Chinese Journal of Chemistry, **2004**, *22*, 283—289 **Article**

Synthesis of Monodisperse Poly(glycidylmethacrylate-co-ethylene dimethacrylate) Beads and Their Application in Separation of Biopolymers

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The monodisperse poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads with macroporous in the range of 8.0—12.0 μm were prepared by a single-step swelling and polymerization method. The seed particles prepared by dispersion polymerization exhibited good absorption of the monomer phase. The pore size distribution of the beads was evaluated by gel permeation chromatography and mercury intrusion method. By using this media, a weak cation exchange (WCX) stationary phase for HPLC was synthesized by a new chemical modification method. The prepared resin has advantages of biopolymer separation, high column efficiency, low column backpressure, high protein mass recovery and good resolution for proteins. The measured bioactivity recovery for lysozyme was (96±5)%. The dynamic protein loading capacity of the synthesized WCX packings was 21.3 mg/g. Five proteins were completely separated in 8.0 min using the synthesized WCX stationary phase. The experimental results show that the obtained WCX resin has very weak hydrophobicity. The WCX resin was also used for the rapid separation and purification of lysozyme from egg white in 8 min with only one step . The purity and specific bioactivity of the purified lysozyme was found more than 92.0% and 70184 U/mg, respectively.

Keywords monodisperse poly(glycidyl methacrylate-co-ethylene dimethacrylate) resins, weak cation exchange chromatography, protein separation, egg white

Introduction

Since 1954[1] the polymeric separation media has attracted much attention due to their chemical stability over the entire pH range. The rigid, highly cross-linked styrene copolymers were first used for chromatography by Moore.[2] The macroporous copolymers currently available are not only chemically stable but also more resistant to mechanical forces prevailing in a column and therefore are comparable to the traditional packings based on silica gel.

Most polymer separation media are still prepared by a classical suspension polymerization.[3] This technique affords beads that have rather broad particle size distributions and, therefore, can not be used directly for chromatography. In the search for uniformly sized beads as chromatographic stationary phases, Ugelstad *et al.*[4] developed a technique named "activated multi-step swelling and polymerization" method. Uniform beads were prepared by Ugelstad's method from a great variety of monomers, such as styrene,[5] methylmethacrylate,[6] 2-hydroxyethylmethacrylate,[7] glycidylmethacrylate,[8,9] vinylphenol[10] and chloromethylstyrene.[11] This method is excellent, but it seems rather complex because at least two steps are needed in the swelling process. The first step is the activation of the seed beads by the absorption of a water-insoluble compound. The subsequent step is the absorption of monomer, cross-linker and diluent.

Ogino *et al.*[12] reported the preparation of the uniform styrene-co-divinylbenzene (PS-DVB) beads by a single-step swelling and polymerization method in 1995. However, due to PS-DVB having strong hydrophobicity, the subsequent chemical modification of PS-DVB for protein separation is difficult. In this paper, we report the preparation of poly(glycidyl methacrylate-co-ethylene dimethacrylate ($P_{GMA/EDMA}$) beads by a single–step swelling and polymerization method in the presence of solvents, linear polystyrene as a porogen, and a new method for chemical modification of $P_{GMA/EDMA}$ for the synthesis of weak cation exchange (WCX) stationary phase. The chromatographic properties of the WCX stationary phase for biopolymer separation are discussed in detail. The WCX resin was also used for the rapid separation and purification of lysozyme from egg white in 8.0 min. The purity of the purified lysozyme was more than 92.0%.

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Received March 14, 2003; revised and accepted November 5, 2003.
Project supported by the National Natural Science Foundation of China (Nos. 39880003 and 20175016).
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Experimental

Materials

Glycidyl methacrylate (GMA) (Aldrich, USA) was distilled under vacuum. Ethylene dimethacrylate (EDMA) (Aldrich, USA) was extracted three times with 10% aqueous sodium hydroxide and distilled water, and then dried with anhydrous magnesium sulfate. Poly-(vinylpyrrolidone, k-30) (PVP, k-30) was purchased from Aldrich (USA). Azobisisobutyronitrile (AIBN), dibutyl phthalate and cyclohexanol were bought from Shanghai Chemical Reagent Co. Ltd (Shanghai, China). Polyvinyl alcohol (PVA) and sodium dodecyl sulfonate (SDS) were obtained from Beijing Chemical Reagent Co. Ltd (Beijing, China). Benzyl peroxide (BPO) was obtained from Xi′an Chemical Reagent Co. Ltd (Xi'an, China). All chemicals were purified by normal methods.

Lysozyme (chicken egg white, Lys), ribonuclease A (bovine pancreatic, RNase-A), myoglobin (horse skeletal muscle, Myo), α-chymotrypsinogen A (bovine pancreatic, α-chy-A), and cytochrome c (horse heart, Cyt-c) were purchased from Sigma (St. Louis, USA).

All Chromatographic tests were carried out by using an LC-10A chromatographic system (Shimadzu, Japan) including a pump and a multiple-wavelength UV detector. Samples were injected through a Rheodyne 7125 valve and detected at 280 nm.

Dispersion polymerization

According to the method reported by Paine *et al.*,[13] monodisperse polystyrene seed beads with low molecular weight were prepared by dispersion polymerization of styrene in alcohol media in the presence of the inhibitor, AIBN and the stabilizer, PVP (k-30) under a nitrogen atmosphere. After centrifugal separation, the seed beads obtained were dispersed in an aqueous solution of PVA (w_{PVA}=0.01) so that the content was 0.1 g/mL. The size of the prepared beads was measured to be 3.2 μm. The conditions for preparing the seed beads are listed in Table 1.

Table 1 Conditions for preparing the seed of polystyrene beads[a]

Substance	Concentration[b] (w)	Amount/g
Styrene	0.2	5.0
Alcohol	0.8	20.0
PVP, k-30	0.02	0.5

[a] Polymerization temperature, 70 ℃; polymerization time, 24 h; initiator, AIBN; 0.2 g (w=0.04, based on styrene). [b] Based on total recipe (25 g).

Preparation of uniform porous beads

The monodisperse polystyrene seed beads were swollen by emulsifying mixture of glycidyl methacrylate, ethylene dimethacrylate, benzyl peroxide and diluents in an aqueous solution containing PVA and SDS at room temperature. After the monomer mixture was completely absorbed by the seed beads, the temperature was kept at 70 ℃ for 24 h. The beads obtained were washed with water and methanol. The porogens were removed by extraction with toluene. The beads were washed with methanol again and dried in air.

Modification of the beads with weak cation ion exchange media

Firstly, the epoxide groups of the $P_{GMA/EDMA}$ beads **I** were completely hydrolyzed to become diol beads **II**. Secondly, after the reaction of the hydroxyl group of diol beads **II** and epichlorohydrin, beads **III** containing 2.2 mmol/g epoxide groups were obtained. Thirdly, the beads **III** reacted with tetraethylenepentamine to obtain the aminated beads **IV**. Finally, The beads **IV** reacted with succinic anhydride to obtain the designed WCX HPLC stationary phase (beads **V**). Figure 1 shows the chemical modification scheme for the preparation of the new WCX packings in this paper. The “P” in the scheme denotes the polymer frame.

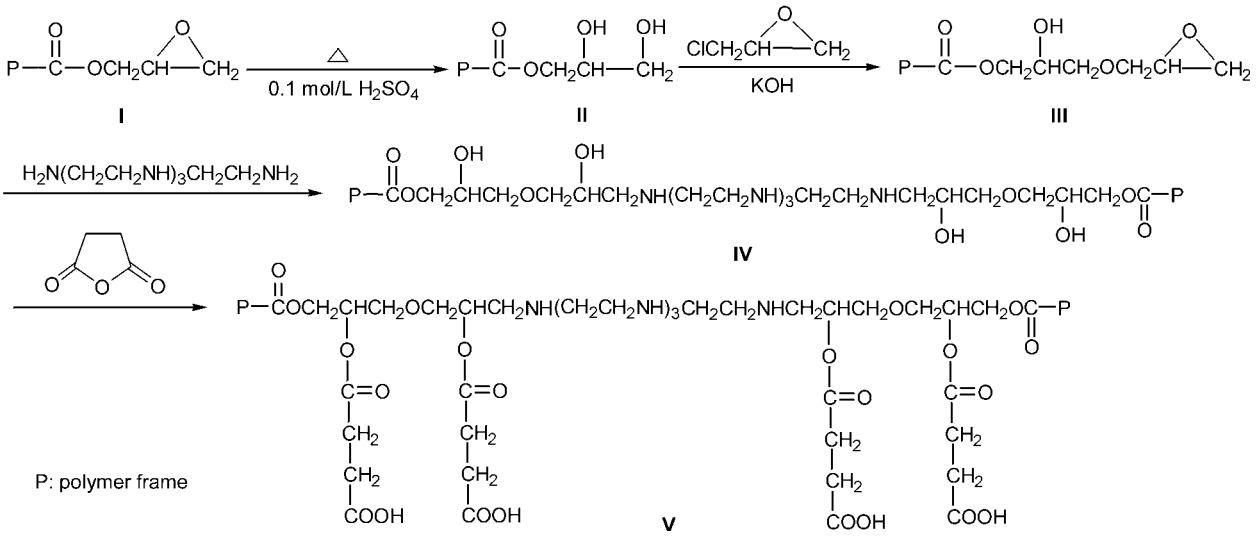

Figure 1 Chemical modification scheme for preparation of the weak cation ion exchange packings.

Characterization of polymeric beads

The particle size and surface morphology, and the specific surface area and pore distribution of the synthesized $P_{GMA/EDMA}$ resins were measured by scanning electron microscopy and the mercury intrusion method, respectively. The pore structure of the resins was also characterized by gel permeation chromatography (GPC). The $P_{GMA/EDMA}$ beads were packed in a stainless steel column by the slurry method with ethanol. The experiments of GPC were carried out in the HPLC instrument with tetrahydrofuran (THF) as eluent, polystyrenes of standard molecular weight as sample, UV-254 nm detection at room temperature.

Determination of epoxy groups

The $P_{GMA/EDMA}$ beads were dispersed in 0.1 mol/L acetic acid solution of tetraethylammonium bromide and titrated with 0.1 mol/L perchloric acid solution until the crystal violet indicator changed to blue-green.

Determination of the capacity of carboxyl group

The titration of carboxyl groups was as follows: Exactly 10.0 mg of WCX packings were titrated in 10 mL of 1 mol/L NaCl. Enough 0.1 mol/L hydrochloric acid was initially added to adjust the pH to 2. Then 50 μL portions of 0.1 mol/L sodium hydroxide were added and the pH was recorded until it approached 12.

Mass recovery

According to Bradford method,[14] Coomassie Blue G250 was used as development reagent to measure the absorbance at 595 nm, using pure BSA as the calibration curve for the determination of protein concentration and calculation of mass recovery.

Determination of the bioactivity of lysozyme

The bioactivity of lysozyme was determined by following the decrease in absorbance at 450 nm of 0.25 mg/mL *Micrococcus lysodeikticus* suspension in 0.067 mol/L phosphate buffer, pH 6.2.[15]

Purification of lysozyme from egg white by the WCX resin

Egg white was obtained from fresh egg and dissolved in sodium phosphate buffer at 1∶4 dilution. The 5.0 cm×0.4 cm I.D. WCX column was used to isolate lysozyme from egg white. The egg white sample was loaded with equilibrium buffer (20 mmol/L sodium phosphate buffer, pH = 7.5). Then the column was washed with a salt gradient and the fractions were collected and assayed.

Results and discussion

Preparation of monodisperse porous beads

Because of a good miscibility of both solvents of cyclohexanol and dibutyl phthalate with the monomers, the mixture of the two solvents was chosen as the porogen diluents for the preparation of $P_{GMA/EDMA}$ resins in this study. In order to increase the content of the epoxide groups in the polymer which are necessary for the subsequent chemical modification, a high percentage of GMA must be used. The ratios of monomer to porogen (35∶65, *V*∶*V*) and GMA to EDMA (60∶40, *V*∶*V*) were selected. Such proportions provide the resins with not only macroporosity and high mechanic intensity, which is required for protein separation by HPLC, but also a quite high content of epoxide groups.

The size of the final beads was well controlled by the seed diameter and the existing amount of organic phase. As organic phase consisting of GMA, EDMA and diluents is fully adsorbed by the seeds in an effective swelling range, the final particle diameter could be calculated according to the following simple equation.[16]

$$\log D = \log d + 1/3 \log \frac{M+m}{m} \quad (1)$$

where d and D are the diameters of the seeds and the final beads, respectively, M and m are the amounts of organic phase and the seeds, respectively; the value of $(M+m)/m$ is the swelling multiple. The experiment results showed that when 3.2 μm of seed beads were used and the swelling multiple was controlled to be in the range of 20—50, a series of monosized $P_{GMA/EDMA}$ resins with particle diameter in the range of 8—12 μm could be obtained.

Figure 2a and 2b show the scanning electron micrographs of the prepared beads, indicating that the prepared beads in this study are uniform in size and have macroporous structure.

Physical properties of the beads

Figure 3 shows the pore size distribution of the synthesized resin in the dry state, which was measured by mercury intrusion method. It is obvious that macroporous (diameter >30 nm) and super-macroporous (diameter >100 nm) structures account for most of the pores within the porous beads. Table 2 shows the properties of the beads. The volumes of porogen diluents used account for about 65% of the total organic phase. It means that the expected porosity and pore volume of the final beads obtained should be approximately 65% and 1.84 mL/g, respectively. The value of the pore volume in Table 2 is very close to the calculated one.

Table 2 Properties of porous $P_{GMA/EDMA}$ beads

Particle size/μm	8.0
Epoxide groups/(mmol•g^{-1})	2.8
Pore volume[a]/(mL•g^{-1})	1.74
Median pore diameter of GPC[a]/nm	89.1
Median pore diameter of mercury porosimetry/nm	110.0
Polystyrene exclusion limit (molecular weight)[a]	1.8×10^6

[a] According to the gel permeation chromatography (GPC).

Figure 4 shows that the dependence of the back pressure of the packed column with the synthesized resin on the flow rate is directly proportional to the flow

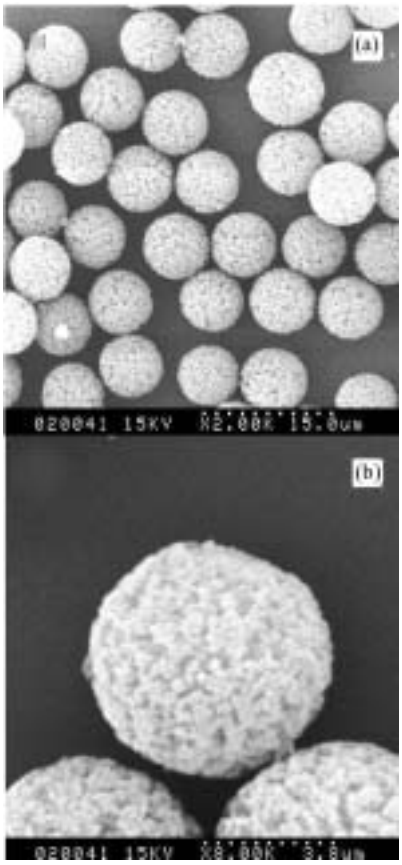

Figure 2 Scanning electron micrographs of the monosized porous beads (a) and their surface structure (b).

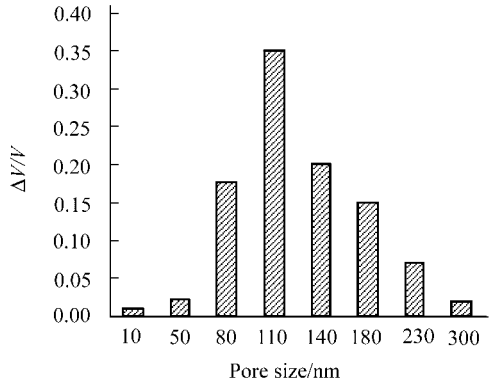

Figure 3 Pore size distribution of the synthesized $P_{GMA/EDMA}$ resin by mercury instrusion method.

rate of the mobile phase in the range from 1.0 to 4.0 mL/min, and the backpressure is only about 3.5 MPa at the flow rate of 4.0 mL/min. This result indicates that the synthesized beads possess high permeability, which is very favorable to the chromatographic applications under the condition of high flow rate.

Chemical Modification of $P_{GMA/EDMA}$ beads

Because of the hydrophobicity of the surface of the macroporous $P_{GMA/EDMA}$ resin，it is difficult to use in the separation of biopolymers. With the chemical modification of the hydrophobic surface by using a hydrophilic

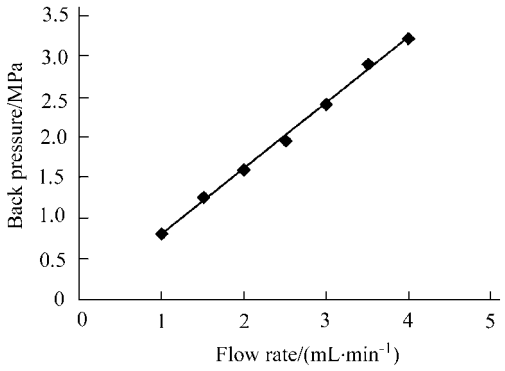

Figure 4 Effect of flow rate on column back-pressure. Column: 50 mm×8 mm I.D.; Mobile phase: 0.02 mol/L sodium phosphate buffer (pH 7.0).

reagent, the irreversible adsorption on the surfaces of the beads and changes in the molecular conformation of protein could be avoided, or diminished. Many reactions can be used for the chemical modification of the epoxide groups existing on the surface of the $P_{GMA/EDMA}$ resin. In Figure 1, the reaction path designed for the preparation of the WCX packings includes an additional hydrophilization step consisting of the reaction of the hydroxyl groups of diol beads (beads **II**) with epichlorohydrin followed by another aminating of the newly introduced epoxide groups with tetraethylenepentamine. This additional hydrophilization step not only results in a better shielding of the hydrophobic main chains of the polymer thereby preventing them from contact with the protein molecules, but also obtains four hydroxy groups which are advantageous to next step reaction with succinic anhydride.

Carboxylic capacity was determined to be 0.60 mmol/g. This result proves that the carboxyl groups were really bound to the surface of the prepared polymer.

Separation of biopolymer by WCX

In order to test the resolution property of the synthesized WCX column, experiment was performed to resolve proteins with different isoelectric points (p*I*). The protein mixture of Myo (p*I* 7.0), RNase-A (p*I* 8.9), α-Chy-A (p*I* 9.5), Cyt-c (p*I* 10.3) and Lys (p*I* 11.1) was chromatographed on the column at a flow rate of 1.0 mL/min (Figure 5).

When the experiment was done at a flow rate of 4.0 mL/min, a baseline separation of these proteins was obtained as shown in Figure 6, demonstrating that the packings can be operated efficiently at high-rates. When α-Chy-A was used as a solute, the theoretical plate number (N) of the column was obtained more than 9000/m at a flow rate of 1.0 mL/min. This result is comparable to that of porous silica-based WCX column.[17] To our knowledge, such a high resolution had not been reported previously on polymer-based porous WCX packing materials designed for protein separations.

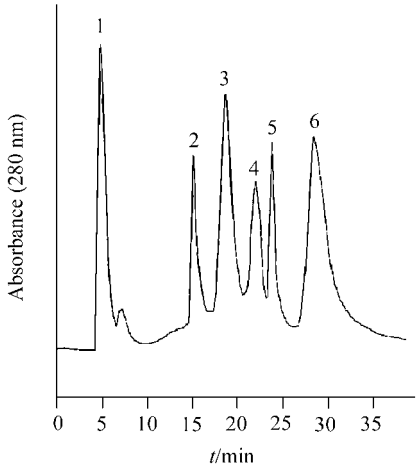

Figure 5 Chromatogram of standard proteins separated by the WCX column (10 cm×0.8 cm I.D.). The linear gradient elution was from 100% solution A (20 mmol/L phosphate, pH 7.5) to 100% solution B (20 mmol/L phosphate-0.5 mol/L NaCl, pH 7.5) at a flow rate of 1.0 mL/min for 35 min with a delay for 5 min. AUFS, 0.08, UV detection at 280 nm. Proteins: 1, solvent+Myo; 2, RNase-A; 3, α-Chy-A; 4, 5, Cyt-c; 6, Lys.

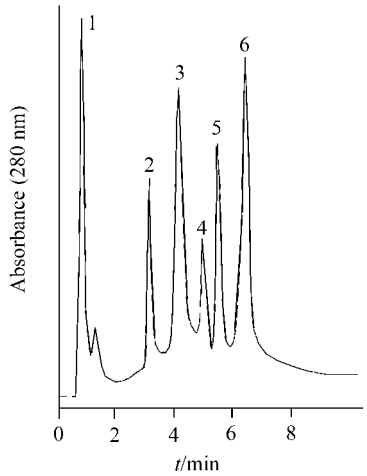

Figure 6 Chromatogram for a fast separation of standard proteins by the WCX column (5 cm×0.4 cm I.D.). Except the linear gradient in 8 min and flow rate of mobile phase being 4.0 mL/min, other conditions are the same as that indicated in Figure 5.

The effect of hydrophobicity on the biopolymer retention was investigated by adding 5% (*V/V*) 2-propanol into the mobile phase. It was found that compared with the absence of any organic solvent in the mobile phase, the retention of Myo, RNase-A, α-Chy-A and Cyt-c decreased by less than 4%, while that of Lys was shortened by about 8%. This fact indicates that the hydrophobic interaction between protein and stationary phase is very weak, and electrostatic interaction dominates the retention behavior of proteins, otherwise, the retention of proteins would obviously decrease under the investigated conditions. The hydrophilicity of the resin was proved to increase greatly after the chemical modification. Therefore, the three dimensional structure of the separated protein molecules under these circumstances should not be changed. This point was also proved by the high bioactive recovery of (96±5)% for Lysozyme.

The mass recoveries of five proteins with three continuous individual measurements obtained from the WCX column are listed in Table 3. It is seen that all mass recoveries are greater that 85%. The relative standard deviations of recoveries of five proteins in three parallel tests are all less than ±5%. This result shows that a high mass recovery of proteins by using the WCX column was obtained in this study.

Table 3 Mass recovery of five proteins by using the synthesized WCX column[a]

Protein	Recovery/%
Myo	89.0+4.3
RNase-A	88.3+4.5
α-Chy-A	94.5+2.7
Cyt-c	95.2+2.2
Lys	96.3+2.0

[a] The linear gradient elution was from 100% solution A (20 mmol/L phosphate, pH 7.5) to 100% solution B (20 mmol/L phosphate-1.0 mol/L NaCl, pH 7.5) at a flow rate of 1.0 mL/min for 25 min with a delay for 5 min. AUFS, 0.2, UV detection at 280 nm.

Effect of pH of moble phase on the protein retention

As shown in Table 4, the pH value of the mobile phase has an important influence on the protein retention in WCX. The retention time of proteins was reduced gradually with increasing pH in the range of 5.5—8.0, which is consistent with the phenomenon observed in the literature,[17] indicating that the modified packings are weak cation exchange packings.

Table 4 Effect of pH of mobile phase on the protein retention[a]

pH	Myo	RNase-A	α-Chy-A	Cyt-c	Lys
5.5	4.1	13.5	14.9	18.7	19.2
6.5	3.75	13.0	13.7	17.1	18.5
7.0	3.2	10.5	11.7	14.1	16.2
7.5	3.2	9.3	11.0	12.5	14.9
8.0	3.2	8.7	10.2	11.7	13.7

[a] Buffer, 20 mmol/L phosphate; linear gradient in 20 min from 0 to 1.0 mol/L NaCl, then at 1.0 mol/L NaCl for 5 min, pH (5.5—8.0), UV detection at 280 nm.

Stability and reproducibility

The WCX column was washed with 1000 mL of 1.0 mol/L NaOH and 1000 mL of 0.5 mol/L H_2SO_4, and then was used for proteins separation. The results showed that the column can be used from pH 0 to 14

and its resolution does not change. When the WCX column was used repeatedly (up to fifty times) for the separation of biopolymers, its separation efficiency was not found to decrease.

Dynamic capacity of the WCX packings

The capacity of WCX packings for adsorption of Lys was determined by the dynamic method.[18] A 5.0 cm× 0.2 cm I.D. WCX column was used; the dynamic capacity of the column for Lys is 21.3 mg/g.

Separation and purification of lysozyme from egg white by the WCX column

Egg whites account for about 58% of the entire egg mass, with 10%—12% of the mass being water. Among these proteins, ovalbumin, ovomucoid, globulins and conalbumin are the major components constituting 54%, 11%, 10% and 13%, respectively, while lysozyme is only as a minor component of about 3.5%.[19] Therefore, egg white is an ideal feed stock for the separation of multiprotein. Levison *et al.*[20] reported large-scale separation of ovalbumin from egg white using Whatman DE92 anion-exchange cellulose. In this study, the 5 cm ×0.4 cm I.D. WCX column was used for the separation of lysozyme in a single step at a flow rate of 4.0 mL/min with a linear gradient of 8.0 min. Figure 7 shows the chromatogram for the fast purification of lysozyme from hen egg white on the WCX column in 8 min. Egg white lysozyme has a p*I* of 11.1, and possesses net positive charge under the experiment conditions, whereas ovomucoid (p*I* 4.0), ovalbumin (p*I* 4.6), globulin (p*I* 5.5—5.8) and conalbumin (p*I* 6.6) possess net negative charge under the given conditions, so they did not retain on the WCX column. In Figure 8 sodium dodecylsulfonate-PAGE analysis shows one main band of purified lysozyme from egg white and the purity of the purified lysozyme is more than 92% after a single-step purification by the WCX column. The specific activity of the purified lysozyme was determined to be 70184 U/mg (Sigma, 71286 U/mg).

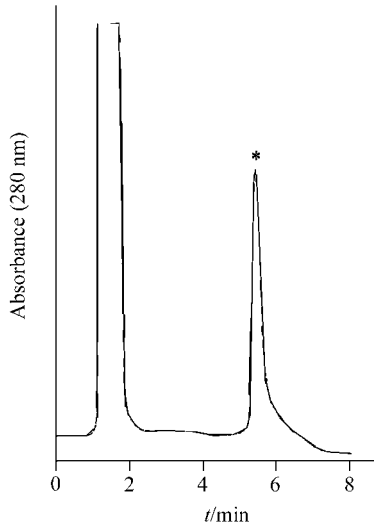

Figure 7 Chromatogram for fast purification of lysozyme from egg white. All chromatographic conditions are the same as those listed in Figure 6 (* lysozyme).

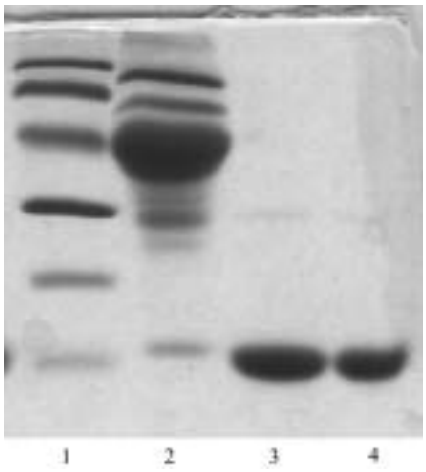

Figure 8 SDS-PAGE analysis of egg white. 1—Marker (14400, 20100, 31000, 43000, 66200, 97400 Da); 2—egg white; 3—purified lysozyme from egg white; 4—standard lysozyme.

Conclusion

The monodisperse poly(glycidyl methacrylate-co-ethylene dimethacrylate) resin with macroporous structure was synthesized by a single-step swelling and polymerization method. The physical properties of the resin were measured and discussed in detail. The results show that the beads have the uniformity in particle size, strong particle rigidity and desired macroporousity. By using this medium, one kind of WCX resin was synthesized by a new chemical modification method. Compared to the silica-based packings, the advantages of the synthesized WCX packings are: (1) it can be used in a wider pH range and also has a higher column loading, and as shown in Figure 5, a comparable resolution;[17] (2) with compared to dextran and agarose, it has a strong chemical and mechanical stabilities; (3) compared to PS-DVS-based WCX packings, it has better resolution and a stronger hydrophilicity, resulting in a high mass recovery of proteins.[11,21,22] The WCX column was also used for the fast separation and purification of lysozyme from egg to obtain a satisfactory result.

Acknowledgments

We thank Prof. SU, Tian-Sheng (Institute of Chemistry, Chinese Academy of Sciences, Beijing) for his helpful comments and suggestions.

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(E0303142 SONG, J. P.)