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Effects of organic modifiers on the chiral recognition by different types of silica-immobilized bovine serum albumin

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Abstract

We prepared three columns containing bovine serum albumin immobilized on silica by different means and the effects of organic modifiers in the eluent on chiral separation were studied using N-substituted amino acids. Adsorption on silica, covalent immobilization to diol-silica with carbonyldiimidazole (CSP-II) and covalent immobilization to amino-silica with glutaraldehyde were studied. CSP-II had the highest stereoselectivity and was the most affected by organic modifiers in the eluent. The hydrophobicity of amino acid moiety affected the chiral recognition of N-benzoylamino acids and the aromaticity of the N-substituted group was important.

Keywords: Enantiomer separation; Mobile phase composition; Chiral stationary phases, LC; Enantioselectivity; Albumin; Amino acids; Isopropanol

1. Introduction

Chiral separation is intimately associated with the therapeutic efficiency of drugs. A variety of compounds has been separated by means of protein-based chiral phases. The reversed-phase mode has been used to separate on the chiral phases and many elements in the mobile phase affect the enantiomeric separation. However, the mechanistic aspects of separation have not been fully investigated.

On the other hand, protein immobilization methods such as ionic bonding [1], adsorption [2] and covalent bonding [3–9] have been described and some investigators have indicated that the method of fixation affects enantiomeric selectivity [2,10]. Anderson et al. have reported the influence of cross-linking reagents upon enantioselectivity [4]. They considered that during the immobilization procedure, proteins assume a variety of conformations with cross-linking reagents, which affects the availability of some chiral binding sites.

In this study, the effects of organic modifiers on chiral separation were compared using columns containing bovine serum albumin (BSA) fixed by different methods and the optimal immobilizing protocol was determined. The influence of solute hydrophobicity on chiral recognition was also studied using N-acyl amino acid derivatives.

2. Experimental

2.1. Chemicals

Spherical silica (Si) (pore size 25 nm, particle diameter 10 μm) was supplied by Tosoh (Tokyo,

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Japan). 3-Glycidoxypropyl trimethoxysilane was obtained from Petrarch Systems (USA). 1,1-Carbonyl diimidazole, 3-aminopropyl ethoxysilane and 25% glutaraldehyde were purchased from Nakalai Tesque (Kyoto, Japan). BSA (fraction V) was purchased from Sigma (St. Louis, MO, USA) and purified by Sephadex G-150 column chromatography.

Amino acid derivatives were prepared using benzoyl chloride (Kishida Chemicals, Tokyo, Japan), enancyl chloride (Tokyo Kasei Kogyo, Tokyo, Japan) or cyclohexanecarbonyl chloride (Tokyo Kasei Kogyo), from DL- or L-amino acids.

2.2. Preparation of sorbents immobilized with BSA

Three chiral stationary phases (CSP-I, II and III) were prepared. CSP-I was prepared by adsorbing BSA onto Si [2] as follows. The Si was poured into a column (150×4 mm I.D.) in chloroform–methanol (2:1). The column was washed with water and equilibrated with 0.05 *M* phosphate buffer (pH 5.0). A solution of 1 mg BSA/ml in 0.05 *M* phosphate buffer (pH 5.0) was pumped through the column until BSA breakthrough was detected at 280 nm. From this point, we calculated the amount of BSA immobilized on the column. The amount of BSA per gram of Si was also determined from an elemental analysis of nitrogen in the Si. The amount of BSA adsorbed was 109 mg/g.

CSP-II was prepared by covalently bonding BSA to diol-silica derived from Si as follow. Si (3 g) and (3-glycidoxypropyl)trimethoxysilane (11.25 ml) were heated in toluene (60 ml) for 20 h at 70°C, then the epoxy-silica was hydrolyzed with 10% perchloric acid [11]. The diol-silica was packed into a column (150×4 mm I.D.) in a manner similar to that described above. After washing with dry dioxane, the diol phase was activated with 1,1-carbonyldiimidazole (CDI) [3]. A solution of CDI (1.8 g) in dry dioxane (30 ml) was circulated through the column for 6 h, then the column was rinsed with dioxane and water. A solution of BSA (0.3 g) in 30 ml of 1 m*M* phosphate buffer (pH 7) was circulated through the column overnight. The column was then successively washed with 60-ml portions of KH_2PO_4 (50 m*M*, pH 7); KH_2PO_4 (50 m*M*, pH 7)–NaCl (50 m*M*) (1:1, v/v); NaCl (50 m*M*); water and KH_2PO_4 (50 m*M*, pH 7), then stored at 4°C. The amount of BSA immobilized on diol-Si calculated from elemental analysis of the nitrogen content was 110 mg/g.

CSP-III was prepared by glutaraldehyde-cross-linking BSA into aminopropylsilica obtained from Si [12,13] as follows. Silica (3 g) was suspended in toluene (60 ml) and 3-aminopropylethoxysilane (3 ml) was added. The reaction mixture was refluxed for 4 h [10]. The aminopropylsilica consisted of 1.74% (w/w) nitrogen and 5.41% (w/w) carbon. A mixture of 5 ml of 5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.0) and 0.8 g of aminopropylsilica was reacted at 25°C under vacuum for 1 h. Excess glutaraldehyde was removed by passage through a glass filter and the residue was washed with cold water. Eighty milligrams of BSA in 8.5 ml of 0.1 *M* phosphate buffer (pH 7.0) was added to the cross-linked silica with glutaraldehyde and reacted at 5°C overnight. The derivatized silica was filtered, washed with cold water, 1 *M* sodium chloride followed by cold water, then packed into a 150×4 mm I.D. column. The amount of BSA immobilized on the silica, determined by elemental analysis, was 114 mg/g.

2.3. Preparation of amino acid derivatives

Amino acids were acylated as described [14]. The products were recrystallized from ethanol–water, and identified by elemental analysis of the crystals.

2.4. HPLC conditions

CSP-I, II and III were packed into stainless steel columns (150×4 mm I.D.). Phosphate buffer (0.05 *M*) containing an organic modifier was used as eluent. Samples were injected as 10 μl of a $5 \cdot 10^{-4}$ *M* solution in the eluent. The flow-rate was maintained at 1 ml/min and the effluent was monitored using a UV detector at 235 or 210 nm.

3. Results and discussion

Three columns loaded with similar amounts of BSA were prepared by the different immobilizing methods and their stereoselectivity for amino acid derivatives was compared.

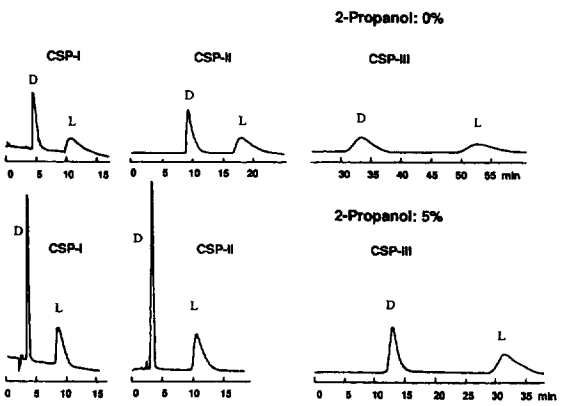

Fig. 1. Chromatograms of N-benzoylisoleucine on various columns. Eluent: 0.05 *M* phosphate buffer (pH 7.0) without or with 5% 2-propanol.

3.1. Influence of organic modifiers

Organic modifiers in the mobile phase affect hydrophobic interactions between the solute and the stationary phase. The hydrophobic region of BSA which is concerned with chiral recognition is also affected by organic modifiers. Chiral separation of N-benzoylamino acids in the three columns under various concentrations of 2-propanol was compared. Chromatograms of N-benzoylisoleucine (Bz-Ile) (Fig. 1) and N-benzoylithreonine (Bz-Thr) (Fig. 2) show the effect of 2-propanol on the separation of the enantiomer. On the CSP-III column, evidence of a powerful achiral interaction caused mainly by the effect of cross-linking of reagent between BSA and the silica matrix [12,15] was shown by the long retention time of solutes. Adding 2-propanol (5%) to the eluent reduced the elution time of enantiomers of Bz-Ile and the distance between two peaks of enantiomer was similar to that of the eluent without 2-propanol. However more 2-propanol (above 5%) gradually reduced the distance between the peaks. The elution time and the distance between Bz-Thr enantiomers using eluent containing 2-propanol decreased compared with those found using eluent without 2-propanol. The enantiometric selectivity value (α) of these compounds on CSP-II was higher than those on CSP-I and CSP-III, because CSP-II had a short retention time and good resolution of the enantiomer peaks.

The value of α was compared at various concentration of 2-propanol (Fig. 3). Because CSP-I exhibited a short column life at a high concentration

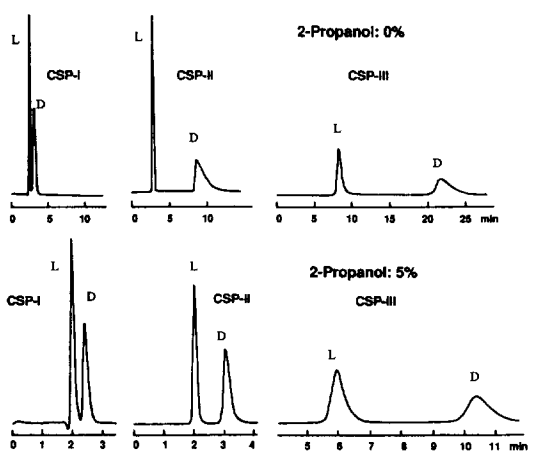

Fig. 2. Chromatograms of N-benzoylthreonine on various columns. Eluent: 0.05 *M* phosphate buffer (pH 7.0) without or with 5% 2-propanol.

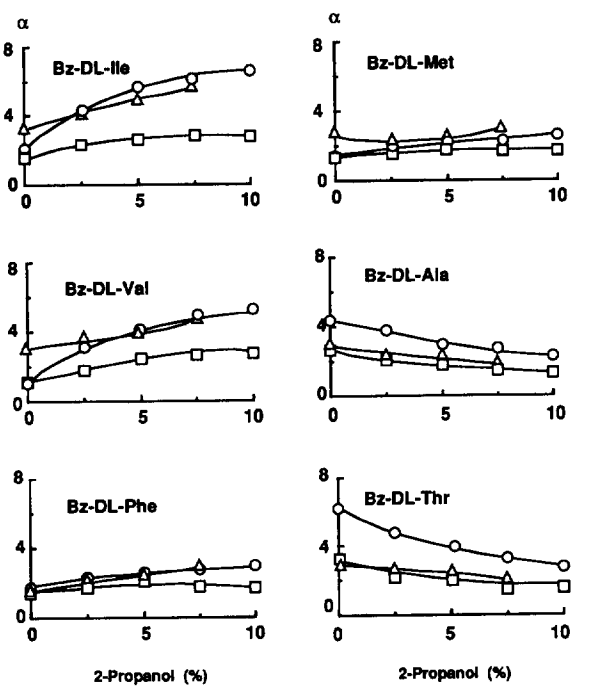

Fig. 3. Effects of 2-propanol in the mobile phase on stereoselectivity of N-benzoylamino acids. Column: (△) CSP-I; (○) CSP-II; (□) CSP-III. Eluent: 0.05 *M* phosphate buffer (pH 7.0) containing 2-propanol.

of the organic modifier, the experiment was performed at concentrations up to 5%. Concentrations up to 10% were investigated using the other columns. The relationship between the concentration of 2-propanol and chiral selectivity was classified into two solute groups. In one case, an increase of the alcohol content increased the enantioselectivity, and in the other, increasing the alcohol content decreased the enantioselectivity. The hydropathy index of Kyte–Doolittle is a hydrophobic parameter of amino acids [16]. The values for isoleucine, valine, phenylalanine, methionine, alanine and threonine are 4.5, 4.2, 2.8, 1.9, 1.8 and −0.7, respectively. Higher values reflect higher hydrophobicity. The hydrophobicity of an N-benzoyl amino acid may parallel that of the original amino acid. Compounds having a value higher than that of methionine increased the α value with increasing proportions of 2-propanol. Compounds with a value lower than that of methionine resulted in the opposite curve. These findings suggested that the hydrophobicity of the solute affects the enantioselectivity of BSA produced by an organic modifier in the mobile phase. Variations in the α value at 0 and 5% 2-propanol in the mobile phase [$\Delta\alpha(5,0)$] were plotted to the Kyte–Doolittle hydropathy index for amino acid constituted N-benzoyl derivatives. The enantioselectivity of highly hydrophobic solutes (having a high Kyte–Doolittle hydropathy index) increased upon adding 2-propanol to the eluent and resulted in a positive $\Delta\alpha(5,0)$ value. It was indicated that the enantiomeric recognition of these compounds was improved by decreasing the hydrophobic interaction between the solute and the BSA binding site. However, the enantioselectivity of less hydrophobic solutes having low Kyte–Doolittle hydropathy indices was decreased by adding 2-propanol to the eluent, resulting in negative $\Delta\alpha(5,0)$ value. Thus, the enantiomeric recognition of hydrophilic compounds became disrupted by decreasing the hydrophobic interaction. The selectivity of the solutes having appropriate hydrophobicity (such as methionine) was not influenced by the concentration of 2-propanol. As shown in Fig. 4, the correlation between the Kyte–Doolittle hydropathy index for amino acids and the value of $\Delta\alpha(5,0)$ was high on each column. This indicated that the extent of the influence of 2-propanol upon the stereoselectivity of the column is associated with the hydrophobicity of the solute. The slope of the line on the CSP-II column was the most steep of the three columns. This BSA immobilizing method of CSP-II is susceptible to the influence of organic modifiers and improved the high chiral recognition, which resulted in a higher α value for all compounds. The extent of the effect of the organic modifier differs according to the immobilization method (CSP-II>III>I) and the tendency for the same solute was similar. It is considered that the immobilization method affected the availability of the chiral recognition site of BSA and thus, the different effects of organic modifiers. The effects of organic modifiers on chiral recognition have been reported by Miwa et al. [6] and Allenmark [17]. In general, an increase in the organic modifier decreases k' and α values. As shown in Fig. 3, k' decreased and the α value increased with highly hydrophobic compounds, which was unusual. Adding 2-propanol in the mobile phase weakened the hydrophobic interaction between the solute and the solid-phase, which affected chiral recognition.

The effect of other alcohols as organic modifiers was also investigated on CSP-II. Methanol, ethanol

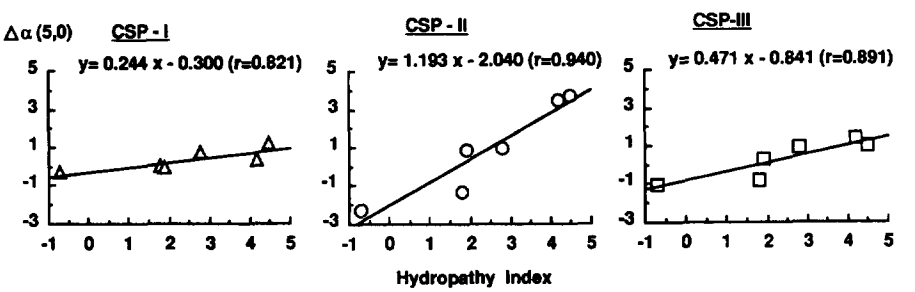

Fig. 4. Correlation of $\Delta\alpha(5,0)$ and the hydropathy indices of amino acids.

and 1-propanol were compared with 2-propanol. The enantioselectivity of N-benzoylamino acids at various concentrations of each alcohol varied in a similar manner to that in the presence of 2-propanol as shown in Fig. 1. Thus hydrophobic amino acids such as Bz-Ile showed an increase of enantioselectivity as the alcohol concentration increased, whereas the selectivity of hydrophilic amino acid such as Bz-Thr decreased. As shown in Table 1, the relationship between the Kyte–Doolittle hydropathy index of amino acids and the value of $\Delta\alpha(5,0)$ was satisfatory with the alcohols tested. The Snyder polarity parameters for methanol, ethanol, 1-propanol and 2-propanol were 5.1, 4.3, 4.0 and 3.9, respectively. The slope of the line on each alcohol parallels the polarity of the alcohol. The effect of methanol, which has low hydrophobicity, upon enantioselectivity, was the smallest and that of 1-propanol having high hydrophobicity was the largest.

3.2. The effect of the substituted group on the chiral separation of N-substituted amino acids

Amino acids without derivation can be chirally separated by ligand exchange chromatography with immobilizing metal ions [18,19]. However, only tryptophan can be separated into enantiomers on the BSA immobilized columns and other amino acids are separated to enantiomers after derivatization [20,21].

Allenmark et al. [22] have studied N-benzoyl and N-naphthoyl derivatives, and the L-form of N-benzoylalanine eluted prior to the D-form. However, the elution order of the N-naphthoyl derivatives changes. N-Naphthoyl derivatives of phenylalanine eluted in a different order from that of N-benzoyl derivatives and their retention times and α values increased. They estimated that hydrophobic interactions are involved in enantiometric separation.

Vindevogel et al. [23] have investigated N-nitroaroyl derivatives and found that the α values of the mononitrobenzoyl derivatives were higher than those of 3,5-dinitrobenzoyl derivatives. In that study, the hydrophobicity of the solute was not a serious factor in chiral separation. The mechanism of chiral separation of amino acid derivatives has not yet been defined.

We have described how the hydrophobicity of amino acids is closely associated with the chiral recognition process of N-benzoyl amino acids. We further investigated the role of the benzoyl group in amino acid derivatives. The aromatic/aliphatic, chain/cyclic and hydrophobic effect of substituted groups having the same carbon number as the enantio separation was studied using N-benzoyl-, N-enancyl and N-cyclohexanecarbonyl derivatives of alanine (Ala) and phenylalanine (Phe). As shown in Fig. 5, benzoyl derivatives had the highest α value

Table 1
Correlation of $\Delta\alpha(5,0)$ and the hydropathy indices of amino acid derivatives in various organic modifiers on a CSP-II column

Organic modifier	Relationship[a]	Correlation factor
Methanol	$y=0.313x-0.964$	0.824
Ethanol	$y=0.516x-1.127$	0.923
1-Propanol	$y=1.354x-2.463$	0.963
2-Propanol	$y=1.193x-2.040$	0.940

[a] x=hydropathy index of amino acid, $y=\Delta\alpha(5,0)$.

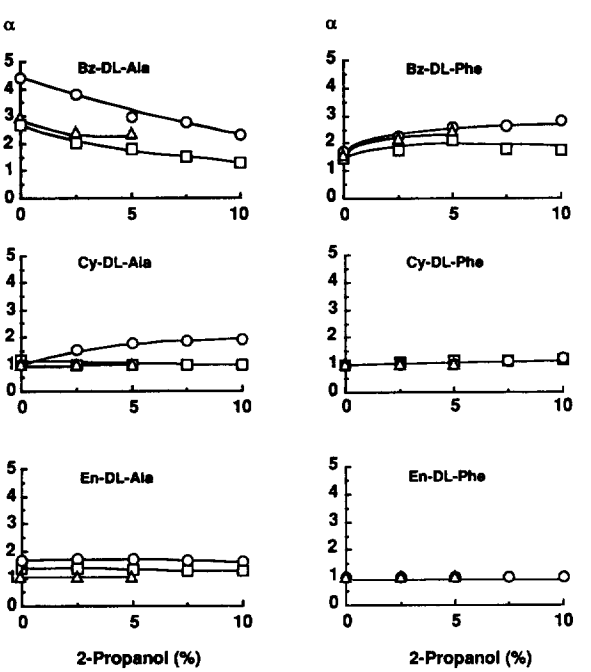

Fig. 5. Effect of 2-propanol in the mobile phase on the stereoselectivity of amino acid derivatives. Column: (△) CSP-I; (○) CSP-II; (□) CSP-III. Eluent: 0.05 *M* phosphate buffer (pH 7.0) containing 2-propanol.

among the three derivatives. The hydrophobicity of benzoyl (Bz), cyclohexanecarbonyl (Cy) and enancyl (En) groups determined by the Rekker method [24] were 0, 2.96 and 3.15, respectively. We supposed that the hydrophobicity of the N-substituted group does not significantly affect the chiral recognition of amino acid derivatives, but that aromaticity is important. The separation factor (α) of Cy-DL-Ala on the CSP-II column increased with increasing concentrations of 2-propanol. Since this profile differed from that of Bz-Ala on the CSP-II column, the chiral recognition site of Cy-Ala may be distinct from that of Bz-Ala. Amino acids having an En group showed high achiral retention and En-Phe was not eluted from the CSP-III column. The elution order of the D- and L- forms was the same among Bz, Cy and En derivatives. Thus the L-form of the alanine derivatives eluted first and the D-form of phenylalanine derivatives eluted first. In a chirally separable compound, a high α value was obtained on the CSP-II column.

3.3. Influence of column load on enantioselectivity

Chiral recognition by a protein based column occurs in a limited area of the protein and therefore, the loading capacity is low [25]. Fig. 6 shows the relationship between sample loading and the α value on three columns. CSP-I showed an almost constant α value but the values of CSP-II and III decreased with increasing sample loading. The sample loading changed to below 3% of the α value obtained on injection of 1.25 nmol of Bz-Ala; the values were 15 (CSP-II) and 5 nmol (CSP-III). The order of the column capacity was CSP-I>CSP-II>CSP-III. The columns containing BSA immobilized by covalent bonding seemed to have a lower capacity than those immobilized by adsorption. The accessibility of the chiral bonding site may be decreased by covalent bonding immobilization. This experiment showed that the amount of the chiral recognition site and the magnitude of the α value reflected the quality of the site.

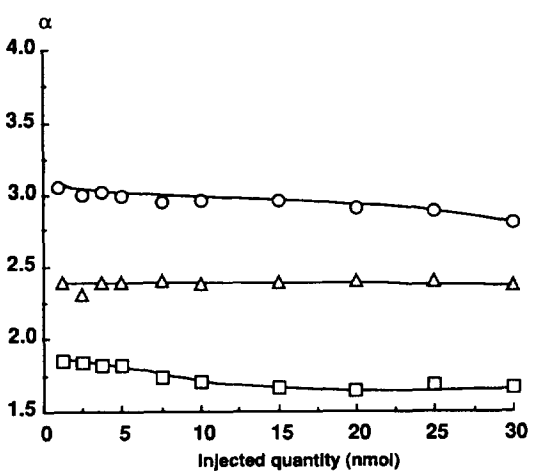

Fig. 6. Influence of column load on the separation factor (α) of N-benzoyl-DL-alanine. Column: (△) CSP-I; (○) CSP-II; (□) CSP-III. Eluent: 0.05 *M* phosphate buffer (pH 7.0) containing 5% 2-propanol.

In contrast to the column capacity, the order of column life was CSP-III>CSP-II>CSP-I. The adsorption type (CSP-I) had the shortest life and did not resist high concentrations of organic modifier. The covalent bonding types had a reasonably long column life and tolerated the repeated use of high concentrations of organic modifier.

4. Conclusion

BSA was immobilized by means of adsorption on silica (CSP-I), covalent immobilization to diol-silica with carbonyldiimidazole (CSP-II) and covalent immobilization to amino-silica with glutaraldehyde (CSP-III). These were prepared from the same silica gel and contained similar concentrations of BSA. We compared the chiral recognition of CSP-I, II and III using organic modifiers. Among them, CSP-II was the most stereoselective and the most susceptible to organic modifiers in the eluent. We showed that the method by which the protein is fixed affects the availability of the chiral recognition site of BSA and causes a difference in the hydrophobic interaction involved in chiral recognition. The order of the availability of the site was shown to be CSP-II> CSP-III>CSP-I. The effect of organic modifiers in the eluent on the chiral recognition of N-benzoylamino acids was correlated with the hydrophobicity of part of the amino acid. The results of the enantioseparation of various N-acyl amino acids revealed that the hydrophobicity of the N-substituted group did not significantly affect the chiral recogni-

tion of amino acid derivatives, but that aromaticity was important.

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