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Preparation of Fe(III)-Chelated Poly(HEMA-MAH) Cryogel for Lysozyme Adsorption

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Abstract

Poly(2-hydroxyethyl methacrylate-N-methacryloyl-(L)-histidine methyl ester) [poly(HEMA-MAH)] cryogel was prepared by bulk polymerization which proceeds in an aqueous solution of monomers frozen inside a glass column (cryo-polymerization). After thawing, the monolithic cryogel contains a continuous polymeric matrix having interconnected pores of 10-50 µm size. Then the monolithic cryogel was loaded with Fe(III) ions to form the metal chelate with poly(HEMA-MAH) cryogel. Poly(HEMA-MAH) cryogel was characterized by swelling studies, FTIR, scanning electron microscopy, and elemental analysis. The equilibrium swelling degree of the poly(HEMA-MAH) monolithic cryogel was 2.82 g H_2O/g cryogel. Poly(HEMA-MAH) cryogel containing 36.4 µmol MAH/g was used in the adsorption/desorption of lysozyme from aqueous solutions. The nonspecific adsorption of lysozyme was very low (1.1 mg/g). The maximum amount of lysozyme adsorption from aqueous solution in phosphate buffer was 40.7 mg/g at pH 7.0. It was observed that lysozyme could be repeatedly adsorbed and desorbed with the poly(HEMA-MAH) cyogel without significant loss of adsorption capacity.

Key Words: Cryogel; lysozyme; immobilized metal affinity chromatography; histidine; poly(HEMA)

Introduction

The development of techniques and methods for the separation and purification of proteins has been essential for many of the recent advancements in biotechnology and biomedical research [1]. The purity of a protein is a pre-requisite for its structure and function studies or its potential application [2]. A wide variety of protein purification techniques are available today, however, different types of chromatography have become dominant due to their high resolving power [3]. In gel filtration chromatography, dye-affinity chromatography, ion-exchange chromatography, immobilized metal-ion affinity chromatography, bioaffinity hromatography and hydrophobic interaction chromatography (HIC), the protein separation is dependent on their biological and physico-chemical properties; molecular size, net charge, biospecific characteristics and hydrophobicity, respectively [4–6].

Immobilized metal affinity chromatography (IMAC) is a sensitive technique for protein separation that enables distinguishing between proteins differing by only a single histidine residue on the surface [7-12]. It is assumed that proteins 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 play important roles in protein binding. Aromatic amino acids and the amino terminal of the peptides also contribute [13]. The low cost of metals and the ability to reuse adsorbents hundreds of times without any detectable loss of metal-chelating properties are the attractive features of metal affinity separation.

Lysozyme (N-acetylmuramide glyconohydrolase) is one of the best characterized hydrolases. Lysozyme is considered to be a self-defense enzyme, which is produced in serum, mucus and many organs of vertebrates. Lysozyme is found in a variety of vertebrate cells and secretions, such as spleen, milk, tears and egg white. Lysozyme lyses certain bacteria by hydrolyzing the linkages between the muramic acid and N-acetylglucosamine of the mucopolysaccharides which are present in the bacterial cell wall. Lysozyme is a commercially valuable enzyme, which has tremendous potential for application in pharmaceutical and food industries. Its common applications are as a cell disrupting agent for extraction of bacterial intracellular products, as an antibacterial agent in ophtalmologic preparations, as a food additive in milk products and as a drug for treatment of ulcers and infections [14]. The potential for its use as an anticancer drug has been demonstrated by animal and in vitro cell culture experiments [15]. Lysozyme has also been used in cancer chemotherapy [16]. In a recent article, it has been reported that lysozyme can be used for increasing the production of immunoglobulin by hybridoma technology [17]. The large-scale applications require more efficient and cost effective techniques for its isolation [18].

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In this study, poly(HEMA-MAH) cryogel, is a copolymer of 2-hydroxyethyl methacrylate (HEMA) and N-methacryloyl-(L)-histidine-methylester (MAH), was prepared by bulk polymerization which proceeds in an aqueous solution of monomers frozen inside a glass column (cryopolymerization). Poly(HEMA-MAH) cryogel was characterized by scanning electron microscopy (SEM), FTIR, elemental analysis, and swelling tests. Then, Fe(III) ions were chelated through imidazole groups on the MAH reactive functional groups of the polymeric structure. The ability of the monoliths to adsorb lysozyme from aqueous solutions containing different lysozyme concentrations at different pHs was investigated. Desorption of lysozyme and material stability were also tested.

Experimental

Materials

Lysozyme (chicken egg white,EC3.2.1.7), L-histidine methylester, methacryloyl chloride and amonium persulfate (APS) were supplied by Sigma (St. Louis, MO, USA). Hydroxyethyl methacrylate (HEMA) obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. N,N,N,N -tetramethylene diamine (TEMED) was obtained from Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731) and then a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

Synthesis of MAH

The synthesis and characterization of MAH were performed as described previously [19]. In the experimental procedure for synthesis of MAH, 5.0 g of L-histidine hydrochloride and 0.2 g of hydroquinone were dissolved in 100 mL of a dichloromethane solution, which was cooled to 0°C. Then, 12.7 g of triethylamine was added to the solution, followed by the addition of 5.0 mL of methacryloyl chloride, which was poured in slowly. Then, this solution was stirred magnetically at room temperature for 2 h, after which hydroquinone and unreacted methacryloyl chloride were extracted with a 10% NaOH solution. The aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was crystallized in an ether-cyclohexane mixture and then dissolved in ethyl alcohol. ^{1}H-NMR, performed in $CDCl_3$ on a JEOL GX-400 300 MHz instrument, was used to determine if the MAH structure was synthesized. The residual nondeuterated solvent ($CHCl_3$) served as an internal reference. Chemical shifts are reported in parts per million downfield relative to $CHCl_3$. The ^{1}H-NMR spectrum shows the characteristic peaks of the groups in the MAH monomer as follows - ^{1}H-NMR ($CDCl_3$): = 1.99 (t; 3 H, J = 7.08 Hz, CH_3), 1.42 (m; 2 H, CH_2), 3.56 (t; 3 H, OCH_3) 4.82-4.87 (m; 1 H, methin), 5.26 (s; 1 H, vinyl H), 5.58 (s; 1 H, vinyl); 6.86 (σ, 1 H, J = 7.4 Hz, NH), 7.82 (σ; 1H, J = 8.4 Hz, NH), 6.86-7.52 (m; 5H, aromatic).

Preparation of poly(HEMA-MAH) cryogel

Poly(HEMA-MAH) cryogel, is a copolymer of 2-hydroxy ethyl methacrylate (HEMA) and N-methacryloyl-(L)-histidine-methylester (MAH), was prepared by bulk polymerization which proceeds in an aqueous solution of monomers frozen inside a glass column (cryo-polymerization). Total concentration of monomers was 6% (w/v). The cryogel was produced by free radical polymerization initiated by TEMED (20 μL) and APS (100 μL). After adding APS (10% (w/v) of the total monomers), the solution was cooled in an ice bath for 2-3 min. TEMED was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into a plastic syringe (5 mL, id. 0.8 cm) with closed outled at the bottom. The polymerization solution in the syringe was frozen at -12°C for 24 h and then thawed at room temperature. Extensive cleaning procedure for removal of unconverted monomers and initiator was performed. Briefly, washing solutions (i.e. a dilute HCl solution and a water-ethanol mixture) were recirculated through the monolithic cryogel column, until to be assured that the cryogel column is clean. Purity of the monolithic cryogel was followed by observing the change of optical densities of the samples taken from the liquid phase in the recirculation system, and also from the DSC thermograms of the cryogel obtained by using a differential scanning microcalorimeter (Mettler, Switzerland). Optical density of the original monolithic cryogel was 1.67. But after the applying of cleaning procedure this value was reduced to 0.02. In addition, when the thermogram of the uncleaned monolithic cryogel was recorded, it has a peak around 50°C. This peak might be originated from TEMED. But after applying of this cleaning procedure, between 30 and 100°C any peak was not observed on this thermogram. After washing, the cryogel was stored in buffer containing 0.02% sodium azide at 4°C until use.

Incorporation of Fe(III) ions

The investigation of Fe(III) chelation was carried out in a recirculating system equipped with a water jacket for temperature control. The monolith was washed with 30 mL of water. Then 40 mL of a Fe(III) solution [50 mg/L (pH 4.1), adjusted with HCl and NaOH] was pumped through the column under recirculation at room temperature for 2 h. A 1000-ppm atomic absorption standard solution (containing 10% HNO_3) was the source of the Fe(III) ions. The concentration of the Fe(III) ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (GFAAS, Analyst 800/Perkin Elmer, USA). The instrument response was periodically checked with known metal solution standards. The experiments were performed in triplicate, as were analyses of the samples. For each set of data, standard statistical methods were used to determine the mean and standard deviation. A 95% confidence interval was calculated for each set of samples in order to determine the margin of error. The Fe(III) concentrations in the initial and final solutions were used to calculate the amount of Fe(III) ions adsorbed.

Fe(III) leakage from the poly(HEMA-MAH) cryogel was investigated in media whose pH varied between 5.0 and 8.0 and also in a medium containing 1.0 M NaCl. The monolith was stirred for 24 h at room temperature. Then the concentration of Fe(III) ions in the supernatants was determined using an atomic absorption spectrophotometer. Note that the metal-chelated monolith was stored at 4°C in a 10 mM Tris buffer (pH 7.4).

Characterization of cryogel

Swelling test

The swelling degree of the cryogel (S) was determined as follows: cryogel sample was washed on porous filter until washing was clear. Then it was sucked dry and then transferred to preweighed vial and weighed ($m_{wet\ gel}$). After drying to constant mass in the oven at 60°C, the mass of dried sample was determined ($m_{dry\ gel}$). The swelling degree was calculated as:

Surface morphology

The morphology of a cross section of the dried cryogel was investigated by SEM. The sample was fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer overnight, postfixed in 1% osmium tetroxide for 1 h. Then the sample was dehydrated stepwise in ethanol and transferred to a critical point drier temperated to +10°C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to +40°C and the pressure to 100 bar. Liquid CO_2 was transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage. Release of the pressure at a constant temperature of +40°C resulted in dried cryogel sample. Finally, it was coated with gold-palladium (40:60) and examined using a JEOL JSM 5600 SEM (Tokyo, Japan).

FTIR

FTIR spectra of MAH and poly(HEMA-MAH) cryogel were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogel (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a tablet, and the spectrum was then recorded.

Elemental analysis

Elemental analysis (Leco elemental analyzer, Model CHNS-932, USA) was performed to evaluate how much MAH was incorporated into the poly(HEMA-MAH) cryogel.

Chromatographic procedures

Lysozyme adsorption from aqueous solutions

Investigation of lysozyme adsorption was carried out in a recirculating system equipped with a water jacket for temperature control. The cryogel was washed with 30 mL of water and then equilibrated with 25 mM phosphate buffer (pH 7.0). Then the prepared lysozyme solution was pumped through the column under recirculation for 2 h. The adsorption was followed by monitoring of the decrease in UV absorbance at 280 nm. The effects of flow rate, lysozyme concentration and pH of the medium on adsorption capacity were studied. The flow rate of the solution (i.e., 50 mL of the aqueous lysozyme solution) was varied in the range of 0.5-2.5 mL/min. To observe the effects of the initial concentration of lysozyme on adsorption, it was varied between 0.5 and 5.0 mg/mL. To determine the effects of pH and temperature on adsorption, they were varied between 5.0 and 8.0 and 4°C and 37°C, respectively.

Desorption and repeated use

In all cases, adsorbed lysozyme molecules were desorbed using 0.1 M phosphate buffer containing 1 M NaCl (pH 8.0). In a typical desorption experiment, 50 mL of desorption agent was pumped through the cryogel at a flow rate of 1.0 mL/min for 30 min. The final lysozyme concentration in the desorption medium was spectroscopically determined. When desorption was achieved, the cryogel was cleaned with 1 M NaOH and then re-equilibrated with 25 mM phosphate buffer (pH 7.0). The desorption ratio was calculated from the amount of lysozyme adsorbed on the cryogel and the final lysozyme concentration in the desorption medium. To test the repeated use of poly(HEMA-MAH) cryogel, lysozyme adsorption-desorption cycle was repeated for 20 times using the same cryogel column. To regenerate and sterilize, after desorption, the cryogel was washed with 1 M NaOH solution.

Results and Discussion

The imidazole nitrogen donor atom incorporated into the MAH group was the most common binding site for metal ions. The amount of chelated Fe(III) on poly(HEMA-MAH) cryogel was measured as 32.2 μmol/g polymer. Mass stoichiometric analysis showed that 1 incorporated MAH molecule interacted around 1 Fe(III) ion (36.4 μmol MAH/g:32.2 μmol Fe(III)/g). Because MAH has two coordinating sites of nitrogen atoms, it could form a ternary complex that was coordinated water molecules at vacant coordination sites of the Fe(III)-MAH complexes. Investigation of leakage of Fe(III) from the poly(HEMA-MAH) cryogel detected no leakage in any of the adsorption and desorption media, suggesting that the washing procedure was satisfactory for the removal of the nonspecific adsorbed Fe(III) ions from the cryogel.

Surface morphology

A supermacroporous cryogel was produced by polymerization in the frozen state of monomers, 2-hydroxyethyl methacrylate (HEMA) and N-methacryloyl-(L)-histidine methyl ester (MAH) in the presence of APS/N,N,N,N - tetramethylene diamine (TEMED) as initiator/activator pair. The SEM of the internal structure of the cryogel is shown in Figure 1. Poly(HEMA-MAH) cryogel have large continuous interconnected pores (10-50 μm in diameter)

that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the protein molecules, allowing them to pass easily. Lysozyme has molecular size of 3.0x3.0x4.6 nm and molecular diameter of 27.3 A°. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible.

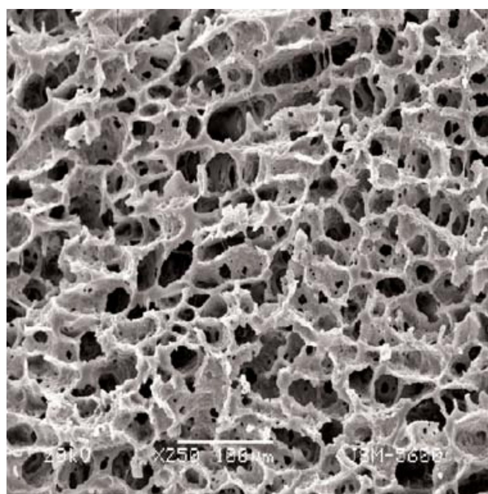

Figure 1. SEM micrograph of poly(HEMA-MAH) cryogel.

Swelling test

The equilibrium swelling degree of the poly(HEMA-MAH) cryogel was 2.82 g H_2O/g dry cryogel. Poly(HEMA-MAH) cryogel is opaque, sponge like, and elastic. This cryogel can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogel was submerged in water, it soaked in water and within 1-2 s restored its original size and shape.

FTIR

The FTIR spectrum of the poly(HEMA-MAH) monolith had the characteristic stretching vibration bands of hydrogen-bonded alcohol, O H, around 3481 cm^{-1} and carbonyl, at 1641 cm^{-1}, and the absorption bands of amide II, at 1516 cm^{-1} (Figure 2).

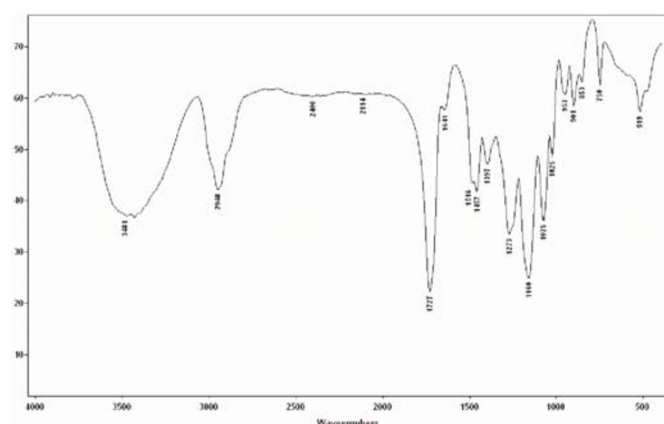

Figure 2. FTIR spectrum of poly(HEMA-MAH) cryogel.

Lysozyme adsorption from aqueous solutions

Effects of pH

Figure 3 shows the effect of pH on the adsorption of lysozyme on Fe(III)-chelated poly(HEMA-MAH) cryogel. The maximum adsorption of lysozyme was observed at pH 7.0. With the increase of pH above and below the pH 7.0, the lysozyme adsorption capacity decreased. The protein adsorption on IMAC is mainly based on chelating bonding between metal ions and amino acid residues. The ionization of amino acid residues at a weakly alkaline pH favors the reaction and therefore induces protein adsorption on the metal-immobilized matrixes [20]. The adsorption of lysozyme on metal-immobilized materials is mainly through chelating bonding between metal ion and histidine residue of lysozyme, and the pKa values of histidine residue in most of proteins are in the range of 5.5–8.5 [21,22].

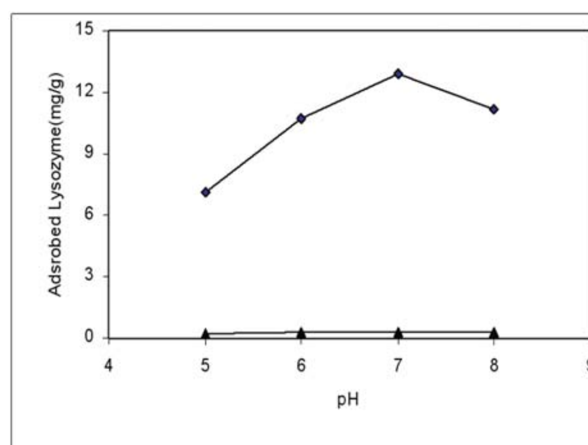

Figure 3. Effects of pH on lysozyme adsorption; MAH content: 32.6 μmol/g; Fe(III) loading, 32.2 μmol/g; initial lysozyme concentration: 0.5 mg/mL; flow rate: 1 ml/min; T: 25°C.

Therefore, the optimal pH for lysozyme adsorption on Fe(III)-chelated poly(HEMA-MAH) cryogel is around 7.0. It is similar to the results of lysozyme adsorption on metal chelated polyamide hollow membrane [23]. It should be also noted that nonspecific adsorption (i.e. adsorption on Fe(III)-chelated poly(HEMA-MAH) cryogel) was independent of pH and it was observed at the same at all the pH values studied.

Effects of lysozyme concentration

Figure 4 shows the lysozyme adsorption isotherm of the plain and Fe(III)-chelated poly(HEMA-MAH) cryogels. Lysozyme adsorption on poly(HEMA-MAH) cryogel was low (about 4.6 mg/g), although adsorption of lysozyme molecules on Fe(III)-chelated poly(HEMA-MAH) cryogel through Fe(III) ions was significant (up to 40.7 mg/g). As expected, the amount of lysozyme coupled to cryogel almost reached a plateau of around 5.0 mg/mL because of saturation of the active binding sites.

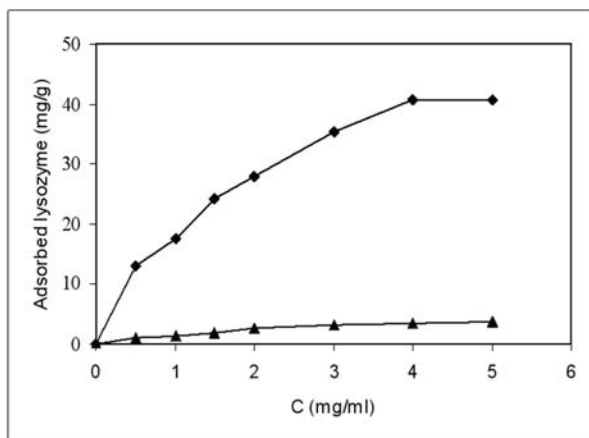

Figure 4. Effects of Lysozyme concentration on adsorption: MAH content: 32.6 µmol/g; Fe(III) loading, 32.2 µmol/g; pH 7.0 (phosphate buffer); flow rate: 1 ml/min; T: 25°C.

Effects of flow-rate

The adsorption amounts at different flow-rates are given in Figure 5. Results show that the lysozyme adsorption capacity on Fe(III)-chelated poly(HEMA-MAH) cryogel decreases when the flow-rate through the column increases. The adsorption capacity decreased significantly from 13.4 to 7.0 mg/g polymer with the increase of the flow-rate from 0.5 to 2.5 ml/min. An increase in the flow rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column. This is due to decrease in contact time between the lysozyme molecules and Fe(III)-chelated poly(HEMA-MAH) cryogel at higher flow rates. These results are also in agreement with those referred to the literature [24]. When the flow-rate decreases the contact time in the column is longer. Thus, lysozyme molecules have more time to diffuse to the pore walls of cryogel and to bind to the ligand, hence a better adsorption capacity is obtained. In addition, for column operation the cryogel is continuously in contact with a fresh protein solution. Consequently the concentration in the solution in contact with a given layer of cryogel in a column is relatively constant.

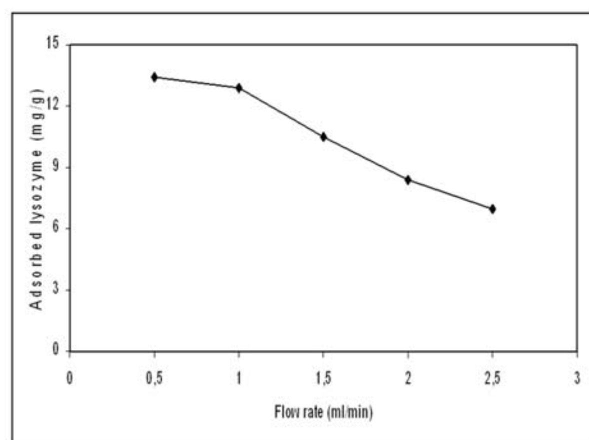

Figure 5. Effects of flow rate on lysozyme adsorption: MAH content: 32.6 µmol/g; Fe(III) loading, 32.2 µmol/g; initial lysozyme concentration: 0.5 mg/mL; pH 7.0 (phosphate buffer); T: 25°C.

Effects of temperature

The effect of temperature on lysozyme adsorption was studied in the range of 4°C-35°C. The equilibrium adsorption of lysozyme on Fe(III)-chelated poly(HEMA-MAH) cryogel decreased significantly with increasing temperature, with maximum adsorption achieved at 4°C (Figure 6). From 4°C to 35°C, the adsorption capacity of the cryogel decreased about 73,7% for Fe(III)-chelated poly(HEMA-MAH) cryogel. A possible explanation for this behavior is the exothermic nature of the adsorption process.

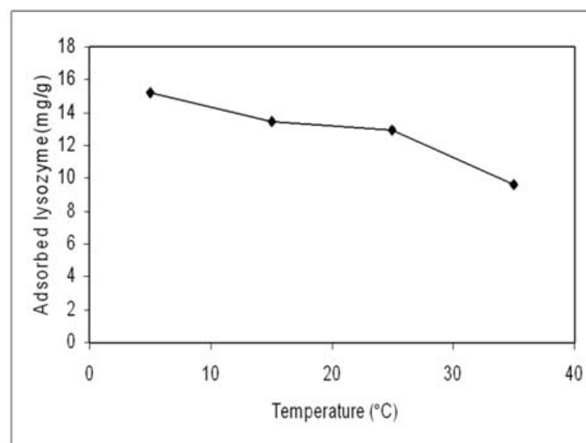

Figure 6. Effects of temperature on lysozyme adsorption: MAH content: 32.6 µmol/g; Fe(III) loading, 32.2 µmol/g; initial lysozyme concentration: 0.5 mg/mL; pH 7.0 (phosphate buffer).

Desorption and reusability of adsorbents

Desorption of lysozyme from Fe(III)-chelated poly(HEMA-MAH) cryogel was also carried out in a column system. The desorption of lysozyme is expressed in percentage of totally adsorbed lysozyme. Up to 97.2% of the adsorbed lysozyme was desorbed by using 0.1 M phosphate buffer containing 1 M NaCl (pH 8.0) as elution agent. The addition of elution agent reduced electrostatic interactions, resulting in the release of the lysozyme molecules from the adsorbent. Note that there was no Fe(III) release from the cryogel. With the desorption data given earlier, we concluded that 0.1 M phosphate buffer containing 1 M NaCl (pH 8.0) is a suitable desorption agent, and allows repeated use of the affinity cryogel used in this study.

To show the reusability of Fe(III)-chelated poly(HEMA-MAH) cryogel, the adsorption-desorption cycle was repeated 20 times using the same cryogel. There was no remarkable reduce in the adsorption capacity of the cryogel. The lysozyme adsorption capacity decreased only 5.2% after 20-cycle.

Conclusion

A wide variety of functional molecules, including enzymes, coenzymes, cofactors, antibodies, amino acid derivatives, oligopeptides, proteins, nucleic acids, and oligonucleotides may be used as ligands in the design of novel adsorbents [25,26–28]. These ligands are extreme-

ly specific in most cases. However, they are expensive, due to high cost of production and/or extensive purification steps. In the process of the preparation of specific adsorbents, it is difficult to immobilize certain ligands on the supporting matrix with retention of their original biological activity [27]. Precautions are also required in their use (at adsorption and elution steps) and storage.

A new metal-immobilized affinity adsorbent can be prepared by chelateing Fe(III) on poly(HEMA-MAH) cryogel. Experimental results indicated that this novel adsorbent can effectively adsorb lysozyme from aqueous solution, and the excellent adsorption–desorption behaviors of the adsorbent promise it to be useful in practical applications. The lysozyme adsorption capacity decreased only 5.2% after 20-cycle. Compared with poly(HEMA-MAH) cryogel, the adsorption capacity of Fe(III)-chelated poly(HEMA-MAH) cryogel is greatly increased. It can be concluded that Fe(III) plays a key role for the adsorption of lysozyme on poly(HEMA-MAH) cryogel. Therefore, the formation of coordinated compound between protein and Fe(III) should be considered to be the major binding mode. However, electrostatic and hydrophobic interactions may be also involved in the adsorption of lysozyme on Fe(III)-chelated poly(HEMA-MAH) cryogel.

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