Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 987 (2003) 323–330
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Separation of proteins on polymeric stationary phases grafted with various amine groups

Seong-Ho Choi, Young-Mi Hwang, Kwang-Pill Lee*
Department of Chemistry Graduate School, Kyungpook National University, Daegu 702-701, South Korea

Abstract

Six polymeric stationary phases with various amine groups were prepared by surface grafting of glycidyl methacrylate on silica gel surface and its subsequent amination. The six kinds of amines, namely, triethylamine, diethylamine, ethylenediamine (EDA), hexaethylenediamine (HEDA), diethylenetriamine (DETA) and triethylenetetraamine (TETA) were used in this study. The separation of bovine serum albumin (BSA) and chicken egg albumin (CEA) on the polymeric stationary phase with various amines was investigated. The affinity degree of BSA was higher than CEA for the EDA, HEDA and DETA columns, whereas the affinity degree of CEA was higher than BSA for the TETA column.

Keywords: Stationary phases, LC; Grafting; Proteins; Glycidyl methacrylate; Amines

1. Introduction

Glycidyl methacrylate (GMA) is one of the monomers which is easily modified into various functional groups. As GMA is polymerized, the epoxy groups of the GMA become useful for the introduction of various functional groups, such as amines [1,2], alcohols [3], phosphoric acid [4], proteins [5], etc. [6,7].

In a previous study [8], the epoxy group of the GMA was introduced onto polypropylene film by radiation-induced graft copolymerization for recovery of urokinase in human urine. The epoxy group was converted to amino acid as affinity group. The adsorbed amounts of urokinase was in the range 9–12 mg/g by L-phenylalanine-modified polypropylene film. On the other hand, the epoxy group of the GMA was also introduced onto a polyethylene membrane by radiation-induced polymerization for recovery of heavy metal ions such as Pb^{2+} and Pd^{2+}. Subsequently, the epoxy group of the GMA was converted to amine groups. It was found that the adsorption of the Pd^{2+} by chelating hollow fiber membrane modified with five kinds of amines was in the following order: diethylenetriamine>hexamethyldiamine > ethylenediamine > dimethylamine > trimethylamine [9].

On the other hand, Chen and Lee published a non-porous particle with an epoxy group by copolymerization of styrene, methylmethacrylate, and glycidyl methacrylate. The dye, Cibacron Blue 3G-A, was introduced to the epoxy group of the non-porous particle. The lysozyme and bovine serum albumin were separated using the non-porous particle with dye [10].

Choi et al. also prepared polymeric micro beads by radiation-induced polymerization of GMA and diethylene glycol dimethacrylate as crosslinking

*Corresponding author. Tel.: +82-53-950-5901; fax: +82-53-952-8104.
E-mail address: kplee@knu.ac.kr (K.-P. Lee).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01840-X

agent, and, subsequently, the polymeric micro beads were immobilized by lipase in an alkaline medium for chiral separation [11]. However, the polymeric micro beads by radiation-induced polymerization were not used on a chiral column because the polymeric stationary phase prepared by radiation-induced polymerization has a soft property.

In order to overcome the soft property, the silica gel was used as support material. Firstly, the silica gel was modified using a monomer with vinyl group, 3-(trimethoxysilyl) propylmethacrylate. Subsequently, the six polymeric stationary phases were prepared by copolymerization of the GMA and vinyl-group modified silica gel and its subsequent amination. The polymeric stationary phase with amines such as triethylamine (TEA), diethylamine (DEA), ethylenediamine (EDA), hexaethylenediamine (HEDA), diethylenetriamine (DETA), and triethylenetetraamine (TETA) were prepared. The polymeric stationary phases with various amines were characterized by FT-IR, FT-Raman, solid state ^{13}C NMR spectroscopy, scanning electron microscopy (SEM), thermal analysis (TGA/DTA) and elemental analysis. Bovine serum albumin (BSA) and chicken egg albumin (CEA) as model proteins were separated using the prepared columns with amines. From the results, the efficiencies of the prepared columns were discussed.

2. Experimental

2.1. Materials

The 3-(trimethoxysilyl) propylmethacrylate (γ-MAPs) was obtained from Tokyo Kasei (Japan). GMA and α,α′-azobis(isobutyronitrile) (AIBN) were obtained from Junsi Chemical. The DETA, TETA, HEDA, EDA, and TEA were purchased from Aldrich. The silica gel (Article 7734, silica gel 60) was obtained from Merck. The analytes BSA and CEA as model compounds were also obtained from Aldrich. The molecular masses of the BSA and CEA are 66 000 and 44 287, respectively. The isoelectric points (p*I*) of BSA and CEA are 4.9 and 4.9, respectively. The toluene and tetrahydrofuran (THF) obtained from Aldrich, were distilled before use.

2.2. Preparation of the polymeric stationary phase

Fig. 1 shows the preparation procedure of the polymeric stationary phase with amines. To a solu-

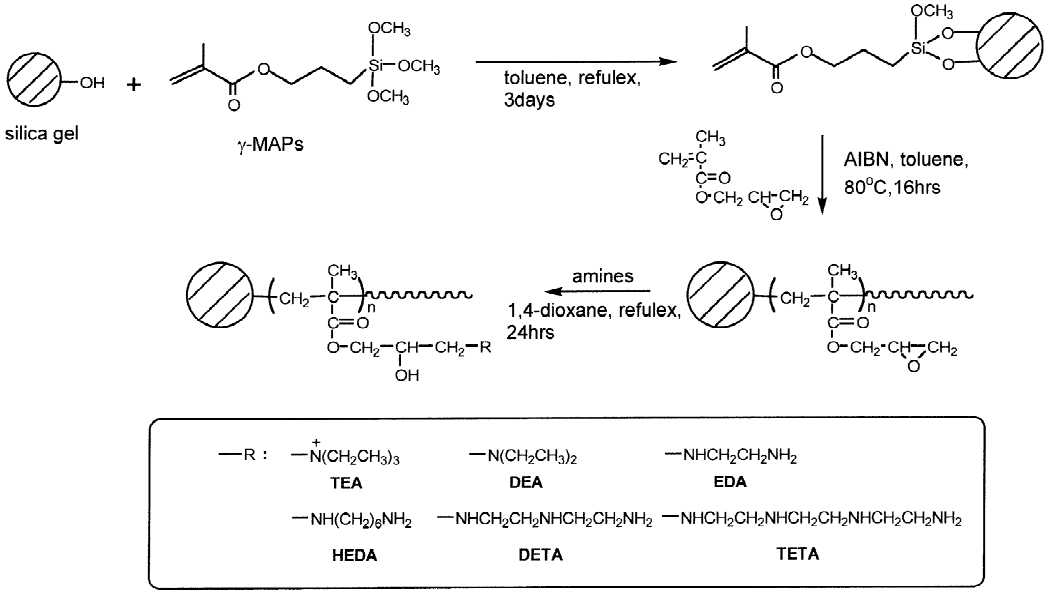

Fig. 1. Preparation procedure for the polymeric stationery phase with various amines.

tion of 10 μm silica gel (2.5 g) in toluene (40 ml), the γ-MAPs (2.5 g, 10.0 mmol) was added under nitrogen at 0 °C. After the mixture was stirred at 80 °C for 3 days, it washed with MeOH. The yield of silica gel with vinyl group was 1.10 g (22.1%). FT-IR: 1736 (C=O) cm^{-1}; FT-Raman: 1640 (C=C) cm^{-1}; Anal. found ($C_{10}H_{20}O_5Si$): C, 3.47. Subsequently, the GMA (1.0 g, 7.0 mmol) was copolymerized with AIBN in toluene at 80 °C for 16 h in the presence of γ-MAPs-modified silica gel (1.0 g). The polymer was precipitated in a large amount of methanol, separated by centrifugation, and dried in a vacuum oven at 50 °C for 3 h.

2.3. Preparation of polymeric stationary phase with amines and separation of model proteins

The epoxy group of GMA-modified silica gel was introduced by amination of TEA, DEA, EDA, HEDA, DETA, and TETA in 1,4-dioxane on refluxing for 24 h as shown in Fig. 1. The polymeric stationary phase obtained was slurry packed into a 5.0 cm×0.46 cm I.D. stainless steel column using a column packer (DSF-122, Haskel, USA). MeOH was used as the slurry mixing agent during the packing procedure.

Chromatographic separations were carried out with a column of size 5.0×0.46 cm using a HP 1100LC/MSD model gradient HPLC system. The samples of CEA and BSA were dissolved in 20 m*M* Tris–HCl buffer (pH 7.4). The separation condition was described in Table 2.

2.4. Characterization

FT-IR spectra of the polymeric stationary phase with amine groups were obtained using Nujol mulls with a Perkin–Elmer Model 983 infrared spectrophotometer. The near-IR (NIR) Fourier transform (FT) Raman spectra were recorded with a Bruker FT-106 Raman module, equipped with a Ge detector cooled by liquid nitrogen and connected to a Brucker FT-IR 66 interferometer. In order to excite the Raman signal, a continuous wave diode-pumped Nd:YAG laser with a radiation wavelength of 1064 nm (9398.4 cm^{-1}) was used. In all cases, the laser power was 300 mW, and the spectral resolution was 2 cm^{-1}. For SEM, the sample was coated with a gold–palladium alloy prior to the measurement. The sputtered sample was then scanned by the electron beam in a scanning electron microscope (JSM-5400, JEOL, Japan). Solid state ^{13}C NMR spectroscopy (Unity Plus, Varian, USA) was carried out. Thermal analysis of the polymeric stationary phase with amines was made on a TG/DTA 320 (Seico, Japan) with a heating rate of 10 °C/min in the temperature range 50–700 °C. Elemental analysis (EA) of the microspheres was carried out with a Fisons EA1110/EA1108 instrument.

3. Results and discussion

In this study, we prepared a polymeric stationary phase with amines for separation of proteins in a gradient HPLC system. In a previous paper [11], the poly(glycidyl methacrylate) polymer prepared by radiation-induced polymerization was not packed in a stainless column because the poly(glycidyl methacrylate) has soft properties. In order to prepare a column for HPLC, the poly(glycidyl methacrylate) needed a rigid polymer as support material. Silica gel is one of the best material supports for HPLC. Silica-based chromatography supports have numerous qualities, such as high mechanical stability, resistance to swelling, and excellent efficiency. In order to use silica gel as support material, γ-MAPs was introduced onto the silica gel surface as shown in Fig. 1.

3.1. Characterization of the polymeric stationary phase with amines

Fig. 2 shows FT-IR spectra for base silica gel (a), γ-MAPs-modified silica gel (b), GMA-modified silica gel (c), and polymeric stationary phase with DETA (d) in 850–950 and 1500–2000 cm^{-1} regions. In Fig. 2a, a characteristic peak at 1636 cm^{-1} is observed due to the Si–O–Si stretching mode. In Fig. 2b, a characteristic peak at 1727 cm^{-1} is seen due to carbonyl (>C=O) group of the γ-MAPs on the silica gel surface. It is clearly indicated that the γ-MAPs was successfully introduced on to the silica gel surface. In Fig. 2c, the characteristic peak at 1735 cm^{-1} due to a C=O stretching is also observed. Furthermore, a characteristic peak appears at 910

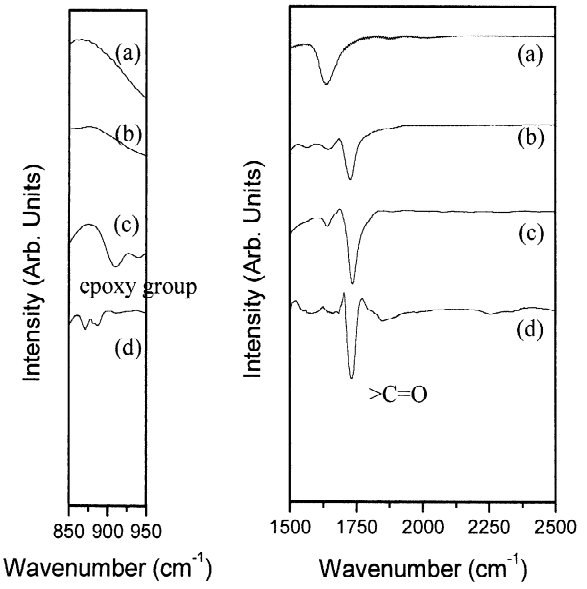

Fig. 2. FT-IR spectra for the polymeric stationary phase with DETA, in the 850–950 and 1500–2500 cm^{-1} regions. (a) Silica gel, (b) vinyl group-modified silica gel, (c) GMA-modified silica gel, and (d) polymeric stationary phase with DETA.

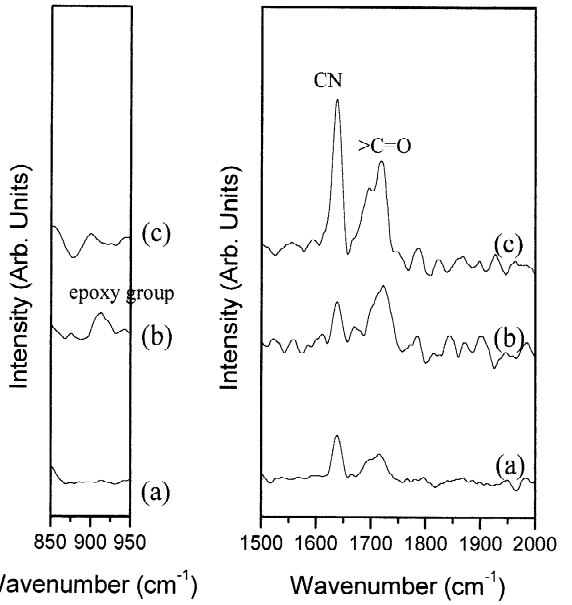

Fig. 3. FT-Raman spectra for the polymeric stationary phase with DETA in the 850–950 and 1500–2000 cm^{-1} regions. (a) Vinyl group modified silica gel, (b) GMA-modified silica gel, and (c) polymeric stationary phase with DETA.

cm^{-1} due to a COC (epoxy group) stretch. From the results, the epoxy group of GMA was grafted onto the silica gel surface. In Fig. 2d, the carbonyl peak at 1734 cm^{-1} is also observed.

In order to define amine groups, the polymeric stationary phase with various amine groups were characterized by FT-Raman spectroscopy. Fig. 3 shows the FT-Raman spectra of the γ-MAPs-modified silica gel (a), GMA-modified silica gel (b), and DETA-modified silica gel (c). In Fig. 3a, the characteristic peak at 1717 cm^{-1} due to carbonyl group of γ-MAPs is observed, and the peak at 1635 cm^{-1} was determined to be due to the C=C stretching peak. It was interpreted that γ-MAPs was successfully introduced onto the silica gel surface. In Fig. 3b, a dominant peak appeared at 1722 cm^{-1} due to the carbonyl group. Characteristic peaks were seen at 1258 and 910 cm^{-1} due to the symmetric epoxy stretch. These results clearly indicate that the epoxy group of GMA had been introduced onto the surface of the silica gel. In Fig. 3c, the carbonyl peak at 1720 cm^{-1} is observed, the epoxy group at 1258 cm^{-1} has disappeared, and the characteristic peak at 1637 cm^{-1} due to C–N peak has appeared. These results clearly indicate that DETA has been introduced onto the surface of the poly(GMA)-modified silica gel.

Fig. 4 shows ^{13}C NMR spectra of γ-MAPs-modified silica gel (a) and DETA-modified silica gel (b). In Fig. 4a, the peaks of γ-MAPs-modified silica gel were assigned as follows: at 166 ppm due to >C=O; at 66 ppm due to $-CH_2-O-$; at 50 ppm due to $-CH_2-$; at 22, 17, and 9 ppm due to $-CH_3$. In Fig. 4b, the peak at 177 ppm due to the carbonyl (>C=O) group and 167 ppm due to C–N were observed. From the results, DETA had been introduced onto the epoxy group of the poly(GMA)-modified silica gel.

Fig. 5 shows TGA/DTA curves for the base silica gel (a), γ-MAPs-modified silica gel (b), poly(GMA)-modified silica gel (c), and DETA-modified silica gel (d). In Fig. 5a, the first mass loss was observed around 100 °C due to moisture on the silica gel surface. In Fig. 5b, the first mass loss around 300 °C was interpreted as the decarboxylation of the γ-MAPs on the silica gel surface, while the second mass loss around 400 °C was interpreted as the

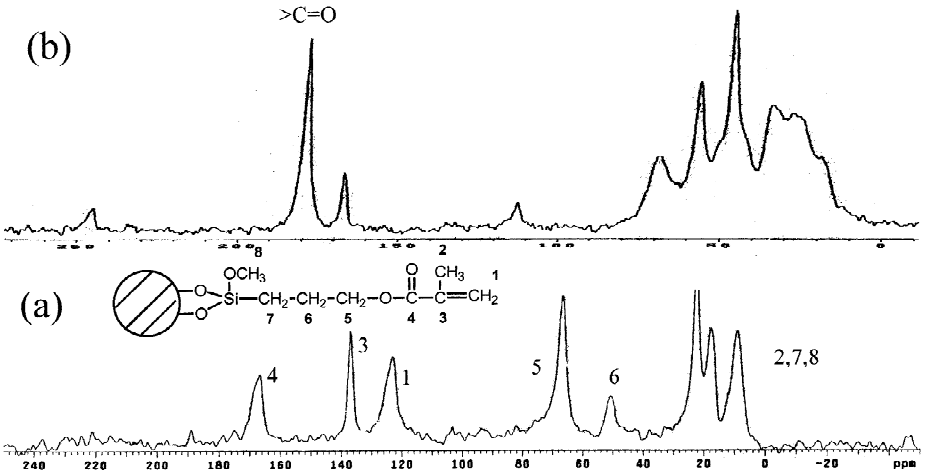

Fig. 4. Solid state ^{13}C NMR for the polymeric stationary phase with DETA. (a) Vinyl group-modified silica gel and (b) polymeric stationary phase with DETA.

elimination of γ-MAPs from the silica gel surface. In Fig. 5c,d, the first mass loss appeared around 340 °C and the second mass loss around 400 °C.

Table 1 shows the structure of the polymeric stationary phase with various amines. The amine group content (mmol/g) determined by elemental analysis was in the range 0.17–1.45. From the results, the amine group had been introduced onto the epoxy group of the poly(GMA)-modified silica gel.

3.2. Separation of BSA and CEA

In order to determine the efficiency of the prepared columns, BSA and CEA as model compounds were separated by the gradient HPLC system. Fig. 6 shows the chromatograms for BSA and CEA using the EDA column (a) and DETA columns (b). In Fig. 6a, the resolution time of CEA was 0.87 min, whereas the resolution time of BSA was 4.20 min. In the DEA column, the affinity degree of BSA was higher than CEA. In Fig. 6b, the resolution time of CEA was 0.52 min, whereas the resolution time of BSA was 0.73 min. In the EDA and DETA columns, the affinity degree of BSA was higher than CEA.

Table 2 shows the results for separation of BSA and CEA using a polymeric stationary phase with various amine groups. The affinity degree of BSA was higher than CEA for the EDA, HEDA and DETA columns, whereas the affinity degree of CEA

Table 1
Structure and EA data for the polymeric stationary phase with amines

No.	Abbreviation	Structure	No. of amine groups	Content of amines[a] (mmol/g)
		+		
1	TEA	$-N(CH_2CH_3)_3$	1	1.25
2	DEA	$-N(CH_2CH_3)_2$	1	0.30
3	EDA	$-NHCH_2CH_2NH_2$	2	1.45
4	HEDA	$-NH(CH_2)_6NH_2$	2	0.17
5	DETA	$-NHCH_2CH_2NHCH_2CH_2NH_2$	3	0.82
6	TETA	$-NHCH_2CH_2NHCH_2CH_2NHCH_2CH_2NH_2$	4	0.45

Amination condition: GMA-modified silica gel [1.5 g, 13.3 mmol/g of GMA content (based on C)]; excess amine; at 80 °C; in 1,4-dioxane; for 24 h.

[a] Determined by EA analysis (based on N).

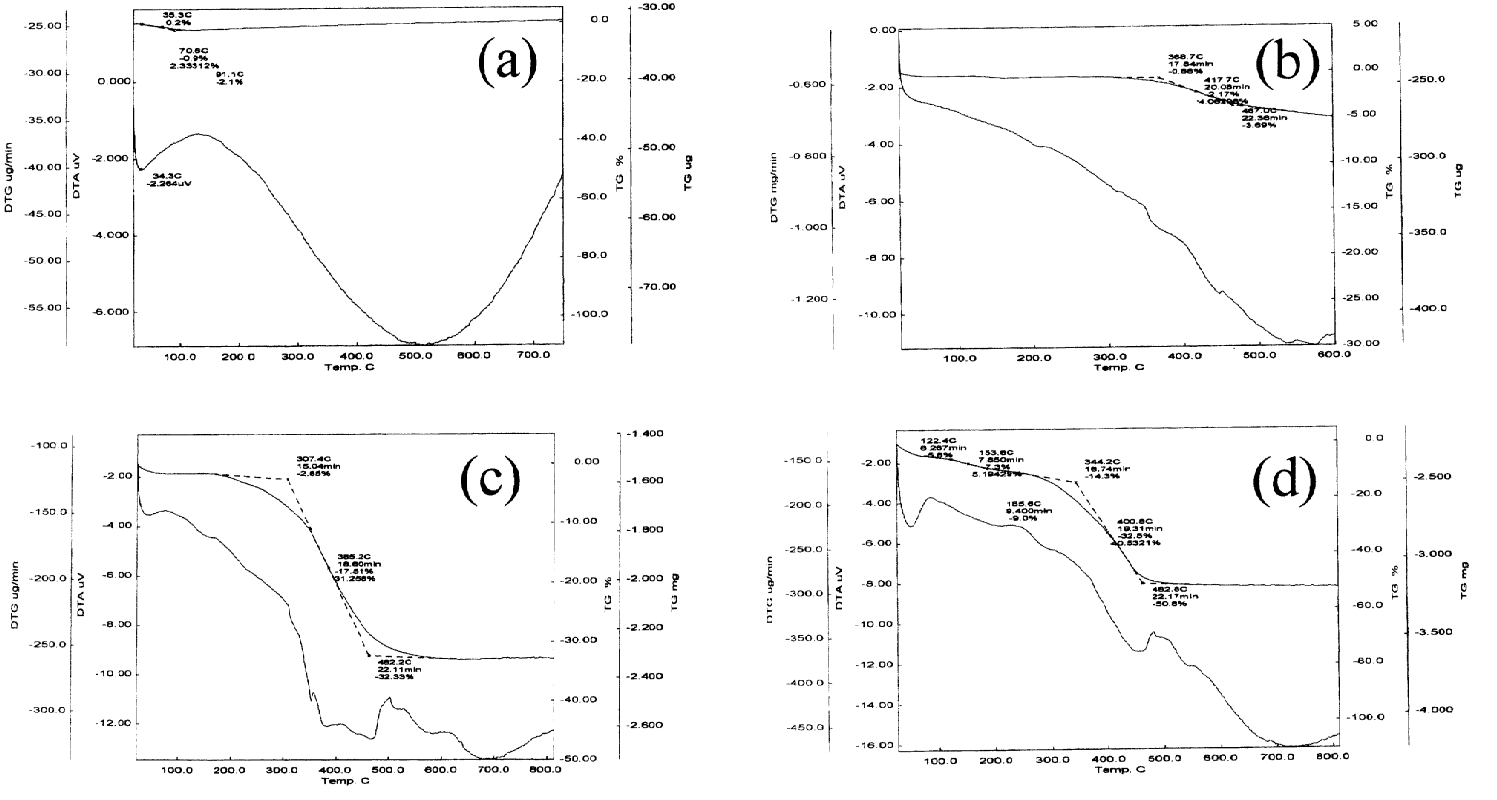

Fig. 5. TGA/DTA curves for the polymeric stationary phase with DETA. (a) Base silica gel, (b) vinyl group-modified silica gel, (c) poly(GMA)-modified silica gel, and (d) DETA-modified silica gel.

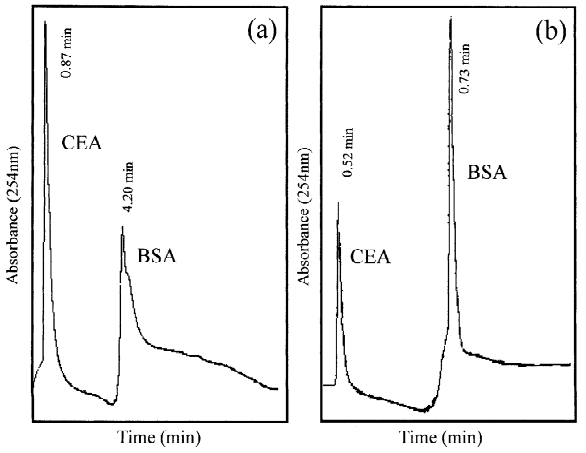

Fig. 6. Separation of CEA and BSA using the EDA column (a) and DETA column (b). Mobile phase: buffer A: Tris–HCl, pH 7.4; buffer B: 1.0 *M* NaCl.

was higher than BSA for the DETA column due to the ionic properties of the functional groups on the stationary phase. On the other hand, BSA and CEA were not separated on the TEA and DEA columns.

4. Conclusions

Six polymeric stationary phases with amine groups were prepared by grafting glycidyl methacrylate onto the silica gel surface and with subsequent amination. The separation of BSA and CEA using polymeric stationary phases with various amines was investigated. From the results, the conclusions were as follows:

1. Polymeric stationary phases with various amine groups were successfully prepared by grafting glycidyl methacrylate onto a silica gel surface and with subsequent amination.
2. The affinity degree of BSA was higher than CEA for the EDA, HEDA and DETA columns.
3. The affinity degree of CEA was higher than BSA for the DETA column. On the other hand, BSA and CEA were not separated for the TEA and DEA columns.
4. BSA and CEA were not separated on a polymeric stationary phase with TEA and DEA.

Table 2
Resolution of CEA and BSA using amine columns by gradient HPLC

No.	Abbreviation	Content of amines (mmol/g)	Separation conditions	CEA (min)	BSA (min)	Separation[a] degree
1	TEA	1.25	Mobile phase: buffer A: 20 m*M* Tris–HCl, pH 7.4; buffer B: 1.0 *M* NaCl; gradient: 0–50% in 3 min; flow-rate: 0.3 ml/min	3.55	3.55	0.00
2	DEA	0.30	Mobile phase: buffer A: 20 m*M* Tris–HCl, pH 7.4; buffer B: 1.0 *M* NaCl; gradient: 0–50% in 3 min; flow-rate: 0.3 ml/min	0.52	0.52	0.00
3	EDA	1.45	Mobile phase: buffer A: 20 m*M* Tris–HCl, pH 7.4; buffer B: 1.0 *M* NaCl; gradient: 100–0% in 5 min; flow-rate: 0.5 ml/min	0.87	4.20	3.33
4	HEDA	0.17	Mobile phase: buffer A: 20 m*M* Tris–HCl, pH 7.4; buffer B: 1.0 *M* NaCl; gradient: 0–50% in 3 min; flow-rate: 0.3 ml/min	0.17 (0.10)[b]	0.40 (0.24)[b]	0.23 (0.14)[b]
5	DETA	0.82	Mobile phase: buffer A: 20 m*M* Tris–HCl, pH 7.4; buffer B: 1.0 *M* NaCl; gradient: 0–18% in 3 min; flow-rate: 0.5 ml/min	0.52	0.73	0.21
6	TETA	1.45	Mobile phase: buffer A: 20 m*M* Tris–HCl, pH 7.4; buffer B: 1.0 *M* NaCl; gradient: 0–70% in 2 min; flow-rate: 0.3 ml/min	1.78 (1.07)[b]	1.32 (1.07)[b]	−0.46 (−0.28)[b]

Column, 5.0 cm×0.46 mm; detection: UV at 254 nm.
[a] Separation degree=detection time of (BSA–CEA).
[b] The value in parentheses is calculated (flow-rate 0.5 ml/min).

Acknowledgements

This study was supported by the Korea–Japan Joint Project Program.

References

[1] S.H. Choi, K.P. Lee, J.G. Lee, Y.C. Nho, Microchem. J. 68 (2001) 473.
[2] K.P. Lee, H.J. Kang, D.L. Joo, S.H. Choi, Radiat. Phys. Chem. 60 (2001) 473.
[3] K. Saito, T. Kaga, H. Yamagishi, S. Hurusaki, J. Membr. Sci. 43 (1989) 131.
[4] S.H. Choi, Y.C. Nho, Kor. J. Chem. Eng. 16 (1999) 725.
[5] M. Malmsten, A. Larsson, Colloid. Surf. B Biointer. 18 (2000) 277.
[6] S.H. Choi, Y.C. Nho, J. Appl. Polym. Sci. 71 (1999) 38.
[7] M. Kim, K. Saito, S. Furusaki, T. Sato, T. Sugo, I. Ishigaki, J. Chromatogr. 586 (1991) 27.
[8] S.H. Choi, K.P. Lee, Y.C. Nho, J. Appl. Polym. Sci. 80 (2001) 2851.
[9] S.H. Choi, Y.C. Nho, G.T. Kim, J. Appl. Polym. Sci. 71 (1999) 643.
[10] C.H. Chen, W.C. Lee, J. Chromatogr. A 921 (2001) 31.
[11] S.H. Choi, K.P. Lee, H.D. Kang, J. Appl. Polym. Sci. (2002) in press.