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Affinity chromatography of proteins on non-porous copolymerized particles of styrene, methyl methacrylate and glycidyl methacrylate

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Abstract

Non-porous particles having an average diameter of 2.1 μm were prepared by co-polymerization of styrene, methyl methacrylate and glycidyl methacrylate, which was abbreviated as P(S–MMA–GMA). The particles were mechanically stable due to the presence of benzene rings in the backbone of polymer chains, and could withstand high pressures when a column packed with these particles was operated in the HPLC mode. The polymer particles were advantaged by immobilization of ligands via the epoxy groups on the particle surface that were introduced by one of the monomers, glycidyl methacrylate. As a model system, Cibacron Blue 3G-A was covalently immobilized onto the non-porous copolymer beads. The dye-immobilized P(S–MMA–GMA) particles were slurry packed into a 1.0 cm×0.46 cm I.D. column. This affinity column was effective for the separation of turkey egg white lysozyme from a protein mixture. The bound lysozyme could be eluted to yield a sharp peak by using a phosphate buffer containing 1 *M* NaCl. For a sample containing up to 8 μg of lysozyme, the retained portion of proteins could be completely eluted without any slit peak. Due to the use of a shorter column, the analysis time was shorter in comparison with other affinity systems reported in the literature. The retention time could be reduced significantly by increasing the flow-rate, while the capacity factor remained at the same level.

Keywords: Immobilized dyes; Affinity adsorbents; Proteins; Lysozyme; Styrene; Methyl methacrylate; Glycidyl methacrylate

1. Introduction

Non-porous sorbents in the micron size range have gained great interest for the rapid separation of biomolecules, especially proteins, since the mid-1980s. A major advantage of the non-porous sorbents is that significant intraparticle diffusion resistances are absent; this is particularly useful for fast protein chromatography through the use of higher flow velocity. Most of the non-porous sorbents are silica- and polystyrene-based particles [1]. This is because these two types of base materials can sustain a high-pressure gradient when the columns are operated in high-performance liquid chromatography (HPLC) mode. However, silica is unstable in extreme pH, and styrene-based polymers are very hydrophobic. In order to yield affinity sorbents these materials need a series of chemical modifications and activation on the particle surface prior to the immobilization of affinity ligands. For polystyrene, the hydrophobic property should be modified before use. Less hydrophobic resins such as poly(2-hydroxyethyl methacrylate–methyl methacrylate) are thus considered in order to avoid using hydrophobic polystyrene. However, these resins are limited to the use of batch adsorption [2]. Column chromatography is not allowed because of their soft property. In the

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present work, non-porous, crosslinked, co-polymerized particles of styrene (S), methyl methacrylate (MMA) and glycidyl methacrylate (GMA) were developed and used for affinity chromatography of proteins. The particles were mechanically stable due to the presence of benzene rings in the backbone of polymer chains and could withstand high pressures. The polymer particles were advantaged by immobilization of ligands via the epoxy groups on the particle surface that was introduced by the monomer GMA.

As a model affinity system, Cibacron Blue 3G-A was immobilized onto the non-porous copolymer beads. Over the past decades, dye-affinity chromatography has gained wide interest because of its high selectivity and efficiency. Cibacron Blue 3G-A, which is also known as Cibacron Blue F3G-A, is the best example of a biomimetic ligand [3]. Molecular modeling of bound dye suggests that it mimics NAD^+, and therefore selectively binds the enzyme that needs this cofactor, for example, yeast alcohol dehydrogenase. Other proteins like hen egg white lysozyme and human serum albumin (HAS) can also bind [4]. Application of this dye ligand for affinity chromatography of proteins has been extensively studied in the past decades [5–18]. Cibacron Blue 3G-A is a monochlorotriazine dye and contains three acidic sulfonate groups and four basic primary and secondary groups, and can interact group-specifically with these proteins. Via the nucleophilic reaction between chloride of its triazine ring and reactive groups of the supports including hydroxyl (–OH) and amino ($–NH_2$), Cibacron Blue 3G-A can be immobilized onto the supports for protein adsorption. Among the solid supports that have been used for dye immobilization, agarose (Sepharose) is the most popular one [5,7,9,12,17]. The use of these dye-immobilized supports for column chromatography, especially in the HPLC mode, is occasionally difficult because of their inherent drawbacks of compressibility and causing the fouling. To overcome these problems, particles with good mechanical properties, especially those having a non-porous structure, are recommended for protein chromatography, which is usually operated in the HPLC mode. The use of non-porous particles also has the advantage of rapid separation and eliminating diffusion limitation. Non-porous silica has been employed for immobilization of Cibacron Blue 3G-A. However, the affinity separation system using dye-immobilized non-porous silica is limited to batch adsorption and frontal elution [19,20]. This paper describes the preparation of non-porous particles containing Cibacron Blue 3G-A on the surface, and their use for dye affinity chromatography of proteins in the zonal elution mode.

2. Materials and methods

2.1. Materials

Monomers of MMA, GMA and styrene were obtained, respectively, from Hayashi Pure Chemical, Aldrich and RDH, and used directly in the polymerization without purification. *N′*,*N′*-Methylenebismethacrylamide (Bis), 2,2′-azobis(isobutyronitrile) (AIBN), poly(vinyl pyrrolidone) (PVP) K-30, cetyl alcohol and methanol are all of reagent grade. Ethenediamine anhydrous (99.8%) was purchased from Tedia. Dye Cibacron Blue 3G-A, turkey egg white lysozyme, and bovine serum albumin (BSA) were obtained from Sigma.

2.2. Preparation of P(S–MMA–GMA) particles and coupling with dye Cibacron Blue 3G-A

Non-porous particles of styrene–glycidyl methacrylate–methyl methacrylate copolymer were prepared by the method of dispersion polymerization. The protocol is similar to a previous one used for the copolymerization of methyl methacrylate and glycidyl methacrylate [21]. The recipe is: 1.0 g styrene, 1.5 g MMA, 1.5 g GMA, 1.0 g PVP, 0.125 g cetyl alcohol, 0.025 g AIBN, 0.005 g Bis and 19.75 g methanol. The specific surface area and pore volume of the resultant particles were determined with an ASAP 2000 instrument (Micromeritics Instruments) and calculated with the BET equation using nitrogen as the adsorbate. The copolymer particles with epoxy groups on the surface were incubated with ethenediamine to yield reactive amino groups. To carry out this reaction, particles (ca. 1.0 g) were ultrasonically dispersed in a 100-ml flask, and mixed with 18 ml of methanol and 2.0 g of ethenediamine. After being sealed, the flask was shaken (125 rpm) at 70°C in a water bath for 24 h. The resultant particles

were cooled and washed with water and methanol. The amino-group-containing particles were then soaked in dye solution for dye immobilization. The dye solution was prepared by mixing 35 ml of methanol with 15 ml of distilled water and 0.02 g of Cibacron Blue 3G-A. After washing and drying, the non-porous, blue-colored affinity particles were obtained.

The epoxy content on the particle surface was determined by the titration method. Particles (0.6 g) were mixed with 50 ml $Na_2S_2O_3$ solution (pH 7) and shaken for 30 min. This suspension was titrated with 0.01 *M* HCl to pH 7. The amount of epoxy groups was calculated from the amount of hydrochloric acid needed in order to maintain neutrality [21].

2.3. Affinity chromatography of protein

The non-porous, dye-immobilized P(S–GMA–MMA) particles were slurry packed into a 1.0 cm× 0.46 cm I.D. stainless steel column using a column packer (Model CPP-085, Chemco, Japan). Aqueous solutions were used as the slurry mixed agent during the packing procedure. The packed column was equilibrated with a 0.02 *M* sodium phosphate buffer prior to sample application. Following the injection of a sample containing lysozyme, the column was eluted at a flow-rate of 0.5 ml/min. The mobile phase strategy was that stepwise change at 1.0 min from a 20 m*M* sodium phosphate buffer to the same buffer containing 1.0 *M* NaCl. The retained component was thus eluted out from the column with the NaCl-containing buffer. Eluted peaks from the HPLC column were detected at 280 nm using a Waters 486 tunable absorbance detector. The peak area was integrated with a Waters PC 800 integrator.

3. Results and discussion

3.1. Property of dye-immobilized non-porous particles

In order to increase the rigidity of the copolymerized particles, styrene was used as the third monomer, in addition to GMA and MMA. The particle diameter of the prepared polymer beads was about 2.1 μm, based on the scanning electron microscopy observation. This copolymer is slightly crosslinked since a very small amount (0.02%, w/w) of Bis was used. According to the measurement using the Micrometritics ASAP 2000, the specific surface area of the copolymer particles was 2.15 m^2/g. The particles were thought to be non-porous due to an extremely small value of pore volume (less than 0.006 ml/g). According to the *Q–e* Scheme of Alfrey and Prince, as cited in a book by Odian [22], the monomer reactivity ratios for styrene and MMA were calculated to be 0.49 and 0.48, respectively. The *Q* and *e* values used for calculation are, respectively, 1.0 and −0.8 for styrene, and 0.78 and 0.4 for MMA. This suggests that styrene and MMA have a similar reactivity. The mass ratio of GMA to the total mass of styrene and MMA is 0.6, according to this recipe. A previous study [21] on the co-polymerization of GMA and MMA concludes that non-porous particles with an average diameter of 2.1 μm could be obtained when the GMA/MMA mass ratio was in the range of 0.5 to 0.75. The results of the present work show the prepared styrene–GMA–MMA copolymer beads have the same diameter as the GMA–MMA copolymer whenever the mass fraction of GMA is the same.

The content of epoxy groups on the copolymerized particles was determined to be 5.1 μmol per gram of particles. After the surface modification with ethenediamine, the non-porous particles were covalently coupled with Cibacron Blue 3G-A on the surface via a nucleophilic reaction. The amount of dye immobilized onto the particles was determined to be 2.74 μmol per gram of particles. This value of dye density was at the same level of dye ligand immobilized on cross-linked agarose beads [13]. In terms of the surface area of particles, the density of dye on the particle surface was calculated to be 1.27 $\mu mol/m^2$. The dye density on the particle surface was determined by monitoring the loss of dye in the solution due to coupling reaction, using a UV–visible spectrophotometer at 630 nm.

Dispersion polymerization was found to be a promising method for the preparation of non-porous particles using styrene, MMA and GMA. The resultant particles were almost spherical and had a narrow distribution in the particle size. The polymeric particles were rigid due to the presence of

styrene as the co-monomer. In contrast to the co-polymer of MMA and GMA, copolymerized particles with the presence of styrene could be well packed into columns for operation in the HPLC mode. Styrene was used to enhance the rigidity of the particles, based on the fact that polystyrene is a highly rigid polymer in aqueous solutions. Since the polymerization was carried out in a polar solvent (methanol) and PVP was used as the stabilizer, the surface of the resultant polymer beads was adsorbed or grafted with PVP. According to the particle formation mechanism proposed by Tseng et al. [23], PVP molecules were adsorbed by the aggregates of growing polymer (oligomer) chains and finally anchored on the mature particles, in order to stabilize the dispersion of hydrophobic particles in the polar medium. The anchored PVP could not be washed out; the reactivity of epoxy on the particle surface was thus probably weakened by the presence of this hydrophilic PVP. Direct immobilization of amino-containing ligands to this epoxy-containing polymer was found to be ineffective (data not shown). However, amination of the co-polymerized particles with ethenediamine could yield surface amino groups, which were ready for covalent coupling of Cibacron Blue 3G-A. The rigid property of co-polymerized beads was proved by the linearity between flow-rate and column pressure. The backpressure increased linearity with the flow-rate up to 2 ml/min. A straight line emerging from the origin was obtained up to 1600 p.s.i., at which the flow-rate was 2 ml/min (1 p.s.i.=6894.76 Pa).

3.2. High-performance affinity chromatography of lysozyme

When pure lysozyme dissolved in a 0.02 *M* sodium phosphate buffer was injected into the affinity column, the retained component can be eluted at ca. 7 min (Fig. 1). Fig. 1 shows a typical elution profile with an injected volume of 20 μl. Results from a blank injection indicate that there was also a baseline shift to the same level as shown in Fig. 1 at ca. 8 min after the mobile phase was changed to the NaCl-containing buffer. The baseline then returned to the original level after the mobile phase was changed back to the NaCl-free buffer. This phenomenon suggests that the baseline shift was simply due

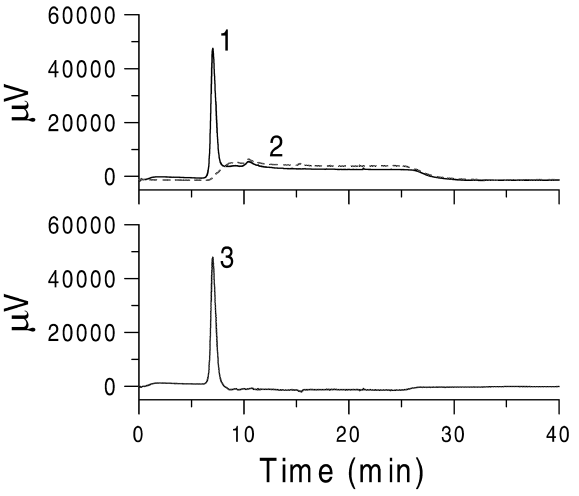

Fig. 1. Affinity chromatography of lysozyme on a 1.0 cm×0.46 cm I.D. column packed with dye-immobilized non-porous absorbents. Stepwise changes in mobile phase are from phosphate buffer (A) to the same concentration of buffer with 1.0 *M* NaCl (B) at 1 min and from B to A at 14 min. Flow-rate: 0.5 ml/min. Sample volume: 20 μl. Lysozyme concentration, 0.4 mg/ml. Keys for elution curves are: 1, the original elution peak; 2, the elution peak for a blank injection (dashed line); 3, the reduced peak.

to the presence of NaCl. These salt ions were bound on the immobilized dye due to the ion-exchange function. A previous report indicates that immobilized Cibacron Blue 3G-A can act as an ion exchanger [9]. The binding of salt ions onto the immobilized ligand displaced the binding of lysozyme, and consequently resulted in the elution of bound lysozyme. Finally as the mobile phase was changed back to a NaCl-free buffer, the bound salt ions were eluted out from the column. To obtain the reduced peak, the peak profile of a blank injection was subtracted from the original elution profile. As shown in Fig. 1, no significant shift of baseline appeared in the reduced elution peak (curve 3).

The retention time of the eluted peak was 7.04 min for sample concentration ranging from 0.1 to 0.4 g/l. Since the elution started at one min after sample injection, the averaging true retention time for lysozyme in the affinity column under elution conditions was 6.04 min. The capacity factor was thus calculated as $k'=(6.04\cdot0.5-0.115)/(0.115-0.031)=34.6$, where 0.031 (ml) is the extra-column dead volume and 0.115 (ml) is the retention volume of the non-retained component.

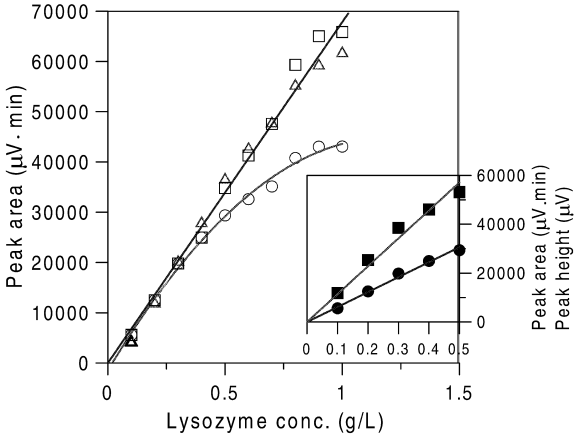

Fig. 2. Peak area (●) and peak height (■) as functions of the loading of lysozyme to the affinity column. Both the total peak area (○) and area of elution peak (□) are presented. The total peak areas (△) resulted from injections to the aged column are also reported. The elution conditions are the same as in Fig. 1.

Fig. 2 shows the total peak area and the peak area for eluted peak as functions of lysozyme concentration in a 20-μl sample. The total peak area, including peak area for the eluted component and that for the non-retained fraction, is directly proportional to the sample load up to ca. 20 μg (corresponding to 1.0 g/l) as shown in Fig. 2. However, the linearity for the elution peak area vs. sample load is no longer valid as the sample concentration exceeds 0.5 g/l. This is because a shorter column (1 cm long) packed with non-porous particles was used. A small fraction of lysozyme did not remain in the column whenever the sample concentration was greater. Although the column capacity is small, this affinity column is suitable for quantitative analysis of lysozyme in the range of small concentrations. With the concentration up to 0.4 g/l, the non-retained peak did not appear and the plot of eluted peak height vs. sample load was also linear. This suggests that the prepared column is most likely useful for micro-analysis. The non-retained peak appeared when the lysozyme concentration in the sample was 0.5 g/l or greater. The non-retained fraction increased with sample concentration. It approached about 35% as the sample containing 1 g/l of lysozyme, corresponding to 20 μg of lysozyme. Even in the presence of a split-peak, the retention time for the eluted peak was not significantly changed by increasing sample load. It was kept at 7 min up to when 1 g/l of lysozyme was applied.

The prepared affinity column was effective for the separation of lysozyme from non-specific proteins. As shown in Fig. 3, mixtures of lysozyme and BSA could be eluted into two split peaks. BSA that has no affinity to dye was eluted at ca. 0.23 min, which is almost the same time of flow through the void space of the column. The results suggest that BSA is the non-retained component with respect to this chromatographic column.

The prepared column was very stable. After reusing several times and storing in a refrigerator for 7 months, the peak profiles for lysozyme elution were reproducible. No significant change in the calibration curve for total peak area, especially in the small concentration range, was observed, as shown in Fig. 2, despite a very small increase in retention time from 7.0 to 7.2 min. This fluctuation in the retention time was possibly due to the different preparations of the mobile phase buffer. Fig. 4 shows the affinity chromatography at different flow-rates (0.5–1.2 ml/min) using the aged column. As expected, high flow-rates could shorten the retention time of the retained component, meanwhile resulting in higher column pressures. However, the capacity factor for lysozyme was not significantly changed by

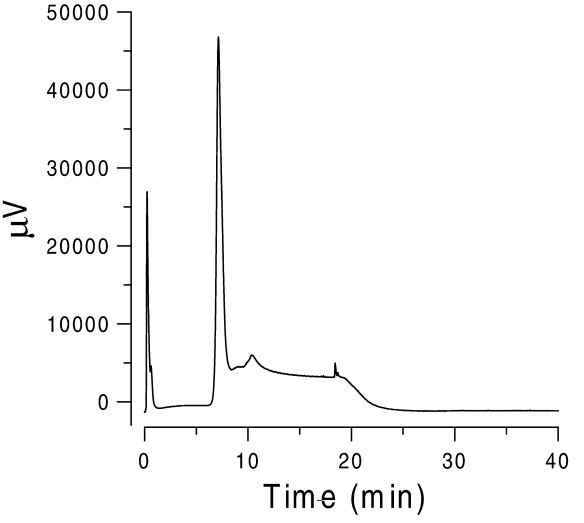

Fig. 3. Affinity chromatography of a protein mixture of lysozyme and BSA. Sample concentration: 0.4 mg/ml lysozyme+0.36 mg/ml BSA. The elution conditions are the same as in Fig. 1.

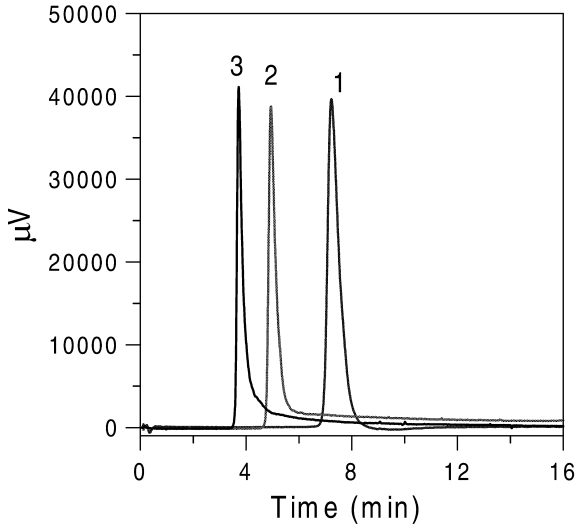

Fig. 4. Affinity chromatography of lysozyme at different flow-rates: 0.5 (1), 0.8 (2) and 1.2 ml/min (3). The sample concentration was 0.3 mg/ml of lysozyme. The elution conditions are the same as in Fig. 1.

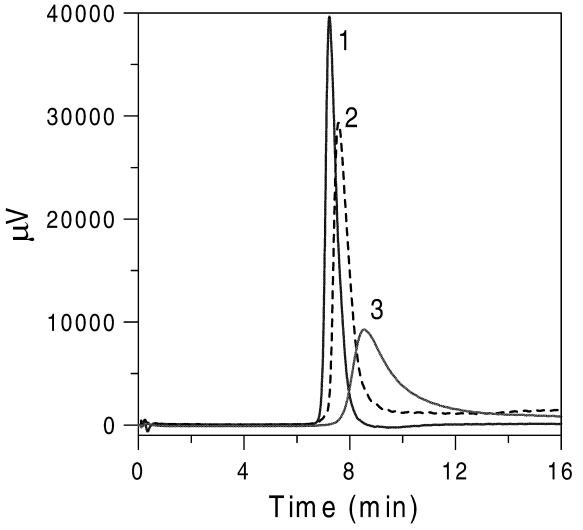

Fig. 5. Affinity chromatography by using the mobile phase with different NaCl concentrations: 1.0 (1), 0.5 (2) and 0.1 *M* (3). The concentration of lysozyme in each sample was 0.3 mg/ml. Other conditions are as in Fig. 1.

the volumetric flow-rate. The retention time decreased significantly from 7.2 to 3.7 min, but the capacity factor changed very slightly from 36 to 37 with the flow-rate varying from 0.5 to 1.2 ml/min. In order to reduce the analysis time, the flow-rate could be carefully increased. The elution peaks in Fig. 4 are all reduced peaks, i.e., the elution profile of a blank injection with the same flow-rate was deducted from each experimental elution profile.

A study on the interaction of Cibacron Blue 3G-A to protein shows that this dye is capable of hydrophobic and electrostatic interactions with protein molecules in the instance of ambient conditions [24]. Increasing the ionic strength of the buffer is the most commonly used method for eluting the bound proteins from the column. Usually 1 *M* of NaCl in the buffer is sufficient, but occasionally 2 or 3 *M* of salt is required for dye-affinity chromatography. The results in Fig. 5 suggest that 1 *M* of NaCl in the buffer is needed in order to obtain a sharp peak. Decreasing the NaCl concentration (ionic strength) in the mobile phase would result in a poor peak shape and an increasing retention time. In other words, the lower the NaCl concentration, the longer the retention time, as shown in Fig. 5.

4. Conclusion

The present paper demonstrates the preparation and application of co-polymerized non-porous beads for affinity chromatography of proteins. Non-porous polymer particles were prepared, chemically modified, and coupled with Cibacron Blue 3G-A. The support, with the presence of benzene rings in the backbone of polymer chains, was mechanically stable and mimics as the rigid matrix for column chromatography. The chromatographic column packed with the dye-immobilized particles was effective for the separation of the mixture consisting of lysozyme and BSA. Elution of the bound lysozyme could be achieved by using 1 *M* of NaCl in the buffer as the mobile phase. Experimental data indicated that samples with lysozyme content up to 8 μg could be analyzed chromatographically using a shorter column (1 cm long) packed with the dye-affinity

non-porous sorbents. The affinity column will also be examined for chromatography of other proteins besides lysozyme, which can specifically interact with Cibacron Blue 3G-A.

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