0.03 ml/min.

time, low flow-rate and a long column. These conditions are convenient for practical utilization. Because the flow-rate is low, long columns can be used without a problem of operating pressure. Supports of very small particle diameters will also be usable. The supports of smaller particle diameters should be effective to achieve higher resolution. However, these conditions have never been employed in the separation of proteins by current HPLC, to our knowledge. Although very long columns and low flow-rates were employed in the first dimension of two-dimensional HPLC [19], it was performed with isocratic elution by size-exclusion chromatography which is not an interactive chromatography like IEC. In the separation of peptides by reversed-phase liquid chromatography, long columns packed with supports of very small particle diameters and relatively long gradient times were employed to attain high resolution [25–27]. However, the flow-rates were in the normal range and operating pressures were extremely high (700–930 bar). An example of separation with high resolution is shown in Fig. 5.

On the other hand, short columns were superior to long ones in the short gradient time region. Higher flow-rates could be applied on short columns within the pressure limit of 100 bar owing to their low-pressure resistances. The higher flow-rates were advantageous to obtain higher resolution at short gradient times even on short columns, as exemplified in Fig. 6. In practice, higher resolutions were obtained on 2-cm long column than on 3.5-cm and longer columns at gradient times of 7.5 min and shorter because 2-cm long column could be operated at higher flow-rates (up to 2 ml/min) than the longer columns. However, 0.5- and 1-cm long columns did not provide the higher resolution than 2-cm long column although a higher flow-rate (4 ml/min) was employed on them. It was probably because the effect of column length was more significant than the effect of flow-rate in the case of very short columns. Therefore, the optimum condition to achieve high resolution in a very short time is a combination of

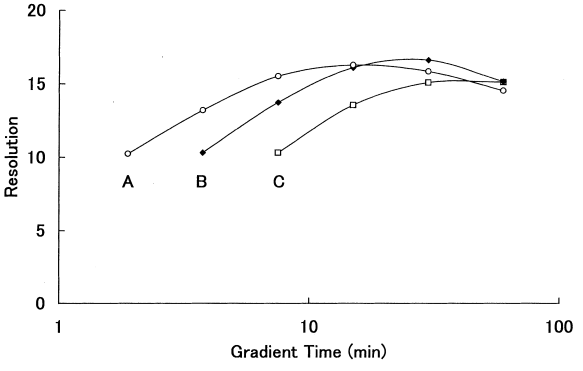

Fig. 6. Dependence of resolution on gradient time in the separation of trypsin inhibitor on a 20×4.6 mm I.D. column by 1.88–60 min linear gradients of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1) at flow-rates of (A) 2 ml/min, (B) 1 ml/min and (C) 0.5 ml/min.

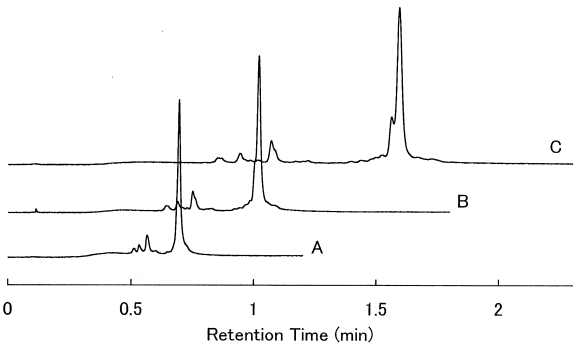

Fig. 7. Examples of very rapid separations of a commercial sample of trypsin inhibitor, which were obtained on a 20×4.6 mm I.D. column by (A) 0.94 min, (B) 1.88 min and (C) 3.75 min linear gradients of sodium chloride from 0 to 0.5 *M* in 20 m*M* Tris–HCl buffer (pH 8.1) at a flow-rate of 2 ml/min.

short gradient time, high flow-rate and short column of around 2 cm. Examples of very rapid separations are shown in Fig. 7. Trypsin inhibitor was separated in less than 1–2 min with fairly high resolution.

4. Conclusions

Two distinct conditions exist that provide high resolution in protein separations by high-performance IEC. One has been widely employed in current high-performance IEC, namely a combination of short gradient time, high flow-rate and comparatively short column. Separation times are usually 5–30 min, and even more rapid separations like in 1–2 min are possible. The other one is the condition that has rarely been employed in current high-performance IEC. It is a combination of long gradient time, low flow-rate and a long column. Although it takes several hours for one separation, very high resolution is attainable.

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