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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