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Chromatographic separation of proteins on metal immobilized iminodiacetic acid-bound molded monolithic rods of macroporous poly(glycidyl methacrylate–co-ethylene dimethacrylate)

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Abstract

Continuous rod of macroporous poly(glycidyl methacrylate–co-ethylene dimethacrylate) was prepared by a free radical polymerization within the confines of a stainless-steel column. The epoxide groups of the rod were modified by a reaction with iminodiacetic acid (IDA) that affords the active site to form metal IDA chelates used for immobilized metal affinity chromatography (IMAC). The efficiency of coupling of IDA to the epoxide-contained matrix was studied as a function of reaction time and temperature. High-performance separation of proteins, based on immobilized different metals on the column, were described. The influence of pH on the adsorption capacity of bovine serum albumin on the Cu^{2+}–IDA continuous rod column was investigated in the range from 5.0 to 9.0. Purification of lysozyme from egg white and human serum albumin (HSA) on the commercially available HSA solution were performed on the naked IDA and Cu^{2+}–IDA continuous rod columns, respectively; and the purity of the obtained fractions was detected by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry.

Keywords: Monolithic rods; Immobilized metal affinity chromatography; Protein; Iminodiacetic acid

1. Introduction

Since its introduction in 1975 [1], immobilized metal ion chelating affinity chromatography (IMAC) has quickly become one of the powerful separation techniques for the purification of proteins and peptides in both analytical and large-scale modes [2–4]. It takes advantage of the selective interaction between immobilized metal ions and the functional groups of amino acids located at the surface of biomolecules, such as the imidazole group of histidine, thiol group of cysteine, and indolyl group of tryptophan [1,2]. IMAC has many advantages over typical methods in affinity chromatography: different metal ions can be immobilized on the same chelating medium after striping off the column and can be easily removed for regeneration by a stronger chelator such as EDTA, for hundreds of times, without any detectable loss of metal chelating properties [2,6]; various carboxymethylated amines, including iminodiacetic acid (IDA) [3–9], tris(carboxymethyl)ethylenediamine (TED) [10–12], nitrolotriacetic acid (NTA) [13–17], and carboxymethylated aspartate (CM-ASP) [2], can be used as chelating

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ligands for immobilization of metal ions. Thus, the optimal conditions for separation of target proteins can be obtained by proper choice of suitable metal ions as well as chelating ligand. Proteins can still keep their biological activity after elution from an IMAC column. Furthermore, IMAC can be used for the removal of metal ions from metalloproteins [18].

At present, most of the matrices used in IMAC are macroporous bead-shaped particles such as agarose, cross-linked dextran and silica. Inherent limitations in bead packed column liquid chromatography include relatively time-consuming and high pressure packing process, high pressure drop of the columns and the slow diffusion of solutes within the pores of the bead matrix. Several approaches have been proposed to overcome these shortcomings. Perfusion chromatographic support developed by Afeyan and co-workers [19,20] is a highly porous matrix with very large pores of up to 1 μm, which greatly reduce the back pressure and improve the kinetics of the separation process. Membrane chromatography (MC) is a ''hybrid'' separation method that combines the advantages of the membrane separation technique and the column chromatography. As a result of the convective flow of the solution through the pores, the mass transfer resistance is tremendously reduced [21–27]. Recently, Fréchet et al. [28–31] developed a novel method for the preparation of ''molded'' monolithic continuous rods of rigid, macroporous polymer column. This new type of chromatographic medium was prepared by an ''in situ'' polymerization within the confines of a chromatographic column or a capillary column, which affords the final product-desired geometry and requires no tedious packing operation. Many applications have been reported for the separation of biomolecules in a variety of chromatographic modes [32–39].

In this work, iminodiacetic acid (IDA)-type adsorbent was prepared by covalently coupling of IDA to the monolithic continuous rods of macroporous poly(glycidyl methacrylate–co-ethylene dimethacrylate). The reaction conditions for coupling of IDA group to the rods were investigated. Different metal ions, including Cu^{2+}, Zn^{2+}, Ni^{2+}, were immobilized on these columns and used for the separation of proteins. The influence of pH on the adsorption capacity of BSA on the Cu^{2+}–IDA column was studied in detail. The naked IDA and Cu^{2+}–IDA columns were used for the separation of lysozyme (Lys) from egg white and commercially available human serum albumin (HSA) for therapeutic usage, respectively. The purity of Lys and HSA fraction was detected by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI–TOF-MS).

2. Experimental

2.1. Materials

Glycidyl methacrylate (Suzhou Chemical Plant, Suzhou, China) and ethylene dimethacrylate (Sigma, St Louis, MO, USA) were distilled under vacuum. IDA was purchased from Sigma. HSA (20%) for therapeutic usage was obtained from Kangbao Biological Product Co. (Shanxi, China). All other proteins were purchased from Sigma. Fresh egg samples were purchased from a local supermarket (Dalian, China). All protein standards were dissolved in buffer containing 75 mmol/l sodium chloride, 20 mmol/l potassium phosphate buffer, pH 7.0. Azobisisobutyronitrile (AIBN) and dodecyl alcohol were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Cyclohexanol was obtained from Beijing Chemical Reagent Co. (Beijing, China). All other chemical reagents were analytical grade. Solutions were prepared in double-distilled water.

2.2. Instruments

A Model 7518-10 peristaltic pump (ColeParmer, USA) and Elite P200II (Dalian, China) were used to carry out the in situ modification of the continuous rod column. A Waters HPLC system consisting of two 515 HPLC pumps, and a 2487 dual-wavelength UV detector (Milford, MA, USA), was used to carry out all the chromatographic experiments. The data were acquired and processed with WDL-97 chromatographic workstation (National Chromatographic R & A Center, Dalian, China).

2.3. Preparation of continuous column

The continuous rod column was prepared by an

''in situ'' polymerization within the confines of a stainless-steel tube of a 50×4 mm I.D. chromatographic column. The free-radical initiator (AIBN, 1%, w/v) was dissolved in 25:75 (v/v) mixture of monomers (glycidyl methacrylate and ethylene dimethacrylate, 3:1, v/v) and porogenic diluents (dodecyl alcohol and cyclohexanol, 1:11, v/v) and the mixture was purged with nitrogen for 15 min. The stainless-steel tube was filled with the above mixture and then sealed at both ends with close column heads. The polymerization was allowed to proceed at 50°C for 12 h. The close heads were removed, the column was provided with fittings, attached to the HPLC system and heated to 40°C. Tetrahydrofuran (THF) (30 ml) was pumped through the column at a flow-rate of 0.25 ml/min to remove the porogenic diluents and other soluble compounds present in the polymer rod.

2.4. Synthesis of IDA-type adsorbents

The scheme for coupling of IDA on poly(glycidyl methacrylate–co-ethylene dimethacrylate) continuous rod column was shown in Fig. 1. The column was attached to a peristaltic pump and washed with water. A 5 g disodium iminodiacetate and 1 g NaCl were dissolved in 50 ml 2 mol/l Na_2CO_3 buffer by adjusting pH to 10.5 with NaOH. The prepared solution was pumped through the column at a flow-rate of 0.3 ml/min under recirculation. After the reaction was completed, the resulting IDA rod column was washed successively with water, aqueous 50 mmol/l EDTA, and again water. The amount of immobilized Cu^{2+}, Ni^{2+} and Zn^{2+} on a gram of IDA rod are about 101.6 μmol, 84.7 μmol and 81.2 μmol, respectively.

2.5. Porous properties

The rod inside the column was pushed out of the tube carefully and dried at 70°C for 24 h and cut to pieces with a razor blade. The porosity of the rod was determined by a mercury intrusion porosimetry and its specific surface area was calculated from nitrogen adsorption/desorption isotherms using a combined BET sorptometer and mercury porosimeter (9310 Mercury Porosimeter, USA).

The piece of rod was placed on a sticky carbon foil that was attached to a standard aluminum specimen stub. The carbon foil used was to increase conductivity. The sample was vapor-deposited with gold (Eiko IB3 Ion Coater, USA). Microscopic analysis of the sample was carried out in an Amary Scanning Electron Microscope (Model 1000B, USA) at 15 keV.

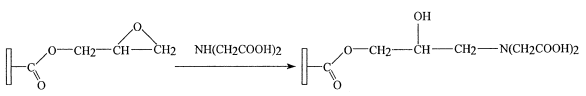

Fig. 1. Scheme for coupling of iminodiacetic acid on poly(glycidyl methacrylate–co-ethylene dimethacrylate) continuous rod column.

2.6. Chromatographic procedures

Columns were washed successively with water, aqueous 50 mmol/l EDTA and water. Thereafter, the stationary phase was loaded with the metal by perfusing the column with 50 ml of 50 mmol/l solution of corresponding metal salt in water. Subsequently, the column was washed with 50 ml water and the equivalent volume of the eluent buffer as used in the subsequent experiments. The metal was removed from the stationary phase by perfusing the column with 50 ml of 50 mmol/l EDTA solution and water prior to loading with other metal solution. All the procedure was performed at a flow-rate of 1.0 ml/min.

A solution of bovine serum albumin (BSA) (0.1 mg/ml) in 20 mmol/l phosphate buffer containing 1.0 mol/l NaCl was used for frontal analysis at a flow-rate of 1.0 ml/min to determine the adsorption capacity of BSA on Cu^{2+}–IDA continuous rod column.

2.7. Purification of lysozyme from egg white and HSA solution

Egg white was obtained from fresh egg and dissolved in phosphate-buffered saline (PBS) at 1:4 dilution. The naked IDA-bound rod column was used to separate lysozyme from egg white. The egg white sample was loaded with equilibrium buffer (20 mmol/l sodium phosphate buffer, pH 7.0). Then the

column was washed with a salt gradient and the fraction were collected and assayed.

IDA continuous rod column was loaded with Cu^{2+} by the method mentioned above. A diluted HSA solution (10 mg/ml) was injected into the column equilibrated with 20 mmol/l phosphate buffer containing 1.0 mol/l NaCl (pH 7.0). The column was eluted with an imidazole gradient and the purified HSA was collected and assayed.

2.8. MALDI–TOF-MS measurements

MALDI–TOF-MS is a very useful tool for qualitative analysis of protein sample, and in some cases the quantitative analysis of proteins can be performed [40]. MALDI–TOF-MS measurements were performed on a BIFLEX time-of-flight mass spectrometer (Bruker–Franzen Analytik, Bremen, Germany) equipped with a SCOUT ion source, operating in the positive linear mode. Ions formed by a pulsed UV laser beam (nitrogen laser, λ=337 nm) were accelerated at 20 keV. The UV laser light (energy about 50 μJ) was forced on to the sample using a focal diameter varying from 100 to 300 μm. In this case, laser power attenuation of 50% was used. The matrix was sinapinic acid, dissolved in acetonitrile–water (50:50, v:v) at a concentration of about 5×10^{-2} mol/l. For MALDI measurements, samples were dissolved in 0.1% trifluoroacetic acid aqueous solution at a concentration of about 6 mg/ml and diluted five times with the same solvent. A volume of 5 μl of the final sample solution were added to the matrix solution and about 1 μl of this mixture was deposited on the stainless-steel multiprobe and allowed to dry before introduction into the mass spectrometer. Mass spectra were obtained and averaged over 100 shots; three independent MALDI measurements were made for each sample to evaluate reproducibility. Mass accuracy ranging from 0.01% to 0.1% was always obtained.

3. Results and discussion

For practical uses, the back-pressure of a column should be as low as possible. A matrix with broad pores can reduce flow resistance and total surface area, matrix with small pores can do it on the contrary. ‘‘Molded’’ continuous rod materials provide broad channels for mobile phase flow-through, so very low flow resistance is observed. However, the adsorption capacity of the matrix is reduced simultaneously with increasing pore size, which will lead to the low surface area. Therefore, a proper balance must be found between the requirements of low flow resistance and high surface area. Many publications indicate that the pore size distributions of the molded monoliths could be controlled through the optimization of the polymerization conditions [41–43].

Porous properties of the monolithic rod prepared in this study were investigated. According to mercury intrusion porosimetry, the average pore size of this rod was 769 nm and its specific surface area was 89.1 m^2/g. The total pore volume was 3.3 ml/g and represented a porosity over 75%,. This result indicates the pore volume and size of the prepared monolithic rod were sufficiently large to ensure a modest resistance to the mobile phase, and the back-pressure of the continuous rod was about 6.5 mPa at the flow-rate of 9.0 ml/min. The morphology of the monolithic rod was closely related to porous structure, which was a direct consequence of the polymerization conditions. The scanning electron micrograph for the internal structure of the rod is shown in Fig. 2.

A significant aspect in the design of chemically bonded phases for separation of proteins by IMAC is to select the chelate ligand for bonding on the

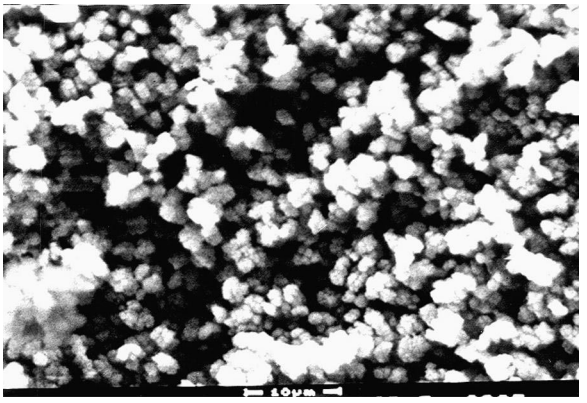

Fig. 2. Scanning electron micrograph of the inner part of the poly(glycidyl methacrylate–co-ethylene dimethacrylate) monolith.

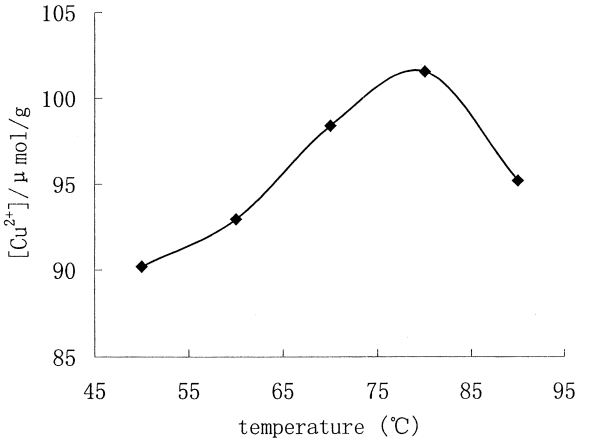

Fig. 3. Influence of reaction temperature on the IDA amount of coupling to the epoxy-contained monolithic rod.

supports. As active site, IDA functionality was chosen because of its high complexation constants toward metals such as Cu^{2+} and previously successful experiments in IMAC [3–5]. The strong binding of metals to the IDA chelating groups minimizes bleeding of metal from the column. The efficiency of coupling of IDA to the epoxide-contained monolithic rod was greatly influenced by the reaction conditions, such as pH, temperature, and reaction time etc. The effect of pH on the coupling efficiency of IDA to epoxide-activated gel was previously studied in detail by Hemdan et al. [3] and the optimum pH

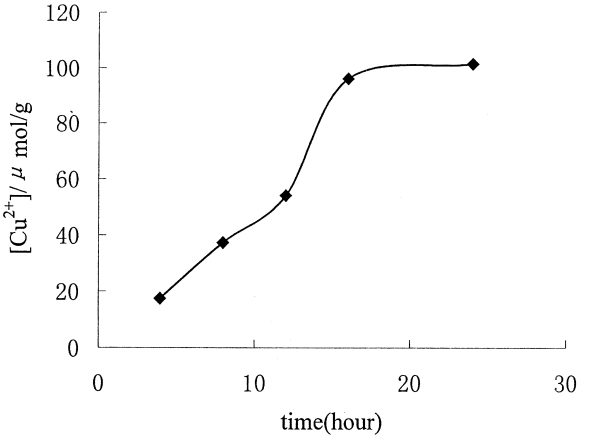

Fig. 4. Influence of reaction time on the IDA amount of coupling to the epoxy-contained monolithic rod.

for directly coupling of IDA to the gel was 10.5. In this study, the effect of temperature and reaction time on the coupling efficiency of IDA to macroporous poly(glycidyl methacrylate–co-ethylene dimethacrylate) monolithic rod column was investigated. As shown in Fig. 3 and Fig. 4, the coupling efficiency reaches maximum at 70–80°C and it increased with prolonging the reaction time from 0 to 16 h, but did not increase much with further prolonging of the reaction time. It means that the reaction time should be around 16 h at the given conditions. Therefore, the optimum conditions for directly coupling of IDA to the monolithic rod were as follows: pH 10–11, temperature 70–80°C and reaction time at least 16 h.

In principle, any metal ion with an excess of d-electrons is suitable for IMAC. By far, the most often used metals are Ni^{2+}, and Cu^{2+} followed by Zn^{2+}. In most cases, $Cu^{2+}>Ni^{2+}>Zn^{2+}$ represents the general decreasing order of proteins binding to the metal–IDA stationary phase [44]. In this study, the retention behavior of proteins on continuous rods bearing metal IDA chelates was investigated using gradient elution with increasing sodium chloride concentration in 20 mmol/l phosphate buffer at pH 7.0. Similar experiments were carried out on a naked IDA column but with 5 mmol/l EDTA in the eluents, and the obtained retention data of standard protein samples were shown in Table 1. The results are consistent with the order of binding strength described above.

Without metal ion, the IDA-bound stationary phase is expected to be a cation-exchanger under the experimental conditions. Therefore, the protein mixture, consisting of BSA (isoelectric point, pI=4.98), α-chymotrypsinogen A (pI=9.5), cytochrome *c* (pI=10.6), and lysozyme (pI=11.0) was chromatographed on a naked IDA column. Typical chromatogram obtained at pH 7.0 was illustrated in Fig. 5. From the retention data listed in Table 1 and the chromatogram presented in Fig. 5, it can be seen that proteins are retained in order of increasing isoelectric point at pH 7.0, which is parallel with that on a typical weak cation-exchangers with fixed carboxylic functions of pK_a 4.0–5.0. However, the retention behavior of proteins is dramatically altered upon chelation of Cu^{2+} on the stationary phase as shown in Table 1. Most proteins under consideration are

Table 1
Retention volumes of standard protein samples on the naked IDA and metal chelated columns

Column	Retention volume (ml)						
	BSA	Lys	Cyt-*c*	Myo	Trypsin	α-Chy	HSA
IDA	**	7.81	5.33	**	3.39	3.42	**
Cu^{2+}–IDA	*	*	5.78	*	4.58	4.66	*
Zn^{2+}–IDA	**	8.58	5.11	**	3.35	3.71	**
Ni^{2+}–IDA	**	10.28	4.94	**	3.99	4.06	**

Experimental conditions: Column, 50×4 mm I.D.; flow-rate, 1.0 ml/min. A volume of 20 μl of the mixed sample solution was injected with loading buffer. Linear gradient in 6 min from 0 to 0.5 mol/l sodium chloride in 20 mmol/l phosphate buffer at pH 7.0, detection wavelength 280 nm. *Not eluted; **Not retained. Cyt-*c*, cytochrome *c*; Myo, myoglobulin; α-Chy, α-chymotrypsin.

retained very strongly by Cu^{2+}–IDA and only α-chymotrypsinogen A, cytochrome *c* and trypsin are eluted under the same conditions. On the other hand, the similar retention behavior of proteins on Zn^{2+}–IDA and Ni^{2+}–IDA with that on naked IDA column was observed. Fig. 6 and Fig. 7 showed the typical chromatograms of proteins on Zn^{2+}–IDA and Ni^{2+}–IDA continuous rod column, respectively. The observed retention values depend not only on the strength of protein–metal chelate interactions but also on the amount of metal bound under operating conditions to the IDA ligates. The amount of immobilized Cu^{2+}, Ni^{2+} and Zn^{2+} on IDA rod was measured and they are about 101.6 μmol, 84.7 μmol and 81.2 μmol/g of IDA rod, respectively. Which means that the amount of Cu^{2+} is higher than that of

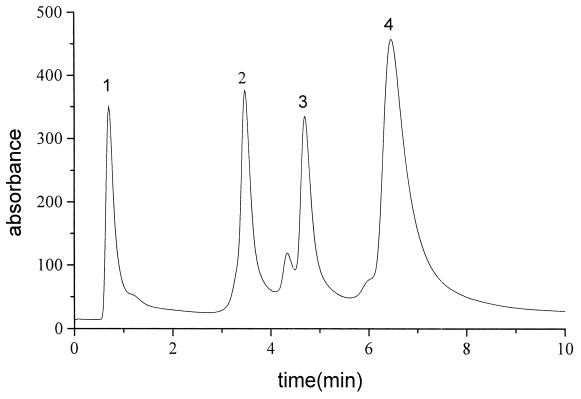

Fig. 5. Chromatogram of proteins on a naked IDA column. Experimental conditions: Column, 50×4 mm I.D.; flow-rate, 1.0 ml/min. A volume of 20 μl of the mixed protein solution was injected with loading buffer. Linear gradient in 6 min from 0 to 0.5 mol/l sodium chloride in 20 mmol/l phosphate buffer at pH 7.0; detection wavelength, 280 nm. Peaks: 1=BSA; 2=trypsin; 3=cytochrome *c*; 4=Lys.

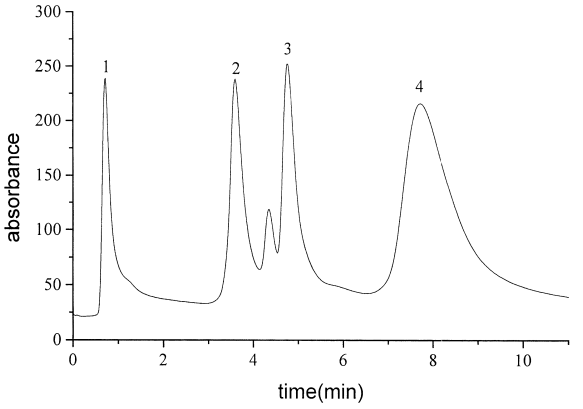

Fig. 6. Separation of proteins by IMAC on Zn^{2+}–IDA column. Experimental conditions were the same as in Fig. 5.

Ni^{2+} and Zn^{2+}. According to the previous reports [4,5,45], the binding constants of IDA to metal ions in free solution and immobilized form are similar,

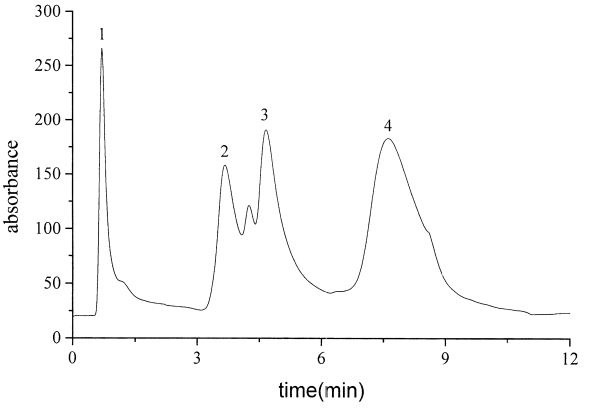

Fig. 7. Separation of proteins by IMAC on Ni^{2+}–IDA column. Experimental conditions were the same as in Fig. 5.

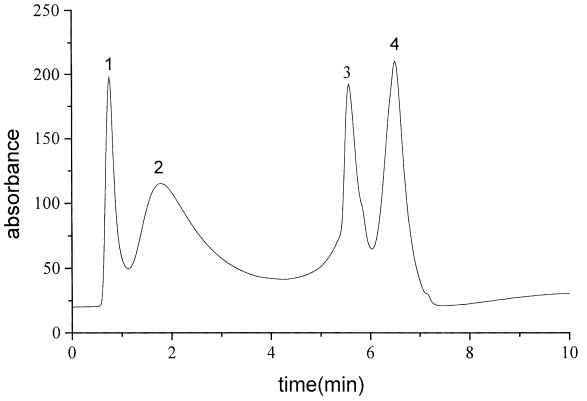

Fig. 8. Separation of proteins by IMAC on Cu^{2+}–IDA continuous rod column. Experimental conditions: Linear gradient in 6 min from 0 to 50% B; eluent A, 20 mmol/l phosphate buffer containing 1.0 mol/l sodium chloride (pH 7.0); eluent B, eluent A containing 100 mmol/l imidazole (pH 7.0). Peaks: 1=trypsin; 2=Lys; 3=BSA; 4=myoglobin.

and the strength of binding follows the order $Cu^{2+}>Ni^{2+}>Zn^{2+}$. The difference in the retention behavior of proteins between Cu^{2+}–IDA and Ni^{2+}, Zn^{2+}–IDA is mainly caused from their binding properties.

The retention behavior of proteins on Cu^{2+}–IDA chelated continuous rod was investigated using gradient elution with increasing imidazole concentration in 20 mmol/l phosphate buffer containing 1.0 mol/l NaCl at pH 7.0. Fig. 8 shows the separation of mixed proteins on Cu^{2+}–IDA continuous rod column. It can be seen that the four proteins were separated within 10 min. Trypsin was almost not retained under the experimental conditions, which may caused from the adoption of mobile phase with high salt concentration and the action of imidazole proton pump [46].

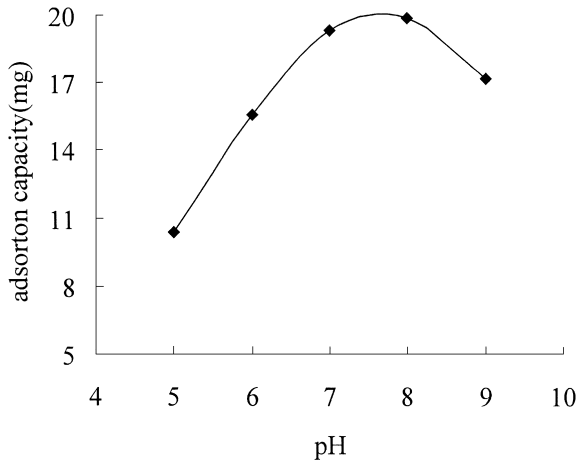

Fig. 9. Influence of pH on the adsorption capacity of BSA on Cu^{2+}–IDA continuous rod column.

The main purpose of the equilibration buffer is to provide optimal conditions for adsorption of the target protein. In IMAC, the most important factor that affects protein adsorption is pH [44,45]. In this study, the effect of pH of the equilibration buffer on the adsorption capacity of BSA on Cu^{2+}–IDA rod column was investigated and obtained results were shown Fig. 9. It can be seen that the adsorption capacity of BSA increased with increasing pH from 5 to about 7.5 and decreased with further increase of pH to 9, which is consistent with the results obtained by Porath et al. [1] and Hansen et al. [45]. At pH values below 7.0, there is weaker binding due to increased protonation of the amino functions of the protein, whereas above 8.0 there may again be reduced affinity, this time caused by the evolution of a negative charge at chelate that is the high affinity binding site of BSA to the metal ions [9].

Chicken eggs are major dairy products rich in protein contents. Egg whites account for about 58% of the entire egg mass, with 10–12% of the mass being water. Among the proteins in egg whites, ovalbumin, ovomucoid, globulins and conalbumin are the major components constituting 54, 11, 10 and 13%, respectively, with lysozyme as the minor component of about 3.5% [47]. Therefore, egg white is an ideal feed stock for the separation of multiprotein. Levison et al. [48] reported large-scale separation of ovalbumin from egg white using Whatman DE-92 anion-exchange cellulose. In this study, the naked IDA continuous rod column was used for the separation of lysozyme in a single step. Fig. 10 showed the chromatogram of hen egg white proteins on the naked IDA monolithic column. As we can see that the non-bound fraction consisted of most of the egg white proteins. Egg white lysozyme has a pI of 11.1, and possesses net positive charge under the experiment conditions. Whereas ovomucoid (pI=4.0), ovalbumin (pI=4.6), globulin (pI=5.5–5.8) and conalbumin (pI=6.6) possess net negative charge under the given conditions, so they did not retain on the naked IDA column. The purity of

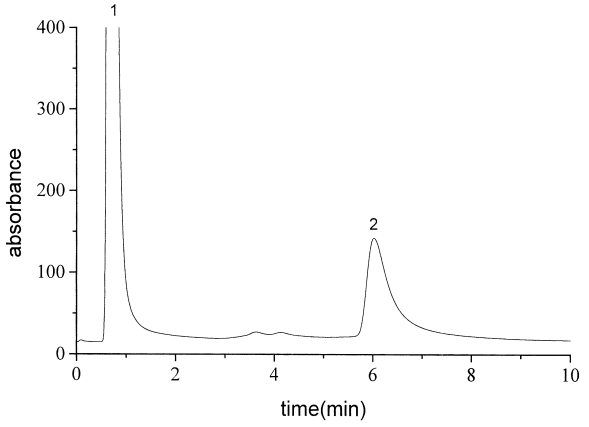

Fig. 10. Chromatogram of egg white on the naked IDA monolithic column. Experimental conditions: 20 μl of the sample solution was injected with loading buffer. Linear gradient in 6 min from 0 to 0.5 mol/l sodium chloride in 20 mmol/l phosphate buffer at pH 7.0, detection wavelength 280 nm. Other conditions were the same as in Fig. 5. Peaks: 1=impurity proteins; 2=Lys.

lysozyme fraction was detected by MALDI–TOF-MS, and the obtained results were compared with the egg white mass spectrum in Fig. 11. The peaks with mass-to-charge ratios about 14 000 and 7000 in Fig. 11b were attributed to the lysozyme with positive charges of 1 and 2, respectively. The results from MALDI–TOF-MS showed that almost all of the impurities in egg white were removed and the purity of the obtained lysozyme was quite high after a single-step purification by naked IDA continuous rod column.

HSA commonly used for therapeutic purposes, such as shock, heavy loss of blood etc., requires relatively high purity for use. Yang et al. [24] and Andersson et al. [49] have reported the purification of HSA for therapeutic usage on the immobilized Ni^{2+}–IDA column. The results of assay by electrophoresis indicated that HSA for therapeutic use was further purified to almost one lane from three lanes after three cycles of chromatography on Ni^{2+}–IDA–Sepharose 6B column. In this study, we purified HSA for injection use obtained from Kangbao Biological Product Co. on Cu^{2+}–IDA continuous rod column and the chromatogram was shown in Fig. 12. The purity of HSA fraction was assayed by MALDI–TOF-MS, and the obtained results were compared with the mass spectrum of crude HSA (as shown in

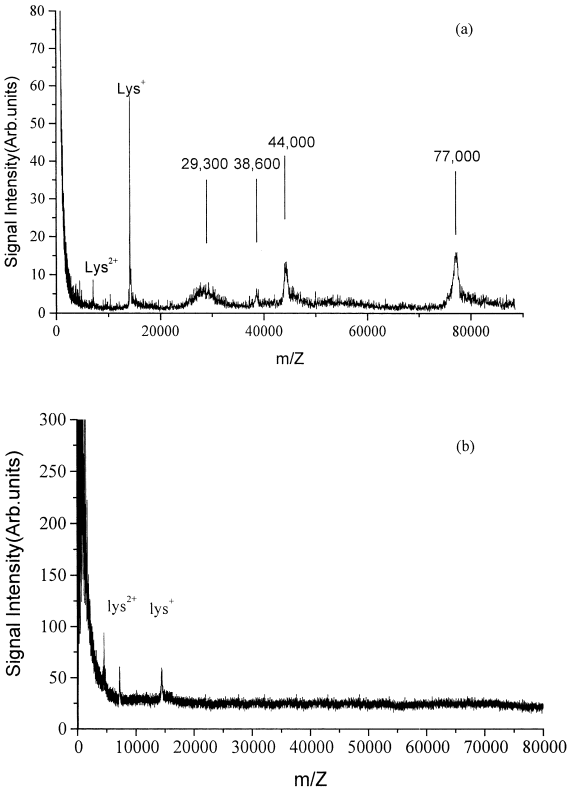

Fig. 11. MALDI–TOF mass spectrum for (a) egg white and (b) fraction of lysozyme purified on the naked IDA continuous rod column.

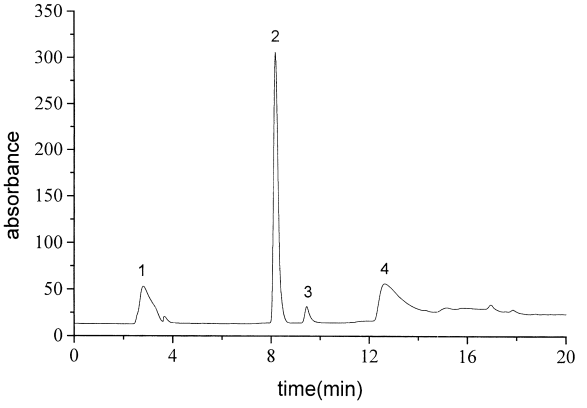

Fig. 12. Chromatogram of crude HSA on the Cu^{2+}–IDA continuous rod column. Experimental conditions: Linear gradient in 15 min from 0 to 50% B in A. Other experiment conditions were the same as in Fig. 7. Peaks: 1, 3, 4=impurities; 2=HSA.

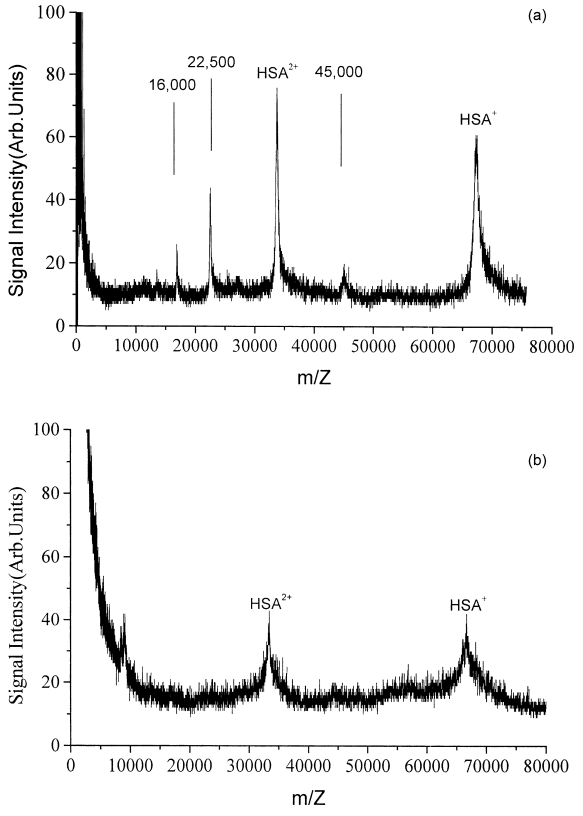

Fig. 13. MALDI–TOF mass spectrum for (a) crude HSA and (b) fraction of HSA purified on Cu^{2+}–IDA continuous rod column.

Fig. 13). The results from MALDI–TOF-MS showed that almost all of the impurities were removed and the crude HSA was purified efficiently.

4. Conclusion

The use of iminodiacetic acid (IDA)-bound macroporous poly(glycidyl methacrylate–co-ethylene dimethacrylate) continuous rod column for HPIMAC was demonstrated. The efficiency of directly coupling of IDA to the epoxy-contained monolithic rod was studied as a function of reaction time and temperature and the optimum conditions were pH 10–11, temperature 70–80°C, and reaction time at least 16 h. The rod was characterized by the specific surface area as 89.1 m^2/g support and the average pore size as 769 nm. The presence of very large pores in the micrometer range allowed all of the mobile phase to flow through at extremely low back pressure. The back pressure of the continuous rod was only about 6.5 MPa when the flow-rate reached 9.0 ml/min. It was much lower than that of the conventional small particles packed column. High-performance separation of proteins, based on immobilized different metals on the column, were described. The influence of pH on the adsorption capacity of BSA on the Cu^{2+}–IDA continuous rod column was investigated in the range from 5.0 to 9.0. The naked IDA and Cu^{2+}–IDA continuous rods were applied to purify the lysozyme purified from egg white and HSA solution, respectively; and purity of the obtained fraction was detected by MALDI–TOF-MS.

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