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Displacement chromatography of proteins on hydrophobic charge induction adsorbent column

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Abstract

Displacement chromatography of protein mixtures is proposed on hydrophobic charge induction chromatography (HCIC). We have used an HCIC medium, MEP-Hypercel as the stationary phase and a quaternary ammonium salt, benzethonium chloride, as the displacer. It was found that the multiple interactions between proteins/displacer and the HCIC sorbent, i.e. hydrophobic interaction and charge repulsion, enabled a greater flexibility for the design of displacement processes and ease of column regeneration by adjustment of pH. The capacity factors of proteins and displacers were used to predict their performances in column displacement, and the experimental results agreed well with the prediction. An isotachic displacement train of lysozyme and α-chymotrypsinogen A was formed with benzethonium chloride as the displacer at pH 5.0 with good yields and purities of the two proteins. Column regeneration was efficiently achieved by charge repulsion between the displacer and the adsorbent at lower pH values (pH 3 and 4). The results indicate that the displacement chromatography on HCIC is a good alternative to traditional hydrophobic displacement chromatography.

Keywords: Displacement chromatography; Proteins; Hydrophobic charge induction chromatography; MEP-Hypercel; Lysozyme; α-Chymotrypsinogen A

1. Introduction

Hydrophobic charge induction chromatography (HCIC) is a new form of protein chromatography that was firstly described by Burton and Harding [1] in 1998. The ligands for HCIC are usually hydrophobic molecules with ionizable groups at certain pH ranges. Unlike traditional hydrophobic interaction chromatography (HIC), where adsorption is promoted by lyotropic salts, HCIC matrices can adsorb proteins at low-salt concentrations owing to its high-ligand density. In addition, desorption of the bound proteins can be readily achieved by electrostatic repulsion via changing some or all the ionizable groups to their charged form by adjustment of pH. Hence, as a new form of protein chromatography, HCIC is advantageous in its salt-independent adsorption, "protein-friendly" elution by charge repulsion, and high capacity. It is anticipated that HCIC could be used as a generic, salt-independent alternative to HIC [2].

MEP-Hypercel is a hydrophobic charge induction adsorbent with 4-mercaptoethylpyridine (MEP, Fig. 1) as its ligand. The high-ligand density of MEP-Hypercel (70–125 μmol/mL) has enabled an adsorption capacity much greater than traditional hydrophobic interaction adsorbents. With a pK_a value of 4.8, it can be easily used for protein separation by adsorption in its uncharged form at neutral pH values and elution at pH 4.0. Up to now, MEP-Hypercel has been widely studied for the purification of antibodies [3–5] as well as other proteins [6–8].

Displacement chromatography is an operational mode of chromatography in which the components to be separated is followed into the system by a continuous stream of a high-affinity substance referred to as displacer [9]. Due to the high affinity of the displacer, it competitively desorbs the adsorbed components and drives them to move through the adsorbent bed. Under appropriate conditions, the components will form an "isotachic displacement train". Displacement of proteins has already been achieved on various adsorbents, such as ion-exchange [10–16], hydrophobic [9,17–19], reversed phase [20], and hydroxyapatite [21,22]. Displacement chromatography can provide simultaneous concentration and purification of bioproducts in a single step

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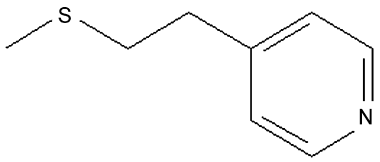

Fig. 1. Structure of 4-mercaptoethylpyridine.

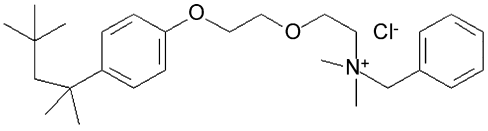

Fig. 2. Structure of benzethonium chloride.

with high capacity and high resolution [13], and is extremely suitable for challenging separations [23].

Despite the various advantages, one of the critical factors that hinder the application of displacement chromatography is the difficulty of column regeneration. As the displacer is generally very strongly adsorbed to the stationary phase, regeneration often requires extreme conditions and the efficiency is usually low. Ruaan et al. [24] achieved high-efficiency column regeneration in hydrophobic displacement by charge repulsion between a bifunctional adsorbent containing both butyl and carboxymethyl groups and a copolymer displacer with negative charges. However, synthesis of the bifunctional adsorbent is cumbersome and adsorption still has to be carried out with high-salt concentrations due to the limited ligand density. By application of displacement chromatography on an HCIC sorbent, the high-ligand density and the charge induction characteristics can enable both low-salt adsorption and charge-facilitated regeneration. Also, accomplishment of such a process will consummate the theoretical and practical systems of displacement chromatography and has great significance.

In this article, displacement chromatography of proteins on HCIC was proposed. The research was carried out with a quaternary ammonium salt, benzethonium chloride (Fig. 2), as the displacer. The performance of proteins and the displacer in column displacement was predicted by their capacity factors according to the stability analysis and the prediction was verified by experimental results. Then, column regeneration was achieved with low pH buffers, and the efficiency of regeneration was compared with that of a traditional regeneration method.

2. Materials and methods

2.1. Materials

MEP-Hypercel from Pall Life Sciences (New York, USA) is packed in a HR5/5 column (50 mm × 5 mm i.d.) (GE Healthcare, Uppsala, Sweden) and a Tricorn 5/100 column (100 mm × 5 mm i.d.) (GE Healthcare). A Sephasil C4 5 μm ST4.6/100 column was obtained from GE Healthcare. Lysozyme, α-chymotrypsinogen A and *Micrococcus lysodeikticus* were from Sigma (St. Louis, MO, USA) and benzethonium chloride was from Aldrich (Milwaukee, WI, USA). Acetonitrile and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Other reagents were analytical grade from local sources.

2.2. Equipment

Isocratic elution and displacement chromatography were carried out on an ÄKTA Purifier 10 chromatographic system from GE Healthcare. RP-HPLC analysis was carried out on an Agilent 1100 HPLC system from Agilent Technologies (Palo Alto, CA, USA) using the Sephasil C4 column. Protein concentration in the finite batch adsorption experiments were determined by UV absorbance at 280 nm on a UV–Vis spectrometer from Perkin-Elmer (Shelton, CT, USA), and fluorescence spectroscopy was carried out on an LS55 luminescence spectrometer also from Perkin-Elmer.

2.3. Determination of linear retention data

Linear retention data of proteins and the displacer at various pH values were determined by isocratic elution on the HR5/5 column. Buffers at various pH values were prepared by blending glycine and hydrochloric acid (pH 3.0), sodium acetate and acetic acid (pH 4.0–5.5), or disodium hydrogenphosphate and sodium dihydrogenphosphate (pH 6.0–7.5) to obtain the desired pH while keeping the concentrations in all the buffers constant (50 mmol/L). Following equilibration of the column with an appropriate buffer, 25 μL injections of the protein samples at ca. 10 mg/mL (ca. 50 mmol/L for the displacer) were injected on the column. The column effluent was monitored at 280 nm for peak detection and retention times were measured at the peak maxima. Each isocratic elution was at least repeated twice and the standard deviations were within 1%. The system dead volume and column void volume were determined by a pulse injection of lysozyme at pH 4.0, when lysozyme does not bind to the stationary phase [25]. The capacity factor k' was calculated using the relation $k' = (V_i - V_0)/V_0$, where V_i is the retention volume for a solute corrected for the system delay and V_0 is the column void volume.

2.4. Determination of adsorption isotherms

The adsorption isotherms of lysozyme, α-chymotrypsinogen A and benzethonium chloride on MEP-Hypercel at pH 5.0 were determined by finite batch adsorption experiments. Before adsorption, MEP-Hypercel was washed with deionized water and pre-equilibrated in 50 mmol/L acetate buffer, pH 5.0 and filtered on a G3 sintered glass funnel. The density of MEP-Hypercel was determined to be 1.072 ± 0.006 g/mL by a pycnometer. A known amount of the adsorbent was then added to 25 mL Erlenmeyer flasks containing protein or displacer solution at different concentrations. The flasks was sealed and shaken for 24 h in a shaking water bath at 25 °C. After that, the solutions in the flasks were centrifuged at 4000 rpm for 10 min, and the protein/displacer concentrations of supernatants were determined by UV absorbance at 280 nm. The amount of protein/displacer adsorbed to the adsorbent was then calculated by mass balance.

2.5. Lysozyme activity assay

Lysozyme activity was assayed using *M. lysodeikticus* as the substrate dispersed in 60 mmol/L sodium phosphate buffer (pH 6.2) at a concentration of 0.25 mg/mL [26]. Samples were diluted to around 25 μg/mL, and 1.4 mL substrate was mixed with 0.1 mL of the diluted sample. The decrease in the absorbance of the mixed solution at 450 nm was then recorded during the first 2 min. The relative activity of a sample is defined as the ratio of the rate of absorbance decrease of the sample to that of native lysozyme of the same concentration. Each sample was measured three times and the average of the three results was used to calculate the relative activity or activity yield.

2.6. Fluorescence spectroscopy

Protein fluorescence emission spectra were obtained using an excitation wavelength of 280 nm and an emission wavelength from 300 to 500 nm using a LS55 luminescence spectrometer.

2.7. Protein and displacer analysis by RP-HPLC

Proteins and the displacer were analyzed by reversed-phase HPLC using the Sephasil C4 5 μm ST4.6/100 column. Buffer A (deionized water with 0.1% (v/v) TFA) and buffer B (acetonitrile with 0.1% (v/v) TFA) were used for this analysis. The gradient design is given in Table 1. Flow rate was 1.0 mL/min and column effluent was monitored at 280 nm.

2.8. Displacement chromatography

Displacement chromatography of lysozyme and α-chymotrypsinogen A was carried out on the Tricorn 5/100 column at a flow rate of 0.1 mL/min. Displacer solution was prepared by blending 50 mmol/L sodium acetate (or disodium hydrogenphosphate) + 50 mmol/L benzethonium chloride and 50 mmol/L acetic acid (or sodium dihydrogenphosphate) + 50 mmol/L benzethonium chloride to obtain the desired pH. The exact flow rate was determined by measuring the weight of pure water pumped by the chromatographic system set at 0.1 mL/min within a fixed period of time. First, the column was equilibrated with the carrier buffer for 10 column volumes, and the protein sample was injected. After that, displacer solution was continuously pumped into the column, and the effluent was collected as fractions of 0.5 mL each for subsequent analysis. After the displacement, the column was perfused with regenerant solutions and re-equilibrated for the next operation.

Table 1
Elution gradient for protein and displacer analysis by RP-HPLC

Time (min)	% Buffer B
0	30
0–2	30–40
2–7	40
7–13	40–70
13–15	70

2.9. Column regeneration test

Displacer solution was pumped continuously into the column at a flow rate of 1.0 mL/min after equilibration with the carrier buffer, and the amount of displacer adsorbed could be calculated by the frontal curve monitored at 280 nm. After breakthrough, the regenerant solution was pumped continuously into the column. Since the effluent absorbance at 280 nm began to level off after 8 mL of the regeneration solution was used, the first 8 mL of the effluent was collected and analyzed by RP-HPLC. The efficiency of regeneration is evaluated by the percentage of displacer eluted in this first 8 mL.

3. Results and discussion

3.1. Retention and adsorption behaviors of proteins and benzethonium chloride

According to the mechanisms of HCIC and displacement chromatography, a good displacer should have several characteristics. First, it must be sufficiently hydrophobic to displace the proteins. Second, it should have a good solubility in water to dissolve in the aqueous-based mobile phase in HCIC. Also, it should have appropriate dissociation properties to be repulsed from the stationary phase at some pH values for the ease of regeneration.

Benzethonium chloride (Fig. 2) is a displacer candidate that satisfies all the above requirements. The molecule has several aromatic rings and alkyl groups that provide hydrophobic interactions with the HCIC ligands as well as a permanent positive charge that enables it to be repulsed from MEP-Hypercel at low pH values, when MEP-Hypercel is also positively charged. Also, the positive charge has greatly increased its water solubility. Lysozyme and α-chymotrypsinogen A are selected here as model proteins for displacement separation.

The actual performance of benzethonium chloride as a displacer was predicted by the capacity factors of the protein and the displacer, following that described by Shukla et al. with hydrophobic displacement chromatography [19]. According to the stability analysis, a stable isotachic zone will be achieved for the solute 1 followed by solute 2 if their capacity factors satisfy the inequality $k_1' < k_2'$. Therefore, if a displacer candidate is to be used for protein displacement, its capacity factor must be greater than those of the proteins.

The capacity factors of proteins and the displacer as a function of pH are given in Fig. 3. It can be seen that retention of lysozyme and α-chymotrypsinogen A are both extremely strong in the pH values close to the neutral condition even at such a low-buffer concentration without any additional salt. The result is due to the high-ligand density of the MEP-Hypercel. Retention of benzethonium chloride is greater than the two proteins at pH 4.0–5.0 but lower than both of them at higher pH values, implying that displacement is only possible around pH 5.0 or lower. It is considered that the different charge-to-hydrophobicity ratios of

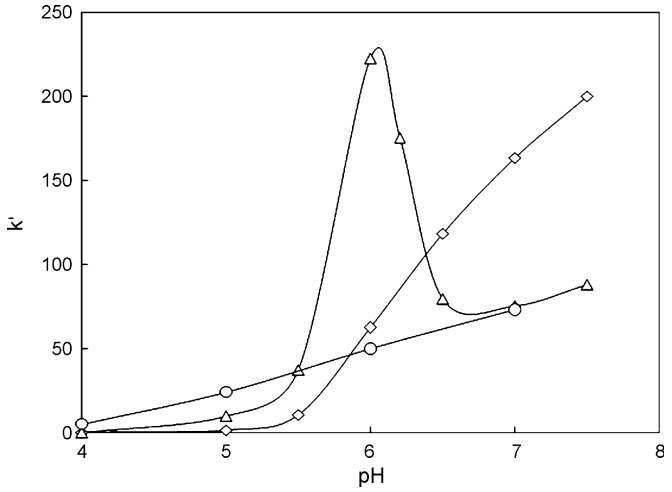

Fig. 3. Capacity factors of proteins and the displacer at various pH values. (◊) Lysozyme; (Δ) α-chymotrypsinogen A; (◯) benzethonium chloride. Column HR5/5, mobile phase: 50 mmol/L acetate buffer (pH 4.0–5.5) or 50 mmol/L phosphate buffer (pH 6.0–7.5), flow rate: 1.0 mL/min.

benzethonium chloride and the proteins have resulted in the complex retention behaviors of the solutes. Benzethonium chloride has weaker hydrophobicity and fewer charges than the proteins. At higher pH values, the effect of hydrophobicity on solute retention is dominant, so benzethonium chloride is less strongly retained on the sorbent. However, at lower pH values, the retention of the proteins decreases rapidly because of the stronger charge repulsion between the protein and the HCIC adsorbent, and the retention of benzethonium chloride exceeds that of the proteins. Therefore, it can be concluded that the multiple interactions between proteins/displacer and the HCIC sorbent, i.e. hydrophobic interaction and charge repulsion, make the retention behaviors of proteins and displacers remarkably changeable with buffer pH. According to the values of capacity factors at pH 7.0, benzethonium chloride may not be a displacer for lysozyme and α-chymotrypsinogen A for traditional HIC sorbents where hydrophobic interaction is the sole mechanism for solute retention. For the HCIC adsorbent, however, it may work at certain pH values. Since protein retention at pH 4.0 is too weak to ensure a sufficient time for separation, pH 5.0 was chosen as the operating condition for displacement.

It is also worth noting that the change of α-chymotrypsinogen A retention with pH is unusual compared with other solutes. With an isoelectric point (p*I*) of 9.7, the positive charge number of α-chymotrypsinogen A should have decreased with increasing pH in the range of the experiment. The positive charge density of MEP-Hypercel also decreases with increasing pH in this range, which should have made the retention of α-chymotrypsinogen A increase with pH. However, the sudden drop of the retention around pH 7.0 does not agree with this general rule of HCIC. Until now, no satisfactory explanation of this phenomenon has been made, but it may imply some kind of conformational change of α-chymotrypsinogen A around pH 7.0, which results in a change in hydrophobicity and/or surface charge number. An accurate explanation of this phenomenon should be a subject of future research.

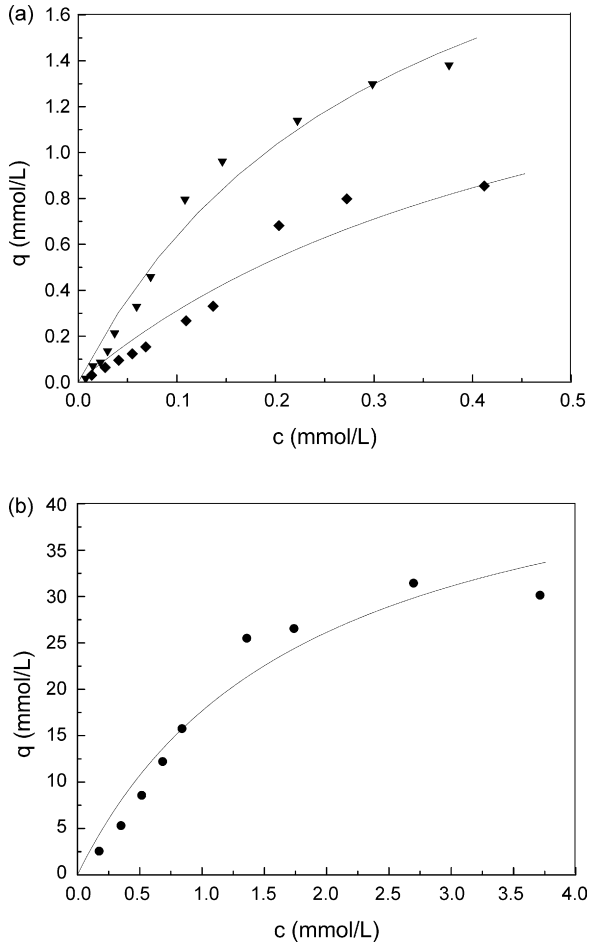

Fig. 4. Adsorption isotherms of (a) (□) lysozyme and (▼) α-chymotrypsinogen A; (b) (●) benzethonium chloride on MEP-Hypercel. Buffer: 50 mmol/L acetate buffer, pH 5.0. Solid lines are fitted by Langmuir model.

The adsorption isotherms of lysozyme, α-chymotrypsinogen A and benzethonium chloride were also determined to further verify the possibility of displacement at pH 5.0, and the results are given in Fig. 4. It can be seen that the order of adsorption capacities of the three solutes are consistent with that of their capacity factors, indicating that benzethonium chloride can possibly be a successful displacer for the two proteins at pH 5.0.

3.2. Analysis of biocompatibility of benzethonium chloride

Fluorescence spectroscopy was employed to study the influence of benzethonium chloride on protein structure. Lysozyme and α-chymotrypsinogen A (each at 5.0 mg/mL) were separately mixed with 50 mmol/L benzethonium chloride in 50 mmol/L acetate buffer (pH 5.0) and incubated at room temperature for 1 h. After that, fluorescence spectra of the protein–displacer mixtures were measured and compared with that of pure protein solutions in the same buffer (Fig. 5). As shown in the two graphs, after incubation with benzethonium chloride for 1 h, the maximum emission wavelength of both lysozyme and α-chymotrypsinogen A remain unchanged, indicating that no structural variation of the two proteins had occurred during incubation.

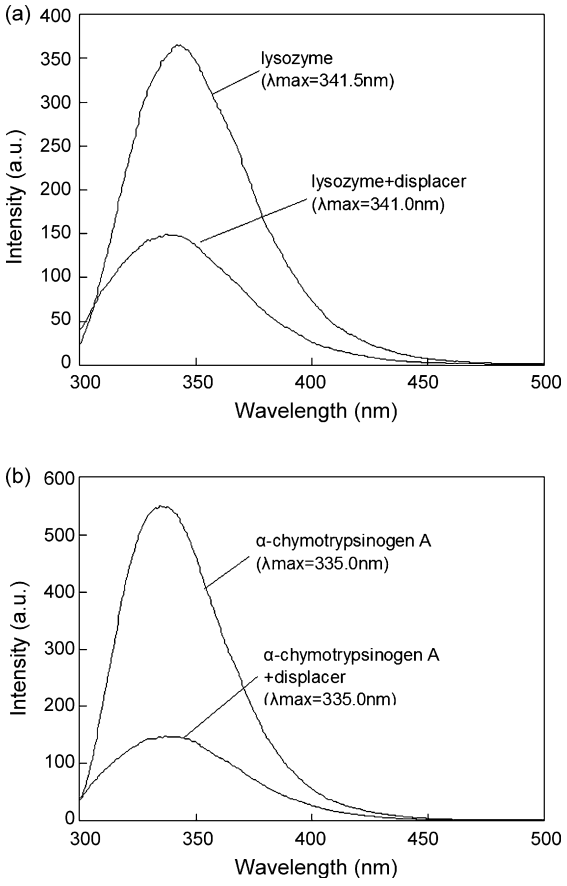

Fig. 5. Fluorescence emission spectra of (a) lysozyme and (b) α-chymotrypsinogen A incubated with benzethonium chloride for 1 h. Protein concentration: 5 mg/mL; displacer concentration: 50 mmol/L; buffer: 50 mmol/L acetate buffer, pH 5.0; excitation wavelength: 280 nm; emission wavelength: 300–500 nm.

The activity of lysozyme incubated with benzethonium chloride for 1 h was also assayed to investigate the influence of benzethonium chloride on lysozyme activity. In the presence of benzethonium chloride, the relative activity of the incubated lysozyme was 95.6% of that of native lysozyme. These results show that the structure and activity of the proteins are practically unchanged upon contacting with the displacer. Therefore, it can be concluded that benzethonium chloride is a "protein-friendly" displacer for displacement operations.

3.3. Displacement of lysozyme and α-chymotrypsinogen A by benzethonium chloride

Displacement of lysozyme and α-chymotrypsinogen A by benzethonium chloride was conducted at pH 5.0 and 7.0 to verify the predictions in Section 3.1, and the results are given in Figs. 6 and 7. It can be seen from Fig. 6 that benzethonium chloride can indeed displace lysozyme and α-chymotrypsinogen A at pH 5.0 in the order of their capacity factors. A typical isotachic displacement train was formed with minimum overlay of the solutes, indicating a successful displacement. RP-HPLC analysis of the pooled fractions of lysozyme and α-chymotrypsinogen A each gave a yield of 92.9% and 83.3% and a purity of 100.0%

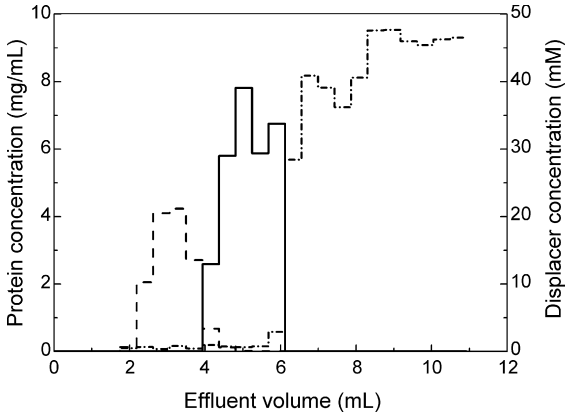

Fig. 6. Displacement of lysozyme and α-chymotrypsinogen A by benzethonium chloride at pH 5.0. (- - -) Lysozyme; (—) α-chymotrypsinogen A; (- · - · -) benzethonium chloride. Column: Tricorn 5/100; mobile phase: 50 mmol/L acetate buffer, pH 5.0; displacer: 50 mmol/L benzethonium chloride; loading: 1 mL protein solution (5 mg/mL lysozyme and 10 mg/mL α-chymotrypsinogen A); flow rate: 0.1 mL/min.

and 95.3%, respectively. The activity yield of lysozyme was 88.4%, approximately the same as its mass yield. The results are comparable with the yields and purities reported in hydrophobic displacement chromatography processes [18,24].

Displacement at pH 7.0 was unsuccessful (Fig. 7), as was predicted by the capacity factors. The zones of proteins and displacer overlaid each other, and the resolution is poor. Lysozyme was too strongly adsorbed to be displaced from the stationary phase, resulting in a very low concentration of it in the effluent. Note that benzethonium chloride still shows some displacement effect to α-chymotrypsinogen A at pH 7.0, as their capacity factors at pH 7.0 is similar (Fig. 3). The good agreement between the prediction by capacity factors and the result of column displacement shows that it is feasible to predict the possibility of displacement by capacity factors on HCIC adsorbents.

Concerning both the protein retention and the displacement chromatography experiments, it can be seen that selectivity reversal has occurred between lysozyme and α-

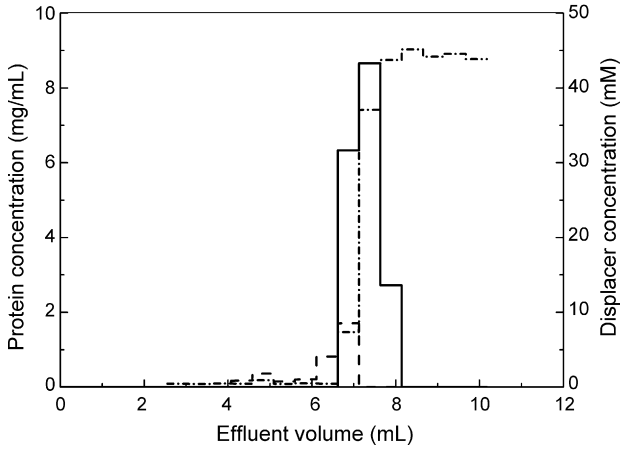

Fig. 7. Displacement of lysozyme and α-chymotrypsinogen A by benzethonium chloride at pH 7.0. (- - -) Lysozyme; (—) α-chymotrypsinogen A; (- · - · -) benzethonium chloride. Mobile phase: 50 mmol/L phosphate buffer (pH 7.0). Other conditions were the same as in Fig. 6.

Table 2
Efficiency of column regeneration with different regenerants

Regenerant	Displacer eluted in the first 8 mL (%)
Glycine–HCl (pH 3.0)	96.3
NaAc–HAc (pH 4.0)	96.0
1.0 mol/L NaOH	50.1

chymotrypsinogen A at different pH values, i.e. retention of lysozyme is stronger than that of α-chymotrypsinogen A at pH 7.0 but lower than that of α-chymotrypsinogen A at pH 5.0. This is due to the complex influence of pH on solute retention and has its own significance. As is often the case in displacement chromatography, the solute at the head of the isotachic displacement train usually have fewer overlays with other solutes while the solutes at the end of the train will be more concentrated. By choosing the appropriate pH and displacer, one can achieve displacement of two proteins in different orders, or selectively displace one of the proteins while letting the other remain on the column. This may bring us more opportunities to tailor the displacement separation process to the specific requirement of the desired product.

3.4. Column regeneration

MEP-Hypercel can become significantly positively charged at pH values lower than its pK_a value (pK_a = 4.8). Hence, by lowering the pH to a certain level, benzethonium chloride can be repulsed from the stationary phase and readily eluted. As the working pH of MEP-Hypercel is 3–12, buffers of pH 3.0 (50 mmol/L glycine–HCl) and pH 4.0 (50 mmol/L NaAc–HAc) were chosen for the column regeneration test. The efficiencies of regeneration at these two conditions were also compared with that by 1.0 mol/L NaOH, which had been used for regeneration in hydrophobic displacement chromatography with quaternary ammonium salts as displacers [19]. The percentages of displacer eluted in the first 8 mL by different regenerants are listed in Table 2. It can be seen that regeneration by pH adjustment is much more efficient than that with NaOH. Since the efficiencies at pH 3.0 and 4.0 are similar, pH 4.0 was chosen as the regeneration condition for the concerns of adsorbent and equipment maintenance.

Fig. 8 shows the frontal analysis curves of benzethonium chloride before and after repeated breakthrough and regeneration with the acetate buffer of pH 4.0. The curves completely overlaid each other, indicating a good result of regeneration [16,19]. This has further demonstrated the simplicity of column generation with HCIC adsorbents in displacement chromatography. Unlike the regeneration steps in traditional hydrophobic displacement chromatography or other modes of displacement chromatography, which usually requires over 10 column volumes of deionized water and regenerant solution [9,15,27,13] and/or extreme regeneration conditions [16,19,28], only four column volumes (8 mL) of regenerant solution in this work had brought very good results.

The achievement of charge-facilitated column regeneration in displacement chromatography has great significance. In traditional modes of displacement chromatography (ion-exchange, hydrophobic, reversed phase, etc.), although separation is achieved by displacement with a strong-affinity displacer instead of altering the magnitude of interactions between solutes and the stationary phase, regeneration still falls into the principles of elution chromatography, which is achieved by weakening the interactions between the displacer and the stationary phase. This has caused a dilemma in the choice of displacers. A displacer with very strong affinity to the stationary phase would be good for displacement, but regeneration will be difficult to achieve. Also, the advantage of high-throughput in displacement chromatography will be offset by the lengthy time for column regeneration. In displacement chromatography with HCIC adsorbents such a problem no longer exists. The possibility of charge repulsion between the stationary phase and the displacer at relatively moderate conditions (i.e. acidic pH) has greatly weakened the affinity of the displacer, providing a good means for effective regeneration.

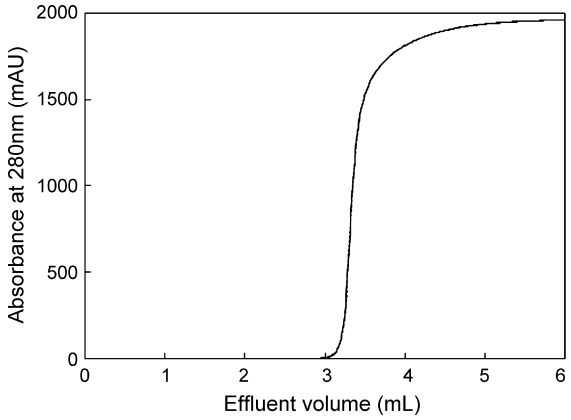

Fig. 8. Frontals of benzethonium chloride before and after repeated breakthrough at pH 5.0 and regeneration at pH 4.0. Column: Tricorn 5/100; breakthrough solution: 50 mmol/L benzethonium chloride in 50 mmol/L acetate buffer (pH 5.0); regeneration solution: 50 mmol/L acetate buffer (pH 4.0); flow rate: 1.0 mL/min.

4. Conclusions

Displacement of lysozyme and α-chymotrypsinogen A was successfully achieved at pH 5.0 with benzethonium chloride as the displacer, as was predicted by their capacity factors according to the stability analysis. Lysozyme activity assay and fluorescence spectroscopy showed that the structure and/or activity of the two proteins remained unchanged upon contacting with the displacer. The yield and purity were comparable with the displacement chromatography processes reported in literature. More importantly, column regeneration was efficiently achieved by charge repulsion between the displacer and the adsorbent at low pH values.

Displacement of proteins on HCIC adsorbents has several advantages over traditional HIC. First, the low-salt operating conditions has simplified the operation steps and enabled a greater productivity. In traditional hydrophobic displacement chromatography, high-salt concentration in the mobile phase is needed to attain the retention appropriate for displacement. Pro-

tein solubility is reduced with increasing salt concentration, and therefore a compromise between solubility and retention must be struck [9]. Although high salt does not seem to hinder the displacement process, the low solubility limits the column load. In displacement chromatography on HCIC no additional salt is needed to promote adsorption, and the high-ligand density of HCIC adsorbents enables even greater capacity at low-salt concentrations. The step of salination before separation and desalination after separation is eliminated, and restrictions on protein solubility have been removed, making the separation process more effective in practical productions.

The second advantage of displacement chromatography on HCIC adsorbents is the greater flexibility resulting from the influence of pH on proteins and displacers. The multiple interactions between proteins/displacers and the HCIC sorbent, i.e. hydrophobic interaction and charge repulsion, make the retention behaviors of proteins and displacers remarkably changeable with the buffer pH. Selectivity reversal between protein and displacer or protein and protein at different pH values is quite possible. The phenomenon of selectivity reversal between protein and displacer can make it possible to use a displacer that may not be hydrophobic enough for the target products in traditional hydrophobic displacement chromatography, thus expanding the range of a displacer's application. Selectivity reversal also enables us to choose appropriate conditions for a certain order of displacement, which may be favorable for the specific requirements of the separation. In a word, the great influence of pH on solute retention in HCIC has offered more opportunities to tailor the displacement process to the user's desire.

Finally, the ease of column regeneration on HCIC adsorbents has made it advantageous compared with any other mode of displacement chromatography. As the displacer is generally very strongly adsorbed to the stationary phase, regeneration in displacement chromatography often requires extreme conditions and the efficiency is usually low. However, since charge repulsion can be utilized for column regeneration in HCIC, it is possible to remove the strongly adsorbed displacer from the stationary phase rapidly and completely. This can further increase the efficiency of displacement chromatography by reducing the time for regeneration and avoiding the use of extreme conditions that may damage the adsorbents and rigs.

The experimental results and all these advantages indicate that displacement chromatography on HCIC adsorbents is a good alternative to traditional hydrophobic displacement chromatography. In order to promote the application of protein displacement in HCIC, some further investigations have to be made. More displacers have to be developed; the displacers should have greater hydrophobicity to displace more hydrophobic proteins at wider range of pH values. Also, separation of real protein mixtures should be studied to optimize the operating conditions and make full use of the potential of HCIC in displacement chromatography.

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