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Hydrophobic interaction chromatography at low salt concentration for the capture of monoclonal antibodies

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Abstract

We evaluated hydrophobic interaction chromatography (HIC) at low salt concentration for the capture of proteins from feed stocks by using monoclonal antibodies as model samples. It was indicated that the HIC at low salt concentration on critical hydrophobicity supports has a potential for capturing hydrophobic monoclonal antibodies directly from large volumes of feed stocks and recovering bound monoclonal antibodies in high yield. On the other hand, the HIC at low salt concentration did not seem so useful for the capture of weakly hydrophobic monoclonal antibodies. The recovery of weakly hydrophobic monoclonal antibodies from columns packed with critical hydrophobicity supports was not quantitative and significantly decreased as the residence time of the monoclonal antibodies in the columns became longer.
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Keywords: Hydrophobic interaction chromatography; Salt effects; Hydrophobicity; Proteins; Monoclonal antibodies

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 to 2.0 M ammonium sulfate to 0 [1–7]. However, the use of eluent containing high concentration of salt is inconvenient particularly in large-scale purification, and it is a major disadvantage of current HIC. If HIC can be performed successfully at low salt concentration, HIC will become more useful.

Shaltiel [8] and Jennissen [9] recommended critical hydrophobicity approach. This approach is based on the use of critical hydrophobicity supports that adsorb target proteins only slightly in buffer solutions of low concentration. The target proteins are adsorbed on the supports in the presence of low concentration of salt but are not essentially adsorbed in the absence of the salt. Alternatively, the target proteins are adsorbed on the supports in the absence of salt but are not essentially adsorbed in the presence of low concentration of polarity-reducing agent like ethylene glycol. This approach seems ideal to separate proteins by HIC at low salt concentration. Thus, we have been confirming the practical feasibility of the idea, and found it possible to successfully separate hydrophobic proteins by using the critical hydrophobicity supports [10,11]. Typical elution conditions are gradients of ammonium sulfate from 0.3–0.5 to 0 M or ethanol from 0 to 10–20% in common buffer solutions of low concentration like 50 mM phosphate buffer (pH 6.8). The HIC with such elution conditions seems useful in protein purification process not only for intermediate purification and polishing steps but also for capturing proteins from feed stocks like cell culture supernatant.

In this paper, we study if the HIC at low salt concentration can be employed for the capture of proteins by using monoclonal antibodies as model samples. First, retention and recovery of monoclonal antibodies are measured on the supports of a wide range of hydrophobicities in order to assess the critical hydrophobicity supports for the individual monoclonal antibodies and preliminarily estimate the possibility of employing HIC for the capture of the monoclonal antibodies. Then, dynamic adsorption capacities for the monoclonal antibodies are determined on the critical hydrophobicity supports to know how much the monoclonal antibodies or feed stocks containing them can be applied. Finally, it is tried to capture the monoclonal antibodies from large volumes of artificial cell culture supernatants.

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2. Experimental

2.1. Measurements of retention and recovery

Chromatographic measurements were carried out with a system consisting of a model CCPM II double-piston pump, a model UV-8010 variable-wavelength UV detector operated at 280 nm and a model SC-8020 system controller/data processor (Tosoh, Tokyo, Japan). The retention and recovery of monoclonal antibodies were measured by isocratic elution with 50 mM phosphate buffer (pH 6.8) and by gradient elution of ethanol from 0 to 20% or ammonium sulfate from 0.5 to 0 M in 50 mM phosphate buffer (pH 6.8) at a flow-rate of 1 ml/min and 25 °C. A 0.1 ml volume of solutions containing 0.16 mg monoclonal antibodies in the initial eluents was injected. The recovery was estimated from areas of eluted peaks. Twelve columns of 75 mm × 7.5 mm i.d. listed in Table 1 were used. 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 in particle diameter (Tosoh). The preparation method has been described elsewhere [10,11]. The G5000PW is also the base material of Ether-5PW and Phenyl-5PW.

2.2. Measurements of dynamic adsorption capacities

Dynamic adsorption capacities were determined by measuring breakthrough curves. A system consisting of two CCPM II pumps, a UV-8010 UV detector operated at 280 nm and a SC-8020 system controller/data processor was employed. The two pumps were connected in parallel with a four-way valve prior to the column. The one was for delivering equilibration and elution buffers, and the other was for delivering monoclonal antibody solutions. The breakthrough curves were measured by applying monoclonal antibody solutions at a flow-rate of 0.64 ml/min and 25 °C to the columns of 40 mm × 6 mm i.d. equilibrated with 50 mM phosphate buffer (pH 6.8) or 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8). The monoclonal antibody solutions were prepared with the equilibration buffers. The concentration of monoclonal antibodies was 0.2 mg/ml or 1 mg/ml. The dynamic adsorption capacities were calculated at 10% breakthrough.

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	0.113
7	Experimentally prepared	0.123
8	Experimentally prepared	0.137
9	Experimentally prepared	0.160
10	Experimentally prepared	0.173
11	Experimentally prepared	0.200
12	Experimentally prepared	0.208

2.3. Capture of monoclonal antibodies from artificial cell culture supernatants

It was carried out with the same system and columns as used in Section 2.2 to capture monoclonal antibodies from artificial cell culture supernatants. The artificial cell culture supernatants were prepared by dissolving monoclonal antibodies in commercial ultra-low protein cell culture medium IS-PRO (Irvine Scientific, Santa Ana, CA, USA) at a concentration of 0.2 mg/ml. IS-PRO is serum-free and contains only 1 μg/ml transferrin as a protein component according to the manufacturer. Some artificial cell culture supernatants were supplemented with 0.5 M ammonium sulfate. The artificial cell culture supernatants were applied for certain periods of time at a flow-rate of 0.64 ml/min and 25 °C to the columns equilibrated with 50 mM phosphate buffer (pH 6.8) or 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8). Then, the columns were washed with equilibration buffers for certain periods of time and bound components were eluted with a 15 min linear gradient of ethanol from 0 to 20% or ammonium sulfate from 0.5 to 0 M in 50 mM phosphate buffer (pH 6.8). Flow-through and bound fractions were collected and subjected to high-performance gel filtration to analyze components contained in the fractions. The gel filtration was performed with the same system as employed in Section 2.1 on a $G3000SW_{XL}$ column of 300 mm × 7.8 mm i.d. (Tosoh) at a flow-rate of 1 ml/min and 25 °C. The eluent was 50 mM phosphate buffer containing 0.3 M NaCl (pH 6.8). A 20 μl volume of fractions was injected.

2.4. Materials

Two monoclonal antibody samples (IgG_1 subclass) obtained in our laboratory were used. According to our definition based on the retention in HIC [10], one of them (MAb-1) is hydrophobic and the other (MAb-2) is weakly hydrophobic.

3. Results and discussion

3.1. Retention and recovery

Retention and recovery in the separations by isocratic elution with 50 mM phosphate buffer (pH 6.8) and by gradient of ethanol from 0 to 20% or ammonium sulfate from 0.5 to 0 M.

The retention and recovery obtained by isocratic elution with 50 mM phosphate buffer (pH 6.8) are summarized in

Table 2
Retention and recovery of MAb-1 and MAb-2 in the separations by isocratic elution with 50 mM phosphate buffer (pH 6.8)

Column	MAb-1		MAb-2	
	Retention time (min)	Recovery (%)	Retention time (min)	Recovery (%)
1	2.72	100		
2	3.51	100		
3	7.50	99		
4	14.70	98	2.65	98
5	74.27	100	2.92	101
6	NE		3.31	100
7			3.73	100
8			4.21	99
9			6.10	100
10			7.77	96
11			33.64	77
12			NE	

NE: not eluted within a practical time frame.

Table 2. The column void volume was ca. 2.5 ml, and therefore retention times of around 2.5 min mean no retention.

MAb-1 was eluted as fairly narrow peaks without being retained so long on columns 1-4. But, it was rather strongly retained on column 5, and was not eluted from column 6 within a practical time frame. These results indicate that columns 4 and 6 are critical hydrophobicity for the separations of MAb-1 by gradients of ammonium sulfate from 0.5 to 0 M and ethanol from 0 to 20%, respectively. The recovery of MAb-1 was always quantitative.

When MAb-1 was separated on column 6 by a 30 min linear gradient of ethanol from 0 to 20%, it was eluted quantitatively at an ethanol concentration of ca. 18% as a reasonably narrow peak. Furthermore, MAb-1 was separated on column 6 by isocratic elution with 50 mM phosphate buffer (pH 6.8) for 500 min followed by a 5 min linear gradient of ethanol from 0 to 20%. MAb-1 was not eluted from the column during the isocratic elution and it was eluted quantitatively by the gradient of ethanol. These results suggest the possibility of applying a large volume of MAb-1 solution containing no ammonium sulfate to column 6 and eluting bound MAb-1 with a common buffer solution containing ethanol with high recovery.

When MAb-1 was separated on column 4 by a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M, it was eluted quantitatively after the ammonium sulfate concentration became zero as a comparatively narrow peak. MAb-1 was also separated on column 4 by isocratic elution with 50 mM phosphate buffer (pH 6.8) containing 0.5 M ammonium sulfate for 450 min followed by a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M. MAb-1 was not eluted from the column during the isocratic elution and it was eluted quantitatively by the gradient of ammonium sulfate. These results suggest the possibility of applying a large volume of MAb-1 solution containing 0.5 M ammonium sulfate to column 4 and eluting bound MAb-1 with a common buffer solution of low concentration with high recovery.

Table 3
Recovery of MAb-2 in the separations by isocratic elution with 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8) followed by a 15 min linear gradient to 50 mM phosphate buffer (pH 6.8) on column 10

Duration of isocratic elution (min)	Recovery (%)
0	82
30	71
60	56
120	39

MAb-2 was eluted early as fairly narrow peaks with quantitative recovery on columns 4–10. But, it was retained rather strongly on column 11 and was not eluted from column 12 within a practical time frame. These results indicate that columns 10 and 12 are critical hydrophobicity for the separations of MAb-2 by gradients of ammonium sulfate from 0.5 to 0 M and ethanol from 0 to 20%, respectively. The recovery decreased down to 77% in the separation on column 11.

When MAb-2 was separated on column 12 by a 30 min linear gradient of ethanol from 0 to 20%, it was eluted at an ethanol concentration of ca. 9% with considerably low recovery, 55%. Furthermore, in the separation of MAb-2 on column 12 by isocratic elution with 50 mM phosphate buffer (pH 6.8) for 60 min followed by a 30 min linear gradient of ethanol from 0 to 20%, the recovery decreased down to around 30%. Therefore, it will not be successful to apply a large volume of MAb-2 solution containing no ammonium sulfate to column 12 and elute bound MAb-2 with a common buffer solution containing ethanol with high recovery.

When MAb-2 was separated on column 10 by a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M, it was eluted after the ammonium sulfate concentration became zero. But, the recover was relatively low, 82%, in this case, too. Moreover, MAb-2 was separated on column 10 by isocratic elution with 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8) for various periods of time followed by a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M. The recovery decreased with increasing the period of isocratic elution, namely with increasing the residence time of MAb-2 in the column, as shown in Table 3. Accordingly, it will not be successful either to apply a large volume of MAb-2 solution containing 0.5 M ammonium sulfate to column 10 and elute bound MAb-2 with a common buffer solution of low concentration with high recovery.

3.2. Dynamic adsorption capacity

Dynamic adsorption capacities are summarized in Table 4. In experiments 1–4, MAb-1 and MAb-2 solutions of the concentration of 0.2 mg/ml were applied to the critical hydrophobicity columns. The dynamic adsorption capacities for both MAb-1 and MAb-2 were less than 10 mg/ml support. These dynamic adsorption capacities were lower than those reported in other modes of liquid chromatography.

Table 4
Dynamic adsorption capacities of MAb-1 and MAb-2

Experiment number	Monoclonal antibody	Solvent[a]	Concentration of monoclonal antibody (mg/ml)	Column	Dynamic adsorption capacity (mg/ml support)
1	MAb-1	1	0.2	6	5.4
2	MAb-1	2	0.2	4	8.7
3	MAb-2	1	0.2	12	8.4
4	MAb-2	2	0.2	10	6.6
5	MAb-1	1	1.0	6	17.0
6	MAb-1	2	0.2	6	19.9

[a] Solvent 1: 50 mM phosphate buffer (pH 6.8); solvent 2: 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8).

Dynamic adsorption capacities of 20 mg/ml support or more have been reported for monoclonal antibodies in other modes of liquid chromatography such as protein A affinity chromatography and ion-exchange chromatography [12–15].

In experiment 5, MAb-1 solution of a concentration of 1 mg/ml was applied to the critical hydrophobicity column. The comparatively high dynamic adsorption capacity of 17 mg/ml support was obtained. Therefore, dynamic adsorption capacities almost equivalent to those in other modes of liquid chromatography can be expected when the monoclonal antibody concentrations of feed stocks are 1 mg/ml or higher. Furthermore, a similarly high dynamic adsorption capacity of ca. 20 mg/ml support was obtained in experiment 6, where MAb-1 solution of the concentration of 0.2 mg/ml containing 0.5 M ammonium sulfate was applied to column 6. Column 6 is more hydrophobic than critical for MAb-1 dissolved in 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8), and it is necessary to add ethanol to elution buffer in order to recover bound MAb-1 from the column. Although this protocol is slightly messy, it will be possible to obtain high dynamic adsorption capacities by adding 0.5 M ammonium sulfate to feed stocks and 20% ethanol to elution buffers.

3.3. Capture of monoclonal antibodies from artificial cell culture supernatants

The artificial cell culture supernatant containing 0.2 mg/ml MAb-1 was applied to column 6 equilibrated with 50 mM phosphate buffer (pH 6.8) for 40 min. The column was washed with the equilibration buffer for 6 min and then bound components were eluted with a 15 min linear gradient of ethanol from 0 to 20%. The flow-through and bound fractions were collected during 1.5–42 and 51–63 min, respectively. The elution profile monitored with UV detector at 280 nm is shown in Fig. 1. Fig. 2 shows the gel filtration chromatograms of the two fractions and applied artificial cell culture supernatant. The applied artificial cell culture supernatant corresponds to 23 column volumes. The amount of applied MAb-1 corresponds to 4.5 mg/ml column volume, which is about 83% of the dynamic adsorption capacity of the column 6 for MAb-1 dissolved in 50 mM phosphate buffer (pH 6.8) at a concentration of 0.2 mg/ml. Nevertheless, MAb-1 was retained in the column and components of IS-PRO only were eluted from the column while the artificial cell culture supernatant was being applied and the column was being washed. Then, MAb-1 was eluted from the column by the gradient of ethanol. The recovery of MAb-1 in the bound fraction was almost quantitative (95%).

The artificial cell culture supernatant containing 0.2 mg/ml MAb-1 and 0.5 M ammonium sulfate was applied to

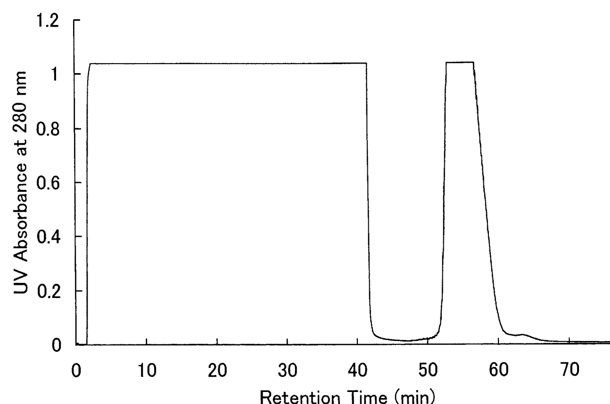

Fig. 1. Capture of MAb-1 from artificial cell culture supernatant. The artificial cell culture supernatant containing 0.2 mg/ml MAb-1 was applied to column 6 equilibrated with 50 mM phosphate buffer (pH 6.8) for 40 min. The column was washed with the equilibration buffer for 6 min and then bound components were eluted with a 15 min linear gradient of ethanol from 0 to 20%. The flow-through and bound fractions were collected during 1.5–42 and 51–63 min, respectively.

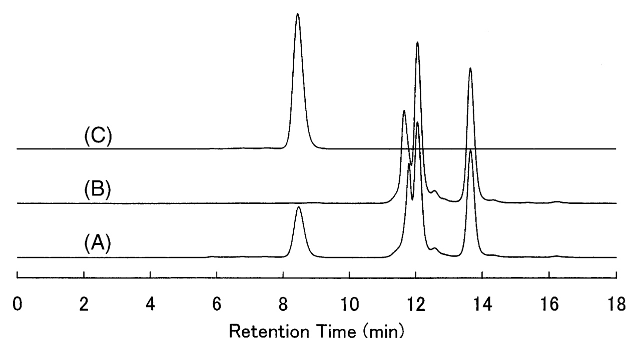

Fig. 2. Gel filtration chromatograms of (A) artificial cell culture supernatant, (B) flow-through fraction and (C) bound fraction applied and collected in the capture in Fig. 1. Peaks eluted at 8.5 min represent MAb-1 and peaks eluted after 11 min are low molecular weight compounds contained in IS-PRO.

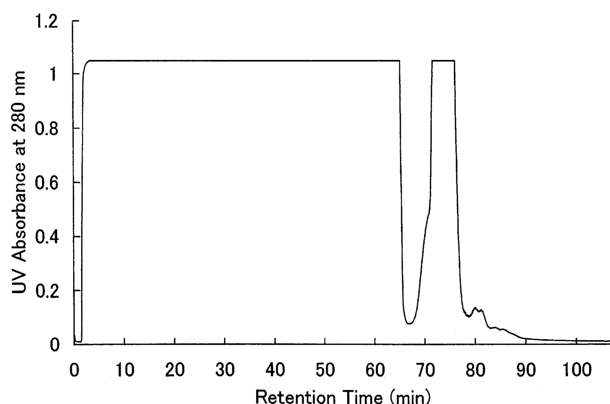

Fig. 3. Capture of MAb-1 from artificial cell culture supernatant. The artificial cell culture supernatant containing 0.2 mg/ml MAb-1 and 0.5 M ammonium sulfate was applied to column 4 equilibrated with 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8) for 64 min. The column was washed with the equilibration buffer for 5 min and then bound components were eluted with a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M. The flow-through and bound fractions were collected during 1.5–66 and 68–80 min, respectively.

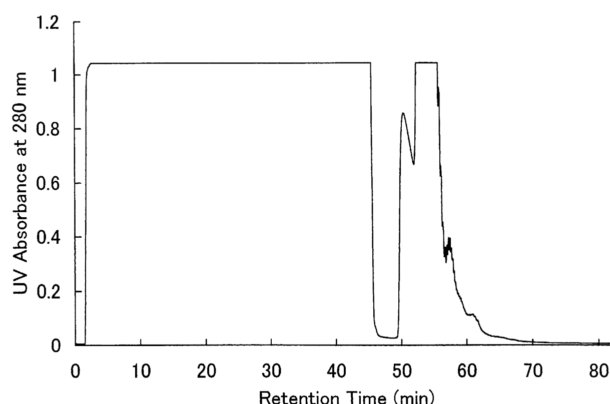

Fig. 4. Capture of MAb-2 from artificial cell culture supernatant. The artificial cell culture supernatant containing 0.2 mg/ml MAb-2 and 0.5 M ammonium sulfate was applied to column 10 equilibrated with 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8) for 44 min. The column was washed with the equilibration buffer for 5 min and then bound components were eluted with a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M. The flow-through and bound fractions were collected during 1.5–46 and 49.5–62 min, respectively.

column 4 equilibrated with 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8) for 64 min. The column was washed with the equilibration buffer for 5 min and then bound components were eluted with a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M. The flow-through and bound fractions were collected during 1.5–66 and 68–80 min, respectively. The elution profile monitored with UV detector at 280 nm is shown in Fig. 3. The applied artificial cell culture supernatant corresponds to 36 column volumes and the amount of applied MAb-1 corresponds to 7.2 mg/ml column volume. The amount of applied MAb-1 seemed a little too much although it was about 83% of the dynamic adsorption capacity of the column 4 for MAb-1 dissolved at a concentration of 0.2 mg/ml in 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8). MAb-1 began to elute from the column before the end of column washing. Therefore, the collection of the bound fraction was begun before the start of the gradient of ammonium sulfate. MAb-1 was contained only in the bound fraction according to the results of gel filtration of the fractions (not shown). The flow-through fraction contained only components of IS-PRO and the bound fraction contained only MAb-1, as in the previous case. The recovery of MAb-1 in the bound fraction was almost quantitative (95%).

The artificial cell culture supernatant containing 0.2 mg/ml MAb-2 and 0.5 M ammonium sulfate was applied to column 10 equilibrated with 50 mM phosphate buffer containing 0.5 M ammonium sulfate (pH 6.8) for 44 min. The column was washed with the equilibration buffer for 5 min and then bound components were eluted with a 15 min linear gradient of ammonium sulfate from 0.5 to 0 M. The flow-through and bound fractions were collected during 1.5–46 and 49.5–62 min, respectively. The applied artificial cell culture supernatant corresponds to 25 column volumes and the amount of applied MAb-2 corresponds to 5.0 mg/ml column volume. The elution profile monitored with UV detector at 280 nm is shown in Fig. 4. Fig. 5 shows the results of gel filtration. The flow-through fraction contained only components of IS-PRO. The bound fraction contained MAb-2 and a small quantity of other component that was originally contained in the MAb-2 sample. Some peaks are seen before and after the main peak in the bound fraction in Fig. 4, which indicates that some components are contained in the fraction and are partially separated. In this experiment, however, whole portion of the peaks was collected as the bound fraction. If only the main peak is collected as the bound fraction, the minor peak seen in the gel filtration chromatogram of the fraction may be removed. The recovery of MAb-2 in the bound fraction was 83%, which was higher than being expected. However, it is anticipated that the recovery of MAb-2 will decrease if it takes longer in practical industrial scale captures.

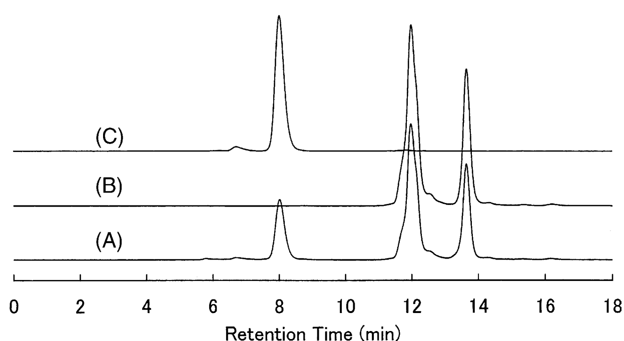

Fig. 5. Gel filtration chromatograms of (A) artificial cell culture supernatant, (B) flow-through fraction and (C) bound fraction applied and collected in the capture in Fig. 4. Peaks eluted at 8.0 min represent MAb-2 and peaks eluted after 11 min are low molecular weight compounds contained in IS-PRO.

As shown above, MAb-1 was successfully captured from large volumes of artificial cell culture supernatants and was recovered quantitatively. MAb-2 was also captured and was recovered in slightly low recovery. It was possible to directly apply the artificial cell culture supernatants to the critical hydrophobicity columns and recover the bound monoclonal antibodies with 50 mM phosphate buffer containing 20% ethanol. It was also possible to apply the artificial cell culture supernatants supplemented with 0.5 M ammonium sulfate to the critical hydrophobicity columns and recover the bound monoclonal antibodies with 50 mM phosphate buffer (pH 6.8). Although these two protocols could be employed practically in capture step, the direct loading of feed stocks to the columns and recovering bound monoclonal antibodies with common buffer solutions containing 20% ethanol seems preferable. In the latter protocol, it is necessary to add ammonium sulfate to feed stocks prior to loading them to the columns. It is probably easier to handle and more economical to add ethanol to the elution buffers than adding ammonium sulfate to larger volumes of feed stocks.

In the experiments above 50 mM phosphate buffer (pH 6.8) or the same buffer containing 20% ethanol was used as elution buffer. However, it is expected that the type, pH and concentration of the elution buffers can be changed within certain limited ranges without affecting the results. Consequently, the bound fractions of the capture step by HIC at low salt concentration could be applied directly to the next purification step by other modes of liquid chromatography such as ion-exchange, hydroxyapatite and affinity chromatography without intermediate treatments. It should be one of advantages of the HIC at low salt concentration for the capture of monoclonal antibodies.

4. Conclusions

HIC at low salt concentration on critical hydrophobicity supports has a potential for capturing hydrophobic monoclonal antibodies directly from large volumes of feed stocks and recovering bound monoclonal antibodies in high yield. Although dynamic adsorption capacities of the supports employed here were almost equivalent to or lower than those reported in other modes of liquid chromatography, it will be possible to increase the dynamic adsorption capacities by improving the supports, e.g., optimizing the pore size. Therefore, HIC at low salt concentration is supposed to be a good alternative to existing capture methods in the purification of hydrophobic monoclonal antibodies. On the other hand, the HIC at low salt concentration does not seem so useful for weakly hydrophobic monoclonal antibodies. The recovery of weakly hydrophobic monoclonal antibodies from columns packed with critical hydrophobicity supports is not quantitative and significantly decreases as the residence time of the monoclonal antibodies in the columns become longer.

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