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ABSTRACT

Comonomer and/or metal-chelating ligand N-methacryloyl-(L)-histidine-methylester (MAH) was synthesized by using methacryloyl chloride and L-histidine methyl ester. Spherical beads with an average diameter of 75–125 μm were produced by suspension polymerization of 2-hydroxyethyl methacrylate (HEMA) and MAH carried out in an aqueous dispersion medium. Poly(HEMA-MAH) beads had a specific surface area of 18.3 m^2/g. Elemental analysis of MAH for nitrogen was estimated as 895 μmol/g of polymer. Then the beads were loaded with different metal ions (i.e. Zn^{2+}, Cu^{2+}, Ni^{2+}) to form the metal chelate. The effect of pH, concentration of lysozyme, and metal type on the

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adsorption of lysozyme to the metal-chelated beads was examined in a batch reactor. Purification of lysozyme from egg-white was also investigated. Maximum lysozyme adsorption capacity of poly(HEMA-MAH) beads was found to be 8.7 mg/g at pH 7.0 in phosphate buffer. Lysozyme adsorption capacity of Zn^{2+}, Cu^{2+}, and Ni^{2+}-chelated beads was higher than that of non-chelated beads. The maximum capacities of Ni^{2+}, Zn^{2+}, or Cu^{2+}-chelated beads were 11.5, 12.6, and 37.1 mg/g, respectively. A significant amount of the adsorbed lysozyme (up to 97%) was eluted in 1 h in the elution medium containing 25 mM EDTA at pH 4.9. Repeated adsorption-desorption process showed that this novel metal chelated beads are suitable for lysozyme adsorption. Purification of lysozyme was monitored by determining the lysozyme activity using *Micrococcus lysodeikticus* as substrate. The purity of the desorbed lysozyme was about 80% with recovery about 75%.

Key Words: Affinity chromatography; Lysozyme separation; Metal-chelate affinity beads; Histidine; Poly(HEMA).

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INTRODUCTION

Immobilized metal ion affinity chromatography (IMAC) has become a widespread analytical and preparative separation method for therapeutic proteins, peptides, nucleic acids, hormones, and enzymes.[1–5] Many transition metals can form stable complexes with electron-rich compounds and may coordinate molecules containing O, N, and S by ion dipole interactions. Metal ion ligands are first-row transition metal ions (Zn^{2+}, Ni^{2+}, Cu^{2+}, and Fe^{3+}) incorporated by iminodiacetic acid, nitrilotriacetic acid, and tris(carboxymethyl)ethylene-diamine. Using IMAC introduces a new approach for selectively interacting materials on the basis of their affinities for chelated metal ions. The separation is based on the interaction of a Lewis acid (electron pair acceptor), i.e., a chelated metal ion, with electron donor atoms (N, O, and S) on the surface of the protein.[6–8] Proteins are assumed to interact mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Cooperation between neighboring amino acid side chains and local conformations plays an important role in protein binding. Aromatic amino acids and the amino terminal of the peptides also have some contributions.[9] The low cost of metals and the reuse of adsorbents for hundred of times without any detectable loss of metal-chelating properties are the attractive features of metal affinity separation.

Lysozyme is found in a variety of vertabrate cells and secretions, such as spleen, milk, tears, and egg white. Lysozyme lyses certain bacteria by

hydrolyzing the β linkages between the muramic acid and N-acetylglucosamine of the mucopolysaccharides, which are present in the bacterial cell wall. Its common applications are as a cell disrupting agent for extraction of bacterial intracellular products, as an antibacterial agent in ophthalmologic preparations, as a food additive in milk products, and as a drug for treatment of ulcers and infections.[10] The potential for its use as an anticancer drug has been demonstrated by animal and in-vitro cell culture experiments.[11] Lysozyme has also been used in cancer chemotherapy.[12] In a recent article, it was reported that lysozyme can be used for increasing the production of immunoglobulin by hybridoma technology.[13] The large-scale applications require more efficient and cost-effective techniques for its isolation.

In this study, we propose N-methacryloyl-(L)-histidine-methylester (MAH) as a metal-chelating ligand for use in the IMAC for lysozyme. The imidazole group of MAH has a chelating property with transition metal ions. Poly(2-hydroxyethyl methacrylate-N-methacryloyl-(L)-histidine methylester) [poly(HEMA-MAH)] beads were produced by suspension polymerization of MAH and HEMA. Three different metal ions Cu^{2+}, Zn^{2+}, and Ni^{2+} were chelated on the beads, then they were tested for their propensity to adsorb lysozyme in a batch system. Then, lysozyme adsorption on the beads from aqueous solutions containing different amounts of lysozyme, at different pHs were performed. Desorption of lysozyme and reusability of these metal-chelate affinity adsorbents were also tested. Finally, the metal-chelated affinity beads were used for the purification of lysozyme from egg white. The purity of the desorbed lysozyme was determined by Bio-LC and the activity of the desorbed lysozyme was measured using *Micrococcus lysodeikticus* as a substrate microorganism.

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EXPERIMENTAL

Materials

Lysozyme (chicken egg white, EC 3.2.1.7), L-histidine methylester dihydrochloride (97%), and methacryloyl chloride were supplied by Sigma (St Louis, MO, USA). 2-Hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. Benzoyl peroxide (BPO) was obtained from Fluka. Poly(vinyl alcohol) (PVAL; MW: 100.000, 98% hydrolyzed) was supplied from Aldrich Chem. Co. (USA). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead

(Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

Preparation of Poly(HEMA-MAH) Beads

Details of the preparation and characterization of the MAH and poly (HEMA-MAH) beads were reported elsewhere.[14] Firstly, N-methacryloyl-(L)-histidine-methylester (MAH) was synthesized by methacryloyl chloride and histidine methylester dihydrochloride. Briefly, 5.0 g of L-histidine methylester dihydrochloride and 0.2 g of hydroquinone (i.e., polymerization inhibitor) were dissolved in 100 mL of dichloromethane. This solution was cooled down to 0°C using an ice-bath, 12.7 g of triethylamine was added to the solution, 5.0 mL of methacryloyl chloride was poured slowly into the solution under nitrogen atmosphere and then this solution was stirred magnetically at 100 rpm at room temperature for 2 h. At the end of the chemical reaction period, unreacted methacryloyl chloride and other ingredients were removed from the medium by extraction with 10% NaOH solution. Aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was dissolved in ethanol.

Poly(HEMA-MAH) beads were prepared by a suspension polymerization. A typical procedure may be summarized as follows: The stabilizer, PVAL, was dissolved in 50 mL deionized water for the preparation of the continuous phase. The dispersion phase was prepared by mixing HEMA (8.0 mL), EGDMA (16.0 mL), MAH (2.0 g), and toluene (24.0 mL) in a test tube. The initiator, BPO (200 mg), was dissolved in this homogeneous solution. The dispersion phase was added to the continuous medium in a glass-sealed polymerization reactor (100 mL) placed in a water bath equipped with a temperature-control system. The polymerization reactor was heated to 65°C within about 30 min by stirring the polymerization medium at 600 rpm. The polymerizaton was conducted at 65°C for 4 h and at 90°C for 2 h. After completion of polymerization, the reactor content was cooled to room temperature. A washing procedure was applied after polymerization to remove the diluent and any possible unreacted monomers and other ingredients from the beads. The polymer beads were filtered and resuspended in ethyl alcohol. The suspension was stirred for about 1 h at room temperature and the beads were separated by filtration. The beads were washed twice with ethyl alcohol and then four times with deionized water using the same procedure. When not in use, the beads were kept under refrigeration in 0.02% sodium azide solution for prevention of microbial contamination.

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Chelation of Metal Ions

Adsorption of Cu^{2+}, Zn^{2+}, and Ni^{2+}, from the single metal ions aqueous solutions was investigated in batch adsorption–equilibrium experiments. Twenty mL of aqueous metal ion solutions were treated with the poly(HEMA-MAH) beads (total mass: 100 mg). The flasks were magnetically stirred at an agitation speed of 600 rpm at room temperature. Initial concentration of metal ions were 30 ppm for Cu^{2+} ions and 40 ppm for both Zn^{2+} and Ni^{2+} ions. Medium pH were 4.0 for Cu^{2+} ions and 5.5 for both Zn^{2+} and Ni^{2+} ions. Copper nitrate [$Cu(NO_3)_2$], nickel nitrate [$Ni(NO_3)_2$], and zinc nitrate [$Zn(NO_3)_2$] were used for preparing standard metal ion solutions. After the predetermined equilibrium time (2 h), the beads were separated from the adsorption medium, and the residual concentration of the metal ions in the aqueous phase was measured by using a graphite furnace atomic absorption spectrophotometer (AAS 5EA, Carl Zeiss Technology, Zeiss Analytical Systems, Germany). Deuterium background correction was used. Pyrolitic graphite coated tubes were used for AAS measurements. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. Each sample was read three times, and a mean value and relative standard deviation were computed. Calibrations were performed in the range of analysis, and a correlation coefficient for the calibration curve of 0.98 or greater was obtained. The instrument response was periodically checked with known metal standard solutions. Adsorption values (mmol/μg) were calculated as the difference in initial and final metal ion concentrations. In order to eliminate contamination, all glassware and plasticware were washed with RBS detergent (Pierce, Rockford, IL) and rinsed with deionized water.

Adsorption of Lysozyme from Aqueous Solutions

The capacity of the adsorbent for lysozyme was determined in batch mode. In short, 100 mg of the adsorbent which prepared as described previously was equilibrated after charging metal ions. Then, the beads were incubated with 10 mL of lysozyme solution for 2 h in flasks agitated magnetically at 150 rpm. Effects of initial concentration of lysozyme and pH on the adsorption capacity were studied. To observe the effects of the initial concentration of lysozyme on adsorption, it was changed between 0.1–2.0 mg/mL. The pH of the adsorption medium was varied between 4.0 and 8.0 using different buffer systems (0.1 M CH_3COONa-CH_3COOH for pH 4.0–6.0, 0.1 M K_2HPO_4-KH_2PO_4, for pH 7.0 and 0.1 M Tris/HCl for pH 8.0). Lysozyme concentration was determined by measuring the absorbance at 280 nm, with a

molar absorptivity of 14.0 for a 1% solution of IgG. The amount of adsorbed lysozyme was calculated as:

$$Q = \frac{(C_o - C)V}{m} \quad (1)$$

Where, Q is the amount of lysozyme adsorbed onto unit mass of beads (mg/g); C_o and C are the concentrations of lysozyme in the initial solution and in the aqueous phase after treatment for certain periods of time, respectively (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the beads used (g).

Desorption and Stability Studies

Lysozyme desorption experiments were performed in a buffer solution containing 25 mM EDTA (pH 4.9). Lysozyme adsorbed beads were placed in the desorption medium and stirred for 1 h at 25°C, at a stirring rate of 100 rpm. The final lysozyme concentration within the desorption medium was determined by spectrophotometry. In the case of metal-carrying adsorbents, desorption of metal ions was also measured in the desorption media by atomic absorption spectrometry. The desorption ratio was calculated from the amount of lysozyme adsorbed on the beads and the amount of lysozyme desorbed by using the following expression:

$$\text{Desorption ratio} = \frac{\text{amount of lysozyme desorbed}}{\text{amount of lysozyme adsorbed}} \times 100 \quad (2)$$

To check reusability of the metal-chelated poly(HEMA-MAH) beads, lysozyme adsorption-desorption operation was done 10 times by using the same adsorbent. For sanitization, the beads were washed with 50 mM NaOH solution for 30 min after each adsorption-desorption cycle. After this procedure, beads were washed with distilled water for 30 minutes, then equilibrated with the phosphate buffer for the next adsorption-desorption cycle.

Adsorption of Lysozyme from Egg-White

Chicken egg white was separated from fresh eggs and diluted to 50% (v/v) with phosphate buffer (100 mM, pH 7.0). The diluted egg-white was homogenized in an ice bath and centrifuged at 4°C, at 10,000 rpm for 30 min. Metal-chelated affinity beads (100 mg) were incubated with 10 mL of diluted egg-white solution for 2 h in flasks agitated magnetically at 150 rpm. The beads

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were next washed and centrifuged to remove unbound proteins. Then, lysozyme adsorbed metal-chelate affinity beads were placed in the desorption medium and stirred for 1 h at 25°C, at a stirring rate of 100 rpm. The desorption of lysozyme from metal-chelated affinity beads was performed with 25 mM EDTA (pH 4.9). In the purification experiments, the activity of lysozyme was determined spectrophotometrically at 620 nm, the decrease in the turbidity of culture of *Micrococcus lysodeikticus* cells suspended in phosphate buffer (0.1 M, pH 7.0) was followed for 5 min after addition of lysozyme. One unit lysozyme activity was defined as the amount of enzyme causing a decrease of 0.001 optical density value per minute at 25°C and pH 7.0, The purity of lysozyme in the purified samples was analyzed using A Dionex Bio-LC system (Dionex Co., Idstein, Germany).

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RESULTS AND DISCUSSION

Properties of Polymer Beads

In this study, we attempted to prepare a specific metal chelate–affinity adsorbent for lysozyme separation. The MAH was used as the metal chelating–affinity ligand to immobilize metal ions for specific binding of lysozyme molecules. The poly(HEMA-MAH) beads were in the spherical form, mostly in the size range of 75–125 μm. Specific surface area of the beads was found to be 18.3 m^2/g. The beads are cross-linked matrices. The equilibrium swelling ratio of the poly(HEMA-MAH) beads is 38%. Concentration of the incorporated MAH chelating groups was found from nitrogen analysis using an elemental analyzer. The incorporation of MAH was found to be 895 μmol/g polymer.

Lysozyme Adsorption from Aqueous Solution

Effects of pH

Figure 1 gives the effect of pH on the adsorption of lysozyme with poly(HEMA-MAH) beads. In all the investigated cases, the maximum adsorption of lysozyme was observed at pH 7.0 with the increase of pH above the pH 7.0 or decrease of the pH under the pH 7.0, the protein adsorption capacity decreased. In general, proteins showed a maximum adsorption efficiency at their isoelectric points since at these points, proteins have no net charge and, therefore, the electrostatic repulsion will be diminished.[15] In this study the maximum adsorption of lysozyme was shifted from the isoelectric point (pH: 11.0) to pH 7.0. This behavior of lysozyme can be attributed to histidine's

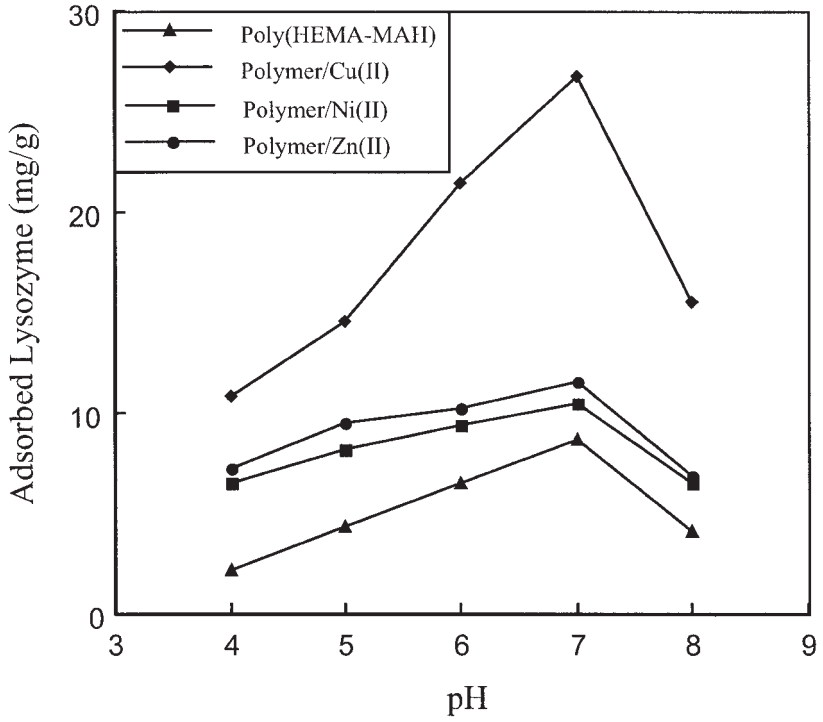

Figure 1. Effect of pH on the lysozyme adsorption: MAH loading: 895 μmol/g; Metal Loading: 135 μmol/g for Cu^{2+} ions; 120 μmol/g for Zn^{2+} ions; 112 μmol/g for Ni^{2+} ions; Lysozyme concentration: 1.0 mg/mL and T: 25°C.

pKa 6.5, because histidines on the lysozyme surface were largely unprotonated and free to coordinate to the metal ions at pH 6.5–7.0, but at alkaline pH, coordination with amino and hydroxyl groups took place and resulted in less effective adsorption of lysozyme.[16] This could also be created from the ionization state of lysozyme and could be caused by repulsive electrostatic forces between lysozyme and the coordinated metal ions. An increase in conformational size of lysozyme and the lateral electrostatic repulsions between adjacent adsorbed lysozyme molecules may also cause a decrease in adsorption efficiency.

Effects of Lysozyme Concentration

To compare the equilibrium adsorption curves of the same ligand derived and three different metals ions chelated affinity beads with lysozyme,

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a series of adsorption experiments were carried out at pH 7.0 and 25°C. The equilibrium adsorption curves for the binding of lysozyme to Zn^{2+}, Cu^{2+}, and Ni^{2+} ions chelated–poly(HEMA-MAH) beads, are shown in Fig. 2. In all the investigated cases, the increases of lysozyme concentration in the adsorption medium led to an increase of the adsorbed lysozyme on the affinity beads. This was linear up to 0.6 mg/mL lysozyme and it became constant when the lysozyme concentration was greater than 0.7 mg/mL. There was, thus, a level in the maximum adsorption capacity for the adsorbents. This could be considered as a typical example of the occupation of all binding sites on the adsorbent surface, which are available for lysozyme adsorption. Thus lysozyme adsorption is favored at a higher initial concentration. Maximum lysozyme adsorption capacity of poly(HEMA-MAH) beads was found to be 8.7 mg/g. Adsorption capacity of the affinity beads was 11.5 mg/g for Ni^{2+}, 12.6 mg/g for Zn^{2+}, and 37.1 mg/g for Cu^{2+}-chelated beads.

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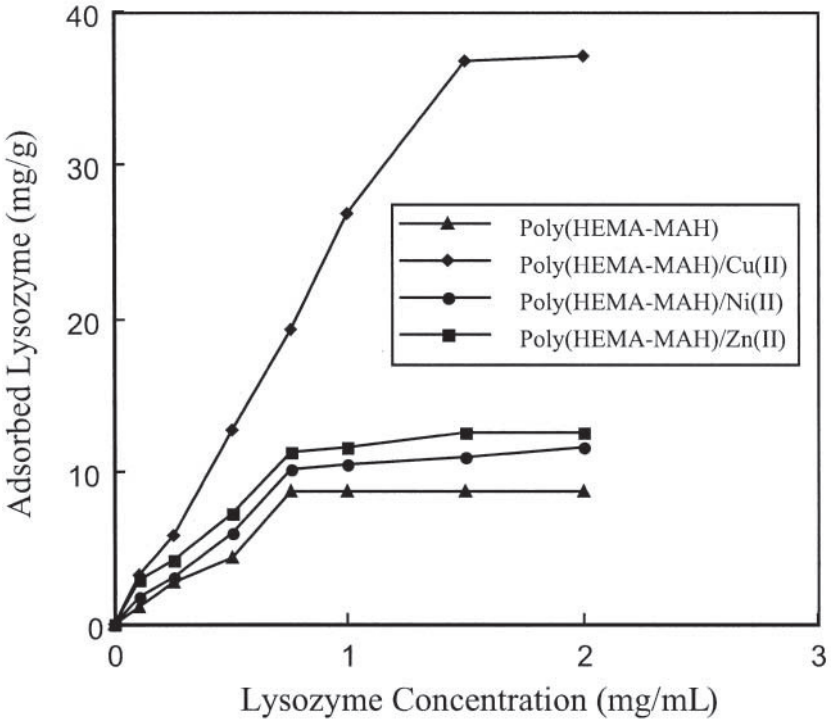

Figure 2. Adsorption isotherms: MAH loading: 895 μmol/g; Metal Loading: 135 μmol/g for Cu^{2+}; 120 μmol/g for Zn^{2+}; 112 μmol/g for Ni^{2+}; pH: 7.0; and T: 25°C.

Desorption and Stability Studies

The desorption of the adsorbed lysozyme from the poly(HEMA-MAH) beads was studied in a batch system. The lysozyme loaded adsorbents were placed within the desorption medium containing 25 mM EDTA at pH 4.9, and the amount of lysozyme released in 1 h was determined. The results show that about 98% of the adsorbed lysozyme molecules were eluted with EDTA eluent. Note that all the Zn^{2+}, Ni^{2+}, or Cu^{2+} ions initially loaded released from the beads. This means that EDTA breaks down the chelates between Zn^{2+}, Cu^{2+}, or Ni^{2+} ions and MAH molecules. Using the desorption data given previously, we concluded that EDTA is a suitable desorption agent for the beads, and allows repeated use of the affinity adsorbents developed in this study.

To be useful in protein purification prossesses, adsorbent materials should be easily regenerated under suitable conditions. To show the reusability of the metal-chelated beads, the adsorption-desorption cycle of lysozyme was repeated ten times by using the same affinity adsorbents. As seen from Fig. 3, adsorption capacities for all the adsorbents did not noticeably change during the repeated adsorption-desorption operations.

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Adsorption of Lysozyme from Egg White

The content of lysozyme in chicken egg white is about 3.4%. The classical lysozyme purification method required several steps, such as precipitation, centrifugation, and affinity adsorption. The purity of the lysozyme desorbed from metal-chelated affinity beads was determined by Bio-LC. The purity of the desorbed lysozyme was about 80% with recovery about 75%. The metal-chelated affinity beads provided an efficient single step method to purify lysozyme from diluted egg white, showing high adsorption capacity and high selectivity for lysozyme. The specific activity of the lysozyme purified with metal chelated beads was 42,000 U/mg.

CONCLUSIONS

Lysozyme is a protein naturally occurring in many organisms such as viruses, plants, insects, birds, reptiles, and mammals. In mammals, lysozyme has been isolated from nasal secretions, saliva, tears, intestines, urine, and milk. Lysozyme lyses certain bacteria by hydrolyzing the β-linkages between the muramic acid and N-acetylglucosamine of the mucopolysaccharides that are present in the bacterial cell wall. It has a large application area; it is used in medicine as a bacteriolytic agent, wound therapeutic agent,

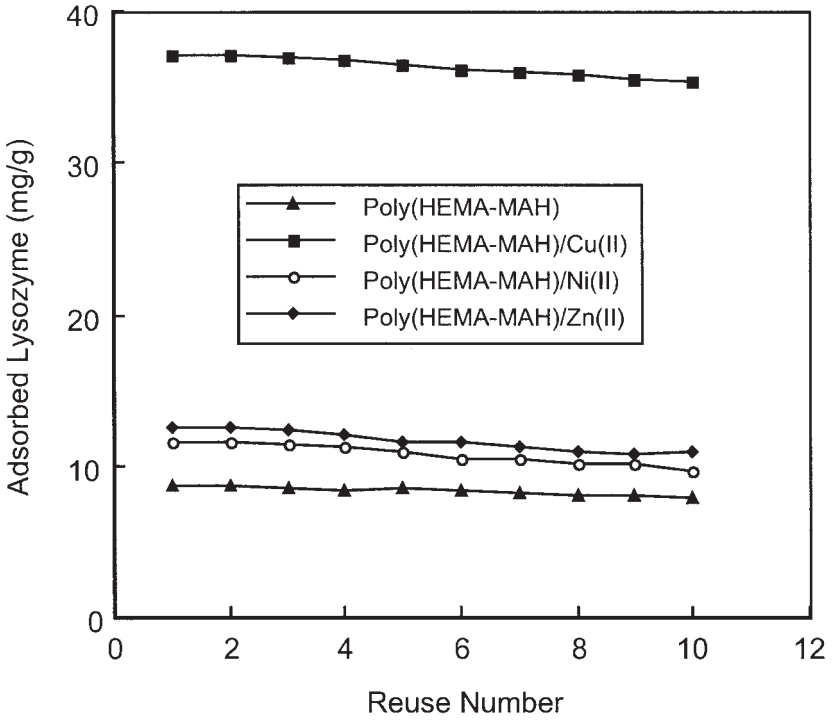

Figure 3. Repeated use of poly(HEMA-MAH) beads. MAH loading: 895 μmol/g; Metal Loading: 135 μmol/g for Cu^{2+} ions; 120 μmol/g for Zn^{2+} ions; 112 μmol/g for Ni^{2+} ions; pH: 7.0; Lysozyme concentration: 2.0 mg/ml; pH: 7.0.

and antibiotic activator.[17] One of the abundent sources of the lysozyme that is used for it is extraction is egg white. The large-scale applications require efficient techniques for its isolation. Bio-ligands are expensive and sensitive to the process operating conditions. They can lose activity or leach into the products by the harsh elution and cleaning conditions commonly used in the separation processes. The time consuming and high cost of chelating procedures have inspired a search for suitable low-cost adsorbents. The main advantage of IMAC consists in its simplicity, universality, stability, and cheapness of the chelating supports.[18,19] In this study, N-methacryloyl-(L)-histidine-methyl ester containing metal chelate–affinity adsorbent for the separation of lysozoyme was prepared. This approach for the preparation of metal chelating–affinity adsorbent has many advantages over conventional techniques. An expensive and critical step in the preparation process of metal-chelating adsorbent is coupling of a chelating ligand to the adsorption matrix.

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In this procedure, comonomer MAH acted as the metal-chelating ligand, and there was no need to activate the matrix for the chelating-ligand immobilization. Another major issue is that of slow release of these covalently bonded chelators off the matrix. Metal-chelating ligand release is a general problem encountered in any IMAC that caused a decrease in adsorption capacity. It is well known that metal-chelating ligand leakage from the adsorbent causes contaminations that will interfere with analysis of the purified biomolecule. The metal-chelating ligand immobilization step was also eliminated in this approach. The MAH was polymerized with HEMA and there was no ligand metal-chelating ligand leakage. The poly(HEMA-MAH) affinity adsorbent was also cheap, reusable, and resistant to harsh chemicals and high temperatures. These results are encouraging and suggest that further studies should be done to develop MAH incorporated metal-chelated adsorbents.

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