Synthesis and Chromatographic Characteristics of Multidentate Ligand-Boned Silica Stationary Phases

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To improve the separation property and stability of metal chelate Cu(II) column, three new kinds of multidentate amino-carboxy silica columns with cation-exchange properties were synthesized using glutamic acid (Glu), glutamic acid-bromoacetic acid (Glu-BAA), glutamic acid-bromosuccinic acid (Glu-BSUA) as ligands and silica gel as matrix. The standard proteins were separated with prepared chromatographic columns. The stationary phases exhibited the metal chelate property after fixing copper ion (II) on the synthesized multidentate ligand silica columns. The binding capacity of immobilized metal ion was related with the dentate number of multidentate ligands. Chromatographic behavior of proteins and the leakage of immobilized metal ion on multidentate chelate Cu(II) columns were affected by the dentate number of multidentate ligands and competitive elution system directly. The results showed that quinquedentate Glu-BSUA-Cu(II) column exhibited better chromatographic property and stability as compared with tridentate Glu-Cu(II) column, tetradentate Glu-BAA-Cu(II) column and commonly used IDA-Cu(II) column.

Key Words: Cation-exchange, Metal chelate, Multidentate ligand, Binding capacity, Leakage

Introduction

Immobilized metal affinity chromatography (IMAC) has been favored by bio-chromatography scientists since it has more advantages as compared with other affinity chromatographic modes.[1] Over the past three decades, IMAC has become an essential tool for the identification, detection, isolation and purification of proteins, especially been active in the areas such as proteomics,[2-5] refolding of recombinant proteins,[6-9] labeling and identification of proteins[5,10-12] as well as cancer diagnosis[13-16] in recent few years. Ion exchangers using aminocarboxy chelating agent as ligand have been widely used in IMAC.[9,17-23] Iminodiacetic acid (IDA) is one of the most commonly used ligands. The characteristics of this sorbent are (1) IDA without immobilized metal ion can be used as cation exchanger to separate positively charged proteins. (2) IDA with metal ion can be used as metal chelate column to isolate proteins with the affinity for metal ions. When transition metal ion, e.g., Ni^{2+}, Co^{2+} or Zn^{2+}, is immobilized to IDA chelating agent, a weak affinity metal chelate column with cation-exchange characteristics is formed. Adsorbed proteins on the column can be easily separated by increasing the ionic strength in phosphate buffer (PB). A typical strong affinity metal chelate column is obtained when IDA ligand is fixed with Cu^{2+}. It is difficult to elute proteins adsorbed on IDA-Cu(II) column with PB-NaCl buffer. The elution of proteins on the column can be achieved only by adding competitive agent in solution or decreasing the pH of the solution.[24] However, the disadvantages of this approach are insufficient separation for proteins and more serious leakage of Cu^{2+} under competitive elution conditions. As a result, the column has shorter lifetime and poor reproducibility, the products and detector will be polluted, and the measurement even can not be performed.[25] Therefore, the popularization and application of metal chelating Cu(II) column have been seriously affected.

According to the mechanism of metal chelate coordination, the authors believe that we should study starting with complex ligands, the dentate number of ligands and competitive agents in order to improve the selectivity and stability of metal chelate Cu(II) column. In this work, tridentate Glu-silica, tetradentate Glu-BAA-silica and quinquedentate Glu-BSUA-silica stationary phases were synthesized using Glu, Glu-BAA, Glu-BSUA as ligands and silica gel as matrix. The cation-exchange properties of proteins on Glu-silica, Glu-BAA-silica and Glu-BSUA-silica columns were investigated. The effect of the dentate numbers of complex ligands on the binding capacity of Cu^{2+} was studied. The leakage of Cu^{2+} and the separation of proteins on metal chelate columns with different dentate numbers were examined under competitive elution conditions.

Experimental

Reagents and chemicals. Silica gel (7 μm, 300 Å) was obtained from Lanzhou Institute of Chemical Physics of the Chinese Academy of Sciences (Lanzhou, China). γ-Glycidoxypropyl-trimethoxysilane (γ-GLDP) was purchased from Gaixian Chemical Engineering Institute (Liaoning, China). Glutamic acid (Glu), bromoacetic acid (BAA) and bromosuccinic acid (BSUA) were purchased from Shanpu Chemical Engineering Company (Shanghai, China). Imidazole (Imid), glycine (Gly) and the other reagents were of analytical grade and purchased from Xi'an Chemical Reagent Company (Xi'an, China). Cytochrome-C (Cyt-C), lysozyme (Lys) and ribonuclease (RNase) were purchased from Sigma Company (St. Louis. Mo., USA). Bovine

serum albumin (BSA) was obtained from Biochemical Reagent Company (Shanghai, China). 2.0 mg/mL protein mixture of BSA, RNase, Cyt-C and Lys were prepared with 20 mM PB (pH 6.0).

Apparatus. ÄKTA purifier 10 (Amersham Biosciences, Sweden) was used for chromatographic experiments. Atomic absorption spectrometry TAS-986 (Puxi General Apparatus Co., Ltd., China) was used for determining binding capacities and leakages of Cu^{2+}. Potentiometer TitraMate 20 (Mettler Toledo, Switzerland) was used for adjusting the pH of the solutions. Slurry packed apparatus 124PP (Chemico, Japan) was used for column packing.

Synthesis of epoxy-activated silica. Epoxy-activated silica was synthesized in acetate-acetic acid buffer (NaAc-HAc, pH 4.0) according to the procedure in reference.[26] 2.0 g dried silica gel was added to 40 mL of 0.1 M NaAc-HAc buffer (pH 4.0), followed by the addition of 2.0 mL γ-GLDP. The reaction was carried out at 90 °C for 2 h with stirring. The obtained epoxy silica was washed with water until neutral pH.

Synthesis of Glu-silica stationary phase. 1.5 g of Glu was added to 30 mL of 1 M sodium carbonate (Na_2CO_3). The pH of the solution was adjusted to 8.5 followed by the addition of 2.0 g dried epoxy silica gel. The reaction was carried out at 65 °C for 12 h with stirring. The obtained Glu-silica was washed successively with water, 10% acetic acid and water again until neutral pH.

Synthesis of Glu-BAA-silica stationary phase. 5.0 g of BAA was added to Na_2CO_3 solution. The pH of the solution was adjusted to 8.5 with 2 M NaOH on an ice bath, followed by the addition of 2.0 g Glu-silica. The reaction was carried out at 25 °C for 24 h with stirring. The resulted Glu-BAA-silica was washed successively with water, 10% acetic acid and water again until neutral pH.

Synthesis of Glu-BSUA-silica stationary phase. 5.0 g of BSUA was added to Na_2CO_3 solution. The synthesis of Glu-BSUA-silica was carried out according to the procedure for the preparation of Glu-BAA-silica.

Column packing. Synthetic stationary phases were packed into 100 × 4.6 mm I.D. stainless-steel columns under 40 MPa pressure.

Immobilization of metal ion. Adopting dynamic chelate method, the packed Glu-silica, Glu-BAA-silica and Glu-BSUA-silica columns (100 × 4.6 mm I.D.) were connected to the chromatographic system respectively, and then perfused with 50 mM copper sulfate in 10 mM NaAc-HAc (pH 5.0) at the flow rate of 0.5 mL/min until saturation. Unbonded copper was washed out with water and 20 mM PB, successively (detection with Na_2S).

Determination of binding capacities for immobilized metal ion. Metal copper ions (II) immobilized to multidentate chelate columns were eluted with 50 mM EDTA-2Na. The eluates were collected into 50 mL volumetric flasks and then diluted to the scale with water. The total concentrations of Cu^{2+} in the diluted eluates were determined by atomic absorption spectrophotometry (AAS) using EDTA-2Na as a blank solution. Binding capacity of Cu^{2+} (q, μmol/g silica) was calculated by the following equation: $q = C·V/M·m$, where C is the total concentration of Cu^{2+} (μg/mL), V is volume of volumetric flask (50 mL),

M is molecular weight of copper (63.55), m is mass of multidentate ligand silica stationary phase (g).

Determination of leakages for immobilized metal ion. Metal copper ions (II) immobilized to multidentate chelate columns were eluted by gradient elution with the selected competitive systems. The eluates were collected into 50 mL volumetric flasks and then diluted to the scale with water. The leakage concentrations of Cu^{2+} in the diluted eluates were determined by AAS using individual competitive eluant as a blank solution. Leakage rate of Cu^{2+} (r, %) was calculated by the following equation: $r = C_0/C × 100$, where C_0 is the leakage concentration of Cu^{2+} (μg/mL).

Chromatographic experiments. The cation-exchange properties of proteins on Glu-silica, Glu-BAA-silica and Glu-BSUA-silica columns were investigated in PB-NaCl system, respectively. The leakages of Cu^{2+} and the separation properties of proteins on Glu-Cu(II), Glu-BAA-Cu(II) and Glu-BSUA-Cu(II) columns with different dentate numbers were studied in different competitive elution systems, respectively. All the chromatographic experiments were performed in accordance with liner gradient elution.

Results and Discussion

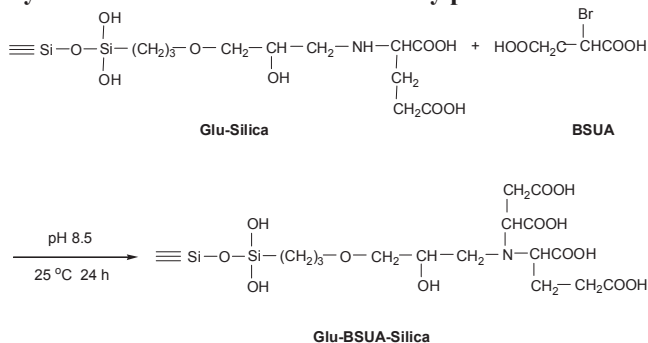**Synthesis of multidentate ligand silica stationary phases.** Multidentate ligand stationary phases consist of silica gel used as matrix, γ-GLDP as spacer arm and ligands. Spacer arm can activate matrix, improve the space freedom of the interaction between ligand and proteins, and regulate the hydrophilicity of stationary phase. In this experiment, spacer arm was attached to silica gel *via* siloxane and to the amino-group of ligand *via* epoxy group. Tridentate Glu-silica packing was firstly synthesized by using Glu as ligand. Then, tetradentate Glu-BAA-silica and quinquedentate Glu-BSUA-silica packings were obtained by Glu-silica bonded with BAA and the BSUA, respectively.

Synthesis of Glu-silica stationary phases.
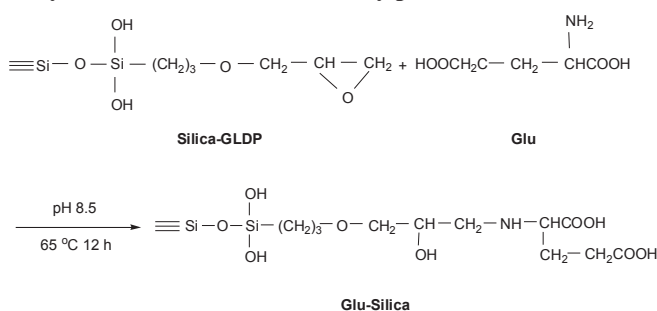
Silica-GLDP　　　　　　Glu

Glu-Silica

Synthesis of Glu-BAA-silica stationary phases.

Glu-Silica　　　　　　BAA

Glu-BAA-Silica
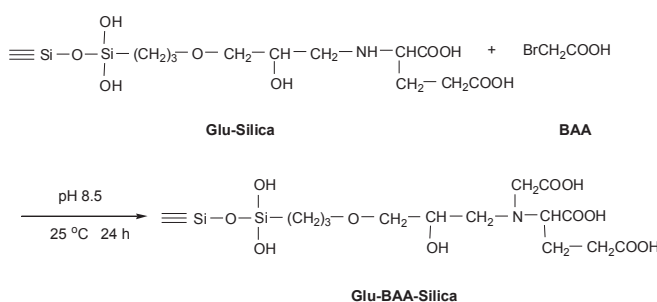
Synthesis of Glu-BSUA-silica stationary phases.

Chromatographic behavior of proteins on multidentate ligand silica stationary phases. Fig. 1 displays chromatograms of proteins on multidentate ligand bonded-silica columns prepared according to above reactions. As seen in Fig. 1, although the dentate numbers of three ligands were different, standard protein mixture could be effectively separated under the same chromatographic conditions. Furthermore, the elution orders of proteins on three columns were consistent, that is, acidic protein BSA (pI 4.9) was not retained, basic proteins, RNase (pI 8.7), Cyt-C (pI 10.1) and Lys (pI 11.0), were eluted in the order of an increase in isoelectric point (pI). The results showed that three kinds of multidentate ligand bonded-silica columns displayed the electrostatic interaction characteristics with cation-exchange properties.

Bonding of Cu^{2+} on multidentate chelate columns. Glu, Glu-BAA and Glu-BSUA contain three, four and five coordination atoms, which can form the cyclic compound with transition metal Cu^{2+}, respectively. When the coordination number of Cu^{2+} is six, tridentate Glu, tetradentate Glu-BAA and quinquedentate Glu-BSUA will form variable octahedral structures with Cu^{2+} in asymmetrical coordination field as the result of Jahn-Teller effect (Fig. 2).

To demonstrate the relationship between the dentate number of ligands and the coordination strength with Cu^{2+}, the effect of ligands with different dentate numbers on the binding capacity of Cu^{2+} was examined in NaAc-HAc buffer system. Binding capacities of Cu^{2+} on Glu-silica, Glu-BAA-silica and Glu-BSUA-silica columns measured by AAS are presented in Fig. 3.

As seen in Fig. 3, binding capacities of Cu^{2+} on multidentate ligand bonded-silica columns were related to the dentate number of ligands. Binding capacities of Cu^{2+} increased with an increase in the dentate number of ligands. Binding capacities of Cu^{2+} on quinquedentate Glu-BSUA-silica column was maximum, and was 23.21 μmol/g silica. However, binding capacities of Cu^{2+} on tridentate Glu-silica column was minimum, and was 11.57 μmol/g silica.

Leakage of Cu^{2+} on multidentate chelate columns. As shown in Fig. 4, metal chelating stationary phase consists of immobilized metal Cu^{2+}, silica matrix and chelating ligand. Chelating ligand is attached to silica *via* spacer arm. As introducing Cu^{2+} to naked column, coordination atoms can form chelate adsorbent with Cu^{2+} through coordination role. The adsorption of protein is attributed to the formation of coordination bond between immobilized Cu^{2+} and coordination atoms from histidine resi-
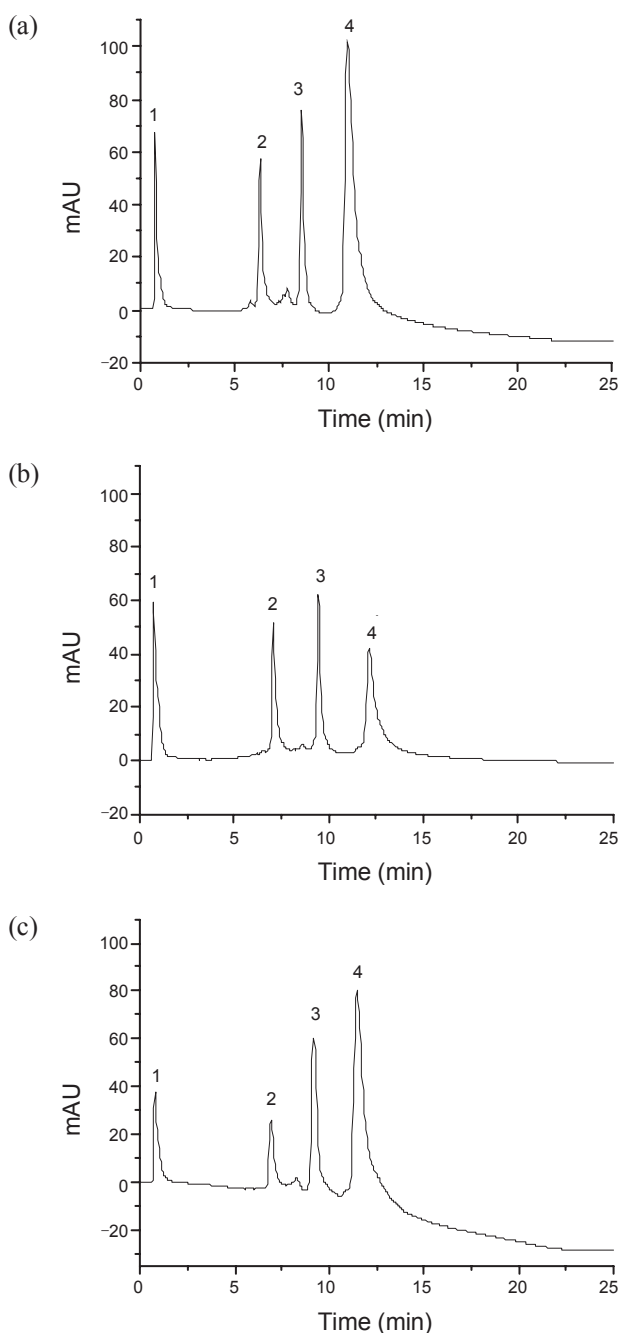

Figure 1. Chromatograms of proteins on multidentate ligand bonded-silica columns. 1, BSA; 2, RNase; 3, Cyt-C; 4, Lys. Columns, (a) Glu-silica column; (b) Glu-BAA-silica column; (c) Glu-BSUA-silica column. Mobile phase, A 0.02 M PB (pH 6.0); B 0.02 M PB + 0.5 M NaCl (pH 6.0). Gradient elution, B from 0 to 100% in 20 min. Flow rate, 1 mL/min. Detector, UV (280 nm). Size of sample, 5 μL. Concentration of each protein, 2 mg/mL.

dues on protein surface, e.g., nitrogen atom on Imid. Absorbed protein on metal chelate Cu(II) column usually can not be eluted with PB, but only by competitive elution.[1]

During the competitive elution, it is possible to cause the leakage of Cu^{2+} if the coordination role of competitive agent with Cu^{2+} is greater than that of multidentate ligand with Cu^{2+}. Based on molecular structure and multidentate chelating me-
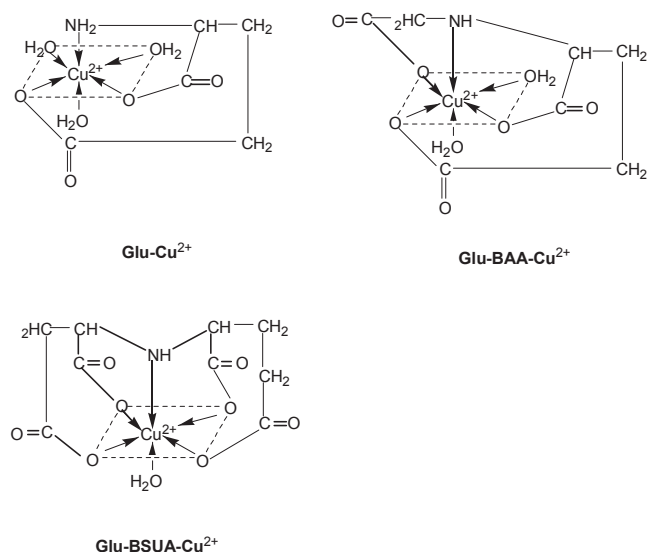
Figure 2. Coordination schematic diagrams of multidentate ligands with Cu^{2+}.
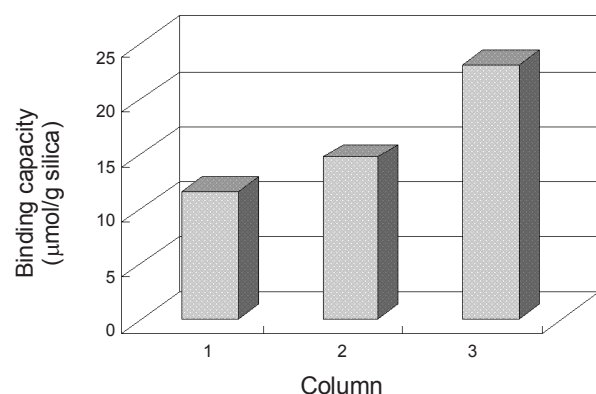

Figure 3. Effect of the dentate number of ligands on Cu^{2+} binding capacities. 1, Glu-silica column; 2, Glu-BAA-silica column; 3, Glu-BSUA-silica column.
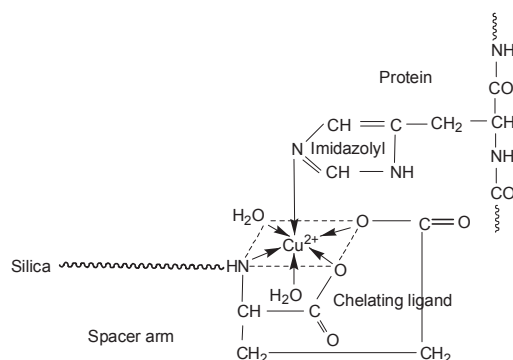

Figure 4. Coordination schematic diagram of Cu^{2+}-Glu-silica with protein.

chanism, we deduce that the more the dentate number of multidentate ligand, the more stable is metal chelate formed with Cu^{2+}, and then the more difficult is the leakage of Cu^{2+}. To
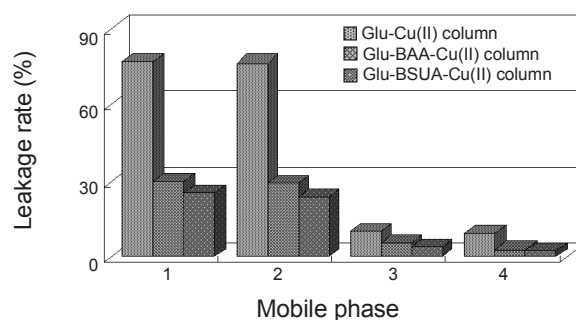

Figure 5. Leakage rates of Cu^{2+} on multidentate chelate Cu(II) columns in different competitive elution systems. Mobile phase 1, A 20 mM PB + 0.5 M NaCl + 1 mM Gly; B 20 mM PB + 0.5 M NaCl + 40 mM Gly (pH 6.0). Mobile phase 2, A 20 mM NaAc + 0.5 M NaCl + 1 mM Gly; B 20 mM NaAc + 0.5 M NaCl + 40 mM Gly (pH 6.0). Mobile phase 3, A 20 mM PB + 0.5 M NaCl + 1 mM Imid; B 20 mM PB+ 0.5 M NaCl + 40 mM Imid (pH 6.0). Mobile phase 4, A 20 mM NaAc + 0.5 M NaCl + 1 mM Imid; B 20 mM NaAc + 0.5 M NaCl + 40 mM Imid (pH 6.0). Other conditions are the same as in Fig. 1.

confirm above viewpoint, the leakage rates of Cu^{2+} on chelating ligands with different dentate numbers were investigated under four kinds of different competitive systems, respectively. The experimental results are presented in Fig. 5.

It can be seen from Fig. 5 that: (1) Selecting Imid as competitive agent, the leakage rates of Cu^{2+} on three kinds of chelating ligands with different dentate numbers were significantly lowered both in PB and NaAc buffer systems as compared with Gly competitive agent. The result indicates that Gly had stronger competitive affinity for Cu^{2+} in comparison with Imid. This is since the stability constants of Gly with Cu^{2+} (lg k' 8.08, 7.24)[27] are obviously greater than those of Imide with Cu^{2+} (lg k' 4.13, 3.48).[28] (2) No matter what kinds of buffer systems and competitive agents were adopted, the leakage rates of metal Cu^{2+} decreased with an increase in the dentate number of ligand. This means that the stability of coordination compound enhances with an increase in coordination atom numbers for the same kind of ligand.

Separation of proteins on multidentate stationary phases. As proteins are separated with multidentate chelate Cu(II) column, the choice of separation conditions should meet two requirements: 1) Adsorbed proteins on chelate Cu(II) column should be efficiently eluted and separated. 2) the leakage of Cu^{2+} should be as low as possible. Therefore, the choice of operating conditions should start with stationary phases, competitive agents and the concentration of agents. Gly and Imid are the commonly used competitive agents. The previous experiment showed that Gly had stronger competitive affinity for Cu^{2+} in comparison with Imid (Fig. 5). It was difficult to control the concentration of Gly. Proteins were not easily eluted with the low concentration of Gly. On the other hand, the leakage of Cu^{2+} became serious at higher concentration of Gly. Consequently, Imid with more moderate affinity for Cu^{2+} was selected for competitive agent. Chromatograms of proteins on Glu-Cu(II), Glu-BAA-Cu(II) and Glu-BSUA-Cu(II) columns in different competitive elution systems are given in Fig. 6. It can be seen from Fig. 6 that: using 20 mM Imid as competitive agent, four kinds of proteins adsorbed on tridentate Glu-Cu(II)
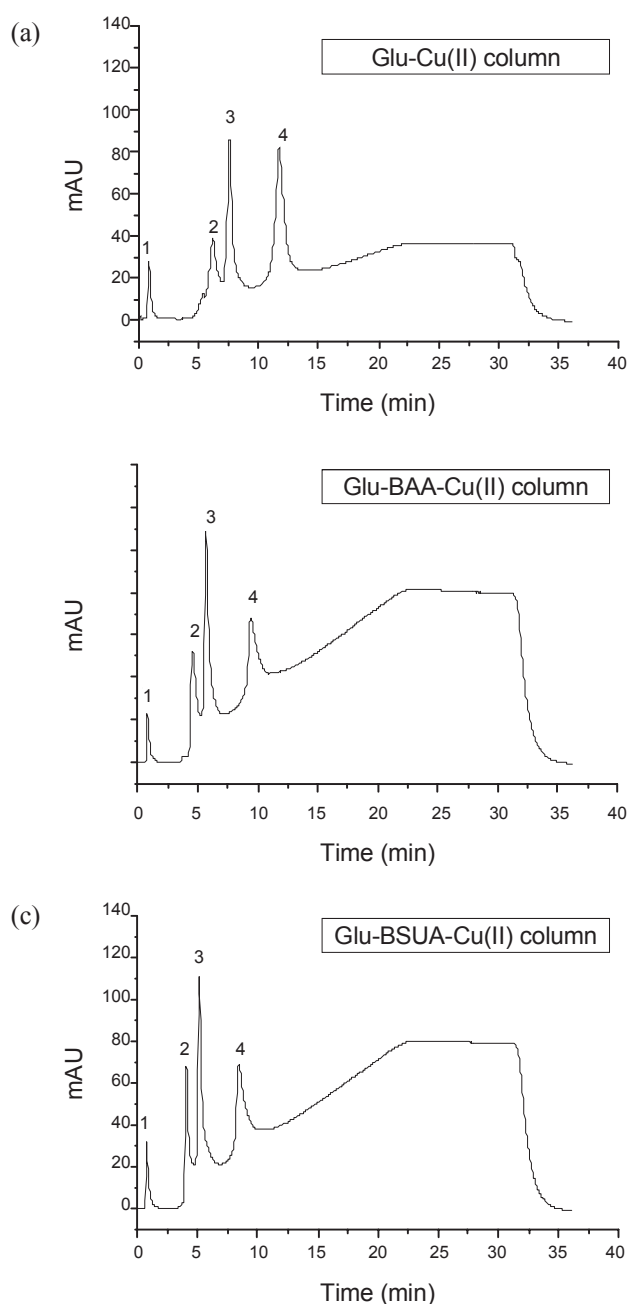
(a)

(c)

Figure 6. Chromatograms of proteins on multidentate chelate Cu(II) columns in different competitive elution systems. 1, BSA; 2, RNase; 3, Cyt-C; 4, Lys. (a) A: 20 mM PB + 0.05 M NaCl; B: 20 mM PB + 0.5 M NaCl + 20 mM Imid (pH 6.0). (b)-(c), A: 20 mM PB + 0.05 M NaCl; B: 20 mM PB + 0.5 M NaCl + 40 mM Imid (pH 6.0). Other conditions are the same as in Fig. 1

column were effectively separated and the leakage rate of Cu^{2+} was 6.96%, which did not affect detection. However, RNase and Cyt-C on tetradentate Glu-BAA-Cu(II) and quinquedentate Glu-BSUA-Cu(II) columns were incompletely isolated due to the stronger adsorption of these two columns for proteins (Figure omitted). Only if the concentration of Imid was increased to 40 mM, these two kinds of proteins adsorbed on tetradentate and quinquedentate Cu(II) columns could be effectively separated. At this concentration, the leakage rates of Cu^{2+} on Glu-BAA-

Cu(II) and Glu-BSUA-Cu(II) columns were 5.28% and 4.08%, respectively. Thus, chelate Cu(II) columns with different dentate numbers all had their own optimum separation conditions. In view of separating efficiency and the leakage rate of Cu^{2+}, quinquedentate Glu-BSUA-Cu(II) column exhibited better chromatographic property as compared with tridentate Glu-Cu(II) and tetradentate Glu-BAA-Cu(II) columns. In addition, separation performance of proteins and the leakage rate of Cu^{2+} on Glu-BSUA-Cu(II) column were also compared with traditional IDA-Cu(II) column. The results indicated that: under the same chromatographic condition, adsorbed proteins on IDA-Cu(II) column could not be effectively eluted and isolated (Figure omitted). The leakage rate of Cu^{2+} on IDA-Cu(II) column was 6.83%, which was higher than that on Glu-BSUA-Cu(II) column. Although the elution and separation of proteins on IDA-Cu(II) column could be slightly improved by increasing the concentration of Imid, the leakage rate of Cu^{2+} was significantly higher. The above results proved that quinquedentate Glu-BSUA is a valuable stationary phase.

Conclusion

Multidentate Glu-silica, Glu-BAA-silica and Glu-BSUA-silica columns with cation-exchange characteristics were prepared, and successfully used for the separation of proteins. Silica gel columns with multidentate ligands had chelate properties for Cu^{2+}. The binding capacity of Cu^{2+} increased with an increase in the dentate number of ligands. The leakage of Cu^{2+} decreased with an increase in the dentate number of ligands, and was related to the strength of competitive elution systems. Three chelate Cu(II) columns with different dentate numbers all could be applied to the isolation of proteins under the competitive elution. The experiment proved that Glu-BSUA-Cu(II) column has better separation property and lower leakage rate of Cu^{2+}.

In summary, besides the leakage of Cu^{2+}, the separation effect of proteins on chelate column also should be taken into account for selecting the metal chelate ligand with appropriate dentate number. These studies may provide reference values for selecting and exploring the novel chelate ligands, improving the separation efficiency of proteins on metal chelate columns, reducing the leakage of immobilized metal copper ion (II) and the pollution of heavy metal in the process of protein separations and purifications.

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