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Preparation for Protein Separation of an Ion-Exchange Polymeric Stationary Phase Presenting Amino Acid and Amine Units Through Surface Graft Polymerization

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Abstract: Ion-exchange polymeric stationary phases presenting amino acid and amino groups were prepared by the surface grafting of glycidyl methacrylate onto a silica gel surface and subsequent amination. Three kinds of amino acids-L-arginine (Arg), D-lysine (Lys), and D-histine (His)-were used in this study. An ion-exchange polymeric stationary phase presenting ethylene diamine (EDA) was also prepared by surface graft polymerization. Separation of the model proteins bovine serum albumin (BSA), chick egg albumin (CEA), and hemoglobin (Hb) was performed using the amino acid- and amine-derived columns. In separating the CEA/BSA mixture, the resolution time of BSA was longer than that of CEA when using the EDA column, whereas the resolution time of BSA was shorter than that of CEA when using the Arg, Lys, and His columns. In the separation of the Hb/BSA mixture, the resolution time of BSA was longer than that of Hb in the EDA column, whereas the resolution time of BSA was shorter than that of Hb in the amino acid columns (D-Lys, L-Arg, and D-His).

Keywords: ion-exchange polymeric stationary phase, glycidyl methacrylate (GMA), amino acid column, ethylene diamine column, hemoglobin (Hb), bovin serum albumin (BSA), chick egg albumin (CEA), resolution time.

Introduction

Different chromatographic techniques were used for the isolation, separation and purification of biomolecules (e.g. proteins, enzymes, hormones, nucleic acid, antigen and antibodies) exploiting the differences in the characteristics of the biomolecules: net electrical charge (ion-exchange chromatography), molecular size (gel permeation chromatography) and hydrophobic interaction chromatography.[1] Among these techniques, ion-exchange chromatography is often used for protein separation.[2]

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

The epoxy group of the GMA was introduced on polypropylene film, polyethylene hollow fiber, polypropylene nonwavon fabric, and core-shell nonwaven fabric by radiation-induced graft copolymerization for the recovery of urokinase in human urine,[8] the removal of heavy metal ion,[9] the application of the battery separator,[10] and the recovery of terephthalic acid in waste water.[11]

C. H. Chen *et al.* published a non-porous particle with an epoxy group by co-polymerization of styrene, methyl methacrylate, and glycidyl methacrylate. The dye, Cibacron Blue 3G-A, was introduced to the epoxy group of the non-porous particle for the separation of protein. The lysozyme and bovine serum albumin as model proteins were separated using the non-porous particle with the dye.[12]

On the other hand, the author prepared polymeric-micro beads by the radiation-induced polymerization (RIP) of glycidyl methacrylate (GMA) and diethylene glycol dimethacrylate as a crosslinking agent, and subsequently the poly-

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meric-micro beads were immobilized by lipase in an alkaline medium for chiral separation.[13] The polymeric-micro beads, however, could not be prepared for a chiral column because the polymeric stationary phase prepared by RIP had a soft property.

In order to overcome softness, the author prepared the amino group-containing silica gel by surface grafting of the GMA on a silica gel surface and its subsequent amination.[14] The aminated silica gel was packed in a stainless steel column. The separation of the bovin serum albumin (BSA) and chicken egg albumin (CEA) as model proteins was carried out using the prepared column. A good resolution of the model proteins was obtained on an ion-exchange polymeric stationary phase. Little, however, has been reported on the preparation of the amino acid column and the comparison between the amine column and amino acid column.

In this study, ion-exchange polymeric stationary phases with amino acids such as L-Arg, D-Lys, and D-His were prepared by the reaction of an amino acid with an epoxy group of poly(GMA)-grafted silica gel. The prepared ion-exchange polymeric materials were characterized by FT-IR, FT-Raman, X-ray photoelectron spectroscopy (XPS), and elemental analysis. The separation of the bovin serum albumin (BSA), chicken egg albumin (CEA), and hemoglobin (Hb) as model proteins was carried out using a prepared column with amino acids. The efficiency of amino acid columns was compared with that of the amine column for the separation of the BSA, CEA, and Hb.

Experimental

Materials. 3-(Trimethoxysilyl)propylmethacrylate (γ-MAP) was obtained from Tokyo Kasei (Japan). Glycidyl methacrylate (GMA) and α,α-azobis(isobutyronitrile) (AIBN) were obtained from the Junsi Chemical Co. Ltd.. EDA, L-Arg, D-Lys, and D-His, were purchased from the Aldrich Chemical Co. Silica gel (Art 7734, silica gel-60) was obtained from Merck. The analytes such as bovin serum albumin (BSA), chicken egg albumin (CEA), and hemoglobin (Hb) were obtained from Aldrich Chemical Co. The molecular weights of the BSA and CEA were 66,000 Dalton and 44,287 Dalton, respectively. The isoelectrical points (pI) of the BSA and CEA were 4.9 and 4.9, respectively. Toluene and 1,4-dioxane obtained from the Aldrich Chemical Co. were distilled before use.

Preparation of Ion-exchange Polymeric Stationary Phases.[14] Figure 1 shows the preparation procedure of the ion-exchange polymeric stationary phases with amino acid and amino groups. To a mixture of 10 μm silica gel (2.5 g) in toluene (40 mL), γ-MAP (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 was washed with MeOH. The vinyl group-modified silica (1.10 g, 22.1%) was obtained. FT-IR: 1736 (C=O) cm^{-1}; FT-Raman: 1640 (C=C) cm^{-1}. Subsequently, the GMA (1.0 g, 7.0 mmol) was polymerized with the AIBN in toluene at 80 °C for 16 hrs in the presence of γ-MAP-modified silica gel (1.0 g). The polymer was precipitated in

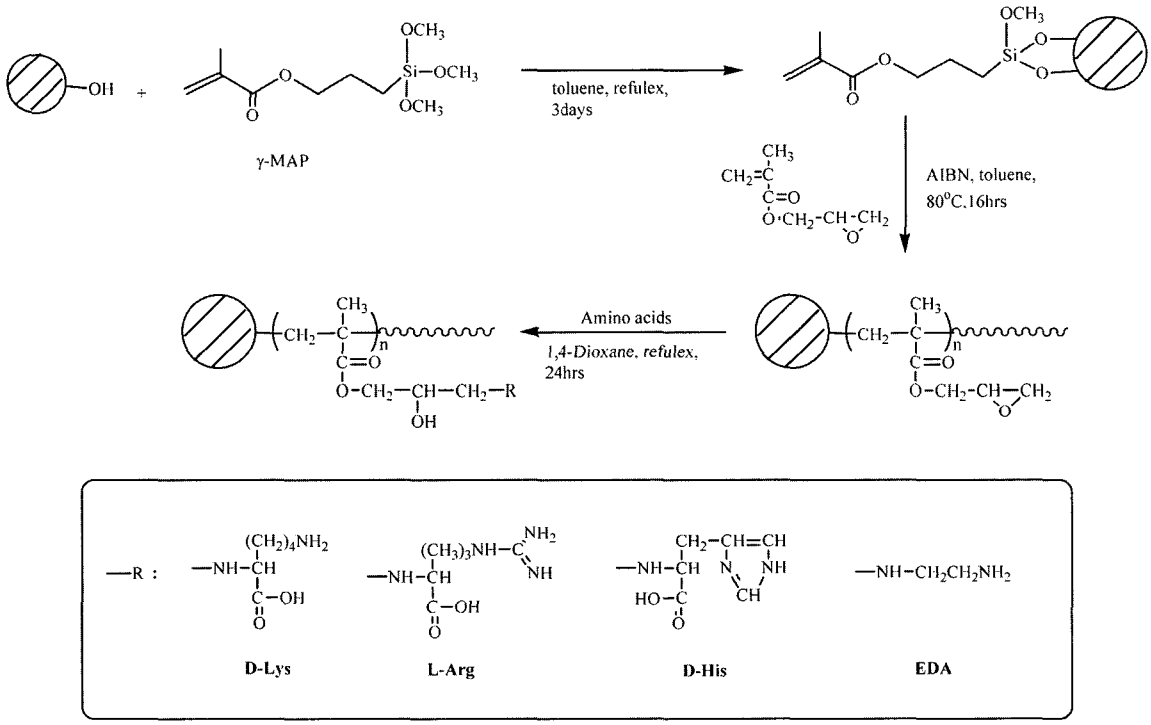


Figure 1. Scheme of preparation procedure of the ion-exchange polymeric stationary phase with amino acid and amine.

a large amount of methanol, separated by centrifugation and dried in a vacuum oven at 50 °C for 3 hrs. The EDA, L-Arg, D-Lys, and D-His was introduced to epoxy group of the GMA-modified silica gel in 1,4-dioxane on refluxing for 24 hrs as shown in Figure 1. The obtained ion-exchange polymeric stationary phase with amino acids was slurry packed into a 5.0 cm× 0.46 cm I.D. stainless steel column using a column packer (DSF-122, Haskel INC, USA). MeOH was used as the slurry mixing agent during the packing procedure.

Chromatographic separation was performed using the gradient HPLC system of the Jasco LC-2000 series with an OR-2090, RI-2031, and UV-2075 detector. A sample of the CEA, BSA, and Hb was dissolved in a 20 mM Tri-HCl buffer (pH=7.4). The separation conditions are described in Table II and III.

Characterizations. The FT-IR spectra of the ion-exchange polymeric stationary phase with amino acids were obtained using Nujol mulls with a Perkin-Elimer Mod. 983 infrared spectrophotometer. The 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. The XRD spectra of the solid powder samples were obtained from an X-ray diffractometer (VG Microtech, MT 500/1 etc). An elemental analysis (EA) of the samples was performed by using an EA1110/EA1108 instrument (FISONS).

Results and Discussion

In this study, we have prepared an ion-exchange polymeric stationary phase with amino acids for the separation of proteins in a gradient HPLC system. In a previous paper,[14] for the purpose of protein separation, poly(GMA)-supported silica was prepared because the poly(GMA) had a softness. 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, the γ-MAP with vinyl group were introduced onto a silica gel surface as shown in Figure 1. The vinyl group can be copolylmerized with various functional monomers including epoxy, amino, alcohol, etc. GMA has vinyl and epoxy groups. Vinyl groups are used for copolymerizations and epoxy groups are easily introduced as the desired functional group, such as amine and amino acid.

Characterization of Ion-exchange Polymeric Stationary Phases with Amino Acids. Figure 2 shows the FT-IR and FT-Raman spectra of base silica gel (a), vinylated silica gel (b), epoxylated silica gel (c), and the ion-exchange polymeric stationary phase with D-Lys (d) in 950~850 and 2000~1500 cm^{-1} regions. In the FT-IR spectrum of Figure 2(a), a peak at 1636 cm^{-1} was observed due to the Si-O-Si stretching mode. The peak at 1727 cm^{-1} (Figure 2(b)) corresponded to a carbonyl (> C=O) group of γ-MAP and indicated that it is attached to a silica gel surface. In Figure 2(c), the peak at 1735 cm^{-1} due to a C=O stretching was also observed. Furthermore, the peak appearing at 910 cm^{-1} is due to a COC (epoxy group) stretching. The peak at 910 cm^{-1} is suggestive of a symmetric epoxy group. These results indicated that the epoxy group of the GMA was introduced onto a silica gel surface. Figure 2(d) shows the carbonyl peak at 1734 cm^{-1} with a disappearance peak of the epoxy peak.

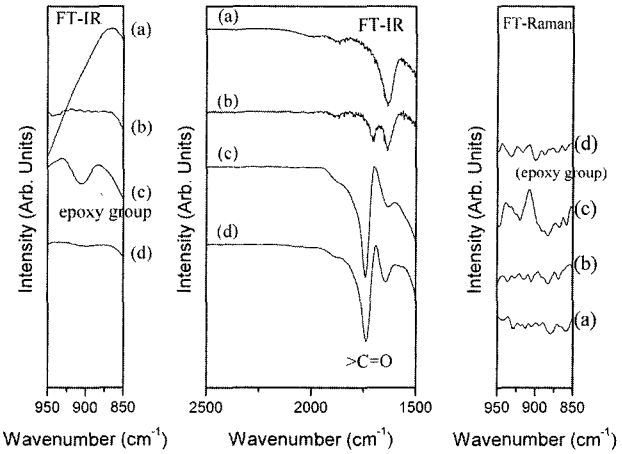


Figure 2. FT-IR and FT-Raman spectra of ion-exchange polymeric stationary phase with D-Lys, in 950-850 and 2000-1500 cm^{-1} regions. (a) silica gel, (b) vinylated silica gel, (c) epoxylated silica gel, and (d) D-Lys modified silica gel.

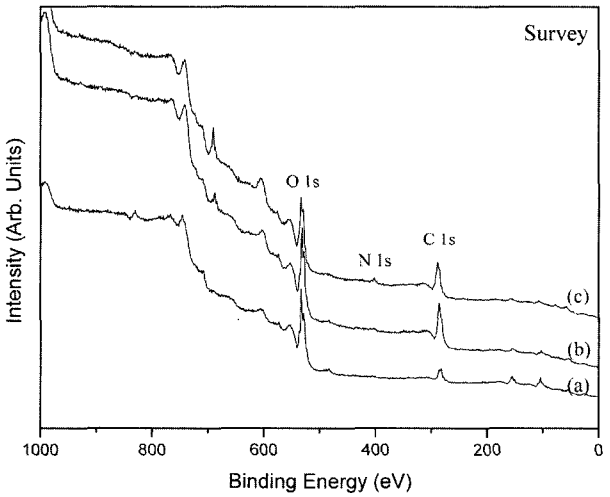
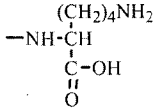
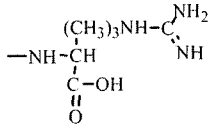
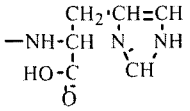


Figure 3. XPS survey scan spectra of ion-exchange polymeric stationary phase with D-Lys. (a) vinylated silica gel, (b) epoxylated silica gel, and (c) D-Lys modified silica gel.

Figure 3 shows the XPS survey scan spectra of (a) vinylated silica gel, (b) epoxylated silica gel, and (c) D-Lys modified silica gel. In Figure 3(c), the N ls peak of the ion-exchange polymeric stationary phase with D-Lys appeared at 400 eV due to D-Lys. The results showed that the D-Lys

Table I. Structure and EA Data of Ion-exchange Polymeric Stationary Phase with Amino Acids and Amine[a]

No.	Abbreviation	Structure	No. of Amine	Content of Functional Group[b] (mmol/g)
1	D-Lys		2	1.10
2	L-Arg		4	0.64
3	D-His		3	1.70
4	EDA	$-NH-CH_2CH_2-NH_2$	2	1.45

[a]Amination condition: GMA-modified silica gel [1.5 g, 13.3 mmol/g of GMA content (base on C)]; excess amine; at 80°C; 1,4-dioxane; for 24 hrs. [b]Determined by EA (base on N).

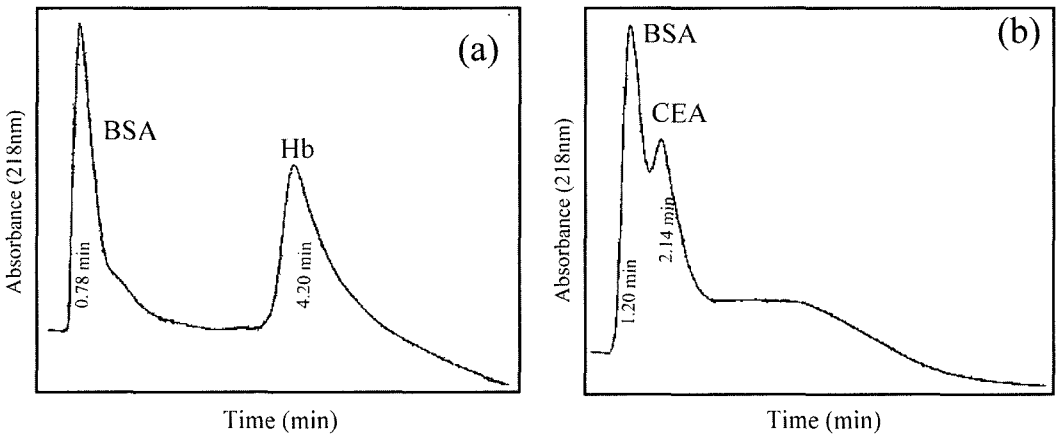


Figure 4. Resolution of the Hb/BSA (a) and CEA/BSA (b) using ion-exchange column with D-Lys in Table I. Mobile phase : buffer A: Tris-HCl pH=7.4; buffer B: 1.0 M NaCl. Conditions: Gradient: 0-60% in 6 min; Flow rate: 0.3 mL/min; Detection: UV at 218 nm.

was successfully introduced to the epoxy group of grafted-poly(GMA).

Table I describes the structures of the functional group of the ion-exchange polymeric stationary phases. The amino acid content (mmol/g) determined by EA was in the range of 0.64~1.70 mmol/g. The EDA content was 1.45 mmol/g.

Separation of Model Proteins by Ion-exchange Polymeric Stationary Phase with Amino Acid and Amino Groups. In order to know the efficiency of the prepared ion-exchange columns, the BSA/Hb and BSA/CEA mixture were separated by the gradient HPLC system. Figure 4 shows the chromatograms of the resolution of the Hb/BSA mixture (a) and the CEA/BSA mixture (b) using an ion-exchange column with the D-Lys. The resolution time of the BSA was 0.78 min, whereas the resolution time of the Hb was 4.20 min in the D-Lys column (Figure 4(a)). The resolution time of the BSA was 1.20 min, whereas the resolution time of the CEA was 2.14 min in the D-Lys column (Figure 4(b)). The resolution time of the Hb was longer than that of BSA in Hb/BSA mixture, and the resolution time of CEA was also longer than that of BSA in CEA/BSA mixture.

Figure 5 shows the chromatograms of the separation of the Hb/BSA mixture (a) and the CEA/BSA mixtures using an ion-exchange column with the EDA. The resolution time of the Hb was 0.39 min, while the resolution time of the BSA was 0.84 min using an ion-exchange column with the EDA (Figure 5(a)). The resolution time of the CEA was 0.87min, whereas the resolution time of the BSA was 4.20 min using an ion-exchange column with the EDA (Figure 5 (b)). The resolution time of the BSA was longer than that of the Hb in the Hb/BSA mixture, and the resolution time of the BSA was also longer than that of the CEA in the CEA/BSA mixture.

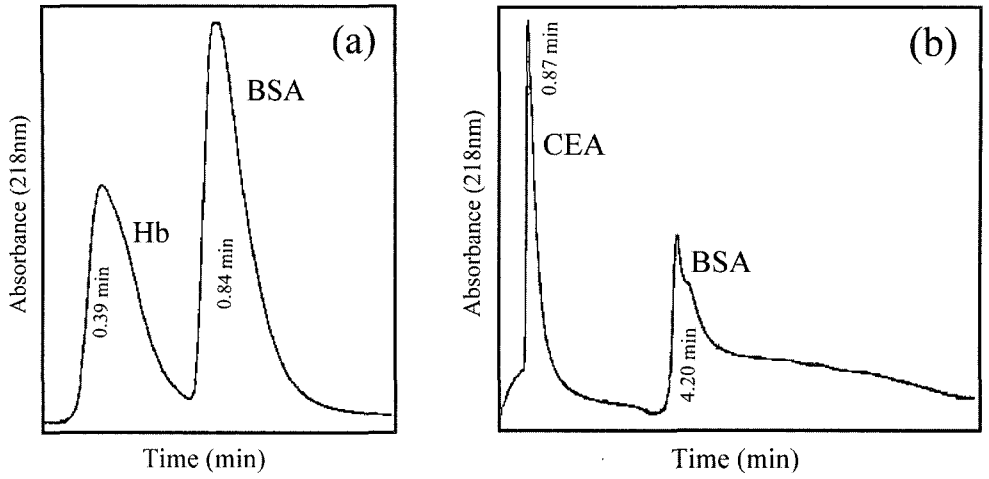


Figure 5. Resolution of the Hb/BSA (a) and CEA/BSA (b) using ion-exchange column with EDA in Table I. Mobile phase : buffer A: Tris-HCl pH=7.4; buffer B: 1.0 M NaCl. Conditions: Gradient: 0-60% in 6 min; Flow rate: 0.3 mL/min; Detection: UV at 218 nm.

Table II. Separation of the CEA/BSA Using Ion-exchange Columns with Amine and Amino Acid by Gradient HPLC[a]

No.	Abbreviation	Content of Functional Group (mmol/g)	Separation Condition	CEA (min)	BSA (min)	Separation Degree[b]
1	D-Lys	1.10	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-60% in 6 min; Flow rate: 0.3 mL/min	2.14	1.20	-0.94
2	L-Arg	0.64	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-100% in 5 min; Flow rate: 0.5 mL/min	6.08(3.65)	1.11(0.67)	-4.97(-2.98)
3	D-His	1.70	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-100% in 5 min; Flow rate: 0.5 mL/min	5.00(3.00)	0.97(0.58)	-4.08(-2.42)
4	EDA	1.45	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-60% in 6 min; Flow rate: 0.3 mL/min	0.87	4.20	+3.33

[a]Column: 5.0 cm × 0.46 mm; Detection: UV at 218 nm. [b]Separetion degree=detection time of (BSA-CEA).
The value of parenthesis is calculated (flow rate 0.3mL/min).

Table II shows the results of the separation of the CEA/BSA mixture using the ion-exchange polymeric stationary phase with the D-Lys, L-Arg, D-His and EDA. The resolution time of the BSA was longer than that of the CEA using the D-Lys, L-Arg, and D-His column, whereas the resolution time of the BSA was longer than that of the CEA using the EDA column. In the D-Lys, L-Arg, and D-His column, the separation degree followed the order: L-Arg > D-His > D-Lys.

Table III shows the results of the resolution of the Hb/BSA mixture using ion-exchange columns with the D-Lys, L-Arg, D-His, and EDA. The resolution time of the Hb was longer than that of the BSA using the D-Lys, L-Arg, and D-His. A longer resolution time of BSA was reached than for that of the Hb using EDA column.

Conclusions

Ion-exchange polymeric stationary phases with amino acid and amine were prepared by grafting GMA onto the silica gel surface and its subsequent amination. Few mixtures of CEA/BSA and Hb/BSA were separated an using ion-exchange polymeric stationary phase with amino acids (D-Lys, L-Arg, and D-His) and amine (EDA). Conclusions are as follows:

(1) An ion-exchange polymeric stationary phase with D-Lys, or L-Arg, D-His, or EDA was successfully prepared by grafting of GMA onto a silica gel surface and subsequent amination.

(2) A longer resolution time of the BSA was reached than for that of the CEA with D-Lys, L-Arg, and D-His column.

Table III. Resolution of the Hb/BSA Using Ion-exchange Columns with Amine and Amino Acid by Gradient HPLC[a]

No.	Abbreviation	Content of Functional Group (mmol/g)	Separation Condition	Hb (min)	BSA (min)	Separation Degree[b]
1	D-Lys	1.10	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-60% in 6 min; Flow rate: 0.3 mL/min	4.20	0.78	-3.42
2	L-Arg	0.64	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-100% in 5 min; Flow rate: 0.5 mL/min	4.42(2.65)	1.11(0.67)	-3.31(-1.98)
3	D-His	1.70	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-100% in 5 min; Flow rate: 0.5 mL/min	4.96(2.98)	0.97(0.58)	-3.99(-2.40)
4	EDA	1.45	Mobile phase: buffer A: 20 mM Tris-HCl pH= 7.4; buffer B: 1.0 M NaCl; Gradient: 0-60% in 6 min; Flow rate: 0.3 mL/min	0.39	0.84	+0.45

[a]Column: 5.0 cm × 0.46 mm; Detection: UV at 218 nm. [b]Separetion degree=detection time of (BSA-Hb).
The value of parenthesis is calculated (flow rate 0.3mL/min).

Resolution time of the BSA was longer than that of the CEA using the EDA column.

(3) The resolution time of the Hb was longer than that of the BSA using the D-Lys, L-Arg, and D-His, while the resolution time of the BSA was longer than that of the Hb using the EDA column.

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