Biporous Polymeric Beads Fabricated by Double Emulsification for High-Speed Protein Chromatography

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ABSTRACT: Rigid biporous beads (BiPB) were fabricated by double emulsification. An aqueous suspension of superfine calcium carbonate granules and organic solvent were used as porogenic agents to create superpores and micropores, respectively. The polymerization of monomers, glycidyl methacrylate, and ethylene glycol dimethacrylate was initiated with benzoin ethyl ether by ultraviolet irradiation. Modified with diethylamine (DEA), the BiPB were derivatized into an anion-exchange medium (which is denoted as DEA–BiPB). The DEA–BiPB with an average diameter of 46.3 μm was characterized to possess two types of pores, that is, micropores (20–200 nm) and superpores (500–5300 nm). Flow hydrodynamic experiments showed that the DEA–BiPB column had a smaller backpressure than that of the conventional microporous beads column at a given flow rate. The static adsorption capacity of the DEA–BiPB was close to that of the DEA–MiPB for bovine serum albumin. However, frontal analysis demonstrated that the dynamic binding capacity of the DEA–BiPB column was two times higher than that of the DEA–MiPB at a flow rate of 1800 cm/h. Moreover, the purification of the molecular chaperone GroEL was carried out with the DEA–BiPB column at two flow rates (150 and 1500 cm/h). This showed that the GroEL purification was nearly the same at the two flow rates tested. These results indicate that the DEA–BiPB column is promising for high-speed protein chromatography.
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Key words: adsorption; macroporous polymers

INTRODUCTION

Chromatography has been one of the most important tools for the purification of biomacromolecules in the last 5 decades because of its high resolution and mild separation conditions.[1,2] The key element of liquid chromatography is the stationary phase, and the progress of the stationary phase promotes its application in bioseparations. Polysaccharides, such as agarose, dextran, and cellulose, are the most widely used chromatographic media for protein chromatography, but their low mechanical strength seriously limits their application at high speed.[3] Because of the inevitable drawbacks of the soft, gel-based stationary phases, organic resins, which have much higher mechanical strengths and are qualified to tolerate extreme environmental conditions, have been widely studied as alternatives.[4]

As a result of the slow diffusion of macromolecules through the interior of the stationary phase, the required operation time for the separations of biomolecules is usually quite long. Thus, perfusion chromatography or flowthrough chromatography using packing materials with large throughpores or superpores have been widely used to overcome the interior mass-transfer resistance[2,5] and to promote intraparticle adsorption by the convective flow through these superpores.[1,2,6–8] The matrix for flowthrough chromatography has primary and secondary sets of pores. Because the convective transport rate of the biopolymer is several orders of magnitude greater than the diffusive rate, the interior binding sites within the pore network of a perfusive support are accessed more rapidly with packing materials that rely solely on diffusive transport. Accelerated mass transport resulting from intraparticle flow minimizes band broadening, which in turn, is recognized as high column efficiency and high capture efficiency at elevated flow rates.[9]

The advances in suspension polymerization technology have made it possible to prepare rigid organic biporous resins with elevated mechanical and chemical stabilities, and thus provide a new approach to efficient chromatographic operations.[3] It has been reported that superporous agarose beads could be prepared by a double emulsification procedure, and the superpores created by the interior oil phase have been proved to greatly improve the intraparticle mass transfer.[10,11] Inspired by this research, we fabricated

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biporous beads (BiPB) by (water/oil)/water ((w/o/)/w) emulsification with a glycerol solution as the interior water phase and toluene and *n*-heptane as the porogenic agents.[12] The new biporous medium was able to overcome the drawbacks of agarose gel in low mechanical strength and took less time to prepare than the polymeric matrices reported previously.[13] Moreover, Wu et al.[9] synthesized a rigid biporous microsphere with calcium carbonate granules and organic solvent as porogenic agents by radical suspension polymerization. In the previous procedures, however, instability in the (w/o/)/w[12] and the leakage of carbonate granules during the suspension polymerization[9] were observed. These unfavorable phenomena affected the recovery of BiPB for the final application. Hence, in this study, we improved the fabrication procedure by combining the methods reported previously.[9,12] That is, the BiPB were synthesized by a double emulsification method with a calcium carbonate suspension as the interior water phase instead of a glycerol solution[12] or calcium carbonate granules.[9] The synthesized BiPB were extensively characterized for their physical properties and chromatographic behavior and were finally used for the high-speed purification of the molecular chaperone GroEL, which is an *in vivo* protein folding helper made of two stacked seven-membered rings of 57 kDa.[14–16]

EXPERIMENTAL

Materials

Glycidyl methacrylate with a purity of 99% was purchased from Suzhou Anli Chemical Co. (Jiangsu, China) and used without further purification. Ethylene glycol dimethacrylate, Coomassie brilliant blue G-250, Coomassie brilliant blue R250, ampicillin, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). A low-molecular-weight calibration kit for sodium dodecylsulfate-polyacrylamide electrophoresis (SDS-PAGE) was purchased from Amersham Biosciences (Uppsala, Sweden). Cyclohexanol and dodecanol were from Tianjin Chemical Co. (Tianjin, China). Benzoin ethyl ether, a free-radical initiator of photopolymerization, was purchased from the Medicine Co., Ltd. (Beijing, China). Sorbitan monooleate (Span 80) and poly(vinyl alcohol) were obtained from Tianjin Kaitong Chemical Co. (Tianjin, China). Diethylamine (DEA) and dioxane were the products of Tianjin Kewei Chemical Co. (Tianjin, China) and were used without further purification. Superfine calcium carbonate (0.3–5 μm) was from Guangdong Enping Chemical Co. (Guangdong, China). An *Escherichia coli* strain harboring the plasmid pND5 was kindly provided by Professor Horowitz (University of Texas Health Science Center). Yeast extraction and tryptone were purchased from Oxoid, Ltd. (Basingstoke, Hampshire, United Kingdom). The other reagents were all analytical grade and were from local sources.

Synthesis of anion exchangers

BiPB were prepared by a radical suspension photopolymerization method.[12] At first, we prepared the calcium carbonate suspension by suspending the superfine calcium carbonate in a 1% Triton X-114 solution in an ultrasonic water bath for 45 min. Then, the carbonate granules were recovered from the Triton X-114 solution by centrifugation and resuspended into deionized water at 20% (w/v). The organic porogenic agent consisted of cyclohexanol and dodecanol in a volume ratio of 5 : 2. The organic porogen (3.5 mL) was added to a mixture of monomers (4.5 mL of glycidyl methacrylate and 3 mL of ethylene glycol dimethacrylate) containing 0.08 g of benzoin ethyl ether and 0.45 g of Span 80. The mixture was degassed and homogenized by ultrasonication for 20 min. Then, the solution was transferred to a glass reactor, and 8 mL of the calcium carbonate suspension was added as the aqueous phase. The two-phase system was rigorously stirred at 8000 rpm for 5 min to prepare the water-in-oil emulsion (emulsification I). The emulsion was then immediately poured into a reactor containing a 120-mL aqueous solution of 2% poly(vinyl alcohol) stirred at 1800 rpm (emulsification II). After 1 min of agitation at 1800 rpm, the (w/o)/w emulsion was transferred to a beaker (i.d. = 70 mm) agitated with magnetic stirrer at 300 rpm and irradiated with a UV lamp (1000 W) for 20 min for polymerization. The white polymeric beads were collected and thoroughly washed with hot deionized water (60–70°C). Then, the organic porogenic agent and the calcium carbonate were removed, as reported previously.[9,12]

Microporous beads (MiPB) were prepared without the procedure of emulsification I as described previously.[12]

Anion exchangers were prepared by the immobilization of DEA to the BiPB and MiPB with the ring-opening reaction of the epoxide groups on the copolymer.[17] After the modification, the anion exchangers were thoroughly washed with distilled water and ethanol and then dried at room temperature. The washing removed almost all of the impurities, so no UV–visible absorbance was detected with the bead-suspending solution.

Analysis and measurements

The surface structure of the adsorbents in the dry state was observed by scanning electron microscopy (SEM; S-3500N, Hitachi, Tokyo, Japan).[12] Particle-size distribution was measured with a Mastersizer 2000 unit (Malvern Instruments, Malvern, UK). The pore size (*D*) distribution was determined by mercury porosimetry with a Quantanchrome Poremaster-60 mercury

porosimeter (Quantanchrome Corp., Boynton Beach, FL). The specific surface area of the dry particles was calculated from the Brunauer–Emmett–Teller (BET) isotherm of nitrogen, measured with a BET Chembet-3000 instrument (Quantanchrome Corp.). The hydrated density of the wet beads was measured with a 25-mL pycnometer. The total ion capacity of the anion exchanger was detected by a method described previously.[12]

Static and dynamic adsorption experiments

A finite batch adsorption experiment was used to determine the static adsorption isotherms of BSA on the DEA–BiPB and DEA–MiPB.[9,12] All of the dynamic adsorption experiments were conducted on an ÄKTA Explorer 100 system controlled by Unicorn 4.11 software (Amersham Biosciences, Uppsala, Sweden) with an HR 5/5 column at room temperature. The dynamic adsorption capacity was determined by frontal analysis conducted in 10 mmol/L Tris-HCl buffer (pH 7.6) and with a 2.0 mg/mL BSA solution. The procedures were the same as those described earlier.[12] The dynamic capacity at 10% breakthrough was calculated from the following equation:

$$q_{10} = \frac{C_0 F(t_{10} - t_0)}{V_B} \quad (1)$$

where q_{10} (mg/mL of bed) is the dynamic binding capacity at 10% breakthrough, C_0 (mg/mL) is the feed BSA concentration, t_{10} (min) is the time at 10% breakthrough, t_0 (min) is the retention time under nonretained conditions, F (mL/min) is the volumetric flow rate, and V_B (mL) is the bed volume.

Purification of the molecular chaperone GroEL

The *E. coli* strain harboring the plasmid pDN5 was used to produce GroEL. The strain was cultured as described previously.[18] The cells were disrupted by ultrasonication and centrifuged at 14,000 g for 30 min to remove cell debris. The supernatant was diluted fivefold with buffer A (50 mmol/L Tris-HCl buffer, pH 7.6, plus 2 mmol/L of ethylenediaminetetraacetic acid and 50 g/L of glycerol) and used as the feedstock for chromatographic separation.

The DEA–BiPB beads were packed into an HR 5/10 column (i.d. = 5 mm, length = 10 cm) and connected to the ÄKTA Explorer 100 system. All chromatography experiments were performed at room temperature. Before the feedstock introduction, the column was equilibrated with buffer A for 10 CVs. Then, the feedstock was loaded onto the column, and the column was washed with buffer A to remove unbound impurities until the UV at 280 nm approached the baseline. Thereafter, the column was developed by stepwise salt-gradient elution with buffer A and buffer B (50 mmol/L Tris-HCl buffer, pH 7.6, plus 1 mol/L KCl, 2 mmol/L ethylenediaminetetraacetic acid, and 50 g/L glycerol). Preliminary experiments with 0.1 mL of the feedstock were performed to determine the proper salt gradients for better resolution. Then, frontal analysis with the feedstock was performed at 150 cm/h, and the effluent fractions were collected for SDS-PAGE analysis. This experiment led to the identification of the breakthrough volume of GroEL from the column. Thus, the loading volume with little GroEL loss was determined. In the frontal analysis and purification experiments with high feedstock loads, a 50-mL superloop (Amersham Biosciences) was used.

After each run, the column was cleaned with 10 CVs of buffer B and then reequilibrated with buffer A until a constant UV baseline was obtained. The total protein concentration in each purification experiments was determined by the Bradford method.[19] The effluent pools were analyzed by discontinuous SDS-PAGE with 12.5% acrylamide in the separation gel.

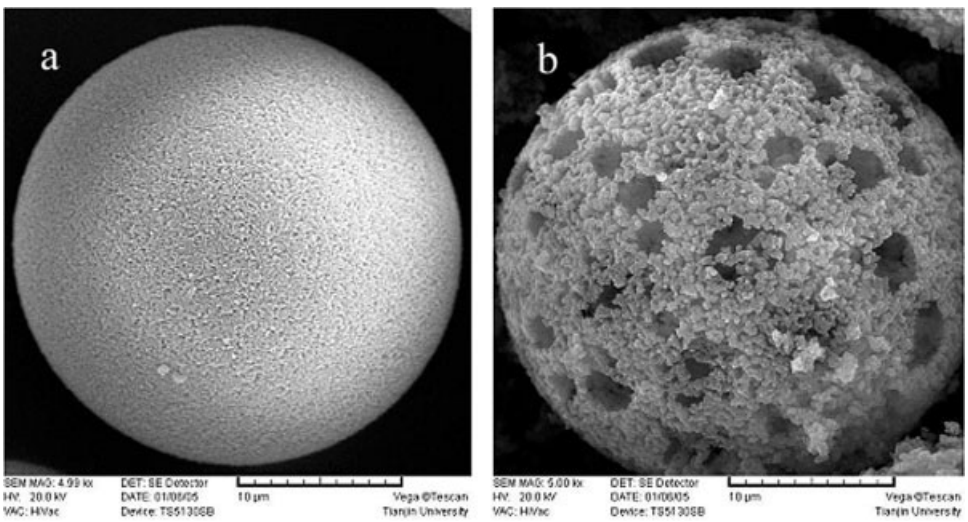

Figure 1 SEM photographs of (a) DEA–MiPB and (b) DEA–BiPB.

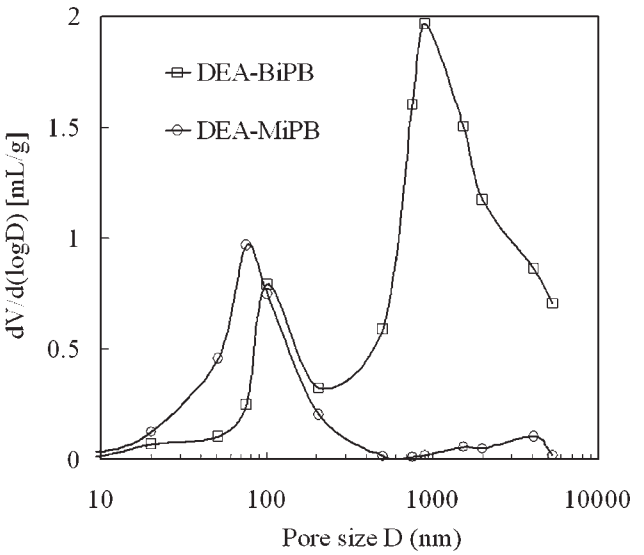

Figure 2 *D* distributions of the two anion exchangers. V, pore volume.

RESULTS AND DISCUSSION

Physical properties of the beads

Figure 1 shows the examples of the SEM photographs of the DEA–MiPB and DEA–BiPB. It was obvious that the surfaces of the two kinds of microspheres were quite different. Compared to the smooth surface of MiPB in Figure 1(a), wide channels with *D*s of 1–3 μm were distributed on the surface of the BiPB [Fig. 1(b)]. These were considered to be the superpores created by the microdroplets of the aqueous suspension of superfine calcium carbonate granules inside the polymeric beads, which was formed by emulsification I. Between the wide pores, there were micropores that were similar to those on the MiPB [Fig. 1(a)]. The micropores were expected to provide interconnected paths for convective flow in chromatography.[9,12]

The *D* distributions of the DEA–BiPB and DEA–MiPB were analyzed by mercury porosimetry. As clearly shown in Figure 2, there was a bimodal *D* distribution in the DEA–BiPB. The micropores mostly ranged from 20 and 200 nm, which were expected to act as the diffusive pores for solute transport. In comparison, the superpores distributed between 500 and 5300 nm, with a peak value at about 900 nm. The size distribution of the superpores was in good agreement with that directly observed by SEM [Fig. 1(b)]. If the superpores were interconnected, convective flow through them could certainly occur during chromatographic operations.[1,2,5] In comparison to the DEA–BiPB, most pores in the DEA–MiPB were in the range from 20 and 200 nm, and only a small amount of micrometer-sized pores were identified. The small amount of the wide pores might have been those between small particles, which were regarded as pores by the mercury porosimetry. Therefore, we concluded that the DEA–MiPB contained mainly micropores through which solutes could transport by diffusion.[7]

Both the micropores and superpores in the DEA–BiPB were larger than those in the BiPB prepared by Wu et al.[9] Hence, it seems that the porogenic method used in this study not only created large superpores but also large micropores. The BET method revealed that the specific surface areas of the DEA–BiPB and DEA–MiPB in this study were 32.2 and 55.3 m^2/g, respectively. The smaller specific surface area of the BiPB, compared to that of the MiPB, was considered due to the existence of the superpores in the BiPB, which contributed less to the specific surface area than the micropores. Moreover, the specific surface area of the DEA–BiPB was only about one-third that of Wu et al.[9] We considered this to be due to the larger pores in this BiPB, as mentioned previously. The hydrated densities of the DEA–BiPB and DEA–MiPB were estimated at 1.08 and 1.10 g/mL, respectively, the same as those reported earlier.[9] The particle size distribution of both the beads was 20–90 μm. The volume-weighted mean diameters of the two kinds of beads were also almost the same, 46.3 μm for the DEA–BiPB and 45.2 μm for the DEA–MiPB, which were about 3–4 μm smaller than those reported by Wu et al.[9] The total ion capacities of DEA–BiPB and DEA–MiPB were determined to be 0.11 and 0.19 mmol Cl^-/mL of wet resin, respectively, which were about one-tenth those of Wu et al.[9]

Flow hydrodynamics

Figure 3 shows the effect of flow velocity on the backpressures of the DEA–BiPB and DEA–MiPB columns.

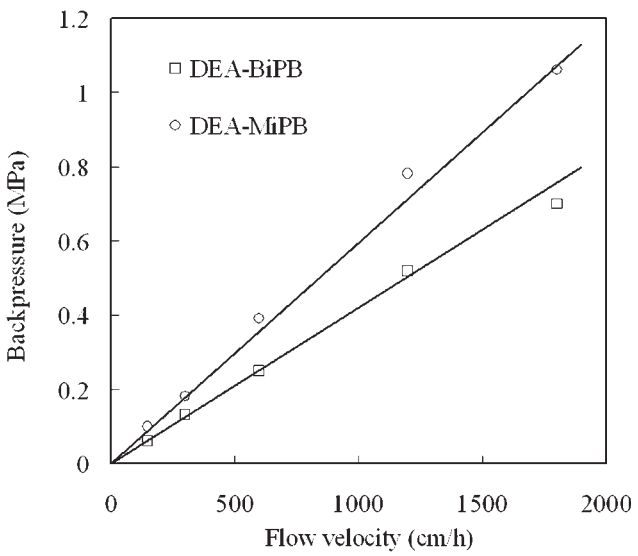

Figure 3 Effect of flow velocity on the backpressure of the DEA–BiPB and DEA–MiPB columns with an HR 5/10 column (i.d. = 5 mm, length = 10 cm) and a mobile phase of 10 mmol/L Tris-HCl buffer (pH = 7.6).

The backpressures of both the columns increased linearly with flow velocity up to 1800 cm/h, but the pressure drop of the DEA–BiPB column was much lower than that of the DEA–MiPB column. The low backpressure of the DEA–BiPB column was evidence for the presence of the flowthrough channels that reduced the flow resistance. The same phenomenon has been observed in previous work.[7–9,12]

Static and dynamic adsorption

Figure 4 shows the adsorption isotherms of BSA to the two anion exchangers. The adsorption equilibria could be approximated by the Langmuir equation, as indicated by the solid lines in Figure 4:

$$q = \frac{q_m c}{K_d + c} \tag{2}$$

where c (mg/mL) is the equilibrium concentration of BSA in bulk solution, q (mg/g of wet resin) is the adsorbed density of protein, q_m is the adsorption capacity, and K_d is the dissociation constant. Least-square fitting of the equation to the experimental data gave the static adsorption capacities of DEA–BiPB and DEA–MiPB at 77.6 and 84.4 mg/g of wet resin, respectively.

It is more important for an adsorbent to show a rather high dynamic or chromatographic capacity than a high static capacity. Frontal analysis by breakthrough experiments can provide the dynamic binding capacity (DBC). The DBC values of the DEA–BiPB and DEA–MiPB columns, calculated from eq. (1), are given in Figure 5 as a function of flow velocity. In Figure 5, a breakthrough curve from the DEA–BiPB column obtained at 1200 cm/h is represented as the inset. At the lowest flow rate tested (300 cm/h), the

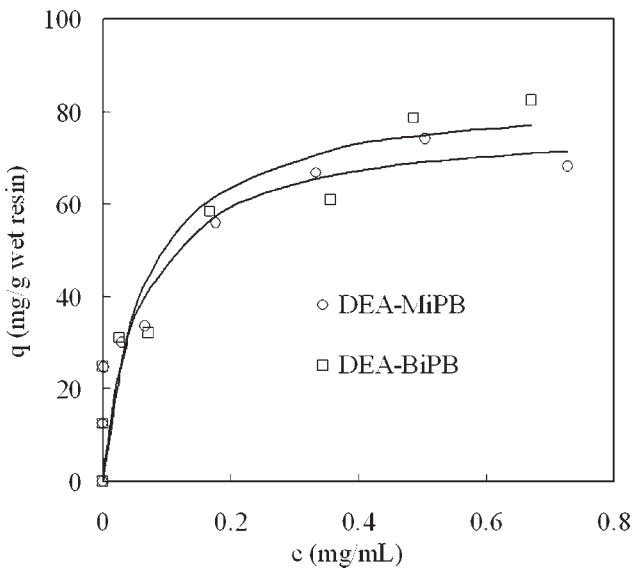

Figure 4 Static adsorption isotherms of BSA to DEA–BiPB and DEA–MiPB.

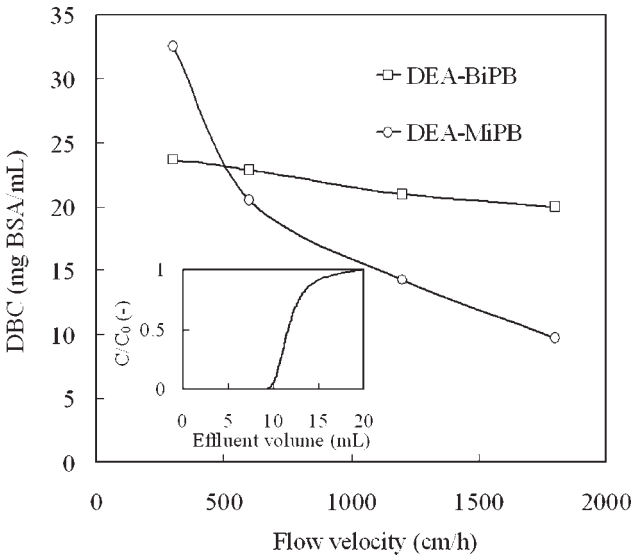

Figure 5 Dynamic binding capacities of BSA on the DEA–BiPB and DEA–MiPB columns. The inset represents a breakthrough curve from the DEA–BiPB column obtained at 1200 cm/h, where C is the outlet protein concentration and C_0 is the feed protein concentration (2 mg/mL). An HR 5/5 column (i.d. = 5 mm, length = 5 cm) was used for the experiments.

DBC of the DEA–MiPB column (32.5 mg/mL of bed) was higher than that of the DEA–BiPB column (23.6 mg/mL of bed). With increasing mobile phase velocity, however, the DBC of the DEA–MiPB column decreased drastically to a value of 9.7 mg/mL of bed at 1800 cm/h. In contrast, the DBC of the DEA–BiPB column decreased slightly and remained at a value of 20.0 mg/mL of bed up to a flow rate of 1800 cm/h, which was two times higher than that of the DEA–MiPB column. When the flow rate was low, the backpressure of the column was low as well (Fig. 3). Thus, convective transport in thee superpores was not obvious, whereas the diffusion in the micropores had a major effect on the dynamic protein adsorption. Increasing the flow rate resulted in an increase in the operation pressure across the column, which promoted the favorable effect of the superpores on the dynamic adsorption. The results indicate that the onset of the convective flow within the superpores accelerated the intraparticle mass transfer and led to the high DBC at an elevated flow velocity.

After the frontal analysis experiments, the adsorbed protein was recovered by elution with 1.0 mol/L NaCl in Tris-HCl buffer (pH 7.6). As a result, the adsorbed BSA was completely eluted by the high-ionic-strength solution, which indicated the absence of nonspecific adsorption of the matrices for the protein.

Purification of the molecular chaperone GroEL

To test the characteristics of the biporous ion exchanger for practical applications, we used it to

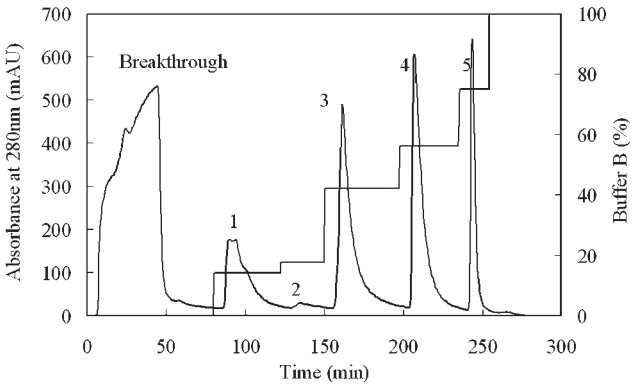

Figure 6 Purification of GroEL with the DEA–BiPB column at 150 cm/h. The feedstock (20 mL) was applied for the purification. The lines parallel to the *x* axis indicate the volume percentage of buffer B in the mobile phase.

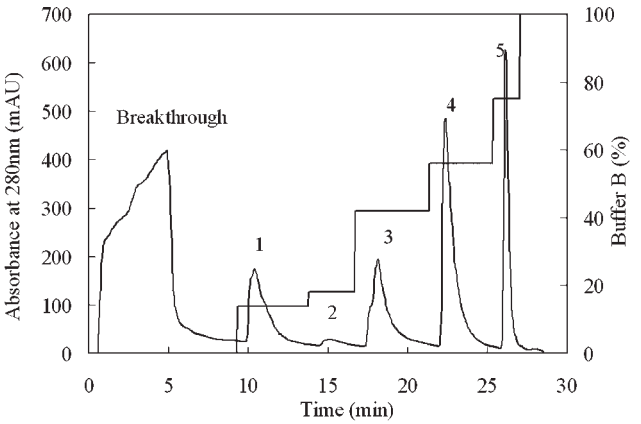

Figure 8 Purification of GroEL with the DEA–BiPB column at 1500 cm/h. The feedstock (20 mL) was applied for the purification. The lines parallel to the *x* axis indicate the volume percentage of buffer B in the mobile phase.

capture the molecular chaperone GroEL from clarified *E. coli* lysate. Preliminary experiments optimized the salt gradient as 14, 18, 42, 56, and finally 100% buffer B for the purification operations at both low (150 cm/h) and high (1500 cm/h) flow rates. Then, frontal analysis at 150 cm/h revealed that GroEL started to breakthrough at about 20 mL. Thus, to give the highest possible loading and to ensure a high recovery yield of GroEL, 20 mL of the feedstock was loaded for the GroEL purification experiments.

Figure 6 shows the chromatographic profiles obtained at 150 cm/h, and Figure 7 displays the electrophoretic analysis of each chromatogram shown in Figure 6. Electrophoresis illustrated that contaminant proteins with different molecular masses were eluted in the first three peaks. Because of the stronger binding between GroEL and the DEA–BiPB resin, GroEL was eluted as the fourth peak at a high salt concentration (0.56 mo1/L). As shown in Figure 7, GroEL existing in the fourth peak showed a single band in SDS-PAGE, which indicated the electrophoretic homogeneity of the purified product. The component of the fifth peak could not be stained by the R-250 dye on the SDS-PAGE, and no protein was detected by the Bradford method. Therefore, it was considered to be nucleic acids. Nucleic acids strongly bind to anion exchanger because they are highly charged with phosphate groups. So, they could only be eluted at high ionic strength (0.75 mol/L in Fig. 7). Similar results were reported by Tong et al.[20] in the purification of GroEL by expanded bed adsorption.

To further identify the advantage of this biporous matrix under a high operation speed, we purified the GroEL at a flow rate elevated 10-fold, 1500 cm/h (Fig. 8). Combined with the electrophoretic analysis

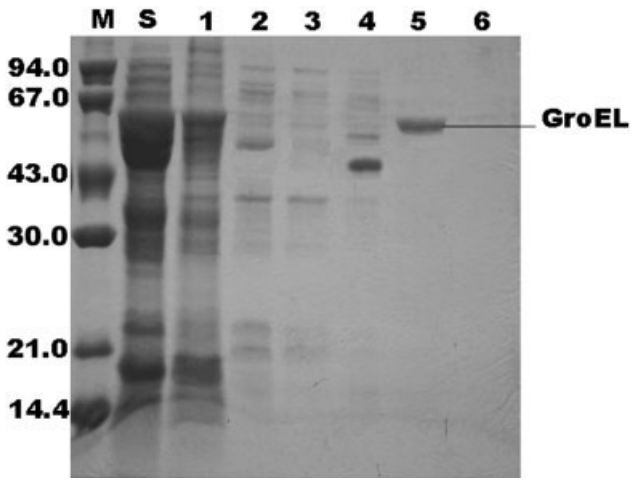

Figure 7 SDS-PAGE analysis of the chromatograms in Figure 6: lanes (M) marker, (F) feedstock without dilution, and (B) breakthrough fraction. Lanes 1–5 correspond to the peaks indicated in Figure 6.

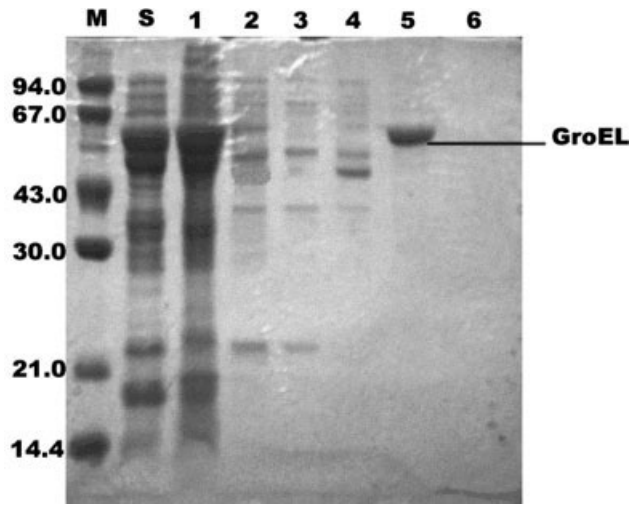

Figure 9 SDS-PAGE analysis of the chromatograms in Figure 8: lanes (M) marker, (F) feedstock without dilution, and (B) breakthrough fraction. Lanes 1–5 correspond to the peaks indicated in Figure 8.

(Fig. 9), we found that the final separation results under such a high velocity showed little difference from those under the low one (Figs. 6 and 7). At this high flow rate, the purification was completed within 30 min, and 10.8 mg of protein was collected from the GroEL peak (peak 4) as detected by the Bradford method. The results demonstrate that the biporous structure of the DEA–BiPB resulted in an obvious increase in the intraparticle mass transfer rate and effectively minimized the negative effects of diffusive mass transfer. Thus, the matrix is promising for application in rapid chromatographic purification of proteins such as GroEL.

CONCLUSIONS

With calcium carbonate suspension and cyclohexanol/dodecanol organic solvent as porogenic agents to create superpores and micropores, respectively, a rigid biporous matrix was prepared by double emulsification and UV-irradiated polymerization. The biporous structure of the medium was identified by *D* analysis. Convective flow of the mobile phase through the superpores in the biporous medium was also confirmed by its low column backpressure and high DBC at a high flow rate. The customized DEA–BiPB was used to purify the molecular chaperone GroEL from a clarified broth of disrupted *E. coli,* and similar results were obtained at flow rates of 150 cm/h and 1500 cm/h. The results indicate that the biporous adsorbent is promising for high-speed protein chromatography. The optimization of the fabrication procedure would need to be conducted to further improve the performance of the biporous stationary phase.

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