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Fibrous polymer grafted magnetic chitosan beads with strong poly(cation-exchange) groups for single step purification of lysozyme

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ABSTRACT

Lysozyme is an important polypetide used in medical and food applications. We report a novel magnetic strong cation exchange beads for efficient purification of lysozyme from chicken egg white. Magnetic chitosan (MCHT) beads were synthesized via phase inversion method, and then grafted with poly(glycidyl methacrylate) (p(GMA)) via the surface-initiated atom transfer radical polymerization (SI-ATRP). Epoxy groups of the grafted polymer, were modified into strong cation-exchange groups (i.e., sulfonate groups) in the presence of sodium sulfite. The MCTH and MCTH-g-p(GMA)-SO_3H beads were characterized by ATR-FTIR, SEM, and VSM. The sulphonate groups content of the modified MCTH-g-p(GMA)-4 beads was found to be 0.53 mmol g^{-1} of beads by the potentiometric titration method. The MCTH-g-p(GMA)-SO_3H beads were first used as an ion-exchange support for adsorption of lysozyme from aqueous solution. The influence of different experimental parameters such as pH, contact time, and temperature on the adsorption process was evaluated. The maximum adsorption capacity was found to be 208.7 mg g^{-1} beads. Adsorption of lysozyme on the MCTH-g-p(GMA)-SO_3H beads fitted to Langmuir isotherm model and followed the pseudo second-order kinetic. More than 93% of the adsorbed lysozyme was desorbed using Na_2CO_3 solution (pH 11.0). The purity of the lysozyme was checked by HPLC and SDS gel electrophoresis. In addition, the MCTH-g-p(GMA)-SO_3H beads prepared in this work showed promising potential for separation of various anionic molecules.

1. Introduction

Ionic interactions are the basis for purification of proteins by ion exchange chromatography [1–4]. The separation is due to competition between proteins with different surface charges for oppositely charged groups on an ion-exchanger adsorbent. Ion exchangers are usually classified as weak or strong [5–7]. Strong ion exchangers have functional groups, e.g., sulphonate and quaternary ammonium [8,9]. On the other hand, weak ion exchangers have functional ionic groups such as carboxylate, primary amine and diethyl ammonium. Much attention has been paid to improving the adsorptive performance of the chromatographic support materials, with the main effort being focused on the introduction of a second functional polymer via grafting [10,11,2,12]. Among the surface functionalization techniques, nano-fibril polymers can be grafted on the adsorbent and the grafted polymers can be readily modified [11,13,14]. Recently, several types of polymer brushes with functional weak and strong ion-exchange groups have been prepared, such as poly(acrylic acid) grafted cotton [15], p(GMA) grafted amine modified resin, p(GMA) grafted polystyrene resin with tertiary amine, p(GMA) grafted and sulphonate groups modified beads, etc., have been shown to have high adsorption capacities [16–18]. These polymer brushes were grafted onto various materials surfaces using surface-initiated atom transfer radical polymerization (SI-ATRP), which has played a significant role in the modification of surface properties [19–21]. Among the above-mentioned polymer brushes, p(GMA) has been the most commonly employed polymeric material for grafting and modification [16,17]. In addition, magnetic separation using magnetic polymeric support is a quick and easy method for the reliable capture of the target protein from crude biological fluids. The magnetic carriers could be conveniently and quickly separated from the medium under magnetic field. Recently, there has been increased interest in the use of magnetic carriers in protein purification [22–24].

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Lysozyme (EC 3.2.1.17) is a glycoside hydrolase which is abundant in egg white. It breaks down the bacterial cell wall by catalyzing the hydrolysis of 1, 4-β-linkages between the muramic acid and N-acetylglucosamine of the mucopolysaccharides. Therefore it is widely used as a cell disrupting reagent and as a potent antibacterial agent due to its bacteriostatic and bactericidal properties. Lysozyme is commonly found in egg white and in secretions of vertebrates such as tears, sweat, and the digestive system of ruminants [25]. It is commercially precious and is commonly used for industrial and medical purposes, such as in bacteria extractions as a cell disruptive agent, for ophthalmologic purposes as an antibacterial agent, in milk products as food additives, and in treatment of ulcers and infections as a drug [26,27]. The anticancer potential of lysozyme was also demonstrated by animal and cell culture studies and it is already being used for cancer chemotherapy [28]. Because of its medical and industrial importance, its separation and purification of lysozyme with high purity has been attracting great attention.

Adsorption and/or purification of lysozyme have been widely studied by using various support materials modified with different weak and strong ion-exchange groups including L-thyroxin ligand immobilized pH-responsive polymer [3]; Magnetic chitosan sorbent [29]; Reactive Red 120 modified magnetic chitosan microspheres [30,5]; Procion Green H-4G immobilized poly(HEMA)/chitosan IPN membranes [31]; magnetic spherical hydroxyapatite microcomposites [24]; Reactive Green 19 dye modified affinity membrane [26]; Reactive Yellow 2 immobilized p(HEMA)/chitosan composite membranes [32]; Cibacron Blue 3GA dye immobilized magnetic chitosan microspheres have been used for the purification of lysozyme [33]. In an interesting application, surface imprinting of lysozyme was demonstrated by magnetic-chitosan submicrospheres [34]. Magnetic nanoparticles conjugated with polyethylene glycol and carboxymethyl chitosan were used as an adsorption carrier for lysozyme [2].

In general, magnetic particles provide significant reductions in operational costs and time compared to conventional methods such as centrifugation, ultra-filtration and chromatography. Magnetic separation systems can recover target proteins at high selectivity in a short time, which are achieved by high surface to volume ratio [35,36]. The separation through magnetic field application facilitates a convenient method without any complicated instrumentation unlike porous gel supports like agarose or polyacrylamide gels [37]. Magnetic separation allows flexibility in sample volume compared to column-based separation. Also, magnetic separation is suitable for high-throughput automation [38].

In this study, a new magnetic strong cation exchange beads was prepared and used for efficient purification of lysozyme from chicken egg white. First, magnetic chitosan beads were synthesized via phase inversion method. The beads were grafted with glycidyl methacrylate via SI-ATRP method, then epoxy groups of the glycidyl methacrylate unit were converted into sulphonate groups in the presence of Na_2SO_3. Strong cation exchange beads containing different amounts of sulphonate groups were obtained by SI-ATRP method, which were synthesized with different ATRP reaction time. The MCTH-g-p(GMA)-SO_3H beads were characterized by analytical studies, FTIR, VSM and SEM. The reversible cation-exchange binding of lysozyme (isoelectric point about: p*I* 10.7) was studied to evaluate the binding properties of the lysozyme on the functional polymer brush. The adsorption parameters such as adsorption conditions (e.g., initial lysozyme concentration, pH, time, ionic strength, and temperature) were varied to evaluate the nature of binding mechanisms of lysozyme on the MCTH-g-p(GMA)-SO_3H cation-exchange beads. Lysozyme adsorption studies from aqueous solutions were performed on the MCTH-g-p(GMA)-SO_3H beads to evaluate the effects of lysozyme concentration at different pHs, temperatures, $-SO_3H$ group densities and ionic strengths. Desorption of the adsorbed lysozyme and reusability of the MCTH-g-p(GMA)-SO_3H beads were also investigated. Lysozyme purification from chicken egg white was then studied under optimized experimental conditions. The purity of the purified lysozyme was determined by HPLC and SDS gel electrophoresis studies.

2. Materials and methods

2.1. Materials

Lysozyme from chicken egg white, chitosan, glycidyl methacrylate (GMA), bromoacetyl bromide, glutaraldehyde, triethylamine, polyvinyl alcohol (PVA; MW: 50,000), bipyridine, CuBr, and tetrahydrofuran were obtained were obtained from Sigma Chemical Co. (St. Louis, USA) and used as received. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of magnetic-chitosan beads

The preparation of chitosan beads was carried out via phase inversion techniques. Chitosan (5.0 g) was dissolved in acetic acid solution (4.0%,) containing $FeCl_3$ (0.3 mol L^{-1}, 100 mL). The resulting chitosan solution was transferred into a pressurized injector and introduced into sodium hydroxide solution (2.5 mol L^{-1}, 250 mL) drop wise under 1.0 bar nitrogen pressure through a nozzle (2.0 cm length, 0.2 mm i.d.) while the alkaline medium was stirred with a mechanical stirrer at 1500 rpm at room temperature, and stirring was continued for a further 30 min after completion of introduction, for curing. The formed chitosan beads were filtered and washed twice with 100 mL purified water. The ferric-chitosan beads were cross-linked with glutaraldehyde by immersing the chitosan beads (5.0 g) into sodium hydroxide solution (pH 8.0, 100 mL) containing glutaraldehyde (1.0%) at 40 °C for 3.0 h while stirring with a mechanical stirrer at 250 rpm. The resulting cross-linked beads were washed with purified water.

For thermal co-precipitation reaction, Fe^{3+} ion containing chitosan beads (about 6.0 g) were transferred into a reactor containing aqueous ammonia (50 mL, 25% w/v), and a solution of $FeCl_2$ in water (4.0 g, 100 mL). The reaction mixture was continuously stirred and refluxed under nitrogen atmosphere at 50 °C for 4.0 h, and then at 90 °C for 2 h. The MCTH beads were separated using a magnet and dried in a vacuum oven at 40 °C and stored at room temperature.

2.3. Grafting p(GMA) fibrous brushes by ATRP onto magnetic chitosan beads

The beads (about 10.0 g), 100 mL tetrahydrofuran and 3.0 mL triethylamine were transferred into a round-bottom flask, and stirred magnetically at 50 rpm. Then, 2.0 mL of bromoacetyl bromide was added in drops within 30 min. The bromination reaction was allowed to proceed for 4 h at room temperature. After the bromination reaction, the MCTH beads were removed and extensively washed with acetone, and purified water, and then dried in a vacuum oven for 18 h prior to grafting. Graft polymerization of glycidyl methacrylate was then achieved through alkyl halide functionalized sites on the surface of the MCTH beads. In a typical procedure, Br-end functionalized beads (5.0 g basis on dry weight) were transferred into a magnetically stirred glass reactor (100 mL) and the following chemicals [monomer GMA (15 mL, 112.5 mmol), CuBr (0.3 g), bipyridine (1.4 g, 9.0 mmol) and dioxane (150 mL)] were added. The solution was purged with nitrogen about 10 min and the reactor was sealed. Polymerization reaction was carried out at 65 °C for 3, 6, 9, 12 or 15 h and referred as MCHT-g-p(GMA)-SO_3H-1-5 beads (Table 1). After this period, the reaction content was transferred into acetone (150 mL) and stirred magnetically