Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 68 (2006) 666–672
Talanta
www.elsevier.com/locate/talanta

# Synthesis of non-porous poly(glycidylmethacrylate-co-ethylenedimethacrylate) beads and their application in separation of biopolymers

Bolin Gong *, Jinxia Zhu, Long Li, Kejuan Qiang, Li Ren

*Key Laboratory of Energy and Chemical Engineering, Ningxia University, Yinchuan 750021, PR China*

Received 14 March 2005; received in revised form 10 May 2005; accepted 10 May 2005
Available online 13 June 2005

**Abstract**

The monodisperse, 5.0 μm non-porous poly(glycidylmethacrylate-co-ethylenedimethacrylate) ($P_{GMA/EDMA}$) beads were prepared by a single-step swelling and polymerization method. The seed particles prepared by dispersion polymerization exhibited good absorption of the monomer phase. Based on this media, a weak cation exchange (WCX) stationary phase for high performance liquid chromatography (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 97 ± 5%. The dynamic protein loading capacity of the synthesized WCX packings was 20.5 mg/g. Four proteins were completely separated in 3.0 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 3.0 min with only one step. The purity and specific bioactivity of the purified lysozyme was found more than 95% and 70.264 IU/mg, respectively.


*Keywords:* Non-porou poly(glycidymethacrylate-co-ethylenedimethacrylate) resins; Weak cation exchange chromatography; Protein separation; Egg white

## 1. Introduction

There is increasing interest in the use of non-porous microparticulate packing materials for fast separation of proteins by HPLC. The main advantage of such packings is that stagnant mobile-phase mass transfer, which leads to band-broadening and a consequent loss of efficiency and resolution has been eliminated [1]. On the other hand, with non-porous packings, the interactions take place on a pure geometrical surface. A maximum surface accessibility to protein adsorption is achieved allowing fast mass transfer kinetics.

In 1984, Unger et al. [2] introduced non-porous silica beads for affinity chromatography. This work was then extended to other applications in HPLC using the same packing material: reversed phase, hydrophobic interaction and anion exchange chromatography [3,4]. Polymeric stationary phases have a number of advantages such as their high ligand flexibility with allows a maximum interaction between the molecule and the stationary phase and avoids denaturing of the protein. Regnier et al. [5,6] have shown that excellent kinetic properties are obtained with non-porous polyethyleneimine (PEI) coated polystyrene-divinylbenzene packings. Sebille et al. [7,8] have developed a new adsorbed coating technology on non-porous silica for protein separations using polyvinylimidazole (PVI) or polyvinylpyrrolidone–polyvinylimidazole copolymers (PVP/PVI).

In the search for uniform sized beads as chromatographic stationary phases, Ugeltad et al. [9] developed a technique named “activated multi-step swelling and polymerization method”. Most of uniform non-porous beads were prepared by Ugelstad’s method [10–12]. This method

* Corresponding author. Tel.: +86 951 2062300; fax: +86 951 2062860.
*E-mail addresses:* gongbl@nxu.edu.cn, gongbolin@163.com (B. Gong).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.05.003

is excellent, but it seems rather complex because at least two steps are needed in the swelling process. Ogino et al. [13] reported the preparation of the uniform styrene-co-divinylbenzene (PS-DVB) beads with macroporous 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 a preparation of 5.0 μm non-porous poly(glycidylmethacrylate-co-ethylenedimethacrylate) ($P_{GMA/EDMA}$) beads by single-step swelling and polymerization method, and a new method for chemical modification of the non-porous $P_{GMA/EDMA}$ beads 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 3.0 min. The purity of the purified lysozyme was more than 95.0%.

## 2. Experimental

### 2.1. 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) was 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), cytochrome C (horse heart, Cyt-C), conalbumin (CON), bovine serum albumin (BSA) and soybean trypsin inhibitor (STI) were purchased from Sigma (St. Louis, USA).

All Chromatographic tests were carried out by using a chromatographic system (Agilent 1100) including a pump and a multiple-wavelength UV detector. Samples were injected through an autosampler (G1313A) and detected at 280 nm.

### 2.2. Dispersion polymerization for preparation of monodisperse polystyrene seed beads

According to the method reported by Pain et al. [14], monodisperse polystyrene seed beads with low molecular weight were prepared by dispersion polymerization. 2.5 mL of styrene, 0.05 g of BPO and 0.25 g of PVP(k-30) were admixed in 22.5 mL of alcohol media under a nitrogen atmosphere. The polymerization was carried out at 70 °C for 24 h with stirring at 300 rpm. After centrifugal separation, the seed beads obtained were dispersed in an aqueous solution containing $w_{PVA} = 0.01$ such that the content was 0.05 g/mL. The size of the prepared beads was measured to be 1.7 μm. To repeat this experiment for three times, the results are same.

### 2.3. Preparation of uniform non-porous $P_{GMA/EDMA}$ beads

0.5 g of 1.7 μm dispersed polystyrene seed beads and 20 mL of 0.1% SDS (w/w) of aqueous solution were placed in a 250 ml flask and the mixture was stirred slowly by a mechanical stirrer. Then 12.5 g of the mixture consisting of 9.0 g glycidyl methacrylate, 3.5 g ethylenedimethacrylate and 2% (w/w) AIBN initiator in terms of the total monomers were added into 120 ml aqueous solution of 0.1% (w/w) SDS and 1.0% (w/w) PVA and then emulsified under ultrasonic condition until the size of oil drops became, at most of 0.5 μm (observed by optical microscope). The emulsion was sequently added into the dispersion solution of the seed beads. The mixture was stirred for 4–6 h at room temperature so that all the emulsified organic phase was completely absorbed by the polymer seeds. This whole process was monitored by an optical microscope until the organic liquid drops completely disappeared. The mixture was degassed by purging with nitrogen for 20 min. The polymerization was carried out at 70 °C for 24 h with continuously stirring. The beads obtained were washed by hot water, methanol and dried in air.

### 2.4. Modification of the non-porous beads for weak cation exchange media

3.0 g of non-porous $P_{GMA/EDMA}$ (beads I) were suspended in 40 mL of 0.1 mol/L sulfuric acid, stirred and kept at 60 °C for 10 h. After that, the beads were filtered, washed with water until neutral and dried under vacuum condition, obtaining the hydrolyzed beads (beads II).

Then 2.5 g dry beads II were dispersed in 15 mL of water and stirred with a magnetic bar for 16 h. The excess water was removed by a fritted-glass filter, and the beads were redispersed in 15 mL 50 wt.% aqueous potassium hydroxide and stirred for 1 h. The liquid was removed, and the beads were transferred to 25 mL of an epichlorohydrin–water (1:1) mixture and stirred at room temperature for 3 h. The product was washed with water and acetone and dried to afford the beads containing 1.5 mmol/g epoxide groups (beads III). The beads III were then hydrolyzed and worked up using the same procedure as described above to afford beads with diol groups (beads IV).

2.0 g of the beads IV with diol groups were dispersed in 50 mL of pyridine solution, into which 3.0 g of succinic anhydride were added and stirred at 60 °C for 16 h. The beads were washed with water, concentrated hydrochloric acid and

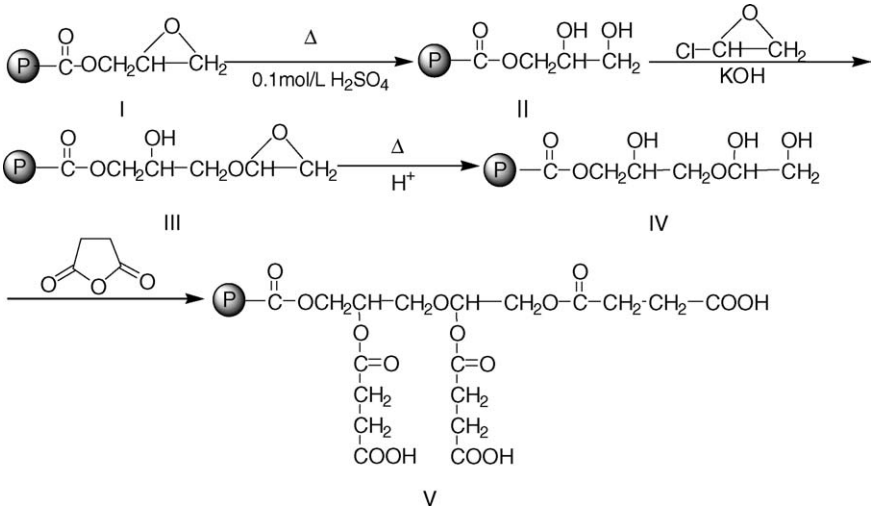


Fig. 1. Chemical modification scheme for preparation of the weak cation exchange packings.

finally, water until to become neutral. Thus, a new non-porous weak cation-exchange HPLC stationary phase was obtained (beads V). Fig. 1 shows the chemical modification scheme for the preparation of the WCX packings in this paper. The "P" in the scheme donotes the polymer frame.

### 2.5. Determination of epoxy groups

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

### 2.6. Determination of the capacity of carboxyl group

The titration of carboxyl groups was as the followings: 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 2. Then 50 μL portions of 0.1 mol/L sodium hydroxide were added and the pH was recorded until it approached 12.

### 2.7. Mass recovery

According to Bradford method [15], 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.

### 2.8. 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 [16].

### 2.9. 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.

## 3. Results and discussion

### 3.1. Preparation of monosized non-porous $P_{GMA/EDMA}$ beads

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. In this paper, the ratio of monomer (GMA) to cross-linking agent (EDMA) (75:25, v/v) was selected.

The size of the final non-porous beads was well controlled by the seed diameter and the existing amount of organic phase. As organic phase consisting of GMA and EDMA is fully adsorbed by the seeds in an effective swelling range, the final particle diameter could be calculated according to the following simple equation [17].

$$\log D = \log d + \frac{1}{3}\log\frac{M+m}{m}$$

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 1.7 μm of seed beads were used and the swelling multiple was 25, 5.0 μm monosized non-porous $P_{GMA/EDMA}$

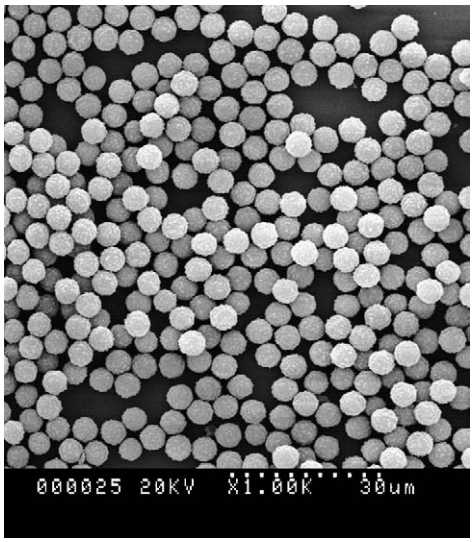


Fig. 2. Scanning electron micrographs of the monosized non-porous beads.

resins could be obtained (repeat this experiment for three times).

Fig. 2 shows the scanning electron micrograph of the prepared non-porous beads, illustrating that the prepared non-porous beads in this study are uniform in size.

### 3.2. Chemical modification of non-porous $P_{GMA/EDMA}$ beads

Because the hydrophobicity of the surface of the non-porous $P_{GMA/EDMA}$ resin, it is difficult to be used for separation of biopolymers. With the chemical modification of the hydrophobic surface by using a hydrophilic 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 non-porous $P_{GMA/EDMA}$ resin. In Fig. 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 hydrolysis of the newly introduced epoxide groups. This additional hydrophilization step not only results in a better shielding of the hydrophobic main chains of the polymer thereby preventing from their contact with the protein molecules [18], but also obtained three hydroxy groups which are advantageous to next step reaction with succinic anhydride.

Carboxylic capacity was determined to be 0.30 mmol/g. The IR spectra of the modified polymer exhibited a large broad adsorption peak at 3421 and 1733 $cm^{-1}$ corresponding to hydroxyl and carbonyl of carboxyl group. These results prove that the carboxyl groups were really bound to the surface of the prepared polymer.

### 3.3. Separation of biopolymer by WCX stationary phase

In order to test the resolution property of the synthesized WCX column, experiment was performed to resolve proteins with differences in their isoelectric points (p*I*). The protein mixture consisting of Myo (p*I* 7.0), RNase-A (p*I* 8.9), Cyt-C (p*I* 10.3) and Lys (p*I* 11) was chromatographed on the column, which was shown in Fig. 3(A–E) at flow rates varied from 1.0 to 5.0 mL/min. When the experiment was done at a flow rate of 5.0 mL/min, a baseline separation of these proteins was shown in Fig. 3(E), which demonstrated that the packings can be operated efficiently at high-rates. In Fig. 3, elution order of the proteins was according to their p*I*. However, the acid protein mixture consisting of CON (p*I* 5.88), BSA (p*I* 4.98) and STI (p*I* 4.5) was chromatographed on the column, which was not retained. This also proved that synthesized resin was WCX stationary phase. When Rnase-A was used as a solute, the theoretical plate number (*N*) of the column was obtained more than 12,000/m at flow rate of 1.0 mL/min. This result is comparable to that non-porous silica-based WCX column [19].

### 3.4. Effect of organic solvent on protein elution

Iso-propanol (IPA) was used as an additive in the mobile phases to detect any hydrophobic adsorption between the solutes and the packing material. If hydrophobic interactions exist, adding a few percent IPA to the mobile phase should reduce the retention significantly. In this paper, the effect of hydrophobicity on the biopolymer retention was investigated by adding 5% (v/v) IPA 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 and Cyt-C decreased by less than 4%, while that of Lys was shortened by about 7%. This fact indicates that the hydrophobic interaction between protein and stationary phase is very weak, and electrostatic interaction dominates the retention behavior of proteins. 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 $97 \pm 5\%$ for lysozyme.

### 3.5. Protein recovery

High yields in the separation of proteins is an essential requirement for industrial downstream processing. The mass recoveries of four proteins with three continuous individual measurements obtained from the WCX column are listed in Table 1. It is seen that all mass recoveries are greater than 90%. The relative standard deviations of recoveries of four proteins in three parallel tests are all less than $\pm 4\%$. This result shows that a high mass recovery of proteins by using the WCX column was obtained in this study.

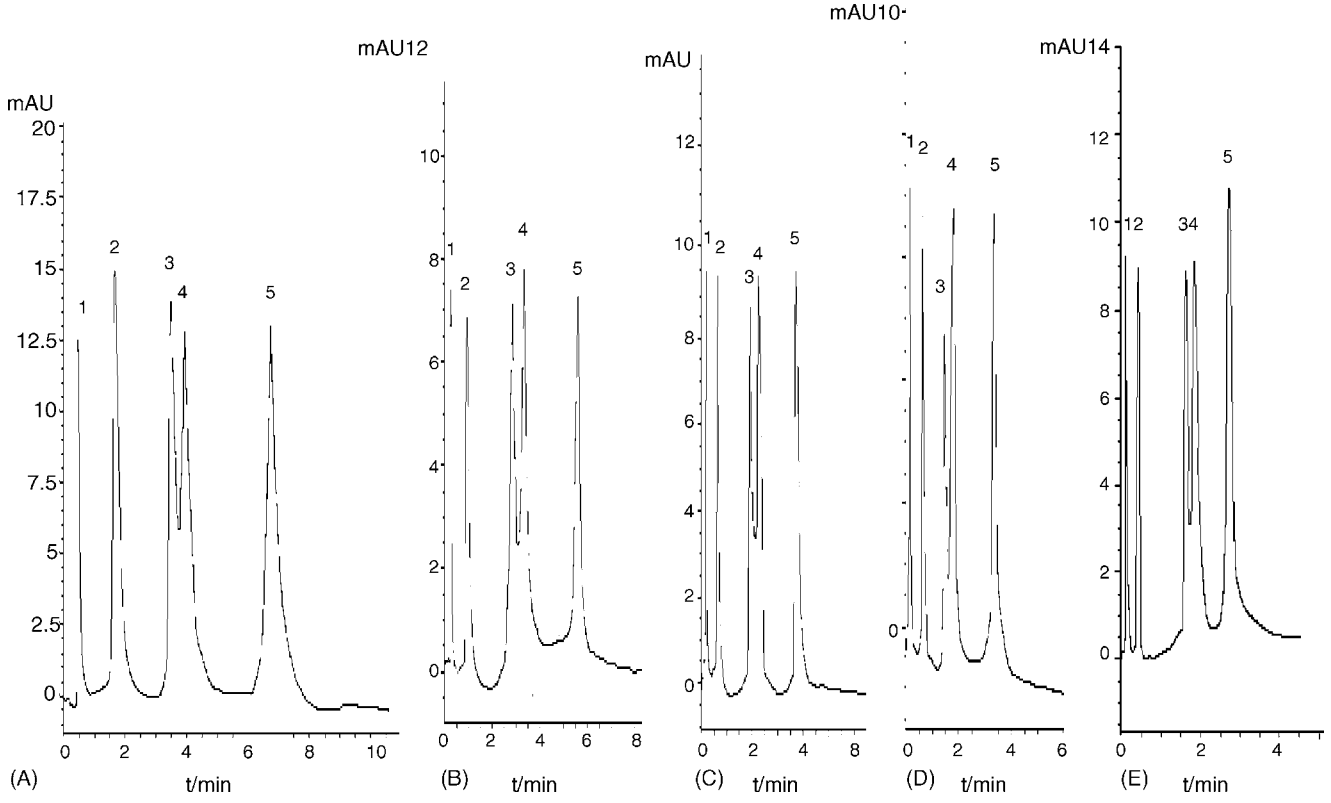


Fig. 3. Chromatogram of standard proteins separated by the WCX column (5 cm × 0.4 cm i.d.). (A) 1.0 mL/min; (B) 2.0 mL/min; (C) 3.0 mL/min; (D) 4.0 mL/min; (E) 5.0 mL/min. The linear gradient elution was from 100% solution A (20 mmol/L of phosphate, pH 7.5) to 100% solution B (20 mmol/L of phosphate–0.5 mol/L NaCl, pH 7.5) at flow rates varied from 1.0 mL/min to 5.0 mL/min for 4–10 min with a delay for 5 min. AUFS, 0.08, UV detection at 280 nm. Proteins: 1, solvent + Myo; 2, RNase-A; 3, 4, Cyt-C; 5, Lys.

### 3.6. Effect of pH of moble phase on the protein retention

One of the more attractive features of separation proteins by ion exchange is the ability to predict chromatographic behavior with respect to the protein's isoelectric point (p*I*). The net charge concept has held that above the p*I* a protein is negatively charged and will be retained on the anion-exchange column, while below the p*I* a protein is positively charged and will be retained on a cation-exchange column. As shown in Fig. 4, the retention time of proteins was reduced gradually with increasing pH in the range of 5.5–8.5, which is consistent with the phenomenon observed in the literature [20]. The elution order of these proteins is directly related to their p*I*. A protein with a higher p*I* was retained longer, as expected in cation-exchange mode.

Table 1
Mass recovery of five proteins by using the synthesized WCX column[a]

| Protein | Recovery (%) |
|---|---|
| Myo | 91.0 ± 3.3 |
| RNase-A | 93.3 ± 3.2 |
| Cyt-C | 95.2 ± 2.2 |
| Lys | 96.3 ± 2.0 |

[a] The linear gradient elution was from 100% solution A (20 mmol/L of phosphate, pH 7.5) to 100% solution B (20 mmol/L of phosphate–0.5 mol/L NaCl, pH 7.5) at a flow rate of 1.0 mL/min for 15 min with a delay for 5 min. AUFS: 0.2; UV detection at 280 nm.

### 3.7. The effect of salt species on the proteins retention

The displacing salt present in the strong buffer is equal importance to the pH in controlling retention, resolution and recovery. Depending on the ion and the charge characteristics of the protein, these quantities could be significantly altered. The effect of different salts on retention could be

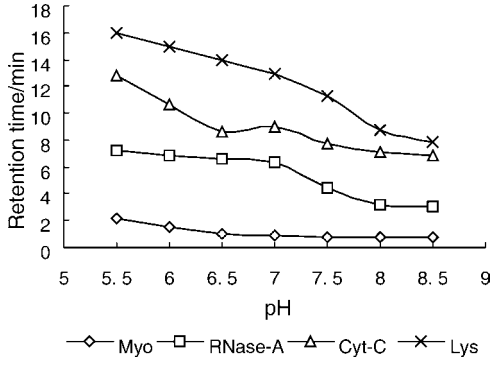


Fig. 4. Effect of pH of mobile phase on the protein retention. Except pH (5.5–8.5), other conditions are the same as that indicated in Fig. 3.

divided into three categories: weak, intermediate, and strong displacing agents [20]. In this paper, we studied the effect of three salts (KCl, $NH_4Cl$, NaCl) on the proteins retention by using the WCX column. The results show that using KCl as displacing agent, the elution volume of RNase-A, Cyt-C and Lys is smaller than using $NH_4Cl$ and NaCl as displacing agent. It demonstrates that the displacing order of the three ions is $K^+ > NH_4^+ > Na^+$, which are according to displacing disciplinarian for cation exchange chromatography.

### 3.8. Stability and reproducibility

Column compressibility is another important feature for HPLC packing materials. This was tested using a 5.0 cm × 0.4 cm i.d. WCX column. We found that the back-pressure of the packed column is about 8.0 MPa at the flow rate of 5.0 mL $min^{-1}$, and the maximum operating pressure is up to 40 MPa. These results demonstrate the beads possess high-pressure endurance ability, which is very favorable to the chromatographic applications under high flow rates. The column operating pressure is much higher than that of columns having the same dimensions packed with most porous polymer-based packing materials.

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 0–14 and its resolution does not change. When the WCX column was used repeatedly (up to 50 times) for the separation of biopolymers, its separation efficiency did not find decrease. After three month, the WCX column was again used for proteins separation. We found that there was no significant difference in retention times or resolution from three month ago.

### 3.9. Dynamic capacity of the WCX packings

Breakthrough curves provide valuable information for the evaluation of the dynamic binding capacity of the separation medium. This is a very important characteristic for large-scale separations. The capacity of WCX packings for adsorption of Lys was determined by the dynamic method [21]. A 5.0 cm × 0.2 cm i.d. WCX column was used; the dynamic capacity at 5% breakthrough for lysozyme at flow-rates of 0.3 mL $min^{-1}$ was 20.5 mg/g.

### 3.10. 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% [22]. Therefore, egg white is an ideal feed stock for the separation of multiprotein. Levison et al. [23] reported large-scale separation of ovalbumin from egg white using

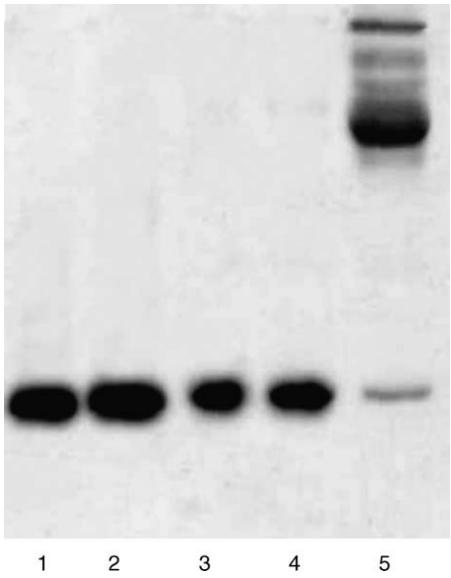


Fig. 5. SDS-PAGE analysis for purification of Lys from egg white. 1 and 2: The standard Lys; 3 and 4: the purified of Lys; 5: the egg white.

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 by a single step at a flow rate of 5 mL/min with a linear gradient of 3.0 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), conalbumin (p*I* 6.6) possess net negative charge under the given conditions, so they did not retain on the WCX column. In Fig. 5 sodium dodecylsulfonate-PAGE analysis shows one main band of purified Lysozyme from egg white and the purity of the purified Lysozyme is more than 95% after a single-step purification by the WCX column. The specific activity of the purified lysozyme was determined to be 70.264 IU/mg (Sigma, 71.286 IU/mg).

## 4. Conclusion

The monodisperse, 5.0 μm non-porous poly(glycidylmethacrylate-co-ethylenedimeth acrylate) resin was synthesized by a single-step swelling and polymerization method. The obtained results show that the beads have the uniformity in particle size and strong particle rigidity. Based on 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, as shown in Fig. 3, a comparable resolution [19]; (2) compared to dextran and agarose, it has a strong chemical and mechanical stabilities [24]; (3) compared to PS-DVS-based WCX packings, it has better resolution and a stronger hydrophilicity, resulting in a high mass recovery of proteins [6]. The WCX column was also used for the fast

separation and purification of lysozyme from egg to obtain a satisfactory results.

## Acknowledgements

Financial support of this work by the National Scientific Foundation in China (No. 20465001), the Minister of Science and Technology Foundation (No. 2004CCA05000), and Program for New Century Excellent Talents in Chinese University (NCET) is gratefully acknowledged.

## References

[1] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York, 1979.
[2] F.B. Anpach, K.K. Unger, H. Giesche, M.T.W. Hearn, Presented at Fourth International Symposium of Proteins, Peptides and Polunucleotides, Baltimore, MD, 1984, paper 103.
[3] G.Y. Lee, C.H. Chen, T.H. Wang, W.C. Lee, Anal. Biochem. 312 (2003) 235.
[4] G. Tishchenko, J. Dybal, K. Meszarsova, Z. Sedlakova, M. Bleha, J. Chromatogr. A 954 (2002) 115.
[5] M.A. Rounds, W.D. Rounds, F.E. Regnier, J. Chromatogr. 397 (1987) 25.
[6] M.A. Rounds, F.E. Regnier, J. Chromatogr. 443 (1988) 73.
[7] B. Sebille, J. Piquion, B. Boussouira, Eur. Pat. Appl. EP 225829 A 2870616 (1987).
[8] R. Lemque, C. Vidal-Madjar, M. Racine, J. Piquion, B. Sebille, J. Chromatogr. 553 (1991) 165.
[9] J.J. Ugelstad, Polym. Sci. Polym. Symp. 72 (1985) 225.
[10] D.J. Burke, J.K. Duncan, L.C. Dunn, L. Cummings, C.J. Siebert, G.S. Ott, J. Chromatogr. 353 (1986) 425.
[11] D.J. Burke, J.K. Duncan, L.C. Dunn, L. Cummings, C.J. Siebert, G.S. Ott, J. Chromatogr. 359 (1986) 533.
[12] Y. Kato, T. Kitamura, A. Mitsui, T. Hashimoto, J. Chromatogr. 398 (1987) 327.
[13] K.J. Ogino, H.Y. Sato, K.R. Tsuchiya, H.S. Suzuki, S.Y. Moriguchi, J. Chromatogr. 699 (1995) 59.
[14] A.J. Pane, W. Luymes, J. McNulty, Macromolecules 23 (1990) 3104.
[15] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[16] E. Michel, R.R. Goldberg, J. Rainer, Biochem. 30 (1991) 2790.
[17] K. Hosoya, J.M.J. Frechet, J. Polym. Sci A: Polym. Chem. 31 (1993) 2129.
[18] V. Smigol, F. Svec, J.M.J. Frechet, Anal. Chem. 66 (1994) 2129.
[19] X.L. Lei, J.D. Wang, R.Z. Cong, J. Instrum. Anal. 18 (1999) 1 (in Chin.).
[20] W. Kopaciewicz, F.E. Regnier, Anal. Biochem. 133 (1983) 251.
[21] I. Mihelic, T. Koloini, A. Podgornik, A. Strancar, J. High Resol. Chromatogr. 23 (2000) 39.
[22] F. Chen, A. Tusak, J. Chromatogr. A 685 (1994) 331.
[23] P.R. Levison, S.E. Badger, D.W. Toome, M.L. Koscielny, L. Lane, E.T. Butts, J. Chromatogr. 590 (1992) 49.
[24] S. Hjerten, J.L. Liao, J. Chromatogr. 457 (1988) 165.