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Protein separation by hydrophobic interaction chromatography using methacrylic block copolymers as displacers

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Abstract

A series of methacrylic triblock copolymers were synthesized. The potential of using these copolymers as displacers or spacers in hydrophobic interaction chromatography (HIC) was discussed. It was found that selected copolymers were able to separate pancreatic trypsin and α-chymotrypsin in an Octyl-Sepharose HIC column. The hydrolytic activities of these two enzymes were not affected by the presence of copolymers. The advantages of methacrylic triblock copolymers were their relatively low molecular mass and the reversible precipitability. These properties make the separation between copolymers and proteins easier. The hydrophobicity of the triblock copolymers could be roughly adjusted by controlling the length of each block. But accurate prediction of their hydrophobicity according to the corresponding chemical structure requires further studies.

Keywords: Displacement; Proteins; Trypsin; Chymotrypsin; Methacrylic block polymers

1. Introduction

Displacement is one of the operational modes of chromatography. Different from elution chromatography that separates sample components based on their differences to compete with the eluent, displacement chromatography separates substances based on direct competition among the adsorbed components [1]. The displacer, the leading element in displacement chromatography, is continuously introduced into the column after sample injection. The displacer has a higher affinity toward the adsorbent than any of the other components in the system. Sometimes, substances with intermediate affinity are also introduced as spacers. Components of lower affinity are successively displaced by the higher affinity ones, which gives rise to a train of continuous bands. A chromatogram of adjacent square zones is the major characteristic of displacement chromatography.

Gathering the efforts of many workers over the last decade, displacement chromatography has been increasingly recognized as a promising technique for preparative protein separation [2,3]. Although displacement chromatography attracts tremendous attention by its high throughput as well as simultaneous separation and concentration, it has not been widely used. The lack of proven displacer/spacer systems is one of the major obstacles [1,4–6]. The search for a suitable displacer/spacer system is the key for a successful displacement operation. Thus far, most displacers for protein purification were natural poly-

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mers of comparable molecular mass of proteins. This makes the separation between the displacer and the protein product difficult [7–9]. Patrickios et al. [10] has suggested using synthetic polyampholytes as protein displacers in ion-exchange chromatography. The polyampholytes they used are low-molecular-mass triblock copolymers of methacrylic acid, (dimethylamino)ethyl methacrylate and methyl methacrylate. The major advantages of this type of polyampholyte are their small size, pre-determinable characteristic charges and the ease of recovery by isoelectric precipitation.

Thus far, the applications of displacement chromatography were mostly carried out either in reverse phase or in ion-exchange systems [4,7,11–14]. The only application of protein displacement in hydrophobic interaction mode was reported by Antia et al. [15]. To explore the possibility of using hydrophobic interaction mode, we intended to separate pancreatic trypsin and α-chymotrypsin by displacement chromatography on an Octyl-Sepharose HIC column. Methacrylic triblock copolymers similar to those used by Patrickios were synthesized and used as displacers. The goal of this study is to discuss the possibility of using these triblock copolymers as displacers or spacers in hydrophobic interaction chromatography. In addition to the displacement operations, the following related subjects were also studied: the recovery of protein products, the pH ranges suitable for operation, and the effect of triblock copolymers on enzymatic activities.

2. Experimental

2.1. Materials

Sodium bicarbonate, sodium chloride, trichloroacetic acid, sodium hydroxide and hydrochloride were purchased from Riedel-de Haën (Chinosol, Seelze, Germany). Trifluroacetic acid and acetonitrile were purchased from Aldrich (Milwaukee, WI, USA). Trypsin (Sigma T7418), α-chymotrypsin, Folin reagent (Sigma C4129) and were purchased from Sigma (St. Louis, MO, USA). Octyl-Sepharose was purchased from Pharmacia Biotech Asia Pacific (Quarry Bay, Hong Kong). Triblock copolymers were synthesized by group transfer polymerization as described by Partickios et al. [14]. Three triblock copolymers were used in this study: $A_{12}M_2B_{12}$, $A_{12}M_4B_{12}$ and $A_{12}M_6B_{12}$, where B designates 2-(dimethylamino)ethyl methacrylate (DMAEMA), M is methylmethacrylate (MMA), and A stands for methacrylic acid (MAA). All the copolymers comprised the same 12 MAA and 12 DMAEMA monomers, but varied in their MMA contents. Therefore, the molecular masses of copolymers $A_{12}M_2B_{12}$, $A_{12}M_4B_{12}$ and $A_{12}M_6B_{12}$ are 3156, 3360 and 3564, respectively.

2.2. Enzyme activity assay

The activities of trypsin or α-chymotrypsin were assayed according to the method of casein hydrolysis modified by Walter [16]. Dissolved casein was digested by the sample for 10 min and the reaction was stopped by trichloroacetic acid. The released product was reacted with Folin reagent and monitored at 578 nm through a Beckman DU-65 UV–Vis spectrophotometer.

2.3. Displacement operation

Displacement chromatography was carried out through a Bio-Rad Econo system (Bio-Rad Labs., Hercules, CA, USA) which includes a Model EP-1 Econo pump, a Model EM-1 Econo UV monitor, a Model MV-6 six-port sample injection valve. The signals were processed through a Chem-Lab data station, SISC, Taipei, Taiwan. The volume of sample loop is 2 ml. Six ml of Octyl-Sepharose beads were packed in a 30×1 cm Econo column at a flow-rate of 1 ml/min. The final bed height was 5.5 cm. The column was first equilibrated with carbonate buffer (pH 10.0) added with 4.0 *M* NaCl. The mixture of trypsin (Sigma T7418) and α-chymotrypsin (Sigma C4129) was loaded in the sample loop and pushed into the column by the displacer solution. Eluent was collected through a fractional collector. Collected samples were analyzed by 0 to 50% acetonitrile gradient elution from a Hamilton PRP3 reversed-phase column. The equipment for gradient formation included a Gilson 305 and a Gilson 306 pump, a Gilson 805 manometric module and a Gilson 811C dynamic mixer. Eluent was monitored through a Gilson 112 UV detector.

2.4. Octyl-Sepharose HIC

The equipment used in these experiments was the same as that used for displacement chromatography. The column was first equilibrated with bicarbonate buffer (pH 10.0) added with 4.0 *M* NaCl. Spacer was mixed with the protein mixture and loaded in the sample loop. The samples were eluted by a 50-min NaCl gradient from 4.0 *M* to 0.0 *M* at a flow-rate of 0.1 ml/min.

2.5. The precipitation of triblock copolymer

The pH ranges for precipitating the copolymers were estimated by turbidity measurement. Various amounts of sodium chloride were added to 20 m*M* bicarbonate buffer (pH 10.0) to make up solutions containing 0, 0.2 and 0.5 *M* NaCl. Triblock copolymer was dissolved in each of the above solutions. The 5.0 mg/ml polymer solution was then titrated by 0.1 *M* HCl solution and the turbidity was measured at 460 nm by an Orbeco-Hellige 975MP spectrophotometer.

2.6. Copolymer recovery by isoelectric precipitation

The percentage of precipitated copolymer at each different pH value was measured by the concentration of the copolymer in the supernatant. Triblock copolymer was dissolved in 20 m*M* bicarbonate buffer at pH 10.0. The correlation of polymer concentration and its absorbency at 215 nm was established through a Beckman DU-65 spectrophotometer. Equal volume of HCl solution (various concentrations) was added to 5 mg/ml of the polymer solution. The sample was centrifuged and the supernatant was collected. The pH value and the polymer concentration of the supernatant were measured.

2.7. Coprecipitation of proteins with copolymers

The percentage of coprecipitated α-chymotrypsin at each different pH value was measured by the concentration of α-chymotrypsin in the supernatant. Both α-chymotrypsin (0.2 mg/ml) and copolymer (5.0 mg/ml) were dissolved in 20 m*M* bicarbonate buffer at pH 10.0. Equal volume of HCl solution (various concentrations) was added to the solution. The sample was centrifuged and the supernatant was collected. The concentration of α-chymotrypsin in the supernatant was estimated by its UV absorbency at 280 nm.

3. Results and discussion

3.1. Enzyme activities were not affected by the presence of triblock copolymers.

The proteolytic activities of trypsin and α-chymotrypsin were measured with or without the presence of the triblock copolymer $A_{12}M_6B_{12}$. It was found that the enzymatic activities were hardly affected by the triblock copolymer. After trypsin (0.5 mg/ml) was mixed with triblock copolymer $A_{12}M_6B_{12}$ (5.0 mg/ml) for 40 min at 25°C, the proteolytic activity of trypsin dropped from 1441.4 to 1409.53 units/ml. The effect of copolymer addition on α-chymotrypsin was also measured under the same condition. The proteolytic activity of α-chymotrypsin dropped from 1811.5 to 1741.4 units/ml. The loss of activity was considered negligible since the losses were only 2.21% and 3.87% for trypsin and α-chymotrypsin, respectively.

3.2. Displacer selection

In order to select an appropriate copolymer as the displacer for displacement chromatography, the adsorption isotherms of all the proteins and block copolymers were measured. The adsorption isotherms of trypsin and α-chymotrypsin are shown in Fig. 1a. The adsorption isotherms of copolymers are shown in Fig. 1b. When the isotherms of both copolymers were fitted by standard Langmuir model, the adsorption capacities (Q_m) and dissociation constants (K_d) were shown in Table 1. The K_d values of both trypsin and α-chymotrypsin were far smaller than those of copolymers, which might indicate that the proteins were adsorbed more strongly than all the copolymers. If this was true, the copolymers could not displace either trypsin or α-chymotrypsin.

Since the molecular masses of copolymers are far smaller than proteins, the hindering effect may not

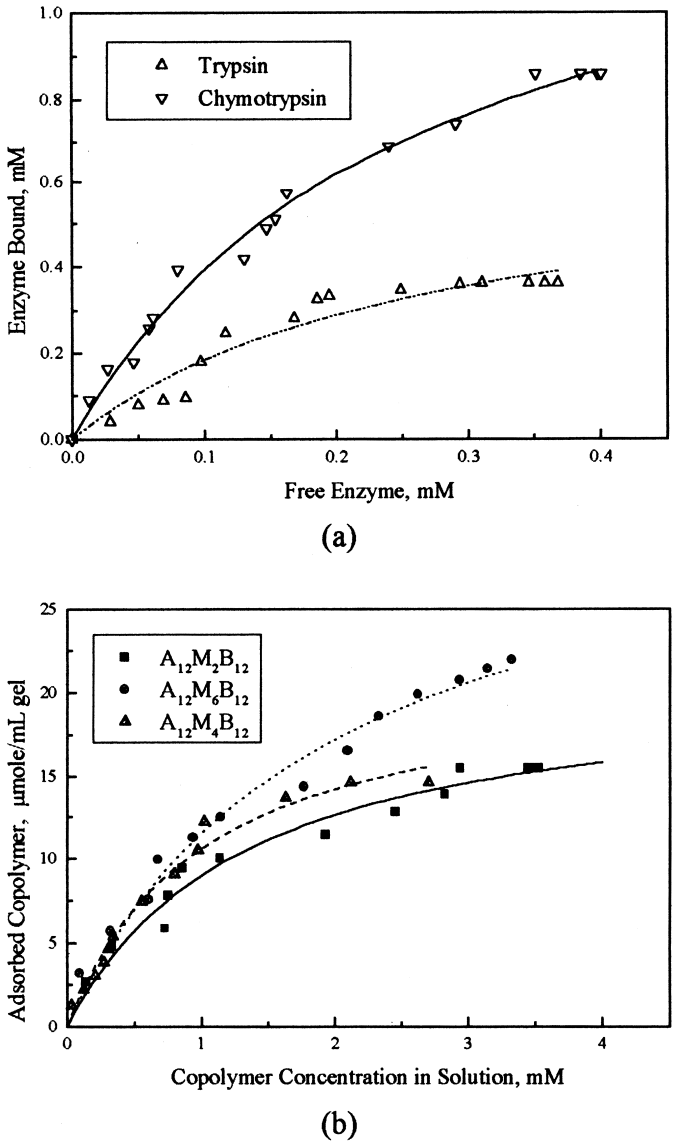

Fig. 1. Batch adsorption isotherms of (a) triblock copolymers and (b) enzymes onto Octyl-Sepharose beads at room temperature.

be neglected. Once one of the hydrophobic sites was occupied, the neighboring sites may no longer be reached by other molecules. Therefore, the following modified Langmuir model was adopted to described the isotherms:

$$[\text{Protein}]_{\text{ads}} = Q_{\text{m}}[\text{Protein}]_{\text{solution}}/(K_{\text{d}} + (1+\delta)[\text{Protein}]_{\text{solution}})$$

where δ is the hindrance factor, K_{d} is the dissociation constant, Q_{m} denotes the maximum capacity, $[\text{Protein}]_{\text{ads}}$ and $[\text{Protein}]_{\text{solution}}$ represent the concentration of bound and free proteins, respectively. If the adsorption of copolymers was considered free from hindrance, the maximum adsorption capacity of all molecules was assumed to be the same as that of copolymer $A_{12}M_4B_{12}$. The fitted K_{d} values were

Table 1
Fitted parameters in Langmuir and modified Langmuir adsorption model

	K_d (m*M*)	Q_m (μmol/ml gel)	δ
Trypsin	0.26	0.67	
	4.64[a]	21.5[a]	44.39[a]
α-Chymotrypsin	0.27	1.45	
	4.09[a]	21.5[a]	13.44[a]
$A_{12}M_2B_{12}$	1.37	21.25	–
$A_{12}M_4B_{12}$	1.04	21.56	–
$A_{12}M_6B_{12}$	1.95	33.91	–

[a] Parameters obtained from the modified Langmuir isotherm.

shown in Table 1. According to this analysis, the dissociation constants of both proteins were larger than those of the copolymers. It meant that the copolymers could displace both trypsin and α-chymotrypsin. However, the adsorption of macromolecules by hydrophobic interaction may not be described by Langmuir type isotherm. The adsorption isotherm cannot give a sure answer for displacer selection. Therefore, all three copolymers were tested in the displacement chromatography.

3.3. Trypsin/α-chymotrypsin separation by displacement chromatography

Trypsin and α-chymotrypsin were separated by an Octyl-Sepharose column, which was operated in the displacement mode. The eluent was collected every 3 min. The collected samples were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). Fig. 2a–c show the results of displacement chromatography using each triblock copolymer $A_{12}M_2B_{12}$, $A_{12}M_4B_{12}$ or $A_{12}M_6B_{12}$ as the displacer. It was found that trypsin and α-chymotrypsin were well separated by $A_{12}M_4B_{12}$ and $A_{12}M_6B_{12}$ with good recovery. It could hardly find any trypsin in the collected α-chymotrypsin fraction when copolymers $A_{12}M_4B_{12}$ and $A_{12}M_6B_{12}$ were used as displacers. In spite of the successful separation, the chromatogram of α-chymotrypsin looked somewhat like the result of elution chromatography instead of displacement. It seemed that neither $A_{12}M_4B_{12}$ nor $A_{12}M_6B_{12}$ was able to fully displace α-chymotrypsin.

It was found from the RI chromatograms that the displacer front of both $A_{12}M_4B_{12}$ and $A_{12}M_6B_{12}$ came out just in front of the α-chymotrypsin peak. This result indicated that trypsin was displaced by copolymer together with α-chymotrypsin, while α-chymotrypsin did not come out of the column by displacement.

The recoveries of trypsin and α-chymotrypsin are listed in Table 2. When $A_{12}M_4B_{12}$ was used as the displacer, 83% of trypsin and almost all the α-chymotrypsin were recovered. However, only 62% of trypsin was recovered when $A_{12}M_6B_{12}$ was used as the displacer.

3.4. Copolymer facilitated protein separation in Octyl-Sepharose HIC

Although they could not displace α-chymotrypsin, the copolymers appeared to be a good displacer of trypsin. When the mixture of trypsin (2 mg) and α-chymotrypsin (2 mg) was eluted from an Octyl-Sepharose column by a 4.0 *M* to 0.0 *M* NaCl salt gradient, as shown in Fig. 3, the two proteins could not be separated. However, when 10 mg of $A_{12}M_4B_{12}$ or $A_{12}M_6B_{12}$ was added, two proteins were well separated from each other. As shown in Fig. 4a Fig. 4b, trypsin was pushed forward while α-chymotrypsin remained in its original position. The improved separation may result from the displacement of trypsin by triblock copolymers. Overloaded trypsin was pushed by high concentration $A_{12}M_4B_{12}$ or $A_{12}M_6B_{12}$ so that the latter part of trypsin peak moved forward and then separated from α-chymotrypsin. α-chymotrypsin adsorbed more strongly than copolymers, therefore, the shape of α-chymotrypsin peak remained unchanged.

3.5. Reversible precipitation of triblock copolymers by pH adjustment

The methacrylic triblock copolymers can be easily precipitated by pH adjustment. A turbidity test was performed for a rough estimation of the pH range of copolymer precipitation. Each of the copolymers was first dissolved in a 20 m*M* bicarbonate buffer at pH 10.0. The polymer solution was titrated with HCl solution and the turbidity was measured. The solution became turbid as precipitation occurred. The

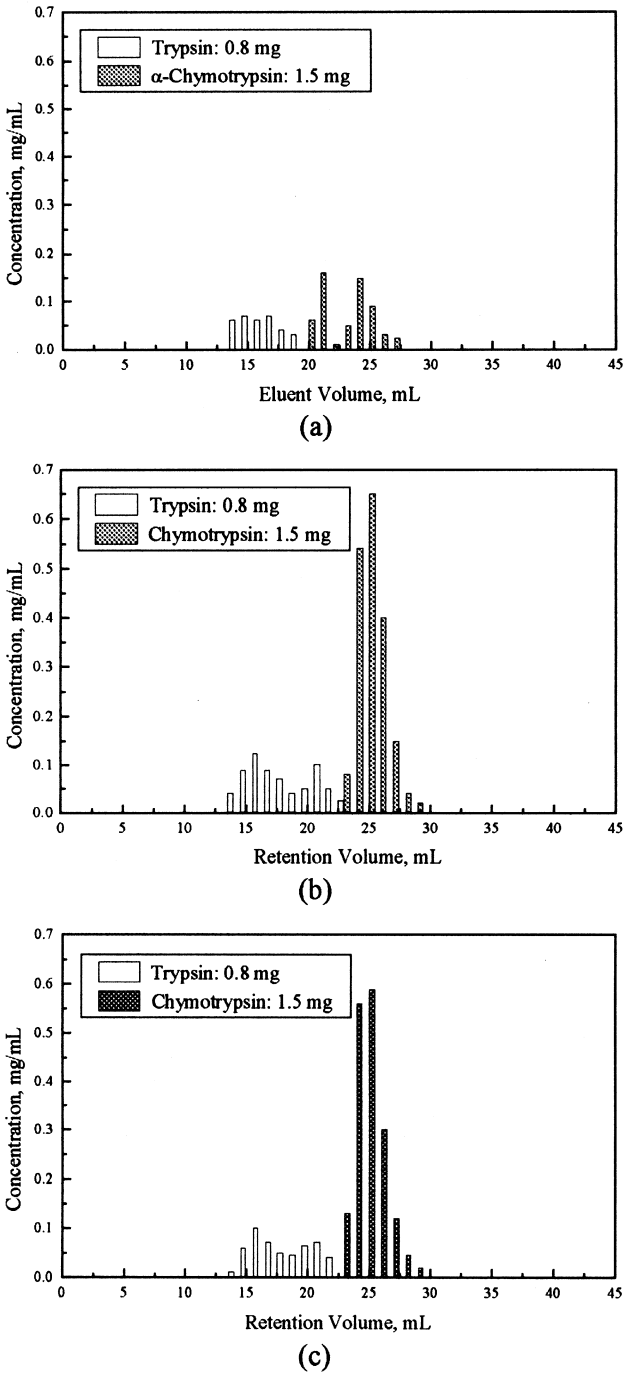

Fig. 2. Displacement chromatography of trypsin and α-chymotrypsin using copolymer (a) $A_{12}M_2B_{12}$, (b) $A_{12}M_4B_{12}$, (c) $A_{12}M_6B_{12}$ as the displacer. Sample mixture of trypsin (0.4 mg/ml) and α-chymotrypsin (0.75 mg/ml) were loaded in a 2-ml sample loop. Displacer solution (10 mg/ml displacer in 20 m*M* bicarbonate–4.0 *M* NaCl solution) continuously flowed through the column at a flow-rate of 0.1 ml/min. Eluent was monitored at UV 280 nm.

Table 2
Protein recovery after displacement operation

Displacer	Recovery (%)	
	Trypsin	α-Chymotrypsin
$A_{12}M_2B_{12}$	48.07	39.49
$A_{12}M_4B_{12}$	83.32	124.59
$A_{12}M_6B_{12}$	62.10	114.13

results were shown in Fig. 5. At low ionic strength, most copolymers precipitated when the pH was adjusted to a value between 4.5 and 8. The copolymer owning shorter hydrophobic section had a narrower precipitation pH range. The copolymer precipitation was affected by the increase in salt concentration. The copolymer $A_{12}M_2B_{12}$ was precipitable at low ionic strength but totally soluble when the salt concentration was higher than 0.2 *M*.

The precipitation of copolymer $A_{12}M_4B_8$ was also quantitatively measured. As the pH value was adjusted between 6.4 and 8.0, more than 70% of copolymer $A_{12}M_4B_8$ was precipitated. The optimum pH for copolymer $A_{12}M_4B_8$ precipitation was around 7.2. As high as 92% $A_{12}M_4B_8$ was precipitated at this pH value.

3.6. Coprecipitation of protein with triblock copolymers

It is important to know how easily the copolymer displacer can be separated from proteins. If the displacer is removed by precipitation, the degree of product coprecipitation with the displacer ought to be tested. Therefore, the mixture of α-chymotrypsin and $A_{12}M_4B_{12}$ was adjusted to various pH values and the amount of precipitated α-chymotrypsin was measured. It was found that 22% of α-chymotrypsin coprecipitated with copolymer $A_{12}M_4B_{12}$ at pH 8.1, 17% coprecipitated at pH 7.4, and 16% coprecipitated at pH 6.7. Therefore, the best condition for α-chymotrypsin–$A_{12}M_4B_{12}$ separation was pH 7.2–7.4 at which around 90% copolymer could be removed with a sacrifice of 17% of α-chymotrypsin.

4. Conclusions

The major advantages of these methacrylic triblock copolymers were their relative small sizes, the ease of precipitation and the controllable block sizes. The small size may make them easily separable from most proteins by ultrafiltration. Precipitation by pH

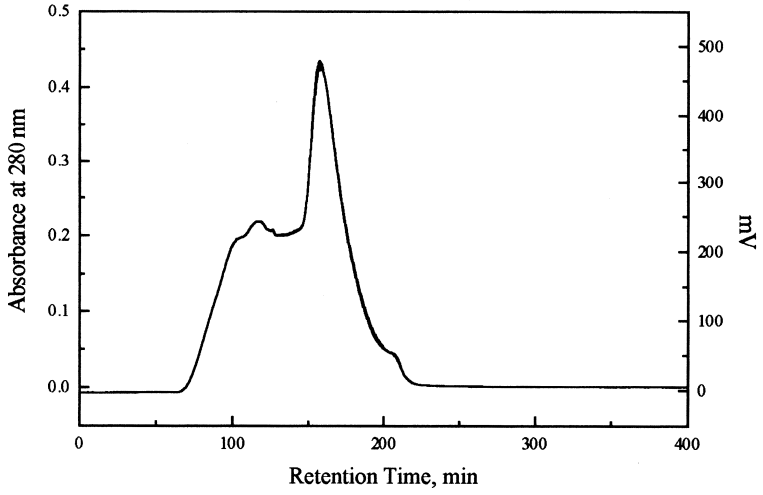

Fig. 3. Hydrophobic interaction chromatography of the mixture of trypsin and α-chymotrypsin. Octyl-Sepharose column was pre-equilibrated in pH 10.0 bicarbonate buffer containing 4.0 *M* NaCl. A 2-ml sample mixture of trypsin (1.0 mg/ml) and α-chymotrypsin (1.0 mg/ml) was injected into the column and gradiently eluted from 4.0 to 0.0 *M* NaCl at a flow-rate of 0.1 ml/min.

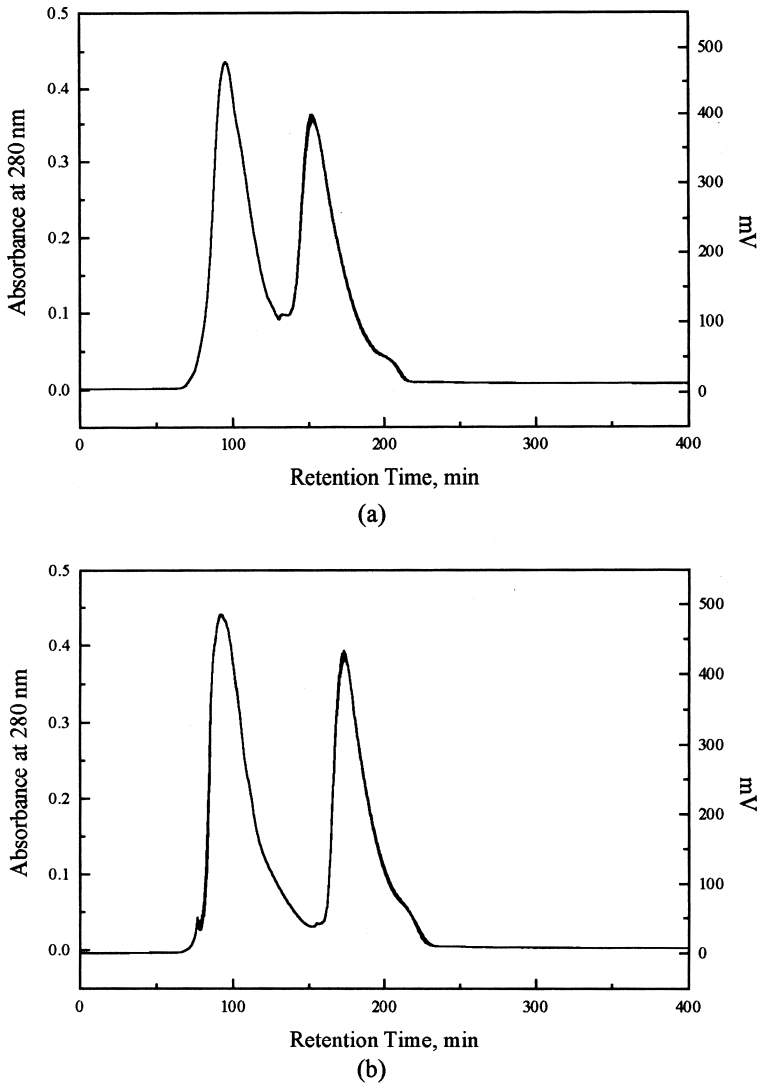

Fig. 4. Hydrophobic interaction chromatography of the mixture of trypsin and α-chymotrypsin after adding (a) $A_{12}M_4B_{12}$ or (b) $A_{12}M_6B_{12}$. Octyl-Sepharose column was pre-equilibrated in pH 10.0 bicarbonate buffer containing 4.0 *M* NaCl. A 2-ml sample mixture of trypsin (1.0 mg/ml), α-chymotrypsin (1.0 mg/ml) and triblock copolymer (5.0 mg/ml) were injected into the column and gradiently eluted from 4.0 to 0.0 *M* NaCl at a flow-rate of 0.1 ml/min.

adjustment makes them easily recovered from dilute solution. The controllable block size makes us able to select the copolymers of desirable isoelectric point (p*I*), characteristic charge, and hydrophobicity. However, triblock copolymers will form micelles in aqueous solution. Whether ultrafiltration is able to separate them from proteins requires further investigation. Besides, more studies on the structure–hydrophobicity relations of triblock copolymers in order to select the correct copolymers as displacers for hydrophobic interaction chromatography are required.

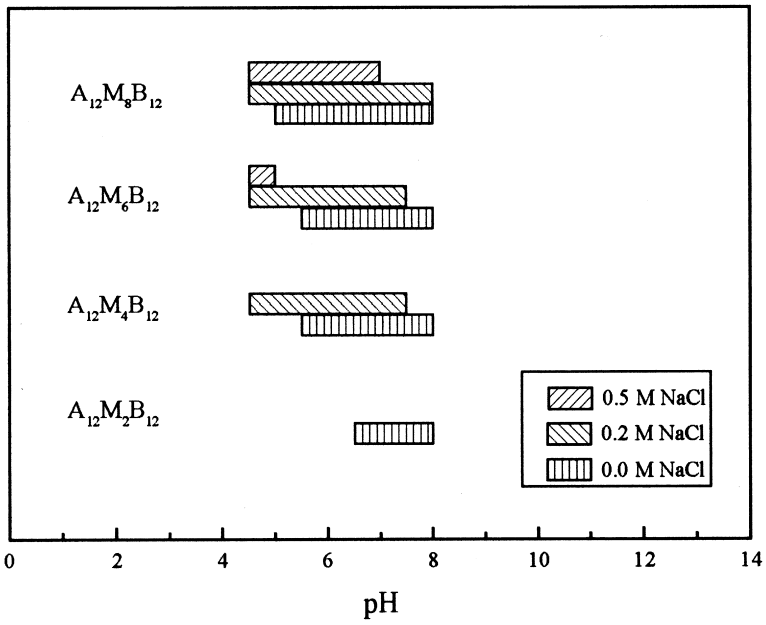

Fig. 5. Effect of salt concentration on the precipitation of triblock copolymers.

Acknowledgements

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