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Hydrophobicity gradient columns for the separation of trypsin inhibitor by hydrophobic interaction chromatography at low salt concentration

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Abstract

We investigated hydrophobicity gradient columns composed of two columns packed with supports of different hydrophobicities in order to save time in protein separation by hydrophobic interaction chromatography at low salt concentration using a crude sample of trypsin inhibitor as a model sample. One of the two hydrophobicity gradient columns was packed with a support whose hydrophobicity was critically controlled for target protein (trypsin inhibitor) and the other was packed with a support which was less hydrophobic than the critically controlled hydrophobicity support. It was found that the hydrophobicity gradient columns are useful to separate samples containing impurities of a wide range of hydrophobicities within a reasonable time.

Keywords: Hydrophobic interaction chromatography; Hydrophobic gradients; Gradient elution; Proteins; Trypsin inhibitor

1. Introduction

Hydrophobic interaction chromatography (HIC) has been widely employed in the separation of proteins. Proteins can be separated rapidly with high resolution in high yield without denaturation by gradient elution with decreasing salt concentration, e.g. from 1.5–2.0 *M* ammonium sulfate to 0 *M* [1–7]. However, the use of eluent containing a high concentration of salt is inconvenient particularly in large-scale purification, and it is a major disadvantage of current HIC. If HIC can be carried out successfully at low salt concentration, HIC will become more useful. Then, we investigated protein separation by HIC at low salt concentration [8]. We found it possible to separate hydrophobic proteins such as recombinant protein A and monoclonal antibody by HIC at low salt concentration using critically controlled hydrophobicity supports. The method seemed useful especially for samples containing only components of a limited range of hydrophobicities. However, when samples contained components of a wide range of hydrophobicities, it took a long time to elute all components from the column.

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In this paper, we investigate solving the problem of long separation time using hydrophobicity gradient columns, which was proposed by Shaltiel [9] and Jennissen [10] and was composed of two or more columns packed with supports of different hydrophobicities.

2. Experimental

2.1. Chromatographic measurements

Chromatographic measurements were carried out with a system consisting of a Model CCPM II double-plunger pump, a Model UV-8010 variable-wavelength UV detector operating at 280 nm and a Model SC-8020 system controller/data processor (Tosoh, Tokyo, Japan). Six columns of 75×7.5 mm I.D. were used, listed in Table 1. Two of them, columns 1 and 4, were commercially available (Tosoh) and others were prepared by packing experimental supports. The experimental supports were prepared by introducing phenyl groups into G5000PW of 1000 Å mean pore diameter and 10 μm particle diameter (Tosoh). The phenyl groups were introduced into G5000PW with ether linkage by the reaction between hydroxyl groups on G5000PW and phenyl glycidyl ethers according to the method of Hjertén et al. [11]. The G5000PW is also a base material of Ether-5PW and Phenyl-5PW. The ligand content was estimated according to the method of Genieser et al. [12] with some modification. The supports were reacted with boron tribromide and phenol, which is a cleavage product of phenyl groups on the supports, was quantified by reversed-phase liquid chromatography (RPLC). RPLC was carried out with the same system as used for HIC at 25 °C on an ODS-120T column of 150×4.6 mm I.D. (Tosoh) at a flow-rate of 1 ml/min by isocratic elution with a mixture of water–methanol (55:45, v/v). The phenol was quantified by gas chromatography in the original method of Genieser et al. The estimated phenyl group contents were in the range of 0–0.173 mmol/ml support, as shown in Table 1. Trypsin inhibitor was separated on a single column or two columns connected in series at 25 °C at a flow-rate of 1 ml/min by gradient elution of ammonium sulfate from 0.3 to 0 *M* in 50 m*M* phosphate buffer (pH 6.8). A 0.1-ml volume of solution was injected containing 0.32 mg sample in the initial eluent.

Table 1
Columns and supports used in experiments

Column	Support	Phenyl group content (mmol/ml support)
1	Ether-5PW	0.000
2	Experimentally prepared	0.038
3	Experimentally prepared	0.055
4	Phenyl-5PW	0.067
5	Experimentally prepared	0.090
6	Experimentally prepared	0.173

2.2. Materials

Trypsin inhibitor used was a commercial sample purchased from Sigma (St Louis, MO, USA) (Type I-S: from soybean, sample code T-9003, lot 27H7055). According to the manufacturer, the sample was chromatographically prepared and lyophilized. However, the sample was rather crude and contained many components, as can be seen later. The main (highest abundance) component was confirmed to be trypsin inhibitor by enzymatic activity test. Other components were not tested for enzymatic activity and therefore it is unknown if they are impurities or variants of trypsin inhibitor.

3. Results and discussion

3.1. Separation of a crude sample of trypsin inhibitor on a single column

Fig. 1 shows separations of a crude sample of trypsin inhibitor obtained with a 9-min linear gradient of ammonium sulfate from 0.3 to 0 *M* in 50 m*M* phosphate buffer (pH 6.8) on columns 1, 2, 3, 4 and 5. Many components including trypsin inhibitor eluted together with no or only slight retention on these columns, which indicates that columns packed with more hydrophobic supports are necessary to separate trypsin inhibitor from other components. On the other hand, some components in the sample eluted late on column 5. Accordingly, if columns packed with more hydrophobic supports are used, it

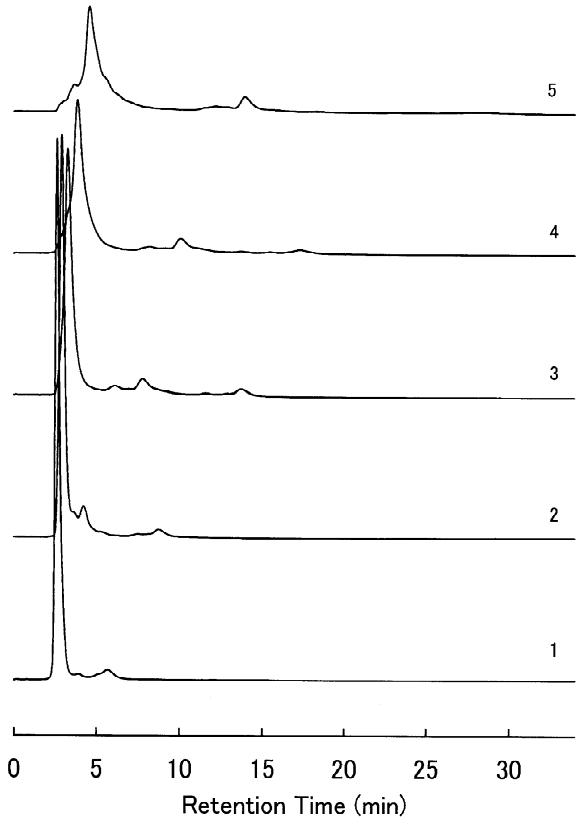

Fig. 1. Separation of a crude sample of trypsin inhibitor on columns 1, 2, 3, 4 and 5 by a 9-min linear gradient of ammonium sulfate from 0.3 to 0 *M* in 50 m*M* phosphate buffer (pH 6.8).

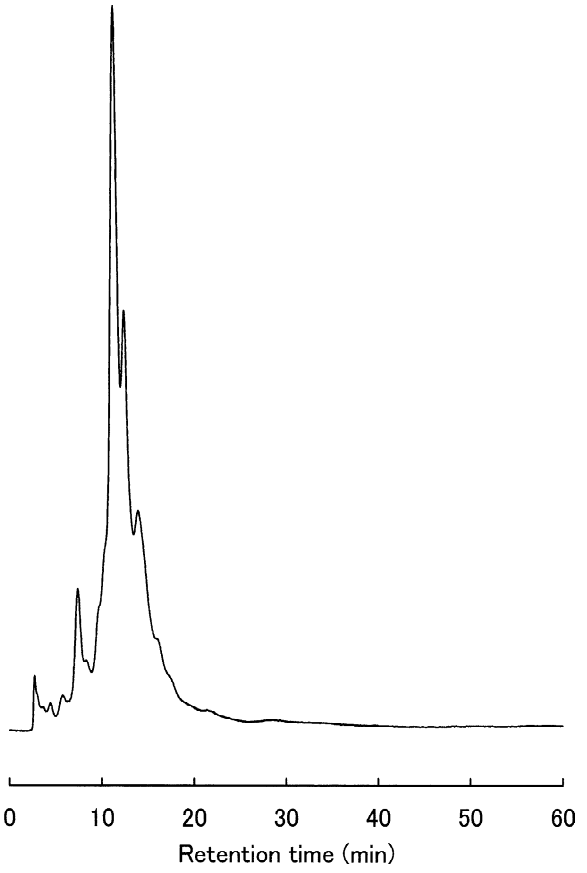

Fig. 2. Separation of a crude sample of trypsin inhibitor on column 6 by a 9-min linear gradient of ammonium sulfate from 0.3 to 0 *M* in 50 m*M* phosphate buffer (pH 6.8).

is difficult to elute components more hydrophobic than trypsin inhibitor within a reasonable time. This is a problem in HIC at low salt concentration on critically controlled hydrophobicity supports. Fig. 2 shows a separation of a crude sample of trypsin inhibitor on column 6. The separation of trypsin inhibitor (the largest peak in the chromatogram) from other components was satisfactory. However, some strongly retained components, e.g. those eluted at around 6, 8 and 14 min on column 3, remained in the column even at 60 min.

3.2. Operation in HIC on hydrophobicity gradient columns

Separation on hydrophobicity gradient columns can conveniently be carried out with such a system as shown in Fig. 3. Column A is for the rough separation between target component and components which are more hydrophobic than the target component. Column B is for the main separation and is packed with a support whose hydrophobicity is critically controlled for target component. Column A is packed with a support which is less hydrophobic than the critically controlled hydrophobicity support packed in column B. Whole sample is injected into column A. Just after the target component has passed through column A and has entered column B, column A is disconnected from the eluent flow line. Then, components retained in column B including the target component are separated. Because components which entered column B are less hydrophobic or very slightly more hydrophobic than the target component, it is supposed that all components

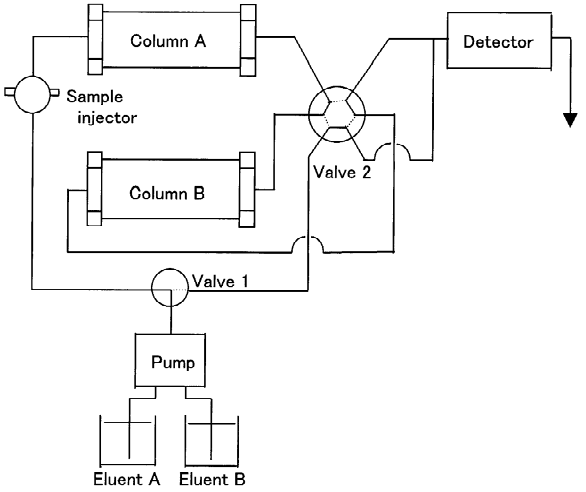

Fig. 3. Schematic diagram of the system for HIC at low salt concentration on hydrophobicity gradient columns. Samples are injected with ''solid line'' positions of valves 1 and 2. Valves 1 and 2 positions are changed to ''broken line'' just after the target components have entered column B. The valve 1 position is returned to ''solid line'' after all components that entered column B have been eluted from column B. The valve 2 position is returned to ''solid line'' after all components remaining in column A have been eluted from column A.

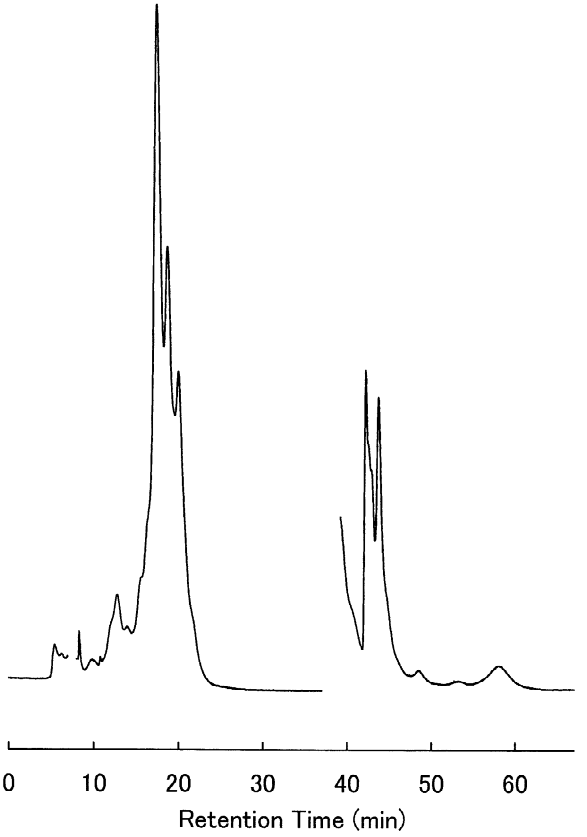

Fig. 4. Separation of a crude sample of trypsin inhibitor on hydrophobicity gradient columns composed of columns 5 and 6.

elute from column B without being retained so long. Components remaining in column A can be washed out from the column and are wasted without being introduced into column B after the separation on column B has been completed. Column A could be washed while the separation on column B is going on using another pump in order to save time of whole separation, if desired. This operation with the system in Fig. 3 could be carried out automatically using a computerized valve controller although we manually made separations on hydrophobicity gradient columns in Section 3.3 without using such a system as shown in Fig. 3.

3.3. Separation of a crude sample of trypsin inhibitor on hydrophobicity gradient columns

Fig. 4 shows a separation of a crude sample of trypsin inhibitor on hydrophobicity gradient columns composed of columns 5 and 6. The sample was loaded onto hydrophobicity gradient columns equilibrated with phosphate buffer (pH 6.8) containing 0.3 *M* ammonium sulfate and was eluted isocratically for 7 min. Then, the pump was stopped and column 5 was disconnected from the line. It took about 1 min for disconnection. The pump was restarted at around 8 min and components retained in column 6 were separated by a 9-min linear gradient of ammonium sulfate from 0.3 to 0 *M* followed by isocratic elution with 50 m*M* phosphate buffer (pH 6.8). After all components in column 6 had been eluted, the pump was stopped again at 37 min and column 6 was replaced with column 5. It took about 1 min for the replacement. Then, the pump was restarted at around 38 min and hydrophobic components remaining in column 5 were eluted by isocratic elution with 50 m*M* phosphate buffer (pH 6.8). A similar sepa-

ration as in Fig. 2 was obtained, while all components in the sample were eluted from columns 5 and 6 in about 1 h.

Fig. 5 also shows a separation of a crude sample of trypsin inhibitor on hydrophobicity gradient columns composed of columns 5 and 6 obtained in the same way as in Fig. 4 except that the pump was stopped during 33–34 min for the replacement of column 6 with column 5 and that column 5 was connected in the reverse direction (inlet-side outlet) when column 6 was replaced with column 5. Column 5 was connected in the reverse direction in order to back-flush column 5. This back-flush operation can conveniently be done by installing a four-way valve in the system in Fig. 3, as illustrated in Fig. 6, although we operated it manually without using such a system. All components remaining in column 5 were eluted much earlier than in the case in Fig. 4 by back-flushing column 5. Therefore, there is a significant advantage in back-flushing column 5 for saving time in HIC at low salt concentration.

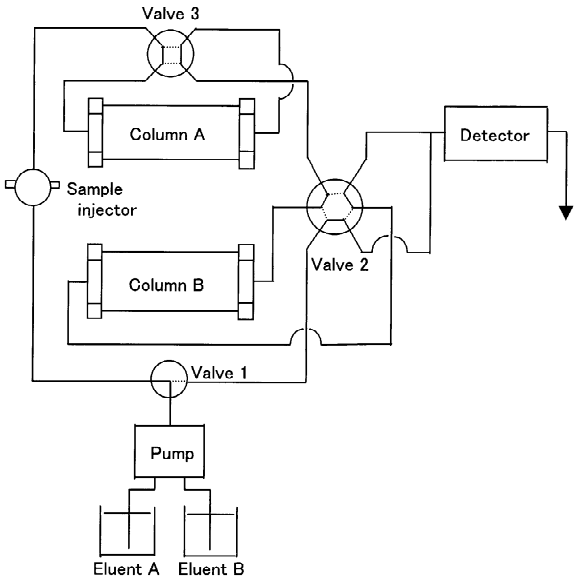

Fig. 6. Schematic diagram of the system 2 for HIC at low salt concentration on hydrophobicity gradient columns. Samples are injected with ''solid line'' positions of all valves. After target components have entered column B, the positions of all valves are changed to ''broken line''. After all components that entered column B have been eluted from column B, valve 1 position is returned to ''solid line''. After all components remaining in column A have been eluted from column A, valves 2 and 3 positions are returned to ''solid line''.

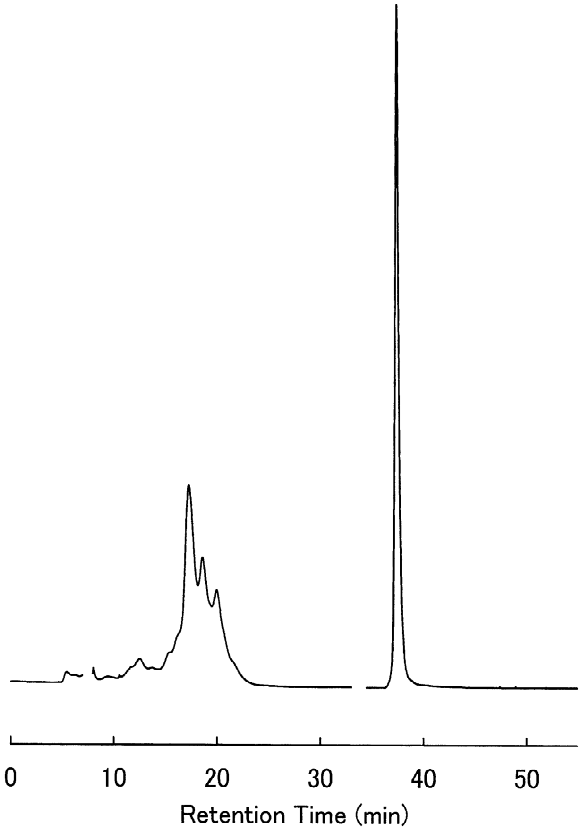

Fig. 5. Separation of a crude sample of trypsin inhibitor on hydrophobicity gradient columns composed of columns 5 and 6. See Section 3.3 for the difference between Figs. 4 and 5.

4. Conclusions

A crude sample of trypsin inhibitor can be separated successfully within a reasonable time in HIC at low salt concentration using hydrophobicity gradient columns composed of two columns packed with supports of different hydrophobicities even if the sample contains components of a wide range of hydrophobicities. One of the two hydrophobicity gradient columns is packed with a support whose hydrophobicity is critically controlled for target protein and the other is packed with a support which

is less hydrophobic than the critically controlled hydrophobicity support.

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