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# Separation of proteins by hydrophobic interaction chromatography at low salt concentration

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**Abstract**

We investigated protein separation by hydrophobic interaction chromatography (HIC) at low salt concentration on the supports of various hydrophobicities. Hydrophobic proteins could be successfully separated with more than 90% recovery by gradient elution of ammonium sulfate from 0.3–0.5 *M* to 0 in 50 m*M* phosphate buffer (pH 6.8) by using supports whose hydrophobicities were properly adjusted individually for each protein. Satisfactory results were also obtained by isocratic elution without ammonium sulfate and gradient elution of ethanol from 0 to 10%. HIC at low salt concentration was compatible with other modes of liquid chromatography like ion-exchange chromatography. On the other hand, it was not successful to separate hydrophilic proteins at low salt concentration. Recoveries of hydrophilic proteins decreased before they were retained enough as support hydrophobicity increased. Therefore, it is inevitable to use a higher concentration of salt, e.g., 1–2 *M* ammonium sulfate, on hydrophilic or moderately hydrophobic support in order to retain hydrophilic proteins without decrease in recovery.


*Keywords:* Hydrophobic interaction chromatography; Proteins

## 1. Introduction

Hydrophobic interaction chromatography (HIC) has widely been 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 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 performed successfully at low salt concentration, HIC will become more useful. In contrast to this current situation, many HIC separations were carried out without using high concentration of salt in the early days although the results were not always satisfactory [8–10]. Proteins were adsorbed on the supports in buffer solutions of low concentration and desorption was conducted by using buffers containing deforming agents like 0.4 *M* imidazolium citrate, polarity-reducing agents like 10–50% ethylene glycol, low concentrations of denaturants like 1 *M* urea, etc. In addition, a critical hydrophobicity approach was recommended [10,11]. This approach is based

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on the use of supports which adsorb target proteins only very slightly in buffer solutions of low concentration. Then the target proteins are adsorbed on the critical hydrophobicity supports in the presence of low concentrations of salt but are not essentially adsorbed in the absence of salt. This approach seems ideal to separate proteins by HIC at low salt concentration although reports on experimental results to confirm the practical feasibility of the idea have been few.

In this paper we investigate protein separation by HIC at low salt concentration. Retention and recovery of various proteins are studied on the supports having a wide range of hydrophobicities.

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 |

## 2. Experimental

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 operated at 280 nm and a Model SC-8020 system controller and data processor (Tosoh, Tokyo, Japan). Retention and recovery were measured for proteins at 25 °C at a flow-rate of 1 ml/min by gradient elution of ammonium sulfate from 0.3 or 0.5 *M* to 0 in 50 m*M* phosphate buffer (pH 6.8), isocratic elution with 50 m*M* phosphate buffer (pH 6.8) or gradient elution of ethanol from 0 to 10% in 50 m*M* phosphate buffer (pH 6.8). A 0.1-ml volume of solutions containing 0.04–0.24 mg proteins in the initial eluent was injected. The recovery was estimated from areas of eluted peaks. Eleven columns of 75×7.5 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 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 Hjerten et al. [12]. 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. [13] 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 performed 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 0–0.200 mmol/ml support, as shown in Table 1.

Some fractions collected in HIC separations were subjected to ion-exchange chromatography (IEC). The IEC separations were carried out with the same system as for HIC at 25 °C at a flow-rate of 0.5 ml/min by a 50 min linear gradient of sodium sulfate from 0 to 0.15 *M* in 50 m*M* phosphate buffer (pH 6.8) on BioAssist S or by a 50 min linear gradient of sodium chloride from 0 to 1 *M* in 50 m*M* Tris–HCl buffer (pH 8.0) on BioAssist Q. BioAssist S and BioAssist Q are, respectively, cation-exchange and anion-exchange columns of 50×4.6 mm I.D. for protein separations (Tosoh).

Antithrombin III, ovalbumin and recombinant (r) protein A were purchased from Welfide (Osaka, Japan), Seikagaku (Tokyo, Japan) and Repligen (Cambridge, MA, USA), respectively. Monoclonal antibody of $IgG_1$ subclass was obtained in our laboratory. All other proteins were purchased from Sigma (St. Louis, MO, USA).

## 3. Results and discussion

### *3.1. Retention and recovery of proteins in the separation by gradient elution of ammonium sulfate from 0.3 or 0.5 M to 0*

Retention and recovery of proteins obtained by a 15 min linear gradient of ammonium sulfate from 0.5 *M* to 0 in 50 m*M* phosphate buffer (pH 6.8) are summarized in Tables 2 and 3. The void volume of a column was ca. 2.5 ml, and therefore retention times of around 2.5 min mean no retention at 0.5 *M* ammonium sulfate. Because a dynamic mixer of 2 ml inner volume was installed between the pump and the sample injector to smooth the salt gradient, ammonium sulfate concentration at column outlet remained constant (0.5 *M*) during 0–4.5 min, then linearly decreased afterward and became 0 at 19.5 min. We tentatively classified proteins into hydrophilic and hydrophobic ones according to the retention on column 4 (Phenyl-5PW) at ammonium sulfate concentration of 0.5 *M*. Definition is as follows: hydrophilic proteins were not retained or very slightly retained and retention times were less than 4 min. Hydrophobic proteins were retained at least to some extent and retention times were more than 4 min. Cytochrome *c*, myoglobin, antithrombin III, conalbumin, β-lactoglobulin, ribonuclease A, ovalbumin, transferrin, α-lactalbumin and lysozyme were classified as hydrophilic proteins. Trypsin, trypsin inhibitor, α-amylase, α-chymotrypsin, α-chymotrypsinogen A, r-protein A and monoclonal antibody were classified as hydrophobic proteins.

Hydrophilic proteins were not retained or only slightly retained in all cases tested here with a few exceptions. Their recoveries decreased before they were retained enough as the support hydrophobicity increased. Although the reason for the decrease in recovery is not clear, it is practically impossible to separate hydrophilic proteins at low salt concentration and it is inevitable to use a high concentration of salt, e.g., 1–2 *M* ammonium sulfate on hydrophilic or moderately hydrophobic support such as Ether-5PW and Phenyl-5PW in order to retain hydro-

Table 2
Retention of proteins in HIC by gradient elution of ammonium sulfate from 0.5 *M* to 0

| Protein | Retention time (min) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Cytochrome *c* | 2.57 | | | 2.55 | 2.65 | 2.69 | | 2.68 | | 2.65 | 2.73 |
| Myoglobin | 2.59 | | | 2.61 | 2.72 | 2.78 | | 2.78 | | 2.79 | 2.92 |
| Antithrombin III | 2.52 | | | 2.54 | 2.72 | 2.81 | | 2.87 | | | |
| Conalbumin | 2.51 | | | 2.57 | 2.76 | 2.85 | | 2.96 | | 3.18 | |
| β-Lactoglobulin | 2.55 | | | 2.62 | 2.76 | 2.85 | | 2.94 | | | |
| Ribonuclease A | 2.72 | | | 2.76 | 2.85 | 2.96 | | 2.99 | | 3.06 | 3.24 |
| Ovalbumin | 2.58 | | | 2.73 | 2.90 | 3.07 | | 3.27 | | 4.00 | 5.83 |
| Transferrin | 2.51 | | | 2.71 | 2.92 | 3.24 | | 3.79 | | 5.86 | 9.23 |
| α-Lactalbumin | 2.68 | 2.75 | | 3.01 | 3.40 | | | | | | |
| Lysozyme | 2.83 | | | 3.38 | 3.53 | 3.91 | | 4.42 | | 5.44 | 7.04 |
| Trypsin | 2.72 | | | 4.20 | 5.32 | 7.34 | | 10.09 | | 13.99 | 20.96 |
| Trypsin inhibitor | 2.81 | | | 5.53 | 6.93 | 9.82 | | 12.72 | | 16.48 | |
| α-Amylase | 2.86 | | | 5.67 | 8.60 | 11.40 | | 13.77 | 16.51 | 17.24 | |
| α-Chymotrypsin | 2.66 | | | 5.06 | 7.59 | 10.92 | | 14.78 | 19.00 | 20.90 | |
| α-Chymotrypsinogen A | 2.79 | | | 7.08 | 11.02 | 15.79 | 18.81 | 21.00 | | | |
| r-Protein A | 2.50 | | | 5.24 | 12.01 | 18.20 | | | | | |
| Monoclonal antibody | 4.57 | 10.94 | 19.68 | | | | | | | | |

Amounts of proteins injected were 0.04 mg (cytochrome *c*, myoglobin), 0.06 mg (antithrombin III), 0.08 mg (lysozyme, α-chymotrypsin, α-chymotrypsinogen A), 0.12 mg (ribonuclease A, transferrin, α-lactalbumin), 0.16 mg (conalbumin, trypsin, trypsin inhibitor, α-amylase, monoclonal antibody) and 0.24 mg (β-lactoglobulin, ovalbumin, r-protein A).

Table 3
Recovery of proteins in HIC by gradient elution of ammonium sulfate from 0.5 *M* to 0

| Protein | Recovery (%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Cytochrome *c* | 99 | | | 100 | 99 | 100 | | 100 | | 99 | 99 |
| Myoglobin | 96 | | | 95 | 93 | 92 | | 88 | | 75 | 61 |
| Antithrombin III | 101 | | | 99 | 74 | 43 | | 22 | | | |
| Conalbumin | 97 | | | 94 | 75 | 66 | | 45 | | 45 | |
| β-Lactoglobulin | 93 | | | 79 | 65 | 52 | | 3 | | | |
| Ribonuclease A | 97 | | | 96 | 98 | 97 | | 96 | | 96 | 96 |
| Ovalbumin | 99 | | | 99 | 96 | 93 | | 90 | | 81 | 51 |
| Transferrin | 97 | | | 95 | 94 | 92 | | 87 | | 74 | 48 |
| α-Lactalbumin | 98 | 90 | | 56 | 9 | | | | | | |
| Lysozyme | 98 | | | 98 | 97 | 98 | | 96 | | 94 | 87 |
| Trypsin | 100 | | | 99 | 99 | 99 | | 100 | | 99 | 97 |
| Trypsin inhibitor | 99 | | | 98 | 100 | 97 | | 98 | | 99 | |
| α-Amylase | 98 | | | 97 | 95 | 90 | | 86 | 80 | 74 | |
| α-Chymotrypsin | 99 | | | 99 | 101 | 101 | | 99 | 98 | 99 | |
| α-Chymotrypsinogen A | 99 | | | 99 | 101 | 100 | 98 | 93 | | | |
| r-Protein A | 95 | | | 97 | 94 | 95 | | | | | |
| Monoclonal antibody | 99 | 97 | 96 | | | | | | | | |

Amounts of proteins injected were the same as in Table 2.

philic proteins without decrease in recovery. On the other hand, retention of hydrophobic proteins increased with almost no decrease in recovery as support hydrophobicity increased. It was possible to elute them at ammonium sulfate concentration below 0.1 *M* with more than 90% recovery although the support hydrophobicity to achieve it differed for each protein. Although α-amylase was an exception and the recovery slightly decreased on the supports of high hydrophobicities, it was still possible to elute it at 0.1 *M* ammonium sulfate with 80% recovery on column 9. Examples of chromatograms are shown in Fig. 1.

Fig. 2 shows separations of hydrophobic proteins obtained with a 9 min linear gradient of ammonium sulfate from 0.3 *M* to 0 on the supports whose hydrophobicities are suitable for each protein. In these separations, ammonium sulfate concentration at column outlet started to decrease from 0.3 *M* at 4.5 min and became 0 at 13.5 min. All proteins except trypsin were eluted at ammonium sulfate concentration below 0.1 *M*. Trypsin was eluted at 0.14 *M* ammonium sulfate, but it is possible to elute it at ammonium sulfate concentration of almost 0 by using column 11. The recovery of α-amylase increased up to 87% here and more increase in recovery was expected by further reducing initial ammonium sulfate concentration. It was indeed confirmed by separating α-amylase by gradient elution of ammonium sulfate from 0.2 and 0.1 *M* to 0 with the same gradient slop. The recoveries increased up to 92 and 95%, respectively.

### *3.2. Retention and recovery of hydrophobic proteins in the separation by isocratic elution with 50 mM phosphate buffer (pH 6.8)*

Retention and recovery of hydrophobic proteins were also studied in the separation by isocratic elution with 50 m*M* phosphate buffer (pH 6.8). Fig. 3 shows chromatograms of α-chymotrypsinogen A. As exemplified here, hydrophobic proteins were successfully separated by isocratic elution with a buffer solution of low concentration not containing ammonium sulfate. They could be retained to a desired extent and eluted quantitatively by using supports of properly adjusted hydrophobicities. It is convenient to separate proteins by isocratic elution although it is practical only for rather pure samples. When samples contain components of a wide range

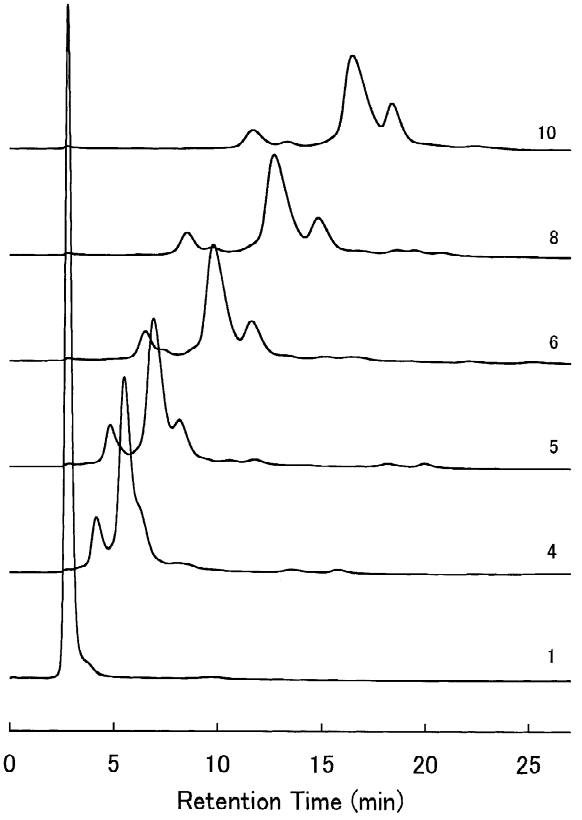


Fig. 1. Separation of trypsin inhibitor (0.16 mg) on columns 1, 4, 5, 6, 8 and 10 by a 15 min linear gradient of ammonium sulfate from 0.5 *M* to 0 in 50 m*M* phosphate buffer (pH 6.8).

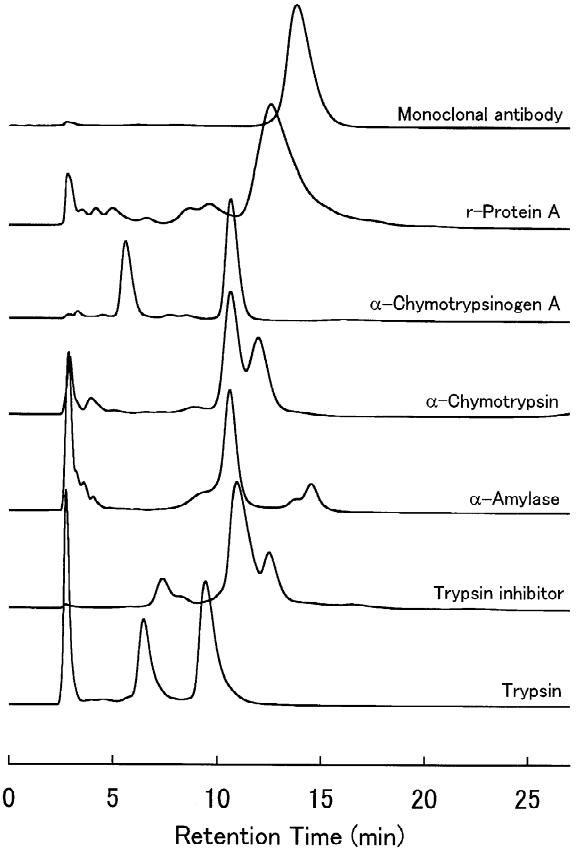


Fig. 2. Separation of hydrophobic proteins by a 9 min linear gradient of ammonium sulfate from 0.3 *M* to 0 in 50 m*M* phosphate buffer (pH 6.8). Trypsin (0.16 mg), trypsin inhibitor (0.16 mg), α-amylase (0.16 mg), α-chymotrypsin (0.08 mg), α-chymotrypsinogen A (0.18 mg), r-protein A (0.24 mg) and monoclonal antibody (0.16 mg) were separated on columns 10, 10, 9, 8, 6, 6 and 3, respectively.

of hydrophobicities, it is difficult to elute all of them from the column within a reasonable time, as can be seen from Fig. 3.

### *3.3. Retention and recovery of hydrophobic proteins in the separation by gradient elution of ethanol from 0 to 10%*

Retention and recovery of hydrophobic proteins were further investigated in the separation by gradient elution of ethanol from 0 to 10% in 50 m*M* phosphate buffer (pH 6.8). Fig. 4 shows chromatograms of α-chymotrypsinogen A. Successful separations of hydrophobic proteins were also achieved by gradient elution of ethanol in buffer solution of low concentration. The retention was decreased and the peaks became more symmetrical as compared to the separation by isocratic elution without ethanol in particular for late eluted components, as usually can be expected in the separation by gradient elution. For example, one of impurities in α-chymotrypsinogen A was eluted at around 25 min as an extremely tailing peak on column 8 in the separation by isocratic elution (Fig. 3), while it was eluted at around 12 min on the same column as a less tailing peak in the separation by gradient elution of ethanol (Fig. 4). Recoveries were also quantitative in these separations. Although the recovery of biological activity was not determined in this study, it is supposed that the risk of protein denaturation by the addition of ethanol less than 10% in the eluent is low. It has

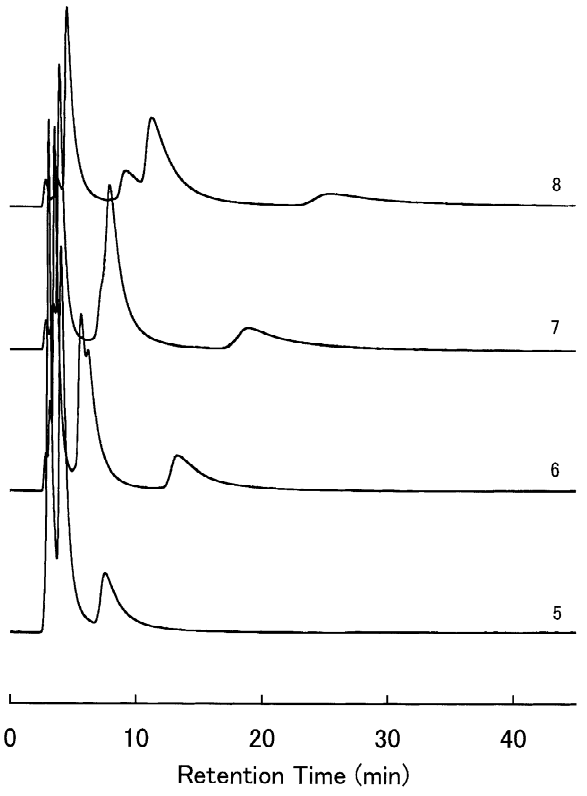


Fig. 3. Separation of α-chymotrypsinogen A (0.08 mg) on columns 5, 6, 7 and 8 by isocratic elution with 50 m*M* phosphate buffer (pH 6.8).

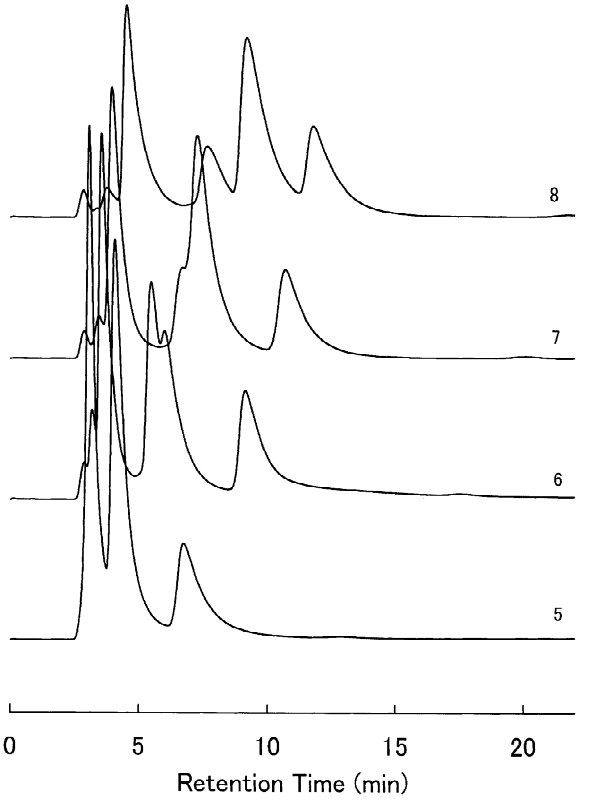


Fig. 4. Separation of α-chymotrypsinogen A (0.08 mg) on columns 5, 6, 7 and 8 by a 15 min linear gradient of ethanol from 0 to 10% in 50 m*M* phosphate buffer (pH 6.8).

even been reported that protein denaturation was suppressed by the addition of 5–10% methanol in the eluent in HIC separation of some proteins [14].

### *3.4. Benefits of HIC at low salt concentration*

Some disadvantages have been claimed for HIC at high salt concentration like 1.5–2 *M* ammonium sulfate. It is not economical to use eluents containing large quantities of salt. For example, ammonium sulfate as much as 264 g is necessary to prepare 1 l of 2 *M* solution. The eluent of high salt concentration is not so friendly to the instrument made of stainless steel. If the eluent leaks, large quantities of solid salt appear after the evaporation of water, which may cause the damage of instrument. It is sometimes difficult by regulation to waste large volumes of ammonium sulfate solution containing nitrogen. It is often problematic to incorporate HIC into multi-step protein purification process. Target proteins are usually eluted in the effluent containing moderate to high concentration of salt and therefore HIC fractions containing the target proteins cannot be applied directly to the next step such as IEC and affinity chromatography (AFC) without intermediate treatment of the fractions such as desalting and buffer exchange. Also, fractions from proceeding step such as IEC and AFC cannot be directly applied to HIC because the fractions do not contain high concentration of salt enough to promote adsorption of proteins on to HIC column. However, if HIC is performed at low salt concentration as described in Sections 3.1–3.3, it is expected that these problems are solved. Fig. 5 shows examples of direct application of HIC fraction to IEC. HIC fractions containing α-chymotrypsin and trypsin inhibitor collected in

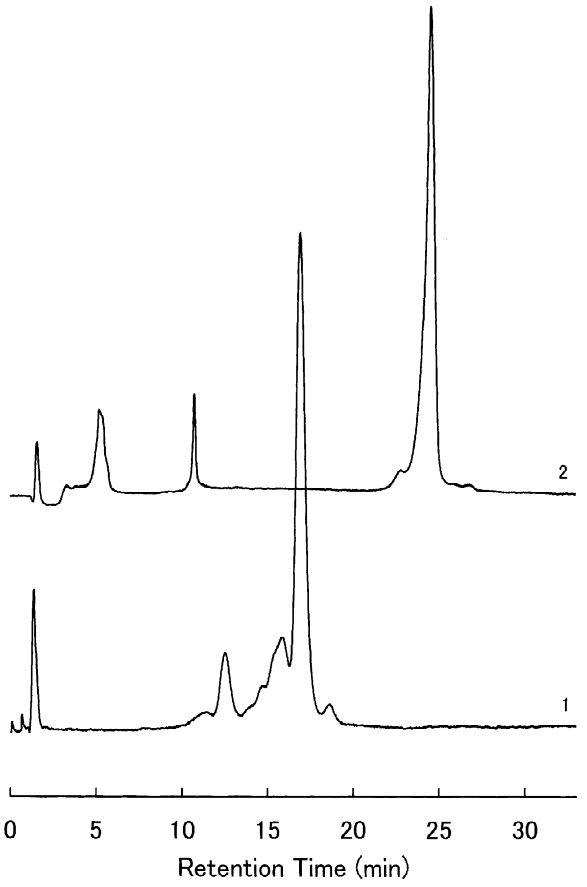


Fig. 5. Direct separations of HIC fractions of α-chymotrypsin and trypsin inhibitor by IEC without intermediate treatment. Peaks of α-chymotrypsin eluted at 10.6 min and trypsin inhibitor eluted at 11.0 min were collected in the separations in Fig. 2. Fractions of 0.5 ml of α-chymotrypsin and trypsin inhibitor were separated on BioAssist S and BioAssist Q, respectively.

separations in Fig. 2 could be successfully applied to IEC without intermediate treatment.

## 4. Conclusions

Hydrophobic proteins such as r-protein A and monoclonal antibody can be successfully separated by HIC at low salt concentration by using supports whose hydrophobicities are properly adjusted individually for each protein. Such elutions as gradient of ammonium sulfate from 0.3–0.5 *M* to 0 in 50 m*M* phosphate buffer (pH 6.8), isocratic with 50 m*M* phosphate buffer (pH 6.8) and gradient of ethanol from 0 to 10% in 50 m*M* phosphate buffer (pH 6.8) can be employed. HIC at low salt concentration is compatible with other modes of liquid chromatography like IEC. On the other hand, it is not successful to separate hydrophilic proteins such as antithrombin III and transferrin at low salt concentration. Recoveries of these proteins decrease before they are retained enough at low salt concentration as support hydrophobicity increases. Therefore, it is inevitable to use a higher concentration of salt, e.g., 1–2 *M* ammonium sulfate, on hydrophilic or moderately hydrophobic support in order to retain hydrophilic proteins without decrease in recovery.

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