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Use of magnetic poly(glycidyl methacrylate) monosize beads for the purification of lysozyme in batch system

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Abstract

The hydrophobic affinity ligand L-tryptophan immobilized magnetic poly(glycidyl methacrylate) [m-poly(GMA)] beads in monosize form (1.6 μm in diameter) were used for the affinity purification of lysozyme from chicken egg white. The m-poly(GMA) beads were prepared by dispersion polymerization in the presence of Fe_3O_4 nano-powder. The epoxy groups of the m-poly(GMA) beads were converted into amino groups with 1,6 diaminohexane (i.e., spacer arm). L-tryptophan was then covalently immobilized on spacer arm attached m-poly(GMA) beads. Elemental analysis of immobilised L-tryptophan for nitrogen was estimated as 42.5 μmol/g polymer. Adsorption studies were performed under different conditions in a batch system (i.e., medium pH, protein concentration and temperature). Maximum lysozyme adsorption amount of m-poly(GMA) and m-poly(GMA)-L-tryptophan beads were 1.78 and 259.6 mg/g, respectively. The applicability of two kinetic models including pseudo-first order and pseudo-second order model was estimated on the basis of comparative analysis of the corresponding rate parameters, equilibrium adsorption capacity and correlation coefficients. Results suggest that chemisorption processes could be the rate-limiting step in the adsorption process. It was observed that after 10 adsorption–elution cycle, m-poly(GMA)-L-tryptophan beads can be used without significant loss in lysozyme adsorption capacity. Purification of lysozyme from egg white was also investigated. Purification of lysozyme was monitored by determining the lysozyme activity using *Micrococcus lysodeikticus* as substrate. It was found to be successful in achieving purification of lysozyme in a high yield of 76% with a purification fold of 71 in a single step. The specific activity of the eluted lysozyme (62,580 U/mg) was higher than that obtained with a commercially available pure lysozyme (Sigma (60,000 U/mg).

Keywords: Magnetic monosize beads; Lysozyme purification; Poly(glycidyl methacrylate)

1. Introduction

The development of protein purification techniques has been essential for many of the recent advancements in biotechnology research [1]. The purity of a protein is a pre-requisite for its structure and function studies or its potential application. A wide variety of protein purification techniques are available today, however, different types of chromatography have become dominant due to their high resolving power [2]. In gel filtration chromatography, dye-affinity chromatography, ion-exchange chromatography, immobilized metal-affinity chromatography, bioaffinity chromatography 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 [3–8].

HIC takes advantage of the hydrophobicity of proteins promoting their separation on the basis of hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of the proteins [9]. The adsorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluent. Therefore, the term of salt-promoted adsorption could be used for this type of chromatography [10]. In fact, HIC has been successfully used for separation purposes as it displays binding characteristics complementary to other protein chromatographic techniques. During the last years, HIC has been studied by many researchers and today it is an established and powerful biosepa-ration technique in laboratory-scale, as well as in industrial-scale

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purification of proteins [11]. The development of a large variety of stationary phases for HIC has promoted a wide range of HIC applications in the purification of biomolecules, like serum proteins, nuclear proteins, hormones, recombinant proteins and enzymes [12].

A lot of different type of hydrophobic molecules which have side chains of non-polar amino acids such as alanine, methionine, tryptophan and phenylalanine on their surface can be used as a ligand in HIC [13,14]. These pseudospecific ligands have low binding constants (10^{-4}–$10^{-6}\,M^{-1}$) and consequently, belong to weak affinity ligands family. Nevertheless, they can exhibit selectivity resulting from the cumulative effects of multiple weak binding events as; electrostatic, hydrophobic, hydrogen binding and van der Waals interactions with fast kinetics. L-phenylalanine has been used as a pseudospecific hydrophobic ligand for the isolation of biomolecules [15].

Lysozyme (mucopeptite *N*-acetylmuramoylhydrolase, EC 3.2.1.17) is a widely distributed enzyme that preferentially hydrolyzes β-1,4-glucosidic linkages between *N*-acetylglucosamine that occur in bacterial cell walls. It is present in tears, saliva, sweat, breast milk of humans and other animals, in plants, microorganisms and viruses [16]. Lysozyme has found wide applications, and is often used in conjunction with other therapeutic drugs, applied topically or administered orally [17]. The antibacterial property has been exploited in a number of other applications such as eye drops and wound healing creams [18]. It is used as a food preservative to inhibit growth of *Clostridia* in cheese [19], spoilage organisms in selected procesed foods [20], and in wine as a substitute for sulfites [21]. It has also been used in gastrointestinal infections and in the treatment of dry-mouth [22]. The potential for its use as an anticancer drug has been demonstrated by animal and in vitro cell culture experiments [23]. Lysozyme has also been used in cancer chemotherapy [24]. In a recent article, it has been reported that lysozyme can be used for increasing the production of immunoglobulin by hybridoma technology [25]. Thus, an efficient, fast, economical and scalable method for its purification is highly desirable.

Micron-sized magnetic beads are currently enjoying a fairly ample range of applications in many fields including among others biotechnology, biochemistry, colloid sciences and medicine [26–28]. The magnetic character implies that they respond to a magnet, making sampling and collection easier and faster, but their magnetization disappears once the magnetic field is removed. Magnetic beads promise to solve many of the problems associated with chromatographic separations in packed bed and in conventional fluidized bed systems [29]. Magnetic separation is relatively rapid and easy, requiring a simple apparatus, composed of centrifugal separation. Recently, there has been increased interest in the use of magnetic carriers in protein purification [30].

In our previous works, the non-magnetic poly(glycidyl methacrylate) beads [poly(GMA)] were prepared in monosize form by modified suspension polymerization. The Cibacron Blue F3GA-modifed non-magnetic poly(GMA) beads were used in affinity depletion of albumin from human serum for proteome studies [31], lysozyme purification from egg white [32] and recombinant interferon-α [33], respectively. Use of magnetic beads in bioprocesses has many advantages. They can be easily separated from reaction medium and stabilized in a fluidized bed column by applying a magnetic field. The use of magnetic beads reduces capital and operation costs [34]. The goal of this study is to prepare a tryptophan containing magnetic monosize poly(glycidyl methacrylate) [m-poly(GMA)] beads with magnetite nano-powder (i.e., Fe_3O_4) for efficient separation of lysozyme from egg white. The monosize m-poly(GMA) beads were obtained by dispersion polymerization of GMA. Lysozyme adsorption properties of the pseudo-affinity beads from aqueous solutions were investigated at different experimental conditions in a batch system. Elution of lysozyme and reusability of the adsorbents were also tested. Finally, the hydrophobic-affinity beads were used for the purification of lysozyme from chicken egg white. The purity of the eluted lysozyme was determined by Bio-LC and the activity of the eluted lysozyme was measured using *Micrococcus lysodeikticus* as a substrate microorganism.

2. Experimental

2.1. Materials

Lysozyme (95% from chicken egg white, EC 3.2.1.17, activity 60000 units/mg protein) and lyophilized *Micrococcus lysopdeikticus* cells were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and used as received. Chicken eggs were purchased from the local market. Glycidyl methacrylate (GMA, Fluka A.G., Buchs, Switzerland) was purified by vacuum distillation and stored in a refrigerator until use. L-tryptophan and magnetite nanopowder (Fe_3O_4, diameter: 20–50 nm) were obtained from Sigma. Azobisisobutyronitrile (AIBN) and poly(vinyl pyrrolidone) (MW: 30,000, BDH Chemicals Ltd., Poole, England) were selected as the initiator and the steric stabilizer, respectively. AIBN was recrystallized from methanol. Ethanol (Merck, Germany) was used as the diluent without further purification. All other chemicals were guaranteed or analytical grade reagents commercially available and used without further purification. Laboratory glassware was kept overnight in a 5% nitric acid solution. Before use the glassware was rinsed with deionised water and dried in a dust-free environment. 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.

2.2. Synthesis of m-poly(GMA) beads

m-Poly(GMA) monosize beads were synthesized as previously described elsewhere [35]. The dispersion polymerization was performed in a sealed polymerization reactor (volume: 500 mL) equipped with a temperature control system. A typical procedure applied for the dispersion polymerization of GMA is given below. The monomer phase was comprised of 40 mL GMA, 250 mg AIBN and 1 g magnetite (Fe_3O_4) particles. The

resulting medium was sonicated for about 5 min at 200 W within an ultrasonic water bath (Bransonic 2200, England) for the complete dissolution of AIBN in the polymerization medium. Four grams of poly(vinyl pyrrolidone) was dissolved in 50% v/v aqueous ethanol solution and placed in a polymerization reactor. The reactor content was stirred at 500 rpm during the monomer addition completed within about 5 min and the heating was started. Then reactor was purged with bubbling nitrogen for about 5 min. Then, the sealed reactor was placed in a shaking water bath at room temperature. The initial-polymerization time was defined when the reactor temperature was raised to 65 °C. The polymerization was allowed to proceed under nitrogen atmosphere at 65 °C for 4 h (stirring rate: 500 rpm). After completion of the polymerization period, the reactor content was cooled down to room temperature and centrifuged at 5000 rpm for 10 min for the removal of dispersion medium. This polymerization reaction led to the formation of white beads. m-Poly(GMA) beads were redispersed within 10 mL of ethanol and centrifuged again under similar conditions. The ethanol washing was repeated three times for complete removal of unconverted monomers and other components. Finally, m-poly(GMA) beads were redispersed within 10 mL of water (0.10%, by weight) and stored at room temperature.

In order to estimate the amount of leached magnetite from the m-poly(GMA) beads, the beads (250 mg) were placed in test tube containing 10 mL of leach media and shaken on a rotary shaker for 24 h. The amount of magnetite leached into the medium was determined by a graphite furnace atomic absorption spectrophotometer (AAS 5EA, Carl Zeiss Technology, Zeiss Analytical Systems, Germany). Three kind of release media were used: 50% acetic acid solution (pH 2.0), 50 mM phosphate buffer solution (pH 7.0) and 50 mM sodium citrate/NaOH buffer solution (pH: 12.0).

2.3. L-tryptophan immobilization

In order to prepare the L-tryptophan immobilized m-poly(GMA) beads following procedure was applied. The epoxy groups of the m-poly(GMA) beads were converted into amino groups with 0.5 M 1,6 diaminohexane (i.e., spacer arm). The m-poly(GMA) beads were incubated with 1,6 diaminohexane solution at 65 °C in a reactor containing 20 g of dry beads and shaked for 6 h. After the reaction, the spacer arm attached m-poly(GMA) beads were washed with distilled water. The spacer arm attached m-poly(GMA) beads were equilibrated in phosphate buffer (100 mL, 10 mM, pH 7.0) for 2 h and transferred to the activation solution containing glutaric dialdehyde (50 mL, 0.25%, v/v). The activation reaction was carried out at 25 °C for 12 h, while continuously shaking the medium. After the reaction period, the excess glutaric dialdehyde was removed by washing sequantially the beads with distilled water and phosphate buffer. The activated beads were incubated in phosphate buffer (10 mM, pH 7.0) for 4 h, then they were transferred to the L-tryptophan solution mixture (3.0 g L-tryptophan/25 mL phosphate buffer). This immobilization reaction was carried out under a constant gentle shaking (250 rpm) at 25 °C for 6 h. At the end of this reaction period, the L-tryptophan-immobilized adsorbents were removed by filtration and washed extensively with methanol and water in order to remove weakly adsorbed L-tryptophan molecules and then dried in vacuum for 24 h. The amounts of 1,6 diaminohexane and L-tryptophan immobilized on the m-poly(GMA) beads were determined by measuring the nitrogen concentration in the polymer structure with elemental analysis (LECO, CHNS-932, USA). FTIR spectra of the m-poly(GMA) beads, 1,6 diaminohexane modified m-poly(GMA) beads and L-tryptophan immobilized m-poly(GMA) beads were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry beads (about 0.1 g) were thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a tablet, and the spectrum was then recorded. When not in use, the resulting adsorbents were kept under refrigeration in 0.02% NaN_3 solution for preventing of microbial contamination.

The leakage of the L-tryptophan from the adsorbents was followed by incubating the fully wetted adsorbents with 10 mL of phosphate buffered saline (PBS, pH 7.4) solution for 24 h at room temperature. The leakage experiments were carried out at 25 °C at a stirring rate of 50 rpm. L-tryptophan released after this incubation was measured in the liquid phase spectrophotometrically.

2.4. Lysozyme adsorption–desorption studies from aqueous solutions

Adsorption of lysozyme on the m-poly(GMA) beads from aqueous solutions was investigated batch-wise. The beads (0.05 g) were incubated with 10 mL of the aqueous solutions of lysozyme for 2 h (i.e. equilibrium time), in flasks agitated magnetically at 150 rpm. Effects of the lysozyme concentration, pH of the medium, temperature and ionic strength on the adsorption capacity were studied. To determine the effect of pH on the adsorption, pH of the solution was changed between 4.0 and 8.0. To observe the effects of the initial concentration of lysozyme on adsorption, it was changed between 0.1 and 2.0 mg/mL. To observe the effects of the temperature on the adsorption, adsorption studies were carried out between 4 and 37 °C. Samples were withdrawn at suitable time intervals and lysozyme concentration was determined by Bradford method at 595 nm. The amount of adsorbed lysozyme was calculated using mass balance. Each experiment was performed in twice for quality control and statistical purposes.

2.5. Desorption and repeated use

The desorption of the adsorbed lysozyme from the m-poly(GMA) beads was studied in batch experimental setup. Lysozyme adsorbed m-poly(GMA) beads were placed in desorption medium, containing 0.1 M ethylene glycol solution and stirred continuously (at stringing rate 150 rpm) for 1 h at room temperature. The final lysozyme concentration in the desorption medium was determined by Bradford method. In order to show the reusability of the beads, adsorption–desorption cycle of lysozyme was repeated ten times by using the same beads.

2.6. Purification 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 homogenised in an ice bath and centrifuged at 4 °C, at 10,000 rpm for 30 min. Hydrophobic 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 hydrophobic beads were then washed and centrifuged to remove unbound proteins. Then, 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 desorption of lysozyme from the beads was performed with 0.1 M ethylene glycol solution. 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 decrase of 0.001 optical density value per minute at 25 °C and pH 7.0, The purity of lysozyme in the purified samples was analysed using A Dionex Bio-LC system.

To examine the purity of the lysozyme samples, a Dionex bioliquid chromatography system (Dionex Co., Idstein, Germany) was used. The BioLC system consisted of a GS50 gradient pump, an LC25 chromatography oven and a PDA-100 phodiode array dedector. Chromatographic separation of proteins was achieved on a MN Nucleosil 4000-7 PEI column (125 mm × 4.0 mm i.d) protected by a guard column (8.0 mm × 4.0 mm i.d). All sample solutions used in chromatographic studies were prefiltered through a syringe membrane filter (0.2 μm Millipore, Bedford, MA, USA) to remove suspended particles and large aggregates. Dionex CHROMELLEION1 software was used and operated under Windows XP Home for data acquisition and integration.

BioLC mobile phases A and B were prepared using Tris–acetate pH 8.0 (20 mm) buffer and buffer A including 1.5 M KCl solutions, respectively. The mobile phases were filtered prior to use. The chromatographic separation was performed using a linear gradient at 1.0 mL/min flow rate in 5 min from 0% to 10% Eluent B and 10 min from 10% to 40% Eluent A. The sample injection volume was 25 μL. The photodiode array dedector was set at 280 nm and the temperature of column was maintained at 25 °C.

3. Results and discussion

3.1. Characteristics of monosize m-poly(GMA) beads

Micron monosize (RSD < 1%, 1.62 μm in diameter) m-poly(GMA) beads were obtained by dispersion polymerization. The physicochemical properties of poly(GMA) beads are presented in Table 1. The morphology and structure of the resulting beads were observed by SEM picture as shown in Fig. 1. As seen here, the m-poly(GMA) beads were highly uniform in size. Polydispersity index (PDI) value of m-poly(GMA) beads was calculated to be around 1.008. m-Poly(GMA) beads are hydrophilic polymer networks capable of imbibing large amounts of water yet remain insoluble and preserve their three-dimensional shape.

Table 1
Some properties of the monosize m-poly(GMA) beads

Particle diameter	1.62 ± 0.01 μm
Polydispersity index	1.008
Specific surface area	3.2 m^2/g
Theoretical epoxy group content	3.46 mmol/g
Experimental epoxy group content	3.00 mmol/g
Swelling ratio	52%
Wet density	1.09 g/mL
Fe_3O_4 incorporation	4.5%
Resonance of magnetic field	2055 Gauss
g factor	2.28
1,6-Diaminohexane content	80.8 μmol/g
L-tryptophan attachment	42.5 μmol/g

Pseudospecific ligand L-tryptophan is covalently attached on 1,6 diaminohexane modified m-poly(GMA) beads. The studies of L-tryptophan leakage from the m-poly(GMA) beads showed that there was no L-tryptophan leakage in any medium used throughout this study, even in long storage period of time (more than 20 weeks).

Fig. 2 shows FTIR spectra of plain and modified m-poly(GMA) beads. The FTIR spectrum of m-poly(GMA) has the characteristic stretching vibration band of hydrogen-bonded alcohol at 3500 cm^{-1}. Among the characteristic vibrations of GMA is the methylene vibration at 2930 cm^{-1}. The epoxide group gives the band at 850 and 910 cm^{-1} (epoxy ring). The vibration at 1740 cm^{-1} represents the ester configuration of GMA. The FTIR spectrum of 1,6 diaminohexane modified m-poly(GMA) beads has the characteristic N–H amine stretching bands at between 3500 and 3300 cm^{-1} (the bands more broadened after attachment of 1,6 diaminohexane). The most important absorption band at 1650 cm^{-1} repseneting N–H bending, is due to 1,6 diaminohexane attachment to the m-poly(GMA) beads. After glutaric dialdehyde activation, the intensity of the N–H band decreased due to the reaction took place between amine and aldehyde groups. In this spectrum, amine peak was decreased, while the C=O band at 1740 cm^{-1}

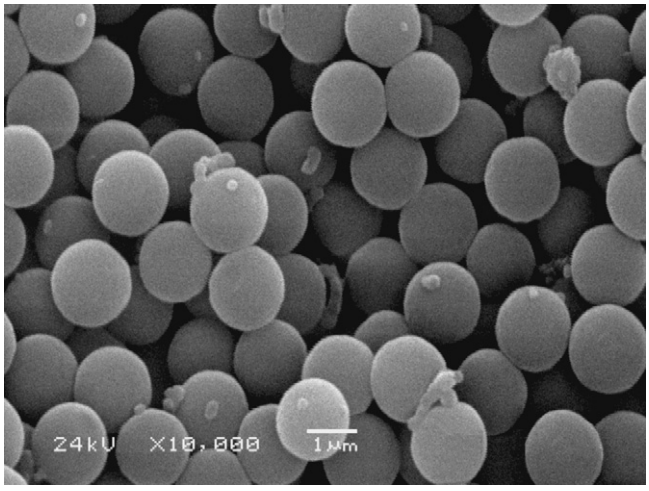

Fig. 1. SEM photograph of monosize m-poly(GMA) beads.

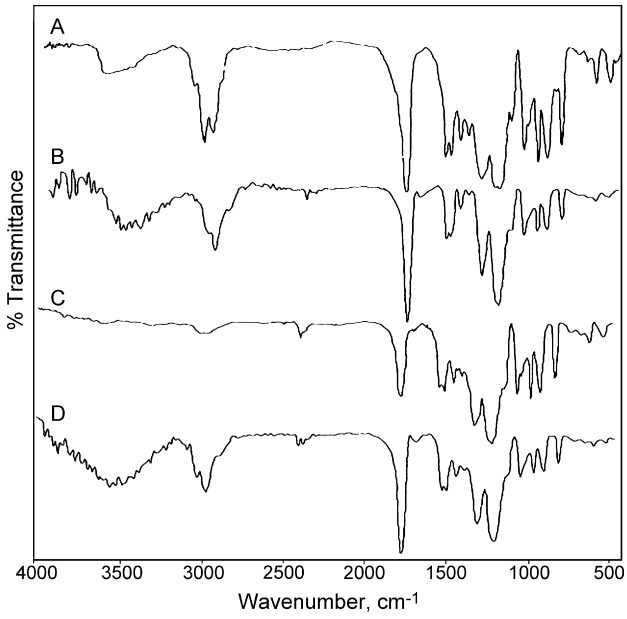

Fig. 2. FTIR spectra of: (A) m-poly(GMA); (B) 1,6 diaminohexane modified m-poly(GMA) beads; (C) glutaric dialdehyde activated m-poly(GMA) beads; (D) L-tryptophan immobilized m-poly(GMA) beads.

as broadened compared to inactivated beads due to reaction with amine groups to yield the C=O bands. The absorption bands of the functional groups of the L-tryptophan can be clearly seen in Fig. 2C, 3400 cm^{-1} and 3100 cm^{-1} absorption bands are due to N–H stretching and aromatic C–H stretching, respectively. The intensive peak 1370 cm^{-1} corresponds to aromatic C–N stretching.

3.2. Adsorption of lysozyme from aqueous solutions

3.2.1. Effect of pH

Fig. 3 shows lysozyme adsorption capacity at different pH values. Maximum adsorption was observed at pH 7. At pH values lower and higher than pH 7, the adsorbed amount of lysozyme drastically decreased. The maximum adsorption capacity was observed at pH 7 indicating that the binding of lysozyme on the m-poly(GMA) beads should involve hydrophobic interactions with a high percentage according to the other interactions. Lysozyme molecule has one histidine, four aspartic acid, two glutamic acid and two tyrosine residues (on the basis of its surface accessible residues). These amino acid side chains of lysozyme molecules could cause other type of interactions with low percentage according to the hydrophobic interactions.

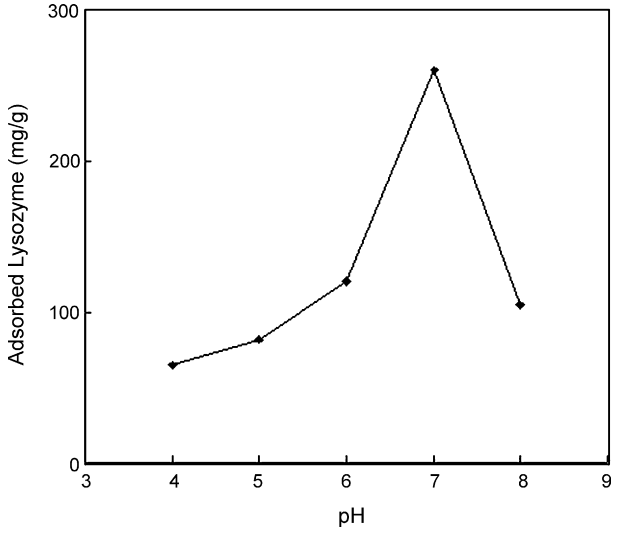

Fig. 3. Effect of pH on lysozyme adsorption; lysozyme concentration: 1.0 mg/mL; incubation time: 2 h; temperature: 25 °C.

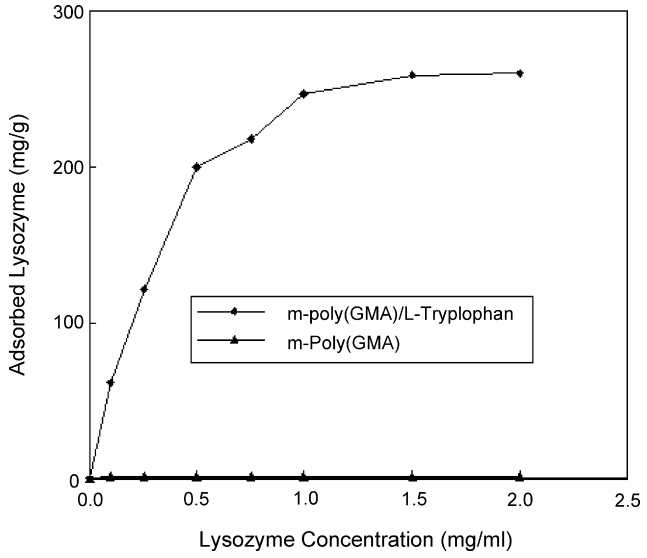

Fig. 4. Adsorption isotherms of lysozyme on m-poly(GMA) monosize beads; pH 7.0; incubation time 2 h; temperature 25 °C.

3.2.2. Effect of lysozyme concentration

Fig. 4 shows the lysozyme adsorption isotherm. A point worth noting that, there was a low non-specific lysozyme adsorption (i.e., the adsorption onto the plain m-poly(GMA) beads) about 1.78 mg/g. There are no reactive binding groups or sites onto m-poly(GMA) which interact with lysozyme molecules. Hence, this non-specific adsorption may be due to weak interactions (van Der Waals interaction and hydrogen binding) between lysozyme and epoxy groups on the surface of m-poly(GMA) beads. L-tryptophan-attachment significantly increased the lysozyme adsorption capacity of the monosize beads (up to 259.8 mg/g). The amount of lysozyme adsorbed per unit mass of the L-tryptophan-affinity beads increased first with the initial concentration of lysozyme then reached a plateau value which represents saturation of the active adsorption sites (which are available and accessible for lysozyme) on the monosize beads. This increase in the lysozyme adsorption capacity may have resulted from hydrophobic interactions caused by the L-tryptophan and by hydrophobic amino acids on the lysozyme molecules. It should be mentioned that L-tryptophan is hydrophobic overall, and it prefers to interact with hydrophobic groups in lysozyme structure.

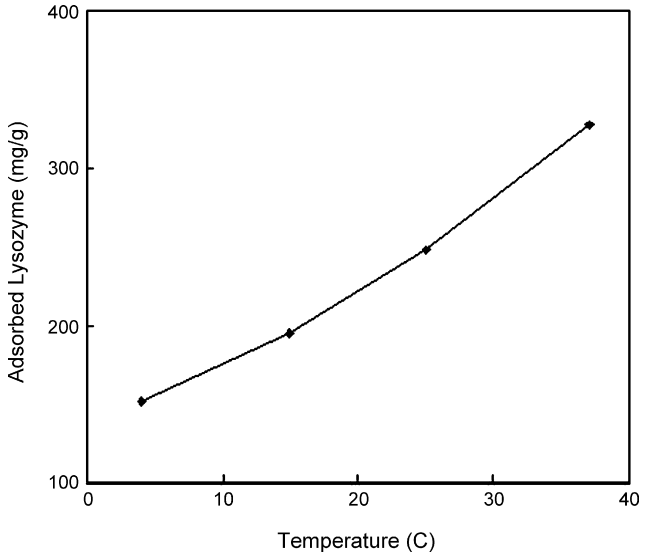

Fig. 5. Effect of temperature on lysozyme adsorption. lysozyme concentration: 1.0 mg/mL; pH 7.0; incubation time 2 h.

3.2.3. Effect of temperature

Effect of temperature on the adsorption of lysozyme onto L-tryptophan immobilized m-poly(GMA) beads was presented in Fig. 5. Adsorption of lysozyme on the m-poly(GMA) beads was significantly increased with increasing temperature indicating that hydrophobic interactions were much more significant in the adsorption. As known, in HIC, increasing the temperature enhances protein retention and lowering the temperature generally promotes the protein elution [36]. In fact, the HIC, is an entropy-driven process [$\Delta G = (\Delta H - T\Delta S) \sim -T\Delta S$]. Since ΔH may be a small positive or negative value, ΔG is controlled by a positive entropy change and thus increases with temperature. The calculated ΔH value of the system for the lysozyme interaction with the L-tryptophan immobilized m-poly(GMA) beads was −2.76 kcal/mol. It is interesting to note that the van der Waals attraction forces, which operate in hydrophobic interactions, also increase with increase in temperature [37].

3.2.4. Adsorption isotherms

Two important physico-chemical aspects for evaluation of the adsorption process as a unit operation are the kinetics and the equilibria of adsorption. Modelling of the equilibrium data has been done using the Langmuir and Freundlich isotherms [38]. The Langmuir and Freundlich isotherms are represented as follows Eqs. (1) and (2), respectively.

$$\frac{1}{q_e} = \left(\frac{1}{q_{max}}\right) + \left[\frac{1}{q_{max} b}\right]\left(\frac{1}{C_e}\right) \quad (1)$$

$$\ln\, q_e = \frac{1}{n}(\ln\, C_e) + \ln\, K_F \quad (2)$$

where b is the Langmuir isotherm constant, K_F is the Freundlich constant, and n is the Freundlich exponent. $1/n$ is a measure of the surface heterogeneity ranging between 0 and 1, becoming more heterogeneous as its value gets closer to zero. The value of q_e gives the theoretical monolayer saturation capacity of monosize beads.Some model parameters were determined by nonlinear regression with commercially available software and are shown in Table 2. Comparison of all theoretical approaches used in this study shows that the Langmuir equation fits the experimental data best.

Table 2
Equilibrium adsorption constants

Langmuir model			Freundlich model		
Q_{max} (mg/g)	b (mL/g)	R^2	K_F	$1/n$	R^2
263.2	38.0	0.9987	260.9	0.150	0.968

3.2.5. Adsorption kinetics modeling

In order to analyze the adsorption kinetics of lysozyme in medium containing salt, the pseudo-first order and the pseudo-second order kinetics models were applied to the experimental data [39]. The first-order equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$\frac{dq_t}{dt} = k_1(q_{eq} - q_t) \quad (3)$$

where k_1 is the rate constant of pseudo-first order adsorption (min^{-1}) and q_{eq} and q_t (mg/g) denote the amounts of adsorbed protein at equilibrium and at time t (min), respectively. After integration by applying boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, gives

$$\log\left[\frac{q_{eq}}{(q_{eq} - q_t)}\right] = \frac{(k_1 t)}{2.303} \quad (4)$$

Eq. (4) can be rearranged to obtain a linear form

$$\log(q_{eq} - q_t) = \frac{\log(q_{eq}) - (k_1 t)}{2.303} \quad (5)$$

A plot of log $(q_{eq} - q_t)$ versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process log q_{eq} should be equal to the interception point of plot of log $(q_{eq} - q_t)$ via t.

In addition, a pseudo-second order equation based on adsorption equilibrium capacity may be expressed in the form

$$\frac{dq_t}{dt} = k_2(q_{eq} - q_t)^2 \quad (6)$$

where k_2 (g/mg min) is the rate constant of pseudo-second order adsorption process. Integrating Eq. (6) by applying boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, leads to

$$\left[\frac{1}{(q_{eq} - q_t)}\right] = \left(\frac{1}{q_{eq}}\right) + k_2 t \quad (7)$$

or equivalently for linear form:

$$\left(\frac{t}{q_t}\right) = \left(\frac{1}{k_2 q_{eq}^2}\right) + \left(\frac{1}{q_{eq}}\right) t \quad (8)$$

A plot of t/q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant

Table 3
The first- and second-order kinetic constants

Initial Conc. (mg/mL)	Exp q_e (mg/g)	First-order kinetic			Second-order kinetic		
		k_1 (1/min)	q_e (mg/g)	R^2	k_2 ($\times 10^{-4}$ (g/mg min)	q_e (mg/g)	R^2
0.25	121.1	0.037	147.1	0.9954	2.79	122.0	0.9975
0.50	197.8	0.036	257.9	0.4430	0.11	226.3	0.9655
0.75	217.6	0.040	284.6	0.7011	0.35	263.6	0.9680
1.00	247.2	0.030	282.2	0.5428	0.14	255.6	0.9783
2.00	259.6	0.050	337.9	0.9307	0.74	317.1	0.9558

(k_2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively.

The comparison of experimental adsorption capacity and the theoretical value estimated from the previous equations, are presented in Table 3. The theoretical q_{eq} value estimated from the first order kinetic model gave significantly different value compared to experimental value, and the correlation coefficient was also found to be lower. These results showed that the first order kinetic model is improper for these affinity beads.

Table 3 lists the computed results obtained from the pseudo-first order and pseudo-second order kinetic models. The correlation coefficients for the pseudo-second order kinetic model are higher than the pseudo-first order kinetic model for all cases. These results suggest that the pseudo-second order mechanims is predominant and that chemisorption might be the rate-limiting step that controls the adsorption process. The rate-controlling mechanism may vary during the course of the adsorption process three possible mechanisms may be occuring [14]. There is an external surface mass transfer or film diffusion process that controls the early stages of the adsorption process.This may be followed by a reaction or constant rate stage and finally by a diffusion stage where the adsorption process slows down considerably [40].

3.2.6. Desorption and reusability of adsorbents

Desorption of lysozyme was studied with 0.1 M ethylene glycol solution in a batch system. Lysozyme adsorbed beads were placed within the desorption medium and stirred continuously (at stringing rate 150 rpm) for 1 h at room temperature. The final lysozyme concentration in the desorption media was determined by Bradford Protein Assay at 595 nm. In order to test the reusability of the beads, lysozyme adsorption–desorption procedure was repeated five times by using the same beads. At the end of ten adsorption–desorption cycle, there was no remarkable reduction in the adsorption capacity (Fig. 6).

3.2.7. Adsorption of lysozyme from egg white

Chicken egg white is a mixture of proteins. Among the proteins in egg whites, ovalbumin, conalbumin and ovomucoid are the major components constituting 54%, 12–13% and 11%, respectively. The content of lysozyme as a minor component in chicken egg white is about 3.5% [41]. Lysozyme has the highest total surface hydrophobicity of the proteins of chicken egg white. The classical lysozyme purification method required several steps, such as precipitation, centrifugation and affinity adsorption [42]. In this study, single step lysozyme purification from egg white was studied in a batch mode. The hydrophobic 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 hydrophobic beads was found to be very high as 62,580 U/mg. Specific activity determined in desorbed fraction was comparable to the commercial egg white lysozyme yielding approximately 71-fold purification in a single step (Table 4). The purity of the lysozyme desorbed from hydrophobic beads was determined by Bio-LC (Fig. 7). The purity of the desorbed lysozyme which was obtained from the integration of Bio-LC peaks was about 85% with overall yield about 76%, which is similar to 77% yield obtained using Streamline SP followed by Dye-Ligand Chromatography [43] and 80% yield in a method where metal-affinity precipitation with Cu^{2+} followed by gel filtration chromatography

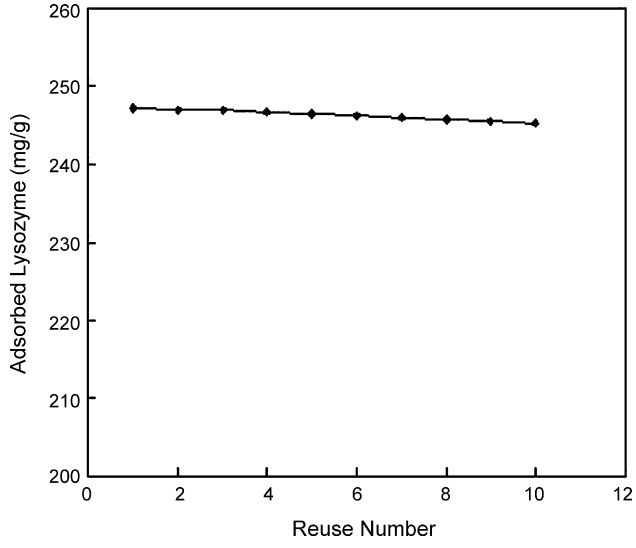

Fig. 6. Reusability of beads; lysozyme concentration: 1.0 mg/mL; pH 10; incubation time 2 h; temperature 20 °C.

Table 4
Specific activity and fold purification of lysoyme isolated from egg white

	Specific activity (unit/mg protein)	Purification fold
Egg white preparation	887 ± 90	1
Batch adsorption	62580 ± 300	71
Commercial lysozyme	60000 ± 280	–

Sample volume: 100 mL; protein content: 32.5 mg/mL.

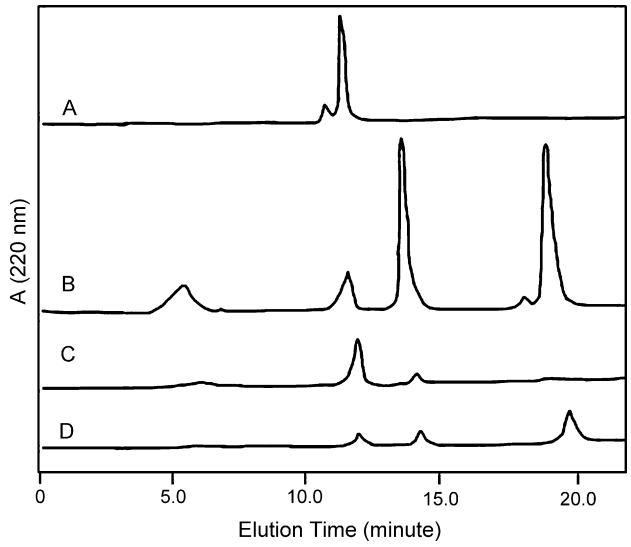

Fig. 7. Bio-LC chromatograms showing the purification of lysozyme with hydrophobic beads: (A) pure commercial lysozyme; (B) whole egg white; (C) eluted lysozyme sample.

on Sephadex G75 [22]. Chang et al showed that a 11-fold purification of the lysozyme was achieved in a high yield of 97.5% with Streamline SP adsorbent in a stirred fluidized-bed [44]. Shen and Cao applied thermo-sensitive *N*-alkyl substituted polyacrylamide polymer carrying Cibacron Blue F3GA in the purification of lysozyme in egg white, it showed a purification fold of 28 and specific activity of lysozyme was 12,000 U/mg [45]. Su and Chiang used the PEG-sulfate aqueous two-phase system for lysozyme purification from chicken egg white and they reported that 70% of lysozyme can be extracted with the specific activity 39,500 U/mg [46]. Odabaşı and Denizli prepared lysozyme-imprinted poly(hydroxyethyl methacrylate-*N*-methacryloly-(L)-histidine methylester) particles for the purification of lysozyme from egg white [47]. They reported 89% purity with recovery about 84%. Compared to the results in literature, the result by this method is so good.

4. Conclusion

Chromatographic techniques for protein purification have a number of drawbacks, such as the compressibility of the column packaging materials (i.e., softgel and macrobeads) and the fouling. Commercially available polymer based porous adsorbents exhibit surface areas 200–500 m^2 or even larger per unit mass of the adsorbent. However, these type of adsorbents have also important disadvantages. First of all the adsorption rates are much slower, because of mainly the pore diffusion resistance. In addition, the high active surface area of these sorbents is mainly due to the fine pores in the matrix, which are not available for large solute molecules. In other words, large molecules cannot penetrate within these pores and therefore cannot use the active surface area available, which means low adsorption capacities for large molecules. In order to increase the protein loading capacity, the particle size has been reduced to 0.1–1.0 μm, but such carriers require high pressure equipments. Due to these reasons, non-porous affinity sorbents of small particle diameter (i.e., micron size) have been gaining more attention since the mid-1980s for the rapid high-performance liquid chromatography (HPLC) of biomolecules. It appears that the monosize m-poly(GMA) beads can be applied for the purification of proteins. Our goal was to find a cost effective and reusable hydrophobic affinity beads having high adsorption capacity for purification of lysozyme from chicken egg white. Based on our evaluations of adsorption capacity, recovery and binding specificity of the hydrophobic-affinity beads offered the promising purification approach.

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