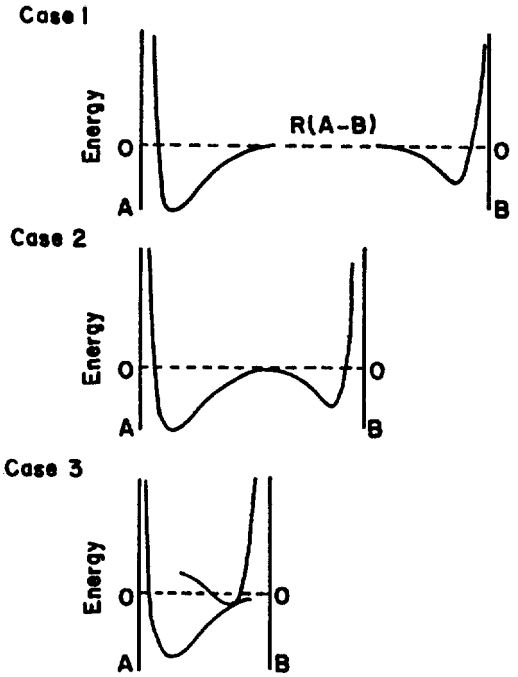

Figure 5. Schematic representation of potential energy profiles for three limiting cases as a function of the distance between proton-donor and proton-acceptor.

energy profile of a proton transfer reaction theoretically, and much careful attention must be paid to the interpretation of the potential energy profiles.

Acknowledgement. This work was supported by the Basic Science Research Grant from the Ministry of Education of Korea and the Research Grant from Korea Science and Engineering Foundation.

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Optical Resolution of Dansyl Amino Acids with Addition of Benzyl-L-Hydroxyproline Copper(II) Chelate by High Performance Liquid Chromatography

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Resolution of enantiomers of DNS-amino acids has been achieved by a reversed phse liquid chromatography with an addition of a copper(II) complex of N-benzyl-L-hydroxyproline to the mobile phase. N-Benzyl-L-hydroxyproline was prepared and used as a chiral ligand of copper(II) chelate for the optical resolution. The pH and the concentration of copper(II) chelate, organic solvent, and buffer agent in the mobile phase all affect the optical resolutions of dansyl amino acids. The elution orders between D and L-DNS-amino acids were different depending on the structure of the side chain of the amino acids. The retention mechanism for the chiral separation of the dansyl amino acids can be illustrated by the equilibrium of ligand exchange and by hydrophobic interaction with C_{18} stationary phase. The chiral separation can be illustrated with cis and trans effect of the ligand exchange reaction.

Introduction

Methods for separating free or derivatized amino acids by reversed phase high performance liquid chromatography (HPLC) consisting of mixed chelate complexes of amino acids have been well described. They are based upon addition of a chiral chelate to the mobile phase[1-15].

The resolution of enantiomers of amino acids by HPLC has been interested especially in the synthesis of peptides and the determination of the chemical structure. Many different kinds of chiral chelate have been developed for the resolution of α-amino acids and dansyl-α-amino acids[1]. Copper (II) chelates have been used as the chiral eluents for the optical resolution. Proline and its derivatives have been used

as the chiral ligands for the optical resolution of the amino acids.[2,15-19] It had been demonstrated that proline and it's derivatives, as chiral additives, are very efficient resolving agents. Generally, the use of L-proline copper (II) chelate as the chiral agent in HPLC gives a different elution order between D and L amino acids depending on the experimental conditions such as the separation method or derivatization of amino acids. The separation selectivity of the optical isomers varies with change in the chemical structure of the derivatized proline in the copper (II) chelate, which leads us that study of separation mechanism of the optical isomers of amino acids is necessary. So we are interested in developing the copper (II) proline derivative chelates as the chiral ligands to enhance the optical separation of amino acids (free or amino acid derivatives).

In this paper, we used N-Benzyl-L-Hydroxyproline (BzHyp) as a chiral chelate additive to the mobile phase to resolve dansylated amino acids. This system can be compared with other systems in terms of retention and selectivity as well as the separation mechanism.

Experimental

Instrument. The liquid chromatograph used in this work was a Waters Associates (Milford, Mass., USA). The chromatography system consisted of various components; a Model U6K loop injector; a Model 6000A high pressure pump; a Model 420 fluoredcence detector; an a Model 730 Data Module. The wavelengths of the excitation and emission filters for detection of the dansylated amino acids were 365 and 495 nm, respectively. The columns used for this wortk were μ-Bondapack C_{18} columns (30 cm × 3.9 mm i.d., 10m). The pH of aqueous solutions was determined by a Fisher Model 292 digital pH meter.

Reagents. Nine L- and D-amino acids used for the optical resolution in HPLC were derivatized with dansyl chloride before the injection. The dansylation was used for enhancing the detectability and the optical resolution.

Seine(Ser), valine(Val), threonine(Thr), alanine(Ala), methionine(Met), leucine(Leu), phenylalanine(Phe), and tyrosine (Tyr) were used for the dansylation and chiral separation. Threonine, phenylalanine and leucine were obtained from Yoneyama(Osaka, Japan). Hydroxyproline(Hyp) and other amino acids were from Sigma(St. Louis MO, USA). Another reagents were from Aldrich (Milwaukee, WI, USA) and solvents for the mobile phase were HPLC grades. Dansylation of amino acids was carried out at 40 °C for 30 min.[1,2,15]

N-Benzyl-L-hydroxyproline(BzHyp) used for the ligand of the chiral chelate was prepared similarly as previously described.[15] 6 m*l* of benzyl chloride and 60 m*l* of ethanol were added to 40 m*l* of the aqueous solution which contains 6.82g of L-hydroxyproline and 6.5g of sodium hydroxide and then refluxed for three hours. To the resulting solution acetic acid was added to control the pH down to 6.0. White powders were obtained by chloroform extraction. The solid residue was dissolved in ethanol and recrystallized by adding ethylether. The resulting product was identified with IR, NMR, and MS spectra. The infrared spectrum showed the aromatic ring system at 1450 and 1600 cm^{-1}, the broad hydrogen bonded OH peak in the range of 3300 and 3400 cm^{-1}, and a strong peak of the carboxylate group at 1620 cm^{-1}. The proton peaks at 7.4ppm from the proton nuclear magnetic resonance spectrum indicates the presence of the benzene ring in the product. A peak at $m/e = 91$ from the mass spectrum appeared as a base peak, with which we can see the benzyl group bonded to proline. The yield was 70%. It was used as the ligand of the copper chelate for the chiral separation.

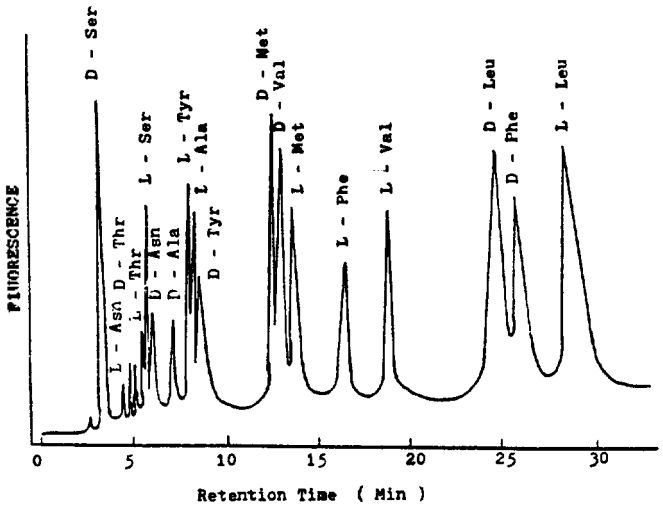

Figure 1. Chromatogram of D,L-dansyl amino acids with Cu(II)-$(BzHyp)_2$ mobile phase. Mobile phase is 25% acetonitrile and 75% aqueous complex solution containing 5×10^{-3}M copper chelate and 1×10^{-2}M NH_4Ac buffer at pH 7.0. The flow rate is 1.0 m*l*/min.

Mobile Phase Preparation. The organic solvent used in this work was acetonitrile. The aqueous portions of the mobile phase were prepared by adding the accurate weight of BzHyp and copper (II) sulfate and a suitable amount of ammonium acetate as a buffer agent and then by adjusting the pH to the desired value with 1 M hydrochloric acid or sodium hydroxide solution. Finally, distilled water was added to the required volume of the solution.

Results and Discussion

Methods for separating D- and L- DNS-amino acids by reversed phase high performance liquid chromatography (HPLC) consisting of mixed Cu(II) chelate complexes of amino acids in the mobile phase have been reported[2,3]. Enantiomers of amino acids are resolved based on a stereospecificity of a chiral Cu(II) complex in the mobile phase in which D- and L-amino acids form two diastereomeric ternary complexes of different stability;

$$Cu(BzHyp)_2 + L\text{-}AA \rightleftharpoons Cu(BzHyp)(L\text{-}AA) + BzHyp \quad (1)$$

$$Cu(BzHyp)_2 + D\text{-}AA \rightleftharpoons Cu(BzHyp)(D\text{-}AA) + BzHyp \quad (2)$$

where *AA* means DNS-amino acids.

The system separates different amino acids one another along with separating their optical isomers. The species in the equilibrium responsible for the formation of the binary complex, $Cu(BzHyp)_2$ would be present in appreciable concentrations at neutral pH. Since the steric effect is important in determining the equilibrium constant of the reactions above, stereoselectivity would be expected when mixed com-

Table 1. The Capacity Ratios(k') and Selectivity Factors(α) of Dansyl Amino Acids as a Function of the Acetonitrile Concentration in the Mobile Phase[a]

DNS-AA		20%		22%		25%		27%		30%[b]	
		k'	α	k'	α	k'	α	k'	α	k'	α
Ser	D	6.74	1.26	2.17	1.37	1.11	1.50	0.55	1.76	0.39	1.46
	L	8.26		2.98		1.67		0.97		0.57	
Asn	D	8.53	0.70	3.15	0.72	1.55	0.79	0.91	0.79	0.50	0.81
	L	6.01		2.27		1.22		0.72		0.41	
Thr	D	8.74	1.26	3.38	1.11	1.66	1.11	0.97	1.07	0.55	1.11
	L	11.06		3.76		1.85		1.04		0.61	
Ala	D	12.59	1.37	4.84	1.31	2.16	1.32	1.47	1.29	0.85	1.26
	L	17.21		6.31		2.85		1.90		1.07	
Tyr	D	20.20	0.78	7.61	0.74	3.54	0.78	2.15	0.79	1.17	0.67
	L	15.87		5.63		2.76		1.71		0.78	
Met	D	28.19	1.14	12.06	1.10	5.67	1.13	3.32	1.14	1.86	1.15
	L	32.20		13.24		6.38		3.78		2.14	
Val	D	43.15	1.46	14.58	1.43	6.70	1.49	4.06	1.42	2.28	1.42
	L	63.21		20.84		9.98		5.76		3.24	
Phe	D	112.21	0.54	37.34	0.53	14.96	0.59	8.39	0.59	4.50	0.59
	L	60.74		19.98		8.82		4.98		2.56	
Leu	D	84.63	1.41	35.04	1.25	14.52	1.37	8.10	1.26	4.31	1.31
	L	119.00		44.10		19.89		10.21		5.66	

[a]The mobile phase contained 5×10^{-3}M Cu(II)-$(BzHyp)_2$ and 1×10^{-2}M NH_4Ac aqueous solution at pH 6.5. [b]Flow rate was 1.5 ml/min. The others 2.0 ml/min.

plexes of enantiomeric solutes, *L-AA* and *D-AA*, are partitioning in the C_{18} stationary phase.

A typcal chromatogram for the separation of dansyl amino acid enantiomers is shown in Figure 1. As can be seen, good resolutions have been achieved for various D,L pairs with relative retention values appoaching 2.0 in certain case. Peak identification was made by separately injecting the dansyl derivatives of the optically pure D- and L-amino acids. The elution orders between D- and L-DNS-amino acids appeared differently according to the functionality of the side chain of the dansyl amino acids.

Generally, D-forms elute before L-forms, but the opposite order was observed for asparagine, phenylalanine, and tyrosine. It is unclear why the elution order for the D,L pairs is dependent upon the side chains of amino acids for a given chelate (see Table 1).

Optical resolution of the dansyl amino acids is dependent upon the composition of acetonitrile in the mobile phase. An increase in the concentration of acetonitrile decreases the retention of the dansyl amino acids as shown in Table 1. The retention was much more affected by the composition of acetonitrile comparing with other cases[2,3,15]. The selectivity between D- and L-pairs showed a little decrease with increasing the concentration of acetonitrile. This results from the facts that the ligand exchange reaction between the binary chelate and the dansyl amino acids gives more selectivity between D and L dansyl amino acids in a low concentration of acetonitrile solution because the polarity of the mobile phase decreases with the increase in the concentration of acetonitrile. The concentration of the BzHyp-copper complex also affected the separation. As the concentration was increased up to 10 mM, the capacity ratio k' increased and the selectivity, α, increased slightly (see Table 2). This behavior indicates

Table 2. The Capacity Ratios(k') and Selectivity Factors(α) of Several Amino Acids as a Function of Complex Concentration in the Mobile Phase[a]

DNS-AA		2.5×10^{-3} M		5×10^{-3} M		1×10^{-2} M	
		k'	α	k'	α	k'	α
Ser	D	0.91	1.34	1.27	1.35	1.71	1.48
	L	1.22		1.71		2.53	
Asn	D	1.15	0.79	1.65	0.78	2.44	0.80
	L	0.91		1.28		1.97	
Thr	D	1.22	1.05	1.78	1.07	2.33	1.27
	L	1.28		1.90		2.76	
Ala	D	1.90	1.13	2.52	1.17	3.58	1.26
	L	2.15		2.95		4.53	
Tyr	D	2.45	0.87	3.53	0.84	4.92	0.84
	L	2.15		2.98		4.17	
Met	D	4.00	1.07	5.76	1.08	7.79	1.17
	L	4.27		6.22		9.11	
Val	D	4.18	1.29	6.47	1.35	8.70	1.45
	L	5.38		8.72		12.07	
Phe	D	9.61	0.65	14.41	0.63	19.04	0.63
	L	6.28		9.05		12.07	
Leu	D	8.37	1.14	13.40	1.20	17.78	1.29
	L	9.92		16.10		22.93	

[a]The mobile phase contained 25% acetonitrile and 75% 5×10^{-3}M Cu(II)-$(BzHyp)_2$ and 1×10^{-2}M NH_4Ac aqueous solution at pH 6.5.

that the ligand exchange reaction affects the optical resolution. As can be seen in Table 1 and 2, the selectivity between D- and L- pairs depends on the side groups of the amino

Table 3. The Capacity Ratios(k') and Selectivity Factors(α) of Several Amino Acids as a Function of pH in the Mobile Phase[a]

DNS-AA		pH 6.0		pH 6.5		pH 7.0	
		k'	α	k'	α	k'	α
Ser	D	2.47	1.24	1.07	1.52	0.85	1.58
	L	3.07		1.63		1.34	
Asn	D	8.36	0.76	1.55	0.74	1.37	0.71
	L	6.41		1.15		0.97	
Thr	D	3.15	1.18	1.70	1.12	1.25	1.13
	L	3.74		1.90		1.41	
Ala	D	6.21	1.27	2.18	1.33	1.70	1.35
	L	7.91		2.91		2.30	
Tyr	D	10.41	0.80	3.59	0.79	2.99	0.76
	L	8.33		2.84		2.22	
Met	D	16.24	1.12	5.67	1.13	4.11	1.16
	L	18.91		6.38		4.78	
Val	D	20.22	1.40	6.83	1.44	4.15	1.61
	L	28.41		9.39		6.68	
Phe	D	42.26	0.73	16.56	0.65	10.55	0.53
	L	31.00		10.74		5.55	
Leu	D	44.04	1.33	16.23	1.30	9.61	1.50
	L	58.48		21.18		14.47	

[a]The mobile phase contained 25% acetonitrile and 75% 5×10^{-3}M Cu(II)-$(BzHyp)_2$ and 1×10^{-2}M NH_4Ac aqueous solution.

Table 4. The Capacity Ratios(k') and Selectivity Factors(α) of Several Amino Acids as a Function of NH_4Ac Concentration in the Buffer[a]

DNS-AA		5×10^{-3} M		1×10^{-2} M		2.5×10^{-2} M	
		k'	α	k'	α	k'	α
Ser	D	1.22	1.46	1.26	1.35	1.00	1.41
	L	1.78		1.71		1.41	
Asn	D	1.68	0.76	1.64	0.78	1.50	0.91
	L	1.28		1.27		1.10	
Thr	D	1.70	1.13	1.76	1.09	1.50	1.00
	L	1.92		1.93		1.50	
Ala	D	2.52	1.27	2.49	1.22	2.02	1.18
	L	3.19		3.04		2.39	
Tyr	D	3.91	0.75	3.53	0.85	2.91	0.86
	L	2.96		3.00		2.49	
Met	D	5.76	1.11	5.74	1.08	4.56	1.07
	L	6.38		6.21		4.88	
Val	D	6.78	1.52	6.41	1.41	4.91	1.39
	L	10.28		9.01		6.85	
Phe	D	14.54	0.60	14.33	0.62	11.45	0.59
	L	8.66		8.92		6.72	
Leu	D	14.97	1.32	13.57	1.25	10.50	1.21
	L	19.80		17.01		12.71	

[a]Flow rate was 1.5 ml/min. The mobile phase contained 25% acetonitrile and 75% 5×10^{-3}M Cu(II)-$(BzHyp)_2$ at pH 6.5.

acids. The optical resolution with N-benzyl-L-hydroxyproline copper (II) chelate was not better than that with N-benzyl-L-proline copper (II) chelate[15]. When the optical resolution with an addition of copper (II) proline was compared with that with an addition of copper (II) hydroxyproline, the selectivity of the enantiomeric pairs was increased. However, the copper (II)-BzHyp chelate showed better optical resolution than the copper (II)-BzPro system. As the pH of the eluent was changed from pH 6.0 to 7.0, the chiral selectivity and capacity ratios changed markedly as shown in Table 3. The capacity ratios decrease with increasing the pH of the mobile phase. This behavior was same as in the previous works[2,3,15]. The chiral selectivity of the dansyl amino acids increased with increasing the pH of the eluent. These results illustrate that the reactivity of the BzHyp ligand in the ligand exchange reaction increases more with pH than the dansyl amino acid does due to decrease in protonation.

Table 5. The Capacity Ratios(k') and Selectivity Factors(α) of Several Amino Acids according to the use of Different Ligands

DNS-AA		Hyp[a]		BzHyp	
		k'	α	k'	α
Ser	D	1.06	0.83	6.74	0.81
	L	1.27		8.26	
Thr	D	1.24	0.87	8.76	0.71
	L	1.43		11.06	
Val	D	3.18	0.99	59.61	0.68
	L	3.22		86.69	
Leu	D	5.58	0.97	84.63	0.71
	L	5.76		119.00	
Phe	D	6.73	0.99	112.21	1.85
	L	6.81		60.74	

[a]See Reference (2).

However, the selectivity of Ala, Tyr, and Met was not affected by the pH under these conditions. As previously found[2,3], the efficiency obtained with this metal chelate additive is quite good. We have also found that column stability at neutral pH can be improved with ammonium acetate buffers for the C_{18} column. Columns appear to be stable for five months with continual use. Table 4 showed the effect on the retention and selectivity of the optical isomers according to the concentration of the buffer. The buffer concentration does slightly affect the optical resolution as shown in Table 4. The retention and selectivity decreased with increasing the concentration of the buffer. It indicates that the acetate ion may take a role as a ligand as BzHyp does toward the binary complexes in the ligand exchange reaction. Retention behaviors of the dansyl amino acids with hydroxyproline was compared with benzylhydroxyproline and showed differently as shown in Table 5. Copper (II)-$(BzHyp)_2$ chelate increased the retention time and enhanced the chiral selectivity between D- and L-amino acids. Except phenylalanine, the elution order was the same as the system of copper (II)-$(Hyp)_2$. The N-benzylated hydroxyproline chelate made the optical resolution better than the hydroxyproline chelate did in this chiral additive method for RPLC.

The mechanism for the enantiomeric separation of dansylated amino acids have been reported in the previous paper[2,15]. This work also supports this mechanism to illustrate all the retention behaviors. The copper (II)-$(BzHyp)_2$ chelates in the mobile phase are believed to have trans con-

figuration due to a greater chiral selectivity. The enantiomeric dansyl amino acids, which have intramolecular hydrophobic interaction of the relatively long side chains with the dansyl group, can attack copper (II) chelate to form a ternary complex by SN2 reaction. L-Forms of DNS-amino acids are able to produce both cis and trans configuration due to the steric effect. Therefore, most of D-DNS-amino acids are less retained than L-DNS-amino acids except Phe, Tyr, and Asn. The dansylated phenylalanine and tyrosine whose ternary complexes seem to have a greater hydrophobic interaction in cis product showed the reverse elution order because the proline ring of the chelate has the same plane with the naphthyl ring of the dansylated amino acids. The dansylated asparagine containing the basic alkyl group also showed elution of L-form ahead of D-form because of the hydrophilicity of the side chain.

Acknowledgement. Financial support from the Korea Science and Engineering Foundation is gratefully acknowledged.

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Ionic Conductivity by A Complex Admittance Method

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The ionic conductivity of polycrystalline, glass, and glass-ceramic silicates was measured using two-terminal AC method with blocking electrode over a frequency range of 100 Hz to 100 KHz in the temperature range of 200 °C to 320 °C. Analysing the capacitance (C), susceptance (B), impedance(Z), and conductance (G) under the given conditions, an equivalent circuit containing temperature and frequency dependent component is proposed. Higher capacitance could be observed in the low frequency region and on the improved ionic migration conditions *i.e.*, at higher temperature in a better ionic conductor. Also the electrode polarization built up at the electrode-specimen interface could be sorted out above 10 KHz. However, grain boundary contribution couldn't be extracted from the bulk resistance over the frequency range measured here.

Introduction

The conductivity measurement by AC method in solid electrolytes has advantages since interfacial polarization between the electrode-electrolyte and the grain boundary effects can be sorted out at the proper frequencies. While the electrode polarization can be eliminated by using reversible electrode there are still other problems to find the proper electrode material when there is more than one type of mobile ion and to handle the electrode in molten state.

More complete model using AC measurement is the complex admittance method applied by Bauerle[1]. This method has been used by many scientists for various solid electrolytes[2]. The complex method is originally from Cole and Cole complex permittivity diagrams[3].

The complex admittance (Y) can be expressed as the sum of the coductance (G) and the susceptance (B).

$$Y = G + iB$$

From the plot of susceptance *vs.* conductance, the resistance