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A Novel Silica-Based Metal Chelate Stationary Phase—L-Glutamic Acid–Copper(II)

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A novel silica-based metal chelate stationary phase, L-glutamic acid-copper(II) (L-Glu-Cu(II)), was synthesized. Effects of the immobilized metal ion, the pH of the solution, and competitive agents on chromatographic behavior of proteins were investigated on prepared chelate column. The coordination role of proteins on L-Glu-Cu(II) column was demonstrated. Optimal separation conditions for proteins on L-Glu-Cu(II) column were discussed. According to the established chromatographic conditions, protein mixtures were effectively separated. Separation performance of proteins and the leakage of Cu^{2+} on L-Glu-Cu(II) column were discussed and compared with the traditional iminodiacetic acid-copper(II) (IDA-Cu(II)) column. Separation performance of L-Glu-Cu(II) column for proteins was superior to that of the IDA-Cu(II) column. Under a proper eluting condition, a less amount of Cu^{2+} was leaked from the L-Glu-Cu(II) column as compared with IDA-Cu(II) column.

Keywords competitive agent; coordination role; leakage; metal chelate column; protein

INTRODUCTION

Immobilized metal affinity chromatography (IMAC), also known as metal chelate affinity chromatography (MCAC), was first proposed by Porath et al. (1) in 1975. The method has been widely used in the isolation and purification of proteins due to the selective affinity of metal chelate column for proteins and peptides (2–6). A series of immobilized metal chelate ligands was developed in order to improve the selectivity and stability of metal chelate column (4,7–8). IDA is one of the most commonly used ligands since it has a suitable coordination number of atoms. This characteristic can meet not only with the coordination of immobilized metal ion, but also with the requirement of protein coordination owing to the remainder empty valence electron orbits of immobilized metal ion. When the transition metal ion, e.g., Ni^{2+}, Co^{2+}, or Zn^{2+}, is immobilized on IDA, 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 column is fixed with Cu^{2+}. Adsorbed proteins on the column are difficultly eluted with PB-NaCl buffer. The elution of proteins can be achieved only by adding the competitive agent with a certain concentration or decreasing the pH of the solution (9).

Although adsorbed proteins on the IDA-Cu(II) column with strong affinity can be eluted with the competitive system, the fatal defects of this approach are ineffective separation for proteins and serious leakage of Cu^{2+}. As a result, the performance and life of the column are all diminished, and the column cannot even be applied. To overcome these shortcomings, a new type of metal chelate stationary phase—L-Glu-Cu(II) was developed by using L-Glu as a complexing ligand and Cu^{2+} as immobilized metal ion. In terms of the competitive elution, a good separation of protein mixtures and a less leakage of Cu^{2+} were obtained on the prepared chelate column. The study has certain value for extending the application of IMAC in the purification of protein.

EXPERIMENTAL

Reagents

Silica gel (7 μm, 300 Å) was obtained from Lanzhou Institute of Chemical Physics (LICP) of the Chinese Academy of Sciences (Lanzhou, China). γ-glycidoxypropyltrithoxysilane (γ-GLDP) was purchased from Gaixian Chemical Engineering Institute (Liaoning, China). L-Glu and IDA were purchased from Shanpu Chemical Engineering Company (Shanghai, China). Imidazole (Imid), glycine (Gly) and other reagents were 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

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protein mixtures 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 the sorption capacities and the leakage 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.

Preparation of L-Glu-Cu(II) Column

Glutamic acid-bonded silica (Glu-silica) packing was synthesized according to the method described in our previous work (10). On the basis of the dynamic chelate method, the packed Glu-silica column (100 mm × 4.6 mm I.D.) was connected to the chromatographic system, and then perfused with 50 mM $CuSO_4$ in 10 mM NaAc-HAc (pH 5.0) at the flow rate of 0.5 mL/min until Cu^{2+} saturation. Unbonded Cu^{2+} was first washed out with water, and then with 20 mM PB-NaCl buffer respectively.

Determination of Binding Capacities for Immobilized Metal Ion

The metal ions immobilized on L-Glu-Cu(II) and IDA-Cu(II) columns were eluted with 50 mM EDTA. The eluate was collected into a 50 mL volumetric flask and then diluted to the scale with water. The sorption capacities of Cu^{2+} were determined by atomic absorption spectrophotometry (AAS) using EDTA as a blank solution.

Determination of Leakage for Immobilized Metal Ion

The metal ions immobilized on L-Glu-Cu(II) and IDA-Cu(II) columns were eluted with the selected competitive system in accordance with gradient elution. The eluate was collected into a 50 mL volumetric flask and then diluted to the scale with water. The leakages of Cu^{2+} were determined by AAS using competitive eluant as a blank solution.

Chromatographic Experiments

The effects of the immobilized metal ion, the pH of the solution, and competitive agents on chromatographic behavior of proteins on L-Glu-Cu(II) column were analyzed, and also the selection of optimal separation conditions as well as the separation of protein mixtures on the column were studied according to the chromatographic conditions given in table and figures.

RESULTS AND DISCUSSION

Retention of Proteins on L-Glu-Cu(II) Column

Adsorption and Desorption of Protein on Cu^{2+} Chelate Stationary Phase

L-Glu chelator is attached to activated silica matrix via covalent bonds. After introducing Cu^{2+}, the multidentate chelator and Cu^{2+} can form metal chelate ligand L-Glu-Cu(II). Proteins will be adsorbed due to the coordination role between proteins and immobilized metal Cu^{2+}. The production of the coordination role is attributed to the electro-donating groups from exposed amino acid residues on the surface of protein, e.g., the imidazole group of histidine which can form the coordination bond with Cu^{2+} (11). When competitive elution is adopted, the protein complexes adsorbed on the L-Glu-Cu(II) column can be replaced by competitive agents (eg, Imid, Gly), and then desorbed. Different proteins have different affinities for Cu^{2+}. In most cases, the separation and purification of proteins have been effectively performed by using such a difference (12). Figure 1 illustrates the diagram for the

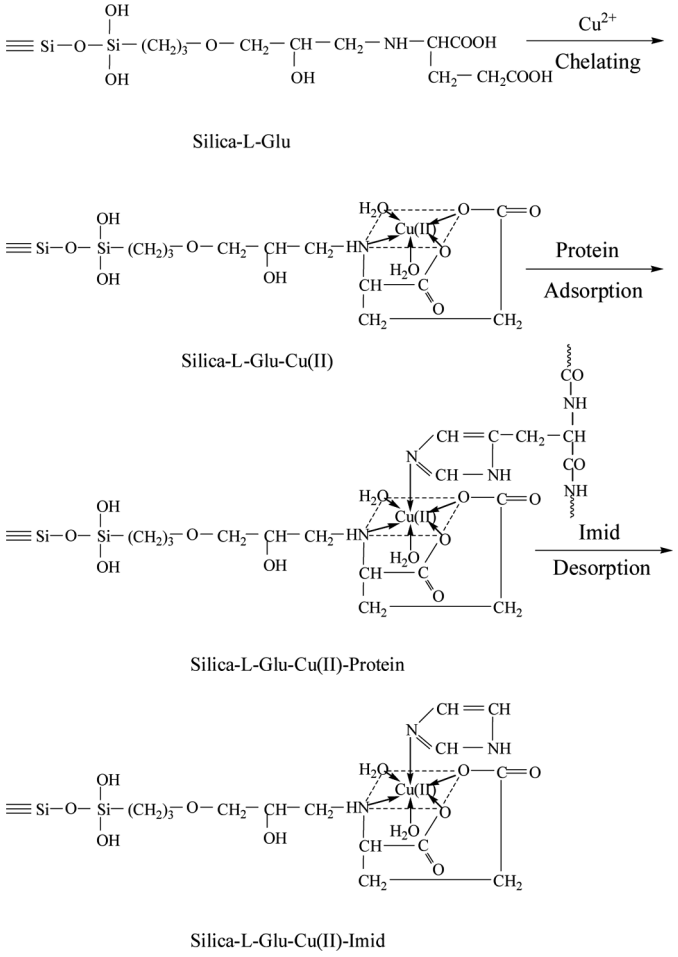

FIG. 1. Adsorption and desorption of protein on L-Glu-Cu(II) column.

adsorption and desorption of protein on L-Glu-Cu(II) stationary phase.

Effect of Immobilized Metal Cu^{2+} on Retention Behavior of Proteins

Chromatograms of proteins on L-Glu and L-Glu-Cu(II) columns determined under the same chromatographic conditions are depicted in Fig. 2a and b, respectively. BSA, RNase, Cyt-C, and Lys adsorbed on the L-Glu column were eluted with PB-NaCl according to a linear gradient elution. As seen in Fig. 2, four kinds of proteins were separated completely in the order of the increasing isoelectric point (pI). The results show that L-Glu column unbound Cu^{2+} displays a typical cation-exchange characteristic. As introducing Cu^{2+} to the L-Glu column, BSA and RNase could not be eluted. Cyt-C and Lys were difficult to be eluted, and their retention times were postponed from 8.15 min and 10.96 min to 10.55 min and 13.74 min, respectively. The results indicate that there exists the coordination role between the immobilized Cu^{2+} and protein.

Effect of Competitive Agent Concentration on Retention Behavior of Proteins

The essence of competitive elution in MCAC is the competitive coordination of the competitive agent and proteins for immobilized metal ion. If LM represents a metal chelate ligand, P is a protein, D is a competitive agent, z is the molecular number of the competitive agent required for replacing one molecular protein, there is an equilibrium between the protein combined with the metal chelate ligand (LM-P) and the competitive agent (D) in solution as follows

$$LM - P + zD = LM - D_z + P$$

If K represents the equilibrium constant of which competitive agent replaces the protein, k_p is a complexing constant of the immobilized metal Cu^{2+} with protein, k_d is a complexing constant of the immobilized metal Cu^{2+} with the competitive agent, then the elution strength of the competitive agent can be expressed as

$$K = \frac{k_d\,[D]^z}{k_p} \tag{1}$$

As shown in Eq. (1), the elution strength of the competitive agent depends on the affinity of the immobilized metal ion for protein and competitive agent, as well as the concentration of the competitive agent. The weaker the coordination role of the immobilized metal ion with protein, the stronger the coordination role of the immobilized metal ion with the competitive agent and the greater the concentration of the competitive agent, so that the adsorbed protein on the stationary phase is more easily eluted. Consequently, the retention time of protein on the chelate column is shorter. As the immobilized metal ion, proteins, and the competitive agent are invariable, the retention of proteins on the metal chelate column only depends on the concentration of the competitive agent. To examine the effect of the concentration of the competitive agent on protein retention, the retention times of proteins on L-Glu-Cu(II) column were investigated by gradient elution using PB buffer containing Imid with different concentrations. Figure 3 is a plot of $\lg k'$ (logarithm of retention factor) vs. $\lg\frac{1}{[D]}$. As seen in Fig. 3, the retention times of proteins decreased with an increase in the concentration of the competitive agent. $\lg k' - \lg\frac{1}{[D]}$ was well in linear relationship at a certain concentration range. Linear correlation coefficients were above 0.996. The results show that the competitive elution of proteins on L-Glu-Cu(II) column is consistent with the stoichiometric displacement theory (13).

Effect of pH Value on Retention Behavior of Proteins

The effect of the pH value on the retention of protein on the metal chelate column is a very complicated issue which

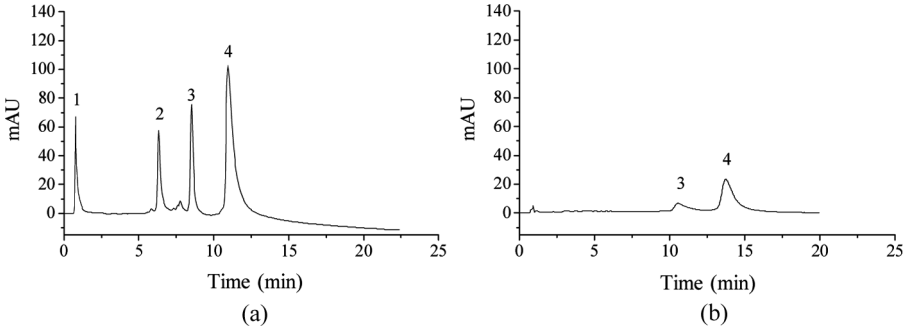

FIG. 2. Chromatograms of proteins on L-Glu and L-Glu-Cu(II) columns 1. BSA; 2. RNase; 3. Cyt-C; 4. Lys Columns: (a) L-Glu column (100 mm × 4.6 mm I.D.); (b) L-Glu-Cu(II) column (100 mm × 4.6 mm I.D.). Mobile phase: A: 20 mM PB (pH 6.0); B: 20 mM PB + 0.5 M NaCl (pH 6.0). Gradient elution: B from 0 to 100% in 20 min. Flow rate: 1 mL/min. Detector: UV ($\lambda = 280$ nm). Size of sample: 5 μL. Concentration of each protein: 2 mg/mL.

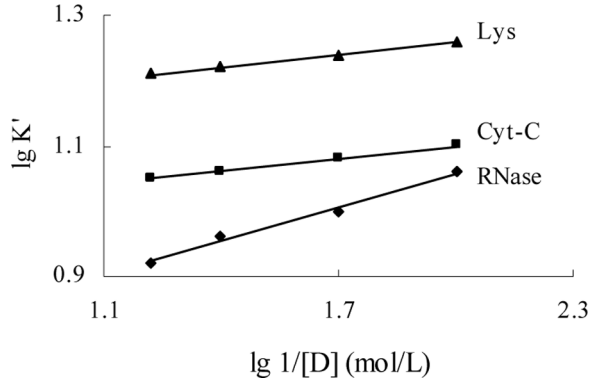

FIG. 3. Relationship between the concentration of Imid and the retention of proteins Column: L-Glu-Cu(II) column (100 mm × 4.6 mm I.D.). Mobile phase: A: 20 mM PB, B: 20 mM PB + 0.5 M NaCl + 10, 20, 40, 60 mM Imid (pH 6.0); Other conditions are the same as in Fig. 2.

can not only involve the nucleophilic behavior of the competitive agent, but also affect the protonation of the complex ligand and coordinate atoms on protein surface as well as the stability of metal ion (11). In order to investigate the effect of the pH value on protein retention, the retention behavior of proteins on L-Glu-Cu(II) column was examined with the different pH values by using gradient elution. The results are shown in Table 1. It can be seen from Table 1 that when the pH is below 5.0, the retention times of proteins on the chelate Cu(II) column decreased with a decrease in the pH values. On the other hand, retention times tended to decrease as pH above 5.0. This is since the competitive agent is in the protonation state at pH below 5.0. The protonation degree of coordination atoms on the protein surface enhances with a decrease in pH values. Therefore, the coordination role of the immobilized metal Cu^{2+} with protein decreases. At pH above 5.0, the protonation degree of the competitive agent Imid weakens with an increase in pH values. The coordination role of Imid with immobilized metal ion can be enhanced. As a result, protein is more easily desorbed. The same result also can be obtained if using Gly instead of Imid. The effect of the pH value on competitive coordination also can be observed from the leakage of the immobilized metal Cu^{2+} (Fig. 4). Experimental results present that the leakages of Cu^{2+} were both reduced with a decrease in the pH values whether in PB or in NaAc system. This is since the decrease of the pH value can strengthen the protonation degree of Gly. As a result, the coordination role of Gly with Cu^{2+} decreases. Since competition elution is controlled by acid-base properties of the solution, efforts should be made to select the pH value in order to minimize the leakage of the immobilized Cu^{2+} as the prerequisite for ensuring the separation of proteins.

TABLE 1
Effect of pH on retention times of proteins (t_R)

	pH			
Protein	3.0	4.0	5.0	6.0
RNase	3.71	5.73	6.79	6.15
Cyt-C	6.67	7.99	8.16	7.54
Lys	8.18	11.28	12.38	11.73

Column: L-Glu-Cu(II) column (100 mm × 4.6 mm I.D.); Mobile phase A: 20 mM PB + 0.05 M NaCl; B: 20 mM PB + 0.5 M NaCl + 20 mM Imid; Other conditions are the same as in Fig. 2.

Separation of Proteins on L-Glu-Cu(II) Column

Selection of Competitive Agents

Once the stationary phase is established, the selection of separation conditions will become important. The chromatographic conditions in MCAC include the buffer system, the pH of the solution, ionic strength, competitive agents, and the concentration of competitive agents, etc. Among these conditions, the choice of competitive agents is the most important because the separation efficiency of the column and the leakage of Cu^{2+} are directly affected by them. Chromatograms of proteins eluted with Imid and Gly in two different buffer systems are depicted in Fig. 5a, b, c, and d, respectively. It can be concluded as follows:

1. Using Imid as competitive agent, BSA, RNase, Cyt-C, and Lys were well separated both in PB and NaAc buffer systems. The difference is that the eluting strength of Imid has been inhibited in the NaAc system. In order to obtain sufficient elution, the concentration of Imid in NaAc system was raised. As a result, the leakage of Cu^{2+} also increased accordingly.
2. The separation effects of Imid were both superior to Gly in PB and NaAc buffer systems, and the eluting strength

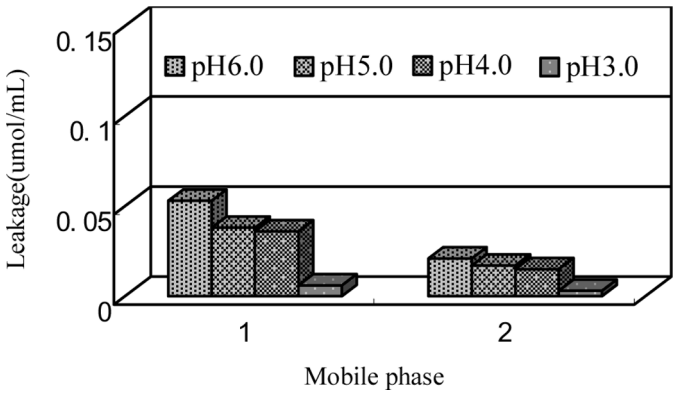

FIG. 4. Leakage of immobilized metal Cu^{2+} Column: L-Glu-Cu(II) column (100 mm × 4.6 mm I.D.). Mobile phase 1: A 20 mM PB + 0.05 M NaCl B; 20 mM PB + 0.5 M NaCl + 4 mM Gly. Mobile phase 2: A 20 mM NaAc + 0.05 M NaCl B; 20 mM NaAc + 0.5 M NaCl + 3 mM Gly. Other conditions are the same as in Fig. 2.

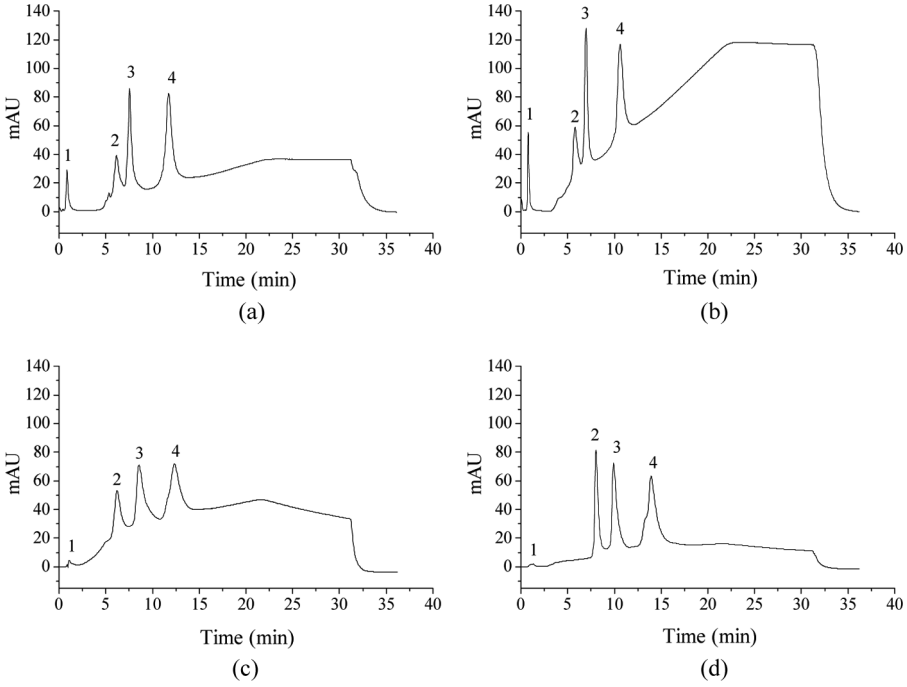

FIG. 5. Chromatograms of proteins on L-Glu-Cu(II) column in the competitive elution system 1. BSA; 2. RNase; 3. Cyt-C; 4. Lys a: A 20 mM PB + 0.05 M NaCl (pH 6.0) B 20 mM PB + 0.5 M NaCl + 20 mM Imid (pH 6.0). b: A 20 mM NaAc + 0.05 M NaCl (pH 6.0) B 20 mM NaAc + 0.5 M NaCl + 60 mM Imid (pH 6.0). c: A 20 mM PB + 0.05 M NaCl (pH 4.0); B 20 mM PB + 0.5 M NaCl + 4 mM Gly (pH 4.0). d: A 20 mM NaAc + 0.05 M NaCl (pH 4.0); B 20 mM NaAc + 0.5 M NaCl + 3 mM Gly (pH 4.0). Other conditions are the same as in Fig. 2.

was also high. Four kinds of proteins were completely separated and "narrow and sharp" peaks were obtained. Using Gly as eluant, "short and broad" peaks were obtained. BSA could not be eluted (3). Using Gly as a competitive agent, the concentration of Gly and the pH of the solution were all lowered as compared with Imid.

This is because Gly is a stronger eluting agent. The complexing constant of Gly with Cu^{2+} ($lgk_{Gly\text{-}Cu(II)} = 8.08$) (14) is much greater than that of Imid with Cu^{2+}($lgk_{Imid\text{-}Cu(II)} = 4.13$) (15). Therefore, the leakage of the immobilized metal Cu^{2+} is serious if Gly is used as eluant. The leakage of Cu^{2+} can be reduced by decreasing the concentration of Gly and the pH of the solution. However, too low pH and eluent concentration will make the elution efficiency decrease.

In summary, the PB system containing 20 mM Imid (pH 6.0) would be the best choice for operating conditions.

Separation Comparison of Proteins on L-Glu-Cu(II) and IDA-Cu(II) Columns

Figure 6 is a chromatogram of proteins on the IDA-Cu(II) column obtained under the optimum conditions. Under the individual chromatographic conditions, the separation performance of L-Glu-Cu(II) column for proteins (Fig. 5a) was better than that of IDA-Cu(II) column. BSA and Lys on the IDA-Cu(II) column could not be fully eluted. Moreover, RNase and Cyt-C could not be effectively separated. On the other hand, the leakage of Cu^{2+} on L-Glu-Cu(II) column was lower than that on the IDA-Cu(II) column under individual optimum eluting conditions. The leakages of Cu^{2+} on these two columns were 0.029 μmol/mL and 0.043 μmol/mL, respectively. This is

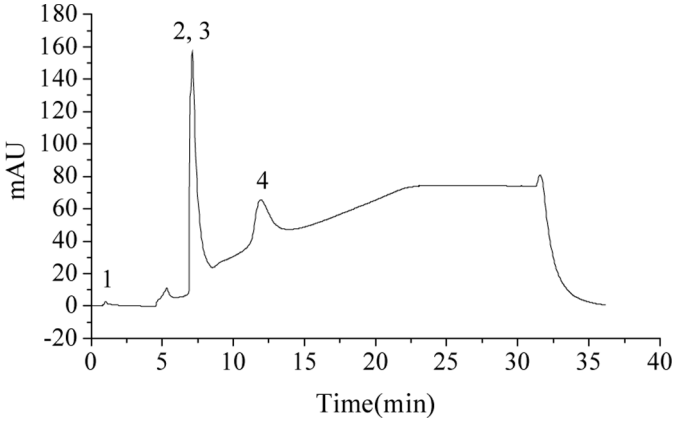

FIG. 6. Chromatogram of proteins on IDA-Cu(II) column in the competitive elution system 1. BSA; 2. RNase; 3. Cyt-C; 4. Lys Column: IDA-Cu(II) column (100 mm × 4.6 mm I.D.). Mobile phase: A 20 mM PB + 0.05 M NaCl (pH 6.0); B 20 mM PB + 0.5 M NaCl + 40 mM Imid (pH 6.0). Other conditions are the same as in Fig. 2.

because IDA is a strong complexing agent, the stability constant of IDA with Cu^{2+} ($lgk_{IDA\text{-}Cu(II)} = 10.62$) (16) is greater than that of L-Glu with Cu^{2+} ($lgk_{L\text{-}Glu\text{-}Cu(II)} = 8.20$) (17). Therefore, the bonding capacity of Cu^{2+} on IDA-Cu(II) column (17.69 $\mu mol/g_{gel}$) was also higher than that of L-Glu-Cu(II) column (11.57 $\mu mol/g_{gel}$). Adsorbed protein on IDA-Cu(II) column was difficult to be eluted in comparison with the L-Glu-Cu(II) column. Consequently, the concentration of Imid should be increased in order to obtain efficient elution. However, the poor separation and high leakage of Cu^{2+} would be caused by increasing the concentration of Imid.

It follows that the copper chelate with higher stability constant is not always the best choice of metal chelate ligand. In order to ensure a lesser leakage of Cu^{2+}, the effective elution and separation of proteins on the metal chelate Cu(II) column, it is ideal that the coordination intensity between the chosen ligand and immobilized Cu^{2+} should be moderated. These studies may provide certain reference values for investigating the retention mechanism in MCAC, screening and application of metal chelate ligands.

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