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Preparation of a novel dual-function strong cation exchange/hydrophobic interaction chromatography stationary phase for protein separation

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ABSTRACT

We have explored a novel dual-function stationary phase which combines both strong cation exchange (SCX) and hydrophobic interaction chromatography (HIC) characteristics. The novel dual-function stationary phase is based on porous and spherical silica gel functionalized with ligand containing sulfonic and benzyl groups capable of electrostatic and hydrophobic interaction functionalities, which displays HIC character in a high salt concentration, and IEC character in a low salt concentration in mobile phase employed. As a result, it can be employed to separate proteins with SCX and HIC modes, respectively. The resolution and selectivity of the dual-function stationary phase were evaluated under both HIC and SCX modes with standard proteins and can be comparable to that of conventional IEC and HIC columns. More than 96% of mass and bioactivity recoveries of proteins can be achieved in both HIC and SCX modes, respectively. The results indicated that the novel dual-function column could replace two individual SCX and HIC columns for protein separation. Mixed retention mechanism of proteins on this dual-function column based on stoichiometric displacement theory (SDT) in LC was investigated to find the optimal balance of the magnitude of electrostatic and hydrophobic interactions between protein and the ligand on the silica surface in order to obtain high resolution and selectivity for protein separation. In addition, the effects of the hydrophobicity of the ligand of the dual-function packings and pH of the mobile phase used on protein separation were also investigated in detail. The results show that the ligand with suitable hydrophobicity to match the electrostatic interaction is very important to prepare the dual-function stationary phase, and a better resolution and selectivity can be obtained at pH 6.5 in SCX mode. Therefore, the dual-function column can replace two individual SCX and HIC columns for protein separation and be used to set up two-dimensional liquid chromatography with a single column (2DLC-1C), which can also be employed to separate three kinds of active proteins completely, such as lysozyme, ovotransferrin and ovalbumin from egg white. The result is very important not only to the development of new 2DLC technology with a single column for proteomics, but also to recombinant protein drug production for saving column expense and simplifying the process in biotechnology.

1. Introduction

With the development of biotechnology and life science, both recombinant protein drug productions and proteomics research depend largely on fast and efficient protein separation technology. Analysis of complex samples has put forward higher and higher requirements to separation science. Therefore, developing new separation material, separation mode and more sensitive detection method should be one of the effective ways to solve these problems.

Two-dimensional liquid chromatography (2DLC) and multi-dimensional liquid chromatography (MDLC) are powerful tools to separate and analyze the complex sample in proteomics [1]. In general, one traditional column in liquid chromatography (LC) can only be employed to separate proteins with single separation mode, such as reversed-phase liquid chromatography (RPLC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), affinity chromatography (AFC) and size exclusion chromatography (SEC). As a result, two or more orthogonal columns are often required in 2DLC or MDLC [2]. Although protein interactions with chromatography sorbents generally are considered in terms of single modes such as ionic or hydrophobic

Abbreviations: SCX, strong cation exchange; HIC, hydrophobic interaction chromatography; 2DLC, two-dimensional liquid chromatography; AFC, affinity chromatography; DMF, *N*,*N*-dimethylformamide; SDT, stoichiometric displacement theory; MMC, mixed-mode chromatography; HILIC, hydrophilic interaction chromatography; MudPIT, multidimensional protein identification technology; TM560, 3-glycidoxypropyltrimethoxysilane; DMAP, 4-dimethylaminopyridine; DIC, *N*,*N*′-diisopropylcarbodiimide; PBS, phosphate buffer solution; 2DLC-1C, two-dimensional liquid chromatography with a single column

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interactions, in fact, protein chromatography often involves multiple modes of interaction with the sorbent bead or spacer–linker structure carrying the nominal ligand [3]. In the 1980s, mixed-mode chromatography (MMC) has been developed to use a sorbent intentionally functionalized with ligands capable of multiple modes of interaction to effect a protein separation process, including binding, washing, and elution [4,5]. The advantages of mixing modes deserve much wider recognition. MMC cannot only enhance selectivity beyond that of chromatography with the same single modes performed separately, but also reduce the number of column and steps needed for protein purification, and indeed, sometimes solves protein purification problems that are otherwise intractable [5].

Although many types of MMC are developed, such as RPLC/IEC [6,7], RPLC/hydrophilic interaction chromatography (HILIC) [8,9] and HILIC/IEC [10,11], etc, in fact, MMC is still dominated by one retention mechanism and assisted by another. As a result, MMC stationary phase can only be used to separate proteins with single mode chromatography.

Regnier's group firstly synthesized an anion exchange stationary phase which had mixed-mode interaction consisting of anion exchange chromatography and hydrophobic interaction chromatography for protein separation [12]. Horvath [13] prepared a single ternary mixed-bed column packed with the a ternary mixture of cation and anion exchangers as well as a mildly hydrophobic stationary phase, which could be used with increasing salt gradient as a cation exchanger for the separation of basic proteins, or as an anion exchanger for the separation of acidic proteins. Furthermore, it could be used as a "bipolar" electrostatic-interaction column with increasing salt gradient and as a hydrophobic interaction column with decreasing salt gradient for the separation of both types of proteins in a single chromatographic run. In 1997, Link et al., packed strong cation exchange (SCX) and RPLC packings into a single microcapillary column and firstly proposed multidimensional protein identification technology (MudPIT) [14]. Such columns are called biphasic or hybrid columns. But so far, it has not been found that any kinds of MMC separation medium can be used to separate proteins by 2DLC with two different modes, such as IEC and HIC, and the resolution and selectivity can be comparable to that obtained from conventional LC with the single mode.

Recently, our group synthesized a new weak cation exchange (WCX)/HIC dual-function column [15]. Because the structure of the ligand has carboxyl and hydrophobic function groups, the employed column displays HIC character in a high salt concentration, and IEC character in a low salt concentration in mobile phase employed. So, it can be used to separate proteins in IEC and HIC modes, respectively, and the resolution and selectivity are comparable to that of the conventional LC column. Because IEC and HIC modes are orthorhombic and the dual-function column can be used to replace two traditional IEC and HIC columns for protein separation, the dual-function column was also vividly called "2D column". Based on it, we also proposed a new 2DLC technology for intact protein separation using only a single column (2DLC-1C) [15] and investigated the mixed-mode retention mechanism of the dual-function column in detail [16].

This special dual-function column must simultaneously satisfy the following three conditions: (1) The ligand must has both ion exchange and hydrophobic functional groups; (2) The hydrophobicity of the ligand is suitable to match the magnitude of the electrostatic interaction; (3) The column has a very good resolution of proteins for both HIC and IEC modes as it is employed alone, and the obtained intact protein must maintain a three-, or four-dimensional molecular structure.

In this paper, we firstly designed and prepared a novel dual-function stationary phase containing sulfonic and benzyl groups, which can display strong cation exchange (SCX) and HIC characters. Therefore, it can be used to separate proteins under SCX and HIC modes, respectively. The resolution and selectivity of this new stationary phase was evaluated under both HIC and SCX modes with standard proteins and can be comparable to that of the corresponding conventional single mode column, respectively. The results demonstrated that the novel SCX/HIC dual-function column could be used to replace two individual SCX and HIC columns for protein separation.

2. Experimental

2.1. Materials

Spherical silica (5 μm particle size; 300 Å pore size; 180 m^2/g surface area) was purchased from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences (China); 3-glycidoxypropyltrimethoxysilane (TM560), 4-dimethylaminopyridine (DMAP) and *N*,*N*′-diisopropylcarbodiimide (DIC) was purchased from Aladding Chemical Reagents (China). Myoglobin, ribonuclease B (RNase B), ribonuclease A (RNase A), cytochrome C, α-chymotrypsin A, lysozyme, insulin, α-amylase were purchased from Sigma (St. Louis, USA). All chemical reagents are of analysis grade.

Five millgram standard protein was dissolved in 1.0 mL purified water to obtain the concentration of protein solution to be 5 mg/mL, and then stored at 4 °C.

2.2. Instrumentation

All chromatographic tests were carried out by using a LC-20A chromatographic system (Shimadzu, Japan), including two LC-20AT vp pumps, a SCL-20A vp system controller, a SPD-20A vp UV–vis detector and a CLASS-VP chromatography workstation. Samples were injected through a Rheodyne 7725 valve and detected at 280 nm.

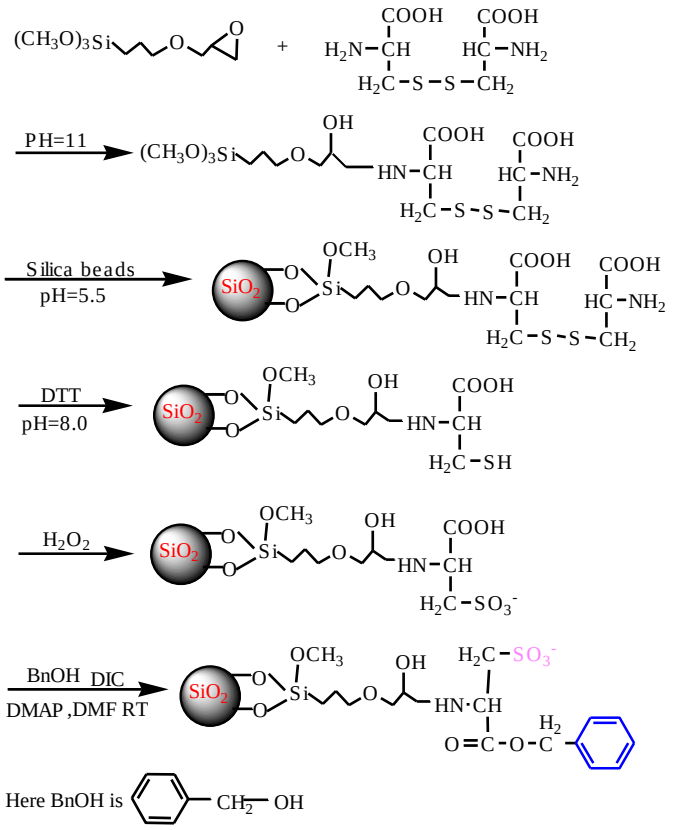
2.3. Synthesis of SCX/HIC stationary phases

The synthetic procedures used in the preparation of SCX/HIC stationary phases were shown in Scheme 1.

Cystine (3.3 g, about 13.7 mmol) was dissolved in the solution of 0.5 mol/L Na_2CO_3 and the pH was adjusted to 11.0 with 1.0 mol/L NaOH. The cystine solution was stirred some minutes in an ice bath and then 3-glycidoxypropyltrimethoxysilane (TM560) (3 mL, 13.7 mmol) was added into the solution slowly. After 30 min of stirring at 0 °C the reaction mixture was allowed to warm up to 65 °C. After 24 h, the reaction solution was cooled to room temperature and the pH was adjusted to 5.5 with acetic acid, filtered, silica (2.0 g) was added to the above filtrate, after 2 h of stirring at 90 °C, silica was filtered and washed with water (2 × 30 mL), methanol (2 × 30 mL) and acetone (2 × 30 mL). The solid product was dried at 50 °C in vacuo for 10 h to afford cystine-silica 2.

Cystine-silica 2 (2.0 g) was added to the solution of DTT (0.2 g) in the buffer (40 mL, 20 mmol/L Tris, pH 8.0) with slowly stirring. The mixture was stirred at room temperature for 1.5 h and then was filtered and washed with water (2 × 30 mL), methanol (2 × 30 mL) and acetone (2 × 30 mL). The solid product was dried at 40 °C in vacuo for 5 h to afford cysteine-silica 3.

Cysteine-silica 3 (2.0 g) was added to the mixture of hydrogen peroxid and methanol (50 mL 35% H_2O_2 + 15 mL methanol) and then three drops concentrated sulfuric acid was dropped with slowly stirring. After 24 h of stirring, filtered, washing with water (2 × 30 mL), methanol (2 × 30 mL) and acetone (2 × 30 mL). The solid product was dried at 40 °C in vacuo for 5 h to afford



Scheme 1. The synthetic procedures of SCX/HIC dual-functional stationary phases.

sulf-group-silica. Then benzyl alcohol (4 mL) or butyl alcohol (4 mL) and DIC (2 mL) was added into 50 mL of anhydrous DMF at room temperature. To this stirred solution was added 2 g of sulf-group-silica followed by catalytic amount of DMAP. The mixture was stirred at room temperature overnight. The solid was filtered and washed with water (2×30 mL), methanol (2×30 mL) and acetone (2×30 mL). The solid product was dried at 50 °C in vacuo for 10 h to afford SCX/HIC packings 5, which were dispersed into a pure methanol packed into a 50×4.6 mm I.D. stainless steel column by multipacking (HY-HPCL-S, made in China) at 30 MPa.

2.4. Chromatographic conditions

HIC mode: mobile phase 1: 3.0 mol/L $(NH_4)_2SO_4$+20 mmol/L PBS, pH 6.5; mobile phase 2: 20 mmol/L PBS, pH 6.5.

SCX mode: mobile phase A: 20 mmol/L PBS, pH 6.5; mobile phase B: 20 mmol/L PBS+1.0 mol/L NaCl, pH 6.5.

2.5. Mass recovery

According to the Bradford method [17], Coomassie brilliant blue G250 was used as development reagent to measure the absorbance at 595 nm, using pure BSA as the calibration curve for the determination of protein concentration and calculation of mass recovery.

2.6. Determination of the bioactivity of lysozyme

The bioactivity of lysozyme was measured by the decrease in absorbance at 450 nm of 0.25 mg/mL micrococcus lysodeikticus suspension in 20 mmol/L phosphate buffer, pH 6.5 [18].

2.7. Static capacity determination

Batch adsorption experiments were carried out by contacting 10 mg of dual-functional packings with 0.3 mg/mL lysozyme solution (with SCX mode: lysozyme dissolved in 20 mmol/L PBS, pH 6.5; with HIC mode: lysozyme dissolved in 20 mmol/L PBS+2.0 mol/L $(NH_4)_2SO_4$, pH 7.0) for different periods of time. The adsorbent and solution were vigorously shaken in a SHZ-82 constant-temperature shaker at 150 rpm and 25 °C. The equilibrated samples were centrifuged for 10 min at 10,000 rpm. The supernatant was diluted in a buffer. The protein concentration in the supernatant was analyzed on a UV spectrophotometer at 595 nm which is similar to 2.4. A mass balance was applied to calculate the protein adsorbed on the SCX/HIC dual-function packings.

2.8. Purification of active proteins from egg white with the synthesized dual-function column with off-line 2DLC

Egg white was obtained from fresh egg and dissolved in 20 mmol/L PBS at 1/4 (V/V) dilution and gently stirred 30 min at 4 °C to give a precipitate which was removed by centrifugation at 10,000 rpm at 4 °C for 15 min. The 50×4.6 mm I.D. SCX column was used to isolate lysozyme from egg white. 500 μL of egg white sample were loaded after the column was equilibrated with mobile phase 2 (20 mmol/L PBS, pH 6.5). Then the column was eluted with linear gradient from 0 to 10% mobile phase 1 (20 mmol/L PBS+3.0 mol/L $(NH_4)_2SO_4$, pH 6.5) for 30 min and the fraction of peak 1 was collected. Then the column was equilibrated with mobile phase 1 for 90 min, during this time the fraction of peak 1 was loaded and then was eluted with linear gradient from 100 to 0% mobile 1 for 30 min. All peaks were collected and assayed by SDS–PAGE.

3. Results and discussion

3.1. Preparation and characterization of SCX/HIC dual-function stationary phase

Because two orthogonal LC separation stages consisting of IEC followed by HIC was reported as an optimized protein purification and the chromatographic conditions are very close to the physiological conditions [19], we designed and prepared one novel dual-function stationary phase for protein separation with the ligand containing ion exchange and hydrophobic function groups. The synthetic procedures of SCX/HIC dual-function stationary phases were shown in Scheme 1. The epoxy group of the TM560 is an active group and can be opened by the amino-group at pH 11.0. Then the pH of the above reacted mixture was adjusted to 5.5 with acetic acid. During this progress, if the unreacted cystine precipitate, the precipitate was filtered, then activated silica was added into the filtrate and reacted to obtain cystine-modified silica of 2, then after reducing disulfide bond with DDT, the thiol of compound 3 was oxidized by hydrogen peroxide to produce sulfonic group of compound 4, which can act as the strong cation exchange function group. Finally, benzyl which can act as hydrophobic functional group was introduced into compound 5 by the reaction between benzyl alcohol and the carboxyl of the silica surface with DMAP and DIC as catalyst leading to producing 6 which was characterized by FT–IR (FT–IR spectra not shown here). Sulfonic function groups were verified by two IR absorption peaks, one is strong and wide at 1205.29 cm^{-1} and the other is strong at 1078.01 cm^{-1}. The absorption at 794.52 cm^{-1} ascribed to the phenyl rings; 1631.48 cm^{-1} is assigned to carbonyl groups and 3486.66 cm^{-1} is corresponding to imine groups.

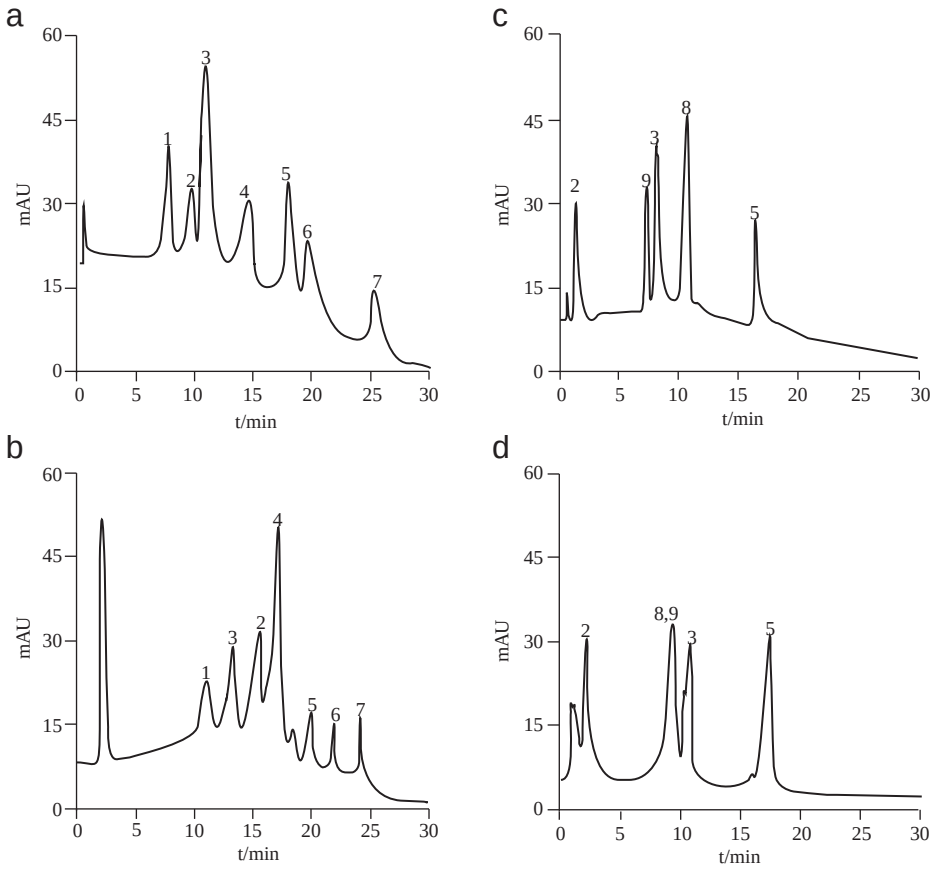


Fig. 1. Chromatograms of standard proteins obtained from dual-function (SCX/HIC) column and comparison with two commercially available columns. ((a) and (c)) Chromatogram of standard protein separation in the HIC and SCX modes using the dual-function column; (b) The HIC column of TSKgel Ether-5PW; (d) The SCX column of PolyLC. The size of the TSK and PolyLC columns were 75 × 7.5 mm I.D and 100 × 4.6 mm I.D, respectively. While that of the (SCX, HIC) column was 50 × 4.6 mm I.D. Mobile phases in the HIC mode: solution A was 3.0 mol/L $(NH_4)_2SO_2$ + 0.02 mol/L PBS (pH 6.5), and solution B was 0.02 mol/L PBS (pH 6.5). Mobile phases in the SCX mode: solution A was 0.02 mol/L PBS (pH 6.5), while solution B was 0.02 mol/L PBS + 1.0 mol/L NaCl (pH 6.5). The flow rate was 1.0 mL/min, gradient: 30 min, 0→100% mobile phase B; UV: 280 nm. Peaks and sample injection amount: (1) cytochrome c (30 μg); (2) myoglobin (20 μg); (3) RNase A (50 μg); (4) OVA (30 μg); (5) lysozyme (15 μg); (6) α-amylase (20 μg); (7) insulin (50 μg); (8) α-chymotrypsin A (25 μg); (9) RNase B (30 μg).

3.2. Protein separation with dual-function (SCX/HIC) column

As mentioned above, the ligand of this dual-function stationary phase contains benzyl and sulfonic functional groups, so the employed column displays HIC character in a high salt concentration, and IEC character in a low salt concentration in mobile phase employed. In order to evaluate the capacity of separating proteins, the dual-function column was used to separate standard proteins under SCX and HIC modes, respectively. The results were shown in Fig. 1.

From Fig. 1a, it can be seen that seven kinds of standard proteins can be separated completely with this dual-function column in HIC mode. The result can be comparable to that of the most popular commercial TSKgel Ether-5PW column for protein separation (Fig. 1b). However, with the comparison of TSK column, the selectivity of the dual-function column was found to change to different extents. A baseline separation between peaks 2 and 4 could be obtained on this dual-function column (Fig. 1a) while that could not on TSK column (Fig. 1b), so the dual-function column can give a better resolution than that of TSK column. In addition, the order in which the proteins were eluted in HIC mode was the same except myoglobin, which was eluted before RNase A on this dual-function column. We think that the electrostatic interaction enhances the selectivity and alters the elution order.

Fig. 1c and d shows the comparison of the resolution and selectivity of standard proteins between the dual-function column and the commercial SCX PolyLC column in SCX mode. The result indicated that five proteins can be separated completely with the former (Fig. 1c), while only four proteins by the latter (Fig. 1d). Furthermore, it can be found that there are some differences in selectivity and chromatographic behaviors of proteins between the two columns. With respect to the order in which the proteins were eluted in the SCX mode, the dual-function column was quite different from the PolyLC column. In the latter, α-chymotrypsin A was eluted before RNase A and overlapped with RNase B completely. The results demonstrated that the resolution and selectivity of dual-function column were much better than that of the PolyLC column.

Table 1
The mass recoveries of proteins eluted with 2D column.

	Mode	RNase B	RNase A	Cytochrome *c*	Lysozyme
Recovery (%)	SCX	97.1	99.5	98.9	97.0
	HIC	98.6	98.9	97.5	98.2

The mass recoveries of proteins on the dual-function column were examined using four proteins in both HIC and SCX modes, as shown in Table 1. Mass recovery of more than 97% was obtained for all the tested proteins. This result indicated that there was non-specific protein adsorption on this new dual-function column. According to the method described by Michel et al. [18], the bioactivity recovery of lysozyme was examined on the

dual-function column and could reach to be 96% for HIC mode and 98% for SCX mode, respectively, which indicated that the protein could maintain its bioactivity in the process of protein separation with dual-function column.

The static binding capacity represents an upper bound on the dynamic binding capacity under the same conditions. It is based on an equilibrium measure of adsorption in which the amount of adsorbed protein is presented as a function of the protein concentration free in solution at equilibrium. The maximum adsorption capacity of lysozyme on this dual-function column was measured to be 160 mg/g for SCX mode and 88 mg/g for HIC mode. Because the concentration of $(NH_4)_2SO_4$ in the solution of equilibrium is 2.0 mol/L, the maximum capacity of lysozyme in HIC mode is lower than that of SCX mode.

In order to evaluate the reproducibility of the SCX/HIC dual-function column, we investigated the separation capacity of the same standard proteins separated with this dual-function column under SCX and HIC mode, respectively, when the column was used for the first time, fiftieth time and two hundredth time (shown in Fig. 2). With the comparison of the changes of the separation capacity, it can be seen that after the two hundredth times, the separation capacity of this dual-function column decreased, but the decrease was little. The results indicated that the reproducibility of the dual-function column was better.

As mentioned above, our group synthesized a new WCX/HIC dual-function column, which can be used to separate proteins in WCX and HIC modes, respectively [15]. Comparison between the WCX/HIC and SCX/HIC dual-function columns, the pH range that can be used under SCX mode is wider than that of WCX. Under the same chromatographic conditions, proteins can be retained stronger under SCX mode than that under WCX mode. The SCX/HIC dual-function column can give better separation capacity under both SCX and HIC modes than that of the WAX/HIC dual-function column [15].

As a result, since the resolution and selectivity are comparable to the conventional IEC and HIC columns, and the bioactivity recovery and mass recovery for protein under both HIC and SCX modes are as good as those of conventional IEC and HIC columns, the single dual-function column can surely replace two individual columns to separate proteins under SCX and HIC modes, respectively. In addition,

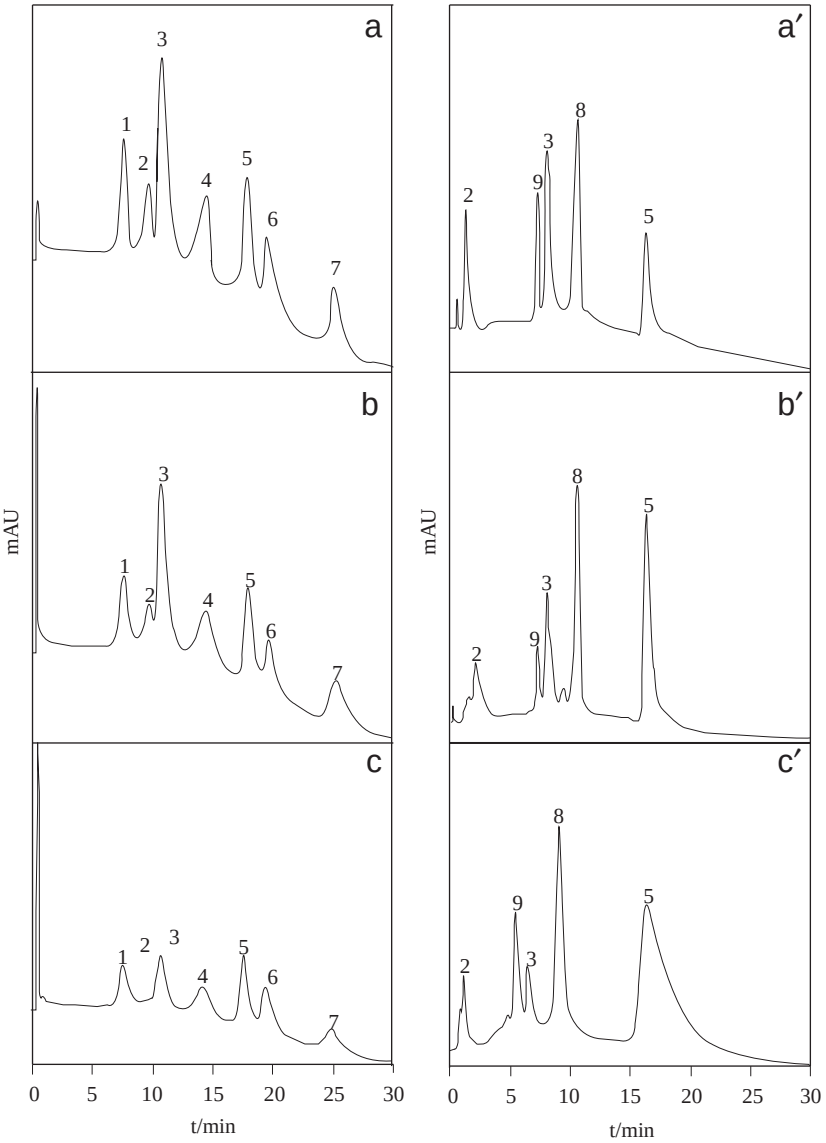


Fig. 2. The effect of using times on the separation capacity of the SCX/HIC dual-function column. Chromatographic conditions are the same as those indicated in Fig. 1. (a-a′) the first time (HIC and SCX modes); (b-b′) the fiftieth time (HIC and SCX modes); (c-c′) the 200th time (HIC and SCX modes). Peaks: (1) cytochrome *c*; (2) myoglobin; (3) RNase A; (4) OVA; (5) lysozyme; (6) α-amylase; (7) insulin; (8) α-chymotrypsin A; (9) RNase B.

HIC and SCX modes are orthorhombic, this dual-function column could be called vividly "2D column", which can be used to set up 2DLC with a single "2D column" as 2DLC-1C.

3.3. The effect of the hydrophobicity of the ligand of dual-function stationary phase on protein separation

There is an inherent orthogonality to the mixed-mode chromatography process, as multiple types of chromatography occur simultaneously. However, interactions between two modes mean the chromatography processes are not independent. Thus, for example, in a mixed-mode ligand containing hydrophobic and ionic elements, increasing ionic strength will disrupt ionic bonds but the increasing salt concentration will promote hydrophobic adsorption. Therefore, the hydrophobicity of the ligand of dual-function stationary phase can seriously influence protein separation. In general, the hydrophobicity of MMC ligand [20,21] is so strong that the proteins adsorbed on MMC stationary phase can not be eluted easily with salt gradient elution in IEC mode, which can only be eluted by reducing the pH of the elution buffer and increasing the magnitude of electrostatic charge repulsion effects, for example, hydrophobic charge induction chromatography (HCIC) [22,23].

In order to investigate the effect of the hydrophobicity of the ligand of this dual-function stationary phase on the protein separation, the butyl as hydrophobic function group was introduced into the ligand. The chromatogram was shown in Fig. 3. In comparison with Fig. 1a and c, only five standard proteins can be separated in HIC mode (Fig. 3a), and the same separation result can be obtained in SCX mode (Fig. 3b). Because the hydrophobicity of butyl is weaker than that of benzyl, it cannot match the electrostatic interaction of the ligand, leading to so bad resolution in HIC mode. The result indicated that the ligand with suitable hydrophobicity to match the electrostatic interaction is very important to the preparation of the dual-function stationary phase.

3.4. The effect of pH on protein separation in SCX mode

A strong cation exchange column can be used at a wider pH range because of containing sulfonic acid groups, which remains fully ionized over a pH range from 2.0 to more than 13.0. The effect of the pH of the eluent on the protein chromatographic behaviors was investigated in detail using five basic proteins such as myoglobin (p*I* 7.0), RNase B (p*I* 8.8), RNase A (p*I* 9.4), α-chymotrypsin A (p*I* 9.5) and lysozyme (p*I* 11.0) and the result was shown in Fig. 4. From Fig. 4, it can be found that the retention time of each tested protein increased monotonously with decreasing the pH of the eluent with the range of 2.5 to 7.0. The result indicated that the synthesized dual-function packings surely had strong cation exchange properties.

In addition, it also can be found that the resolution and selectivity become worse with decreasing the pH of the mobile phase. The result was shown in Fig. 5. The proteins can be retained at a wide pH range, and the five kinds of proteins were separated completely at pH 6.5 and 7.0. When pH decreased to 6.0, the retention times of proteins became longer and the peak height of RNase B become lower than that at pH 6.5 and the separation between RNase A and RNase B is insufficient. Clearly, with decreasing pH of the mobile phase, the resolution became worse and worse. When pH is below 5.5, peaks of myoglobin, RNase B and RNase A overlapped completely and the same result was for α-chymotrypsin A and lysozyme. Therefore, the better resolution and the selectivity can be obtained at pH 6.5 of the mobile phase shown in Fig. 5b.

3.5. Mixed-mode retention mechanism and U-shape elution curve on dual-function column

MMC is a universal phenomenon. Hydrophobic interactions alter retention behavior in ion-exchange and affinity chromatographies [24,25], while electrostatic effects are often present in size-exclusion chromatography [26]. Although mixed interactions are usually to be avoided in traditional chromatography due to their possible counter-actions, a proper combination of different interactions can lead to unique selectivity and facilitate the separation process. We also investigated the mixed mode

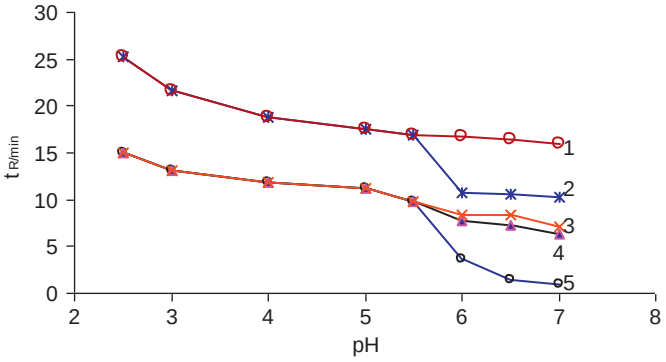


Fig. 4. Effect of pH of mobile phase on the retention times of proteins. (1) lysozyme; (2) α-chymotrypsin A; (3) RNase A; (4) RNase B; (5) myoglobin.

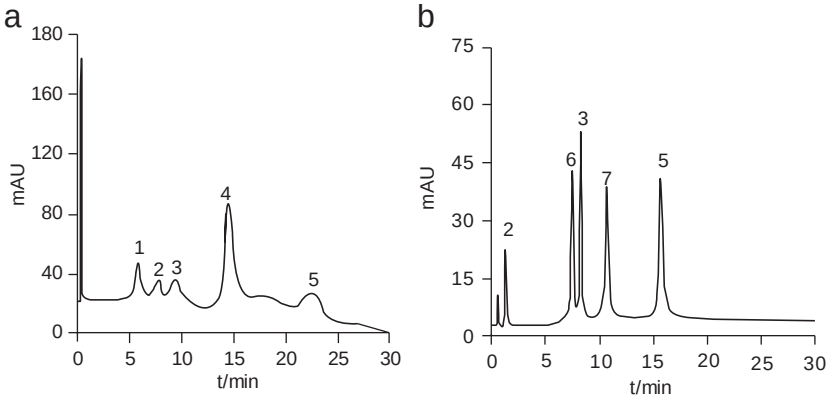


Fig. 3. The chromatograms of five proteins separated by dual-function stationary phase with butyl as hydrophobic functional group in the ligand. Chromatographic conditions are the same as those indicated in Fig. 1. (a) HIC mode; (b) SCX mode. Peaks: (1) cytochrome *c*; (2) myoglobin; (3) RNase A, (4) OVA; (5) lysozyme; (6) RNase B; (7) α-chymotrypsin A.

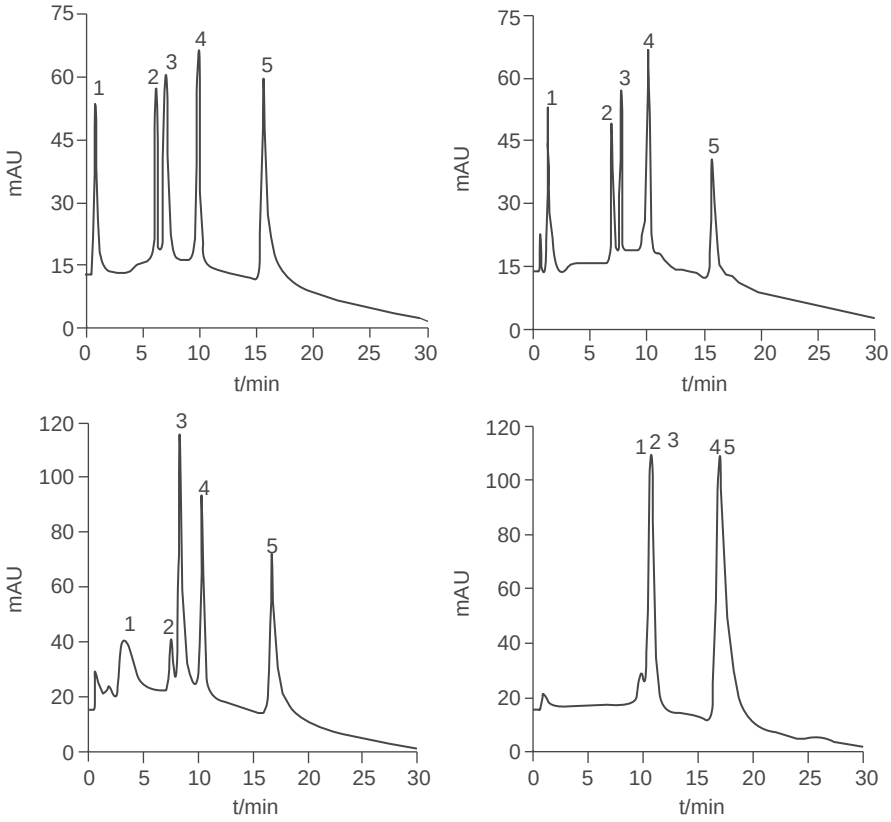


Fig. 5. The effect of pH of mobile phase on protein separation in SCX mode with dual-function column. Except pH, other conditions are the same as those indicated in Fig. 1c. (a) pH 7.0; (b) pH 6.5; (c) pH 6.0; (d) pH 5.5. Peaks: (1) myoglobin; (2) RNase B; (3) RNase A; (4) α-chymotrypsin A; (5) lysozyme.

interactions by which proteins are retained on this synthesized novel column to understand why this dual-function column has such a good resolution in both HIC and SCX modes, respectively, but the previous MMC columns have not. We also need to understand how to find the balance point between electrostatic and hydrophobic interactions so as to obtain the best selectivity and resolution in protein separations.

Many mixed retention mechanisms have been explained by an "U-shape" elution curve of proteins as that a half of this curve to causes from one mode corresponding to one kind of LC and its rest half corresponding to other kind of LC [27].

The effect of salt concentration on protein retention has been investigated under various experimental conditions, and representative plots of the measured logarithmic capacity factor (k') against the salt concentration in the eluent are shown in Fig. 6. The U-shaped elution curves of lysozyme and α-chymotrypsin A which could be retained on dual-function column in both HIC and SCX modes, respectively, were obtained by using isocratic elution with 20 mmol/L PBS buffer containing different concentrations of ammonium sulfate at pH 6.5.

Our group [17] first explained the mixed retention mechanism of proteins with the stoichiometric displacement theory (SDT) alone. When the salt concentration changes, the continuously changing of the orientation of protein molecules to stationary phase accomplishes leading to the continuous changes in retention mechanism from one mode to other mode.

When the salt concentration is very low, corresponding to the top of the left-hand side of the "U-shaped" curve, the electrostatic interactions dominate protein separation; With salt concentration increasing slightly, corresponding to the left side of the "U-shaped" curve, the mobile phase begin to appear weak hydrophobicity, in this salt concentration range, protein retention is mainly controlled by the SCX mode but the HIC mode is also

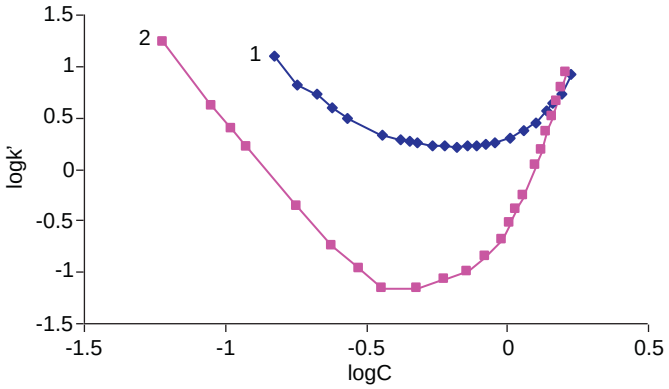


Fig. 6. U-shaped elution curves of α-chymotrypsin A and lysozyme on dual-function column. (1) lysozyme; (2) α-chymotrypsin A.

involved to a small extent. With the salt concentration continuously increasing the two forces are balanced and offset each other, as shown at the bottom of the "U-shaped" curve, protein retention remains unaltered and has a minimum retention. When the salt concentration continuously increases to relatively high extent, corresponding to the right side of the "U-shaped" curve, the mobile phase begin to appear stronger hydrophobicity. In this salt concentration range, protein retention is controlled by HIC mode but the IEC mode is also involved to a slight extent. When the salt concentration is very high, the mobile phase appears very strong hydrophobicity, shown on the top of the right side of the "U-shaped" curve, the protein retention is dominated completely by HIC mode.

The top of the right side and left side of the "U-shaped" curve can fit the following equation of SDT-R to obtain Z and

log I [28,29].

$$\log k' = \log I - Z\log[D] \quad (1)$$

In this case, k' is the capacity factor of the protein; log I is a set of constants (five molecular interactions in HPLC) and relates to the affinity of a protein to the stationary phase; Z is the total moles of water released between the interface of stationary phase and protein in the HIC mode [29] and the total positive charges in SCX mode; and [D] is the molar concentration of the displacer in the mobile phase, water in HIC mode and NH_4^+ in SCX mode [28]. Table 2 shows the linear parameters log I and Z of five proteins obtained from the plot of log k' to log [H_2O] in the HIC mode, and the plot of log k' to log [NH_4^+] in SCX mode. The R values listed in the table indicate that the SDT can also be used to explain mixed mode interactions.

Table 2
Log I and Z of five proteins obtained from SDT in the SCX and HIC modes, respectively.

Protein	SCX mode			HIC mode		
	log I	Z	R	log I	Z	R
RNase B	−3.50	2.83	0.9991	67.63	40.78	0.9903
RNase A	−2.97	2.68	0.9882	66.46	40.02	0.9929
α-Chymotrypsin	−2.27	3.01	0.992	75.30	44.31	0.9948
α-Chymotrypsin A	−2.98	3.45	0.9993	103.58	60.58	0.9987
Lysozyme	−0.60	1.94	0.9910	37.47	21.76	0.9943

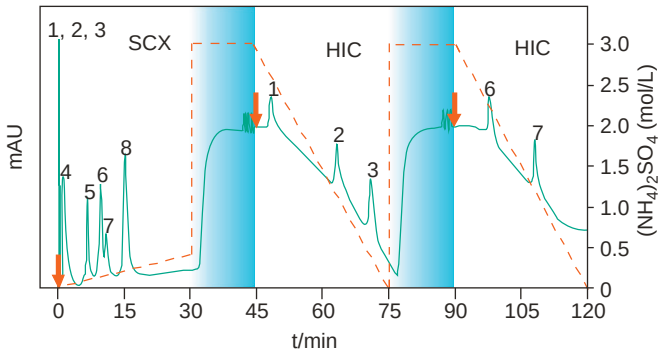


Fig. 7. Chromatogram of eight standard proteins separated by off-line 2DLC with a single dual-function column by cumulative sampling. Mobile phase is the same as that of HIC in Fig. 1, solutions 2 and 1 in the SCX mode and solutions 1 and 2 in the HIC mode. Flow rate, 1.5 mL/min. The gradient elution program is represented by the dashed line in the chromatogram. Peaks: (1) HSA; (2) BSA; (3) insulin; (4) myoglobin; (5) RNase B; (6) α-chymotrypsin; (7) cytochrome c; (8) lysozyme.

3.6. Protein separation by offline 2DLC with a single dual-function (SCX/HIC) column

As mentioned above, dual-function column can be employed to set up 2DLC with a single column. We separated the mixture of eight standard proteins with 2DLC-1C. As shown in Fig. 7, the eight standard proteins were first separated in SCX mode by 2DLC with a single dual-function column. It can be seen that only three of the eight standard proteins, myoglobin, RNase B and lysozyme were completely separated, but fractions 1, 2 and 3 and fractions 6 and 7 could not. The two collected fractions were injected again by cumulative sampling and operated the second separation in HIC mode, respectively. The results indicated that the second separation in the HIC mode led to complete separation of the eight model proteins within 2 h. It demonstrated that 2DLC can really be carried out with only a single dual-function column. This underlines the advantages of 2DLC-1C which can reduce the number of column and steps needed for protein purification and low the separation cost.

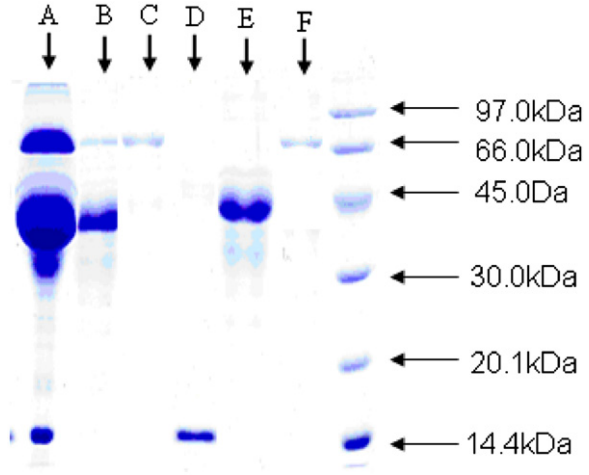


Fig. 9. SDS–PAGE analysis of protein fractions from egg white by the off-line 2DLC with single dual-function column. Lane (a) egg white; (b) fraction of peak 1; (c) fraction of peak 2, ovotransferrin; (d) fraction of peak 3, lysozyme; (e) fraction of peak 4, ovalbumin; (f) fraction of peak 5, ovotransferrin.

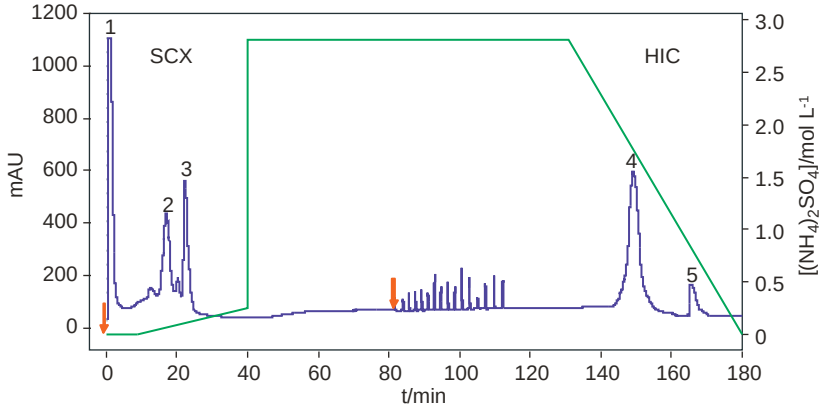


Fig. 8. Chromatogram of egg white separated by the off-line 2DLC with single dual-function column.Chromatographic conditions are the same as those indicated in Fig. 7. Peaks:(1) solvent and unretained acidic proteins; (2) ovotransferrin; (3) lysozyme; (4) ovalbumin; (5) ovotransferrin.

3.7. Separation and purification of active proteins from egg white with the 2DLC-1C based on the dual-function column

To further demonstrate the capacity of separating proteins in complex samples, based on the dual-function stationary phase, 2DLC-1C was set up to separated active proteins from the egg white (see Fig. 8) and the fractions of each peak were collected and analyzed by SDS–PAGE (shown in Fig. 9). It can be seen from Figs. 8 and 9, two proteins were separated completely under first SCX mode, and fraction of peak 1 containing two proteins could not be separated. But after the second separation under HIC mode, another two proteins contained in fraction 1 were also separated completely. We have identified individual proteins using MALDI-TOF MS (not shown here) which indicated that peaks 2 and 5 were both ovotransferrin, peak 3 was lysozyme and peak 4 was ovalbumin.

4. Conclusion

In summary, we described the designation, synthesis, characterization, and chromatographic evaluation of a novel dual-functional mixed-mode silica-based stationary phase that combines both HIC and SCX characteristics. Because the resolution and selectivity are comparable to the conventional SCX and HIC columns, the single dual-function column employed in this study is proved to be able to replace two individual columns for protein separation. The result is very important not only to development a new 2DLC technology with a single dual-function column for proteomics, but also to recombinant protein drug production for saving column expense and simplify the process in biotechnology.

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