

Study on the Adsorption Property of Lysozyme on Weak Cation Exchanger Based on Monodisperse Poly(glycidymethacrylate-co-ethylenedimethacrylate) Beads

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A type of weak cation exchanger was prepared based on poly(glycidymethacrylate-co-ethylenedimethacrylate). The effects of pH and ionic strength on the adsorption behavior were studied, and the results suggested that the adsorption of lysozyme onto a weak cation exchanger is electrostatic interaction, and that the adsorption behavior is in accordance with the Langmuir adsorption model with a correlation coefficient greater than 0.99. It was also found that increasing ionic strength led to a decrease of the adsorption of lysozyme from 49.50 to 28.09 mg/g. Preliminary chromatographic experiments were conducted to test the separation properties of the weak cation exchanger, and the results demonstrated that the retention time of different proteins could be predicted in order of their isoelectric point.

Introduction

The most popular matrix used in high-pressure liquid chromatography is macroporous silica and polymer microspheres. However, silica-based packings can only be used in the pH range from 2.0 to 8.0; under high pH conditions, the silica-based packings are less stable, while the bonded groups on the silica suffer abscission under low conditions. They sometimes cannot meet the requirement for the separation of biomacromolecules. Even worse, other disadvantages of silica-based ion exchanger involve its smaller exchange capacity, stronger non-specific adsorption and the risk of irreversible adsorption of protein, which can even lead to denaturation. Thus, ion exchange chromatographic packings based on polymers, which can be employed for biopolymer separations even in the pH range from 1.0 to 14, are increasingly experiencing rapid development (1, 2). Other merits of polymer-based packings include their hardness, high recovery and strong hydrophilicity that helps reduce nonspecific actions between proteins and packings. Wei and Gong's group have composed a series of chromatographic packings based on poly(glycidymethacrylate) (PGMA) beads, with which separation of several proteins was successfully conducted (3, 4, 5). Ion-exchange chromatography, based on differential adsorption of charged biological macromolecules at oppositely charged surfaces of chromatographic solid supports, is used extensively for the isolation of proteins and biological macromolecules (6, 7, 8).

In this paper, based on monodisperse poly(glycidymethacrylate-co-ethylenedimethacrylate) (PGMA/EDMA) beads, we prepared a weak cation exchanger (WCX). Part of the synthesis route of WCX is virtually the same as that described by Arica

et al. (7, 8), which has been employed to create reverse-phase high-performance liquid chromatography (HPLC) packing materials. The synthesis of WCX based on PGMA and its application in the separation of protein with HPLC has been reported before (3–5). However, the previous reports did not dwell on the mechanism of the adsorption of proteins on the studied WCX. In this study, we use lysozyme as template protein to investigate the related adsorption behavior. The effects on adsorption capacity and retention behavior under different pH and different ionic strength were investigated with the view of elucidating the mechanism between proteins and on the WCX packings.

Experimental

Instruments and chemicals

Instruments

UV-Visible spectrophotometer (TU-1901, Beijing Puxi Tongyong Instrument Co.) and scanning electron microscopy (SEM) images were performed on Quanta 200 (FEI, Eindhoven, The Netherlands); all chromatographic tests were conducted on an LC-20AT chromatograph system (Shimadzu, Tokyo, Japan).

Chemicals

Glycidyl methacrylate (GMA) (Sigma-Aldrich, St. Louis, MO) was distilled under vacuum. Ethylene glycol dimethacrylate (EDMA) (Sigma-Aldrich) was washed with 10% aqueous sodium hydroxide and saturated sodium chloride solution, and then washed with distilled water and dried by anhydrous magnesium sulfate. Poly(vinylpyrrolidone, k-30) (PVP, k-30) was purchased from Pains Chemical Reagent Co. (Zhengzhou, China), and its molecular weight was approximately 10,000–70,000. Azobisisobutyronitrile (AIBN, commercially pure) was purchased from Shanghai Qingxi Chemical Technology Co. (Shanghai, China). Styrene (ST), benzylperoxide (BPO), toluene, cyclohexanol, methanol, anhydrous alcohol, acetone and succinic anhydride were bought from Tianjin Fuchen Chemical Reagent Co. (Tianjin, China). Polyvinyl alcohol (PVA, commercially pure) was bought from Tianjin Tianda Chemical Demonstration Plant (Tianjin, China). Sodium dodecyl sulfonate (SDS) was obtained from Shanghai Yingpeng Chemical Reagent Co. (Shanghai, China). Pyridine (analytical reagent) was purchased from Guangdong Guanghua Chemicals Plant (Guangdong, China). Sodium dihydrogen phosphate (analytical

reagent) and sulfuric acid (analytical reagent) were bought from Guoyao Enterprise Group Chemical Reagent Co. (China). Disodium hydrogen phosphate was obtained from Xi'an Chemical Reagent Co. (Xi'an, China). All water was from a purification system (Millipore, Milford, MA). Myoglobin (horse skeletal muscle, Myo), ribonuclease A (bovine pancreatic, RNase-A), cytochrome C (horse heart, Cyt-C) and Lysozyme (chicken egg white, LYZ) were purchased from Sigma.

Synthesis of WCX packings

Preparation of monodisperse polystyrene seed beads

Dispersion polymerization was adopted to prepare monodisperse polystyrene seed (PS). ST (10 mL), 0.24 g of AIBN and 1.5 g of PVP were added to a mixture medium of 115 mL of ethanol and 6 mL of water, and then the mixture was ultrasonic-assisted blended and degassed with nitrogen. The polymerization was triggered by locating the mixture in the rotary evaporator/evaporation rotator with the temperature set at 70°C, which lasted 24 h. The resulting polymer (PS) was centrifuged and subsequently cleansed with ethanol and water. After cleansing, the PS beads were dispersed in an aqueous solution of 2% (w/w) PVA.

Determination of the content of PS beads

The content of PS (mg/mL) in dispersing medium was determined in order of gravity. A given volume of dispersing medium was dried to constant weight at 60°C, and the calculation was conducted in triplicate.

Preparation of uniform and discrete porous PGMA/EDMA beads

Glycidyl methacrylate (8 mL), 8 mL of ethylene dimethacrylate, 8 mL of cyclohexanol, 8 mL of toluene and 0.6668 g of BPO were mixed and subsequently added to 240 mL of an aqueous solution of 0.1% (w/w) SDS and 1.0% (w/w) PVA, and then the mixture was emulsified under ultrasonic conditions until the size of the oil drop reduced to less than 0.5 μm (observed with optical microscope). The emulsion was then slowly added to 10 mL of PS solution under stirring for 18 h at 30°C so that the emulsified organic phase could be absorbed by PS beads. Following this, the mixture was degassed by purging with

nitrogen for 20 min, and then the polymerization was triggered when temperature was set at 70°C, which lasted for 24 h. Finally, the produced PGMA/EDMA beads were filtered out and cleansed by hot water, methanol and acetone, and left to dry under vacuum at 60°C for 4 h. The synthetic route is shown in Figure 1 (9, 10).

Removal of PS from PGMA/EDMA beads

The PGMA/EDMA beads underwent extraction with toluene in a Soxhlet apparatus for 48 h to remove the PS. The beads were washed with ethanol and acetone, left to dry under vacuum at 60°C for 3 h.

Modification of PGMA/EDMA beads

Dried PGMA/EDMA beads (2.0 g) were added to 50 mL of 0.1 mol/L sulfuric acid under stirring at 60°C; the hydrolysis lasted 24 h. The hydrolyzed product was subsequently cleansed with water and acetone and left to dry under vacuum. Following this, the hydrolyzed product was mixed with 50 mL of pyridine and 3.0 g of succinic anhydride, and the esterification was conducted under stirring at 60°C for 24 h. Finally, the produced WCX was left to dry under vacuum after the residue was removed by cleansing WCX with ethanol, water and acetone, respectively. The synthetic route is shown in Figure 2 (9, 10).

Adsorption experiments of LYZ

The effects of pH and ionic strength on the capacity of adsorption were conducted as follows: 20.0 mg of WCX and a given concentration of LYZ were mixed in a series of pH phosphate buffers (50 mM). Following this step, the mixture was kept under stirring at 25°C for 24 h, and then the WCX in the mixture was separated by centrifugation, allowing the determination of the residual LYZ in supernatants obtained under different pH (6, 7, 8, 9). The determination of LYZ was conducted by measuring its UV absorbance on a spectrophotometer. The amount of equilibrium adsorption capacity on WCX under different pH was determined according to the following formula (11):

$$Q_e = (C_0 - C_e)V/m \quad (1)$$

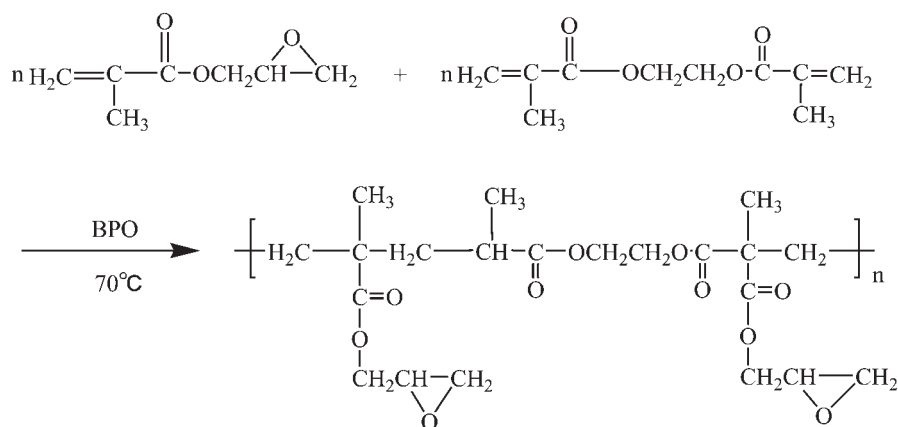


Figure 1. Synthesis routine of PGMA/EDMA beads.

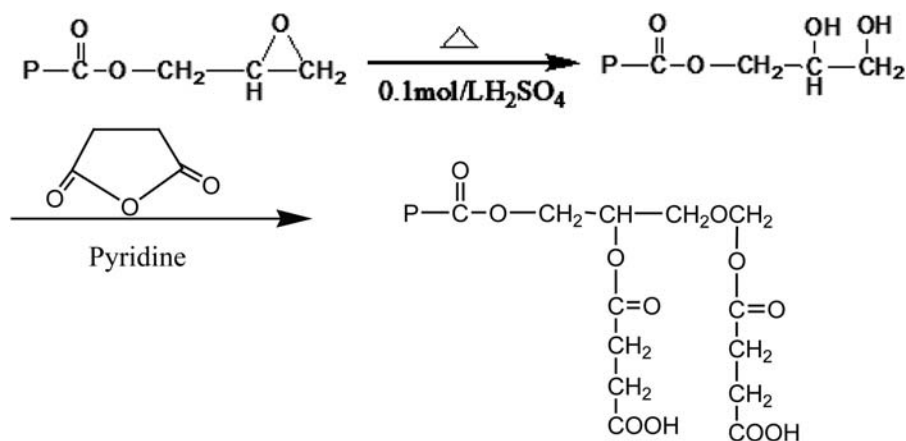


Figure 2. Synthesis routine of WCX.

where Q_e (mg/g) represents equilibrium adsorption capacity of WCX; C_0 (mg/g) and C_e (mg/g) represent the initial and final concentrations of LYZ, respectively; V (mL) is the volume of LYZ solution; and m (g) is the mass of the WCX. The determinations were carried out in triplicate.

Under the optimum pH, the effect of ionic strength on the capacity of adsorption of WCX was conducted in media with different ionic strength (NaCl concentration ranging from 0.05 to 0.30 mol/L). The adsorption, centrifugation and determination were conducted in the same way as described previously.

Preparation of WCX chromatography column

Prepared WCX (0.7 g) was suspended in 50 mL phosphate buffer (pH 6.0) under ultrasonic oscillations and then packed into the chromatographic column (50×4.6 -mm i.d.) at a pressure of 40 MP.

Chromatographic experiments

Mobile phase A was 50 mmol/L phosphate buffer (pH 7.0) and mobile phase B was composed of 1.0 mol/L sodium chloride solution and 50 mmol/L phosphate buffer (pH 7.0). The linear gradient flow was programmed as follows: 0 min (100% A); 20 min (100% B). The flow rate of the mobile phase was set at 1.0 mL/min. The detection wavelength was 280 nm. The injection volume of each sample solution was 20 μ L. Under such conditions, four kinds of alkaline protein with different isoelectric point were analyzed.

Results and Discussion

Synthesis of PS

The preparation of PS seeds

The PS seeds of approximately 2.5 μ m size (Figure 1A) were chosen after this proved the best chromatographic behavior among the three tested sizes (1, 2.5 and 5 μ m) when both the pressure tolerance limit and column efficiency of the chromatographic column prepared were taken into consideration. PS seeds of smaller size lead to unbearably high pressures, and

larger sizes resulted in lower chromatographic column efficiency.

Preparation of PGMA/EDMA beads

The mixed porogen of cyclohexanol and toluene with a ratio of 6:4 (V/V) was chosen so that PGMA/EDMA beads with uniform bore diameter and fewer defects were obtained. Meanwhile, a cross-linking degree of 60% (VEDMA/VGMA + EDMA) was selected to ensure sufficient technical strength and amount of epoxy groups. Finally, the swelling multiple was set at 28 to obtain PGMA/EDMA beads with a diameter of approximately 5 μ m, which could be deduced according to the following equation (12):

$$\log D = \log d + 1/3 \log[(M + m)/m] \quad (2)$$

where D and d are the diameters of PGMA/EDMA beads and PS, respectively; M and m are the masses of organic phase (GMA, EDMA, cyclohexanol, toluene and BPO) and PS, respectively. The value of $(M + m)/m$ is the swelling multiple.

Figure 3 shows SEMs of the prepared PGMA/EDMA beads; these indicate that PGMA/EDMA beads are uniform in size and have a macroporous structure.

Preparation of WCX

The prepared PGMA/EDMA beads had very strong hydrophobicity, which led to irreversible adsorption of biological macromolecules so that it could hardly be employed in the separation of proteins. Therefore, we modified the beads by hydrolyzing epoxy groups with H_2SO_4 and esterifying the produced vicinal diol with succinic anhydride (13). In this way, the WCX beads were made, which promises excellent properties in the separation of biological macromolecules (14).

Adsorption study

LYZ is a kind of protein that can dissolve bacterial cell walls and has many applications in pharmacology. LYZ can not only be used to treat with ulcers, oral mucositis and nasal mucositis, but can also be used as a food additive. In addition, LYZ is of great importance in disease diagnosis (15).

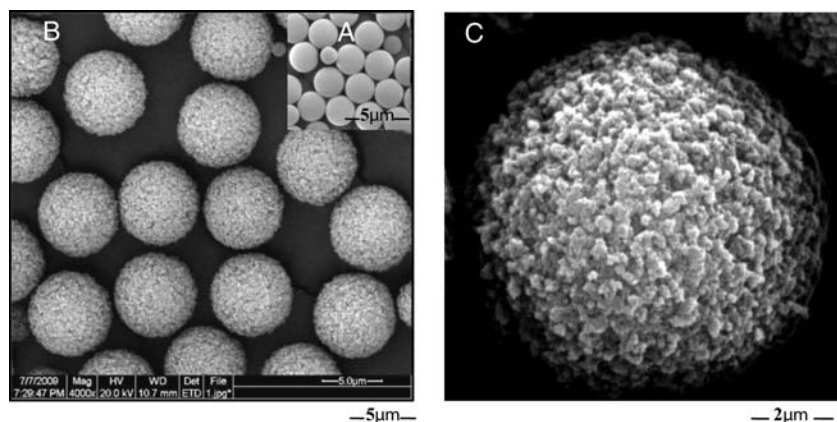


Figure 3. SEMs at 20.0 kv voltage: PS beads ($\times 2,000$) (A); PGMA/EDMA beads ($\times 4,000$) (B); WCX ($\times 16,000$) (C).

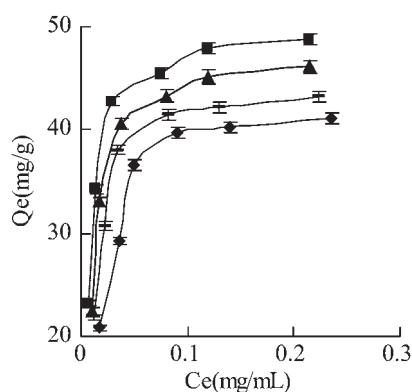
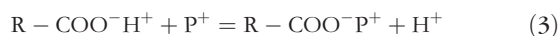


Figure 4. Adsorption isotherms of WCX for LYZ under different pH. Phosphate buffers (50 mM); each point in the isotherm indicates the average values of three replicates; the RSDs for all points were lower than 3.8%. Diamonds indicate pH 6.0, squares indicate pH 7.0, triangles indicate pH 8.0 and dashes indicate pH 9.0.

Effect of pH on LYZ adsorption

The pH of LYZ solution is one of the most significant parameters that affects the net charge on WCX and on the studied protein (16–19), thus largely determining the adsorption behavior of a protein on WCX. The effects of pH of LYZ on its adsorption efficiency and capacity on WCX were investigated between pH 6.0–9.0 and the results are presented in Figure 4. As shown in the figure, the amount of LYZ adsorbed on WCX increased up to pH 7.0. Increasing the pH thereafter caused a decrease in adsorption. The adsorption reaction of LYZ on WCX is as follows:



The rationality of the trend (20, 21) is that too low a pH was unfavorable to the dissociation of charged groups on WCX and therefore decreased the exchange capacity. However, too high a pH led to an apparent decrease in the positive charges on the surface of the LYZ molecule, resulting in weaker electrostatic interaction and the consequent reduction of LYZ adsorption on WCX.

Two theoretical isotherm models (Langmuir and Freundlich) were used to analyze the experimental data, as described by

pH	Fitting Equation	Q_{\max} (mg/g)	K_L (mL/mg)	R
6	$1/Q_e = 0.0002/C_e + 0.0215$	46.51	107.5	0.9983
7	$1/Q_e = 0.0002/C_e + 0.0202$	49.50	101	0.9983
8	$1/Q_e = 0.0002/C_e + 0.0204$	49.02	102	0.9983
9	$1/Q_e = 0.0002/C_e + 0.0207$	48.31	103.5	0.9959

Bayramoglu *et al.* (21), and the result was in accordance with the Langmuir model. The fitting Langmuir adsorption isotherm equations corresponding to different pH were obtained and listed in Table I, along with their maximum adsorption capacities Q_{\max} (mg/g), adsorption equilibrium constant (K_L) (mL/mg) and correlation coefficients (R). The Q_{\max} values were found to be between 46.51 and 49.50 mg/g for LYZ on WCX at different pH. The low K_L values indicate that LYZ has a high binding affinity on WCX and high R values suggest that the Langmuir model is a good fit.

Effect of ionic strength on LYZ adsorption

The effect of ionic strength on LYZ adsorption is presented in Figure 5, which shows that the adsorption capacity decreases from 49.50 to 28.09 mg/g as the salt concentration increases from 0.0 to 0.3 mol/L. The primary effect of salt concentration on protein adsorption onto WCX can be attributed to the competitive interaction of salt cations (Na^+ in this case) with the negatively charged groups of WCX. Increasing ionic strength contributed to the decrease of electrostatic interaction between WCX and LYZ, which resulted in the decrease of the adsorption capacity (22, 23).

The analysis of the data indicated that the adsorption behavior in the presence of NaCl fitted the Langmuir adsorption model. The fitting Langmuir adsorption isotherm equations and their relative parameters are listed in Table II.

The equilibrium adsorption of LYZ onto WCX significantly increased with increasing concentrations of LYZ; the equilibrium adsorption capacity of WCX for LYZ increased by 52% from 0.10 to 0.40 mg/mL under the optimal conditions (pH = 7.0, CNaCl = 0.0 mol/L).

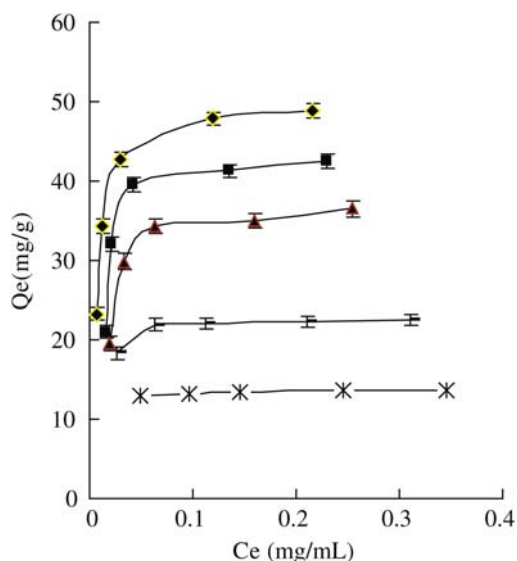


Figure 5. Adsorption isotherms of WCX for LYZ under different ionic strengths. NaCl concentration (from 0.05 to 0.30 mol/L); each point in the isotherm indicates the average values of three replicates; the RSDs for all points were lower than 2.9%. Diamonds indicate 0.0 mol/L, squares indicate 0.05 mol/L, triangles indicate 0.1 mol/L, dashes indicate 0.15 mol/L and x symbols indicate 0.3 mol/L.

Table II
Parameters of Langmuir Adsorption Model under Different NaCl Concentrations

c (mol/L)	Fitting Equation	Q_{\max} (mg/g)	K_L (mL/mg)	R
0.0	$1/Q_e = 0.0002/C_e + 0.0202$	49.50	101	0.9983
0.05	$1/Q_e = 0.0002/C_e + 0.0265$	37.73	132.5	0.9991
0.10	$1/Q_e = 0.0002/C_e + 0.0274$	36.50	137	0.9992
0.15	$1/Q_e = 0.0002/C_e + 0.0310$	32.26	155	0.9994
0.30	$1/Q_e = 0.0002/C_e + 0.0356$	28.09	178	0.9996

Separation performance of WCX-based chromatographic column

Mechanical property of WCX-based chromatographic column

The relationship between column pressure and flow rate is of great importance in the evaluation of the performance of chromatographic columns. The backpressure was found to be directly proportional to the flow rate of the mobile phase; the regression equation is $y = 3.53x - 0.6567$; where y is the back pressure (MP), and x is the flow rate. The linear range is from 1.0 to 5.0 mL/min with a correlation coefficient of 0.999. This result demonstrates that WCX beads possess high permeability. The good linearity indicates that WCX beads have uniform particle size and macroporous structure. Therefore, the WCX-based chromatographic column is eligible for separations of biomacromolecules with complex matrices.

Separation property of WCX-based chromatographic column

To test the separation property of the WCX column, experiments were conducted to separate four proteins with different isoelectric points (pI) in a mixture. The mixture included Myo (pI 7.0), RNase-A (pI 8.9), Cyt-C (pI 10.3) and LYZ (pI 11). The results are shown in Figures 6A–6D at different pH with a flow rate of 1.0 mL/min. A baseline separation of these proteins

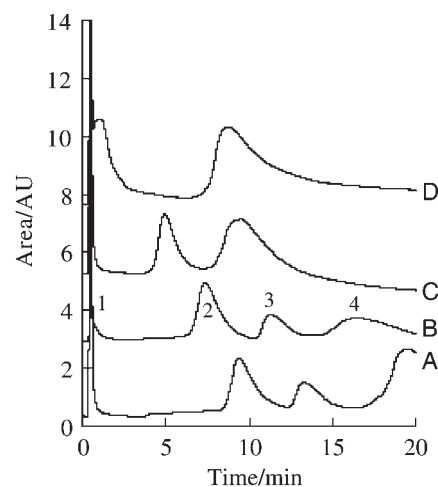


Figure 6. Chromatograms of WCX packings under different pH. Column, 5 × 0.46 cm (i.d.); mobile phase, A (0.05 mol/L, phosphate buffer), B (1.0 mol/L NaCl in 0.05 mol/L of phosphate buffer; flow rate, 1.0 mL/min; detection wavelength, 280 nm. A: pH 6, B: pH 7, C: pH 8, D: pH 9; 1, Myo; 2, RNase-A; 3, Cyt-C; 4, LYS.

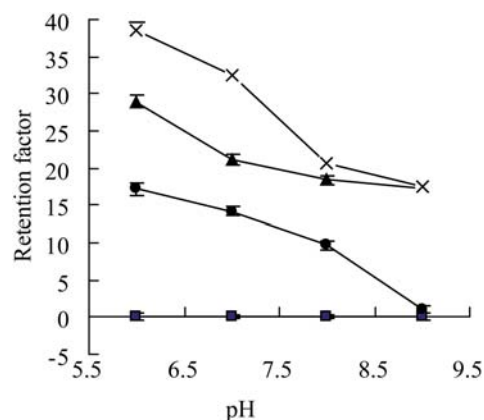


Figure 7. Effect of different pH on the retention of four kinds of proteins: Myo (squares); RNase-A (circles); Cyt-C (triangles); LYS (x symbols); each point in the isotherm indicates the average values of three replicates; the RSDs for all points were lower than 2.9%. Other conditions are the same as in Figure 6.

was obtained at pH 7.0 in Figure 6B, which demonstrated that the WCX-based chromatographic column was eligible for separations of biomacromolecules. In Figure 6, when RNase-A was used as a solute, the theoretical plate number (N) of the column obtained was approximately 3,200/m at pH 7.0 with a flow rate of 1.0 mL/min.

pH effect on individual 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 pI. The net charge concept (20) holds that the alkaline proteins are negatively charged above their pI and can be retained on the anion exchange column, while the acidic proteins are positively charged below their pI and can be retained on a cation exchange column. As is shown in Figure 7, the retention factors of proteins reduced

with increasing pH in the range of 7.0–9.0, which was consistent with the phenomenon observed in the literature (24). The elution order of these proteins is directly related to their pI, and a protein with a higher pI was retained longer as expected. The peaks of Cyt-C and Lys overlapped each other to a certain extent at pH 8.0, which was verified by comparing the retention times of the individually obtained proteins instead of in a mixture at different pH.

Conclusion

The WCX obtained by the modification of the surface of PGMA/EDMA had uniform particle size (approximately 5.0 μm) and macroporous structure. The study on pH confirmed the adsorption of LYZ onto WCX as electrostatic interaction, and the adsorption behavior was in accordance with the Langmuir adsorption model; the maximum adsorption amount of LYZ on WCX was observed at pH 7.0. Also, it was found that increasing ionic strength led to the decrease of the adsorption of LYZ. The chromatographic experiments demonstrated that the retention times of different proteins could be predicted in order of their pI.

Acknowledgments

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References

- Luo, Q.Z., Wei, Y.M., Liu, T., Geng, X.D.; Preparation of hydrophobic interaction chromatographic continuous rod column and its characterization; *Chinese Chemical Letters*, (1999); 10: 215–218.
- Wei, Y.M., Huang, X.D., Chen, Q., Geng, X.D.; Preparation of continuous rod column of weak cation exchange and its chromatographic behaviour for proteins; *Chinese Journal of Analytical Chemistry*, (2000); 28: 1194–1198.
- Gong, B.L., Wang, L.L., Wang, C.Z., Geng, X.D.; Preparation of hydrophobic interaction chromatographic packings based on mono-disperse poly(glycidylmethacrylate-co-ethylenedimethacrylate) beads and their application; *Journal of Chromatography A*, (2004); 1022: 33–39.
- Wei, Y.M., Huang, X.D., Liu, R.X., Shen, Y.H., Geng, X.D.; Preparation of monolithic column for weak cation exchange chromatography and its application in the separation of biopolymers; *Journal of Separation Science*, (2006); 29: 5–13.
- Gong, B.L., Ke, C.Y., Geng, X.D.; Preparation of weak cation exchange packings based on monodisperse poly(glycidylmethacrylate-co-ethylene dimethacrylate) beads and their chromatographic properties; *Analytical and Bioanalytical Chemistry*, (2003); 375: 769–774.
- Bayramoglu, G., Arica, M.Y.; Procion green H-4G immobilized on a new IPN hydrogel membrane composed of poly(2-hydroxyethyl-methacrylate)/chitosan: Preparation and its application to the adsorption of lysozyme; *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, (2002); 202: 41–52.
- Arica, M.Y., Bayramoglu, G.; Invertase reversibly immobilized onto polyethylenimine-grafted poly(GMA-MMA) beads for sucrose hydrolysis; *Journal of Molecular Catalysis B: Enzymatic*, (2006); 38: 131–138.
- Arica, M.Y., Yilmaz, M., Yalçın, E., Bayramoglu, G.; Affinity membrane chromatography: relationship of dye-ligand type to surface polarity and their effect on lysozyme separation and purification; *Journal of Chromatography B*, (2004); 805: 315–323.
- Šmigol, V., Švec, F.; Synthesis and properties of uniform beads based on macroporous copolymer glycidyl methacrylate-ethylene dimethacrylate: A way to improve separation media for HPLC. *Journal of Applied Polymer Science*, (1992); 46: 1439–1448.
- Šmigol, V., Švec, F., Fréchet, J. M. J.; High-performance liquid chromatography of complex mixtures using monodisperse dual-chemistry polymer beads prepared by a pore-size-specific functionalization process. A single column combination of hydrophobic interaction and reversed-phase chromatography; *Analytical Chemistry*, (1994); 66: 2129–2138.
- Bautista, L.F., Plata, M.M., Aracil, J., Martínez, M.; Application of an effective diffusion model to the adsorption of aspartame on functionalised divinylbenzene-styrene macroporous resins; *Journal of Food Engineering*, (2003); 59: 319–325.
- Hosoya, K., Fréchet, J.M.J., Polym, J.; Influence of the seed polymer on the chromatographic properties of size monodisperse polymeric separation media prepared by a multi-step swelling and polymerization method; *Journal of Polymer Science: Part A: Polymer Chemistry*, (1993); 31: 2129–2141.
- Gong, B.L., Zhu, J.X., Li, L., Qiang, K.J., Ren, L.; Synthesis of non-porous poly(glycidylmethacrylate-co-ethylenedimethacrylate) beads and their application in separation of biopolymers; *Talanta*, (2006); 68: 666–672.
- Chang, J.H., Liu, X.H.; The Synthesis of packing of weak cation exchange chromatography for protein separations by reaction of cycloanhydride to silicon with end hydroxy group; *Chinese Journal of Chromatography*, (1994); 12: 436–437.
- Ghosh, R.; Purification of lysozyme by microporous PVDF membrane-based chromatographic process; *Biochemical Engineering Journal*, (2003); 14: 109–116.
- Shentu, J., Wu, J., Song, W., Jia, Z.; Chitosan microspheres as immobilized dye affinity support for catalase adsorption; *International Journal of Biological Macromolecules*, (2005); 37: 42–46.
- Mondal, K., Gupta, M.N.; The affinity concept in bioseparation: Evolving paradigms and expanding range of applications; *Biomolecular Engineering*, (2006); 23: 59–76.
- Bayramoglu, G., Senkal, F.B., Celik, G., Arica, M.Y.; Preparation and characterization of sulfonyl-hydrazine attached poly(styrene-divinylbenzene) beads for separation of albumin; *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, (2007); 294: 56–63.
- Ma, Z.Y., Guan, Y.P., Liu, H.Z.; Affinity adsorption of albumin on cibacron blue F3GA-coupled non-porous micrometer-sized magnetic polymer microspheres; *Reactive & Functional Polymers*, (2006); 66: 618–624.
- Shen, H., Douglas, D.F.; Charge regulation in protein ion-exchange chromatography: Development and experimental evaluation of a theory based on hydrogen ion Donnan equilibrium. *Journal of Chromatography A*, (2004); 1034: 55–68.
- Bayramoglu, G., Ekici, G., Besirli, N., Arica, M.Y.; Preparation of ion-exchange beads based on poly(methacrylic acid) brush grafted chitosan beads: Isolation of lysozyme from egg white in batch system; *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, (2007); 310: 68–77.
- Bayramoglu, G., Logoglu, E., Arica, M.Y.; Cytochrome C adsorption on glutamic acid ligand immobilized magnetic poly(methylmethacrylate-co-glycidylmethacrylate) beads; *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, (2007); 297: 55–62.
- Jiang, X.Y., Bai, S., Sun, Y.; Fabrication and characterization of rigid magnetic monodisperse microspheres for protein adsorption; *Journal of Chromatography B*, (2007); 852: 62–68.
- Kopaciewicz, W., Regnier, F.E.; Mobile phase selection for the high-performance ion-exchange chromatography of proteins; *Analytical Biochemistry*, (1983); 133: 251–259.