Enantiomeric Separation of Dansyl-Derivatized DL-Amino Acids by β-Cyclodextrin-Modified Micellar Electrokinetic Chromatography

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β-Cyclodextrin (CD)-modified micellar electrokinetic chromatography (MEKC) was applied to enantiomeric separation of Dns (dansyl-derivatized)-DL-amino acids with sodium dodecyl sulfate (SDS). Increase in the pH value and SDS concentration of the background electrolyte (BGE) buffer solution was found to prolong the migration time and improve the resolution (Rs) of Dns-DL-amino acids. Rs of each DL-amino acid was <1.0 when 100 mM SDS/75 mM β-cyclodextrin/250 mM borate (pH 8.3) was utilized as BGE buffer. However, as the pH value of the BGE buffer was increased to 9.5, all DL-amino acids, except for DL-serine and DL-alanine, were completely separated into two individual peaks. The separation was further enhanced when the SDS concentration was raised to 150 and 200 mM. Dns-D-amino acids were found to be eluted initially when compared to the corresponding Dns-L-amino acids, indicating the relatively stronger interaction between Dns-D-amino acids and β-CD.

Keywords: *Enantiomeric separation; Dns-DL-amino acid; β-cyclodextrin; micellar electrokinetic chromatography*

INTRODUCTION

Capillary electrophoresis (CE) is a newly developed separation technique that allows rapid separation with high resolution and high efficiency, requiring only small amounts of buffer and sample. Micellar electrokinetic chromatography (MEKC), based on the differential partitioning of analytes between an ionic micelle and the surrounding aqueous phase (Terabe et al., 1985, 1993), has proven to be effective in the separation of enantiomers (Ozaki et al., 1995). Enantiomeric separation of racemic mixtures of amino acids and drugs is important in the biochemical and pharmaceutical fields, since enantiomers of the same drug often show different pharmacological or bioactive effects. Chiral control of drugs, pharmaceutical studies and understanding of the drug metabolism are of great importance. Racemization, cross-linking reactions, degradation, and Maillard reactions occur during food processing, especially when protein-containing foods are treated with alkali (Masters and Friedman, 1979; Friedman and Masters, 1982; Liardon and Hurrel, 1983). Factors affecting racemization include pH, alkali treatment time and temperature, amino acid residues, and protein structures (Friedman and Liardon, 1985; Friedman et al., 1981). Racemization of free amino acids is insignificant compared to that of the amino acids in the proteins (Liardon and Ledermann, 1986), with the decreasing liability of racemization of the amino acid found to be as follows: aspartic acid, phenylalanine, glutamic acid, alanine, leucine, valine, and proline (Masters and Friedman, 1979).

The different types of chiral selectors employed for the CE separation of optical isomers have recently been reviewed (Fanali, 1995). Organic modifiers, such as methanol, acetonitrile, and 2-propanol, are frequently applied to modify the complex formation in the chiral separation (Cole et al., 1991; Katsuta et al., 1995). Wan et al. (1995) indicated that during the separation of 9-fluorenylmethyl chloroformate (FMOC)-amino acids, 2-propanol was indispensable and such method appeared to be complicated and inconvenient. However, with the most common derivatization of dansyl chloride, amino acids could be detected directly with UV at 254 nm (Nergo et al., 1987).

When β-cyclodextrin (CD) is used, the chiral resolution is based on selective complexation with the analytes. The most stable complex formed moves with a lower effective mobility. Recently, Terabe et al. (1993) used CDs for separating hydrophobic compounds, which is normally considered to be difficult by MEKC or other related electrophoretic techniques, and found CD to be very effective in the corresponding separation. In the present study, the enantiomers of amino acids, such as aspartic acid, phenylalanine, glutamic acid, alanine, leucine, and valine, which are liable to cause racemization during alkali treatment (Masters and Friedman, 1979), are dansylated and separated by MEKC in a model system, by using β-CD, to establish the experimental conditions for enantiomeric separation of amino acids. Some polar amino acids, such as serine, threonine, and methionine, and nonpolar amino acid, such as isoleucine, are compared with the separation results of the above amino acids.

MATERIALS AND METHODS

Chemicals. Dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride], boric acid, β-CD, and DL-amino acids were purchased from Sigma (St. Louis, MO), while sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Richmond, CA). Sodium hydrogen carbonate and sodium hydroxide were purchased from E. Merck (Darmstadt, Germany).

Apparatus and Electrophoretic Conditions. All experiments were carried out on a CE instrument P/ACE System

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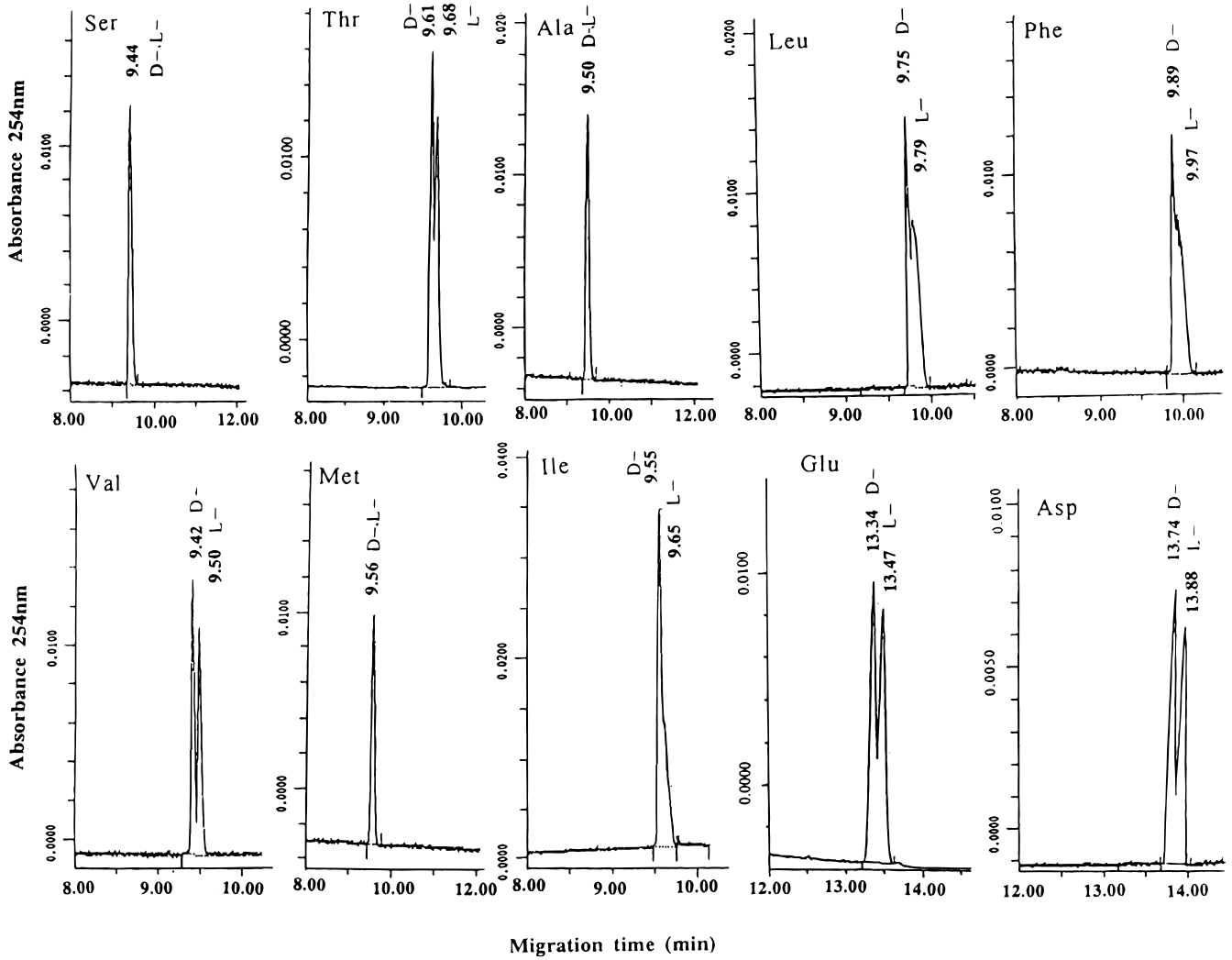

Figure 1. Enanitomeric separation of Dns-DL-amino acids by MECC with β-CD. Experimental conditions: capillary, 75 mm i.d. × 47 cm (40 cm to detector); separation solution, 100 mM SDS/75 mM β-CD/250 mM borate buffer (pH 8.3); applied voltage, 15 kV.

5500 (Beckman, Palo Alto, CA), equipped with a diode array detector with a detective wavelength of 254 nm. An uncoated fused silica capillary (Beckman, total length 47 cm, effective length 40 cm, i.d. 75 μm) was pretreated successively with 0.1 M hydrochloric acid and 0.1 M sodium hydroxide for 10 min each and then rinsed with deionized water and background electrolyte (BGE) solution prior to use. The column was kept at a constant temperature of 25.0 ± 0.1 °C by means of continuous circulation of the fluorocarbon liquid through the cartridge. The applied voltage was 15 kV, unless stated otherwise. Sample introduction was performed using the pressure option for 5 s. Data collection was carried out with the Gold chromatography data system version 8.1.

The BGE composition was 100, 150, or 200 mM SDS, 75 mM β-CD, and 250 mM borate buffer solution (pH 8.3 or 9.5, adjusted with 0.1 N NaOH solution). BGE was filtered through a 0.45 μm membrane prior to use. Deionized water was obtained from the Mili-Q system (Millipore, Japan).

Derivatization of Amino Acids. Dansyl chloride was used to derivatize the DL-amino acids according to the method described by Nergo et al. (1987). Usually, 100 μL of 500 mM $NaHCO_3$ in deionized water and 100 μL of 20 mM dansyl chloride in acetone were added to 10−40 μg of free amino acid dissolved in 100 μL of deionized water in a screw-capped Pyrex tube. The samples were reacted in the dark for 40 min at 65 °C. Dansyl chloride solution was always freshly prepared.

The pertinent parameters, resolution (Rs), separation efficiency ($N/m \times 10^5$), and theoretical plate height (H) were all calculated in the usual manner (Wan et al., 1995; Terabe et al., 1989). Rs value was used to express the separation results of the enantiomers (Wan et al., 1995). Δt value was calculated according to the method described by Gassmann et al. (1985) and Gozel et al. (1987).

RESULTS AND DISCUSSION

Effect of the pH Value of BGE on Separation of Dansyl-Derivatized (Dns) DL-Amino Acid. BGEs with alkaline pH values, i.e., higher than pI of any amino acid, were selected to conduct the separation in order to shift all of the amino acids to negative charge. Figure 1 illustrates the separation results of enantiomers using 100 mM SDS/75 mM β-CD/250 mM borate buffer (pH 8.3) as BGE, which shows that the electropherograms of enantiomers of Dns-DL-serine, Dns-DL-alanine, Dns-DL-methionine, and Dns-DL-isoleucine are completely overlapped, and the separation results for Dns-DL-leucine and Dns-DL-phenylalanine are also unsatisfactory; that is, only a slight separation can be observed on the tops of the corresponding peaks. Other enantiomers showed better separation results but still did not attain the level of separation at the baseline. Terabe et al. (1993) also indicated that the Rs values of most of Dns-DL-amino acids were <0.1 by using 100 mM SDS/60 mM β-CD/0.1 M borate buffer (pH 8.6) as BGE. In Table 1, the migration times, Rs values, and Δt

Table 1. Migration Time (t_D, t_L), Resolution (Rs), and Δt Value of Dns-DL-Amino Acid at pH 8.3[a]

Dns-DL-AA	migration time (min)			
	t_D[b]	t_L[b]	Rs[c]	Δt[d]
Ser	9.44	9.44	0.00	0.0000
Thr	9.61	9.68	0.67	0.0073
Ala	9.50	9.50	0.00	0.0000
Val	9.42	9.50	0.87	0.0085
Met	9.56	9.56	0.00	0.0000
Ile	9.55	9.55	0.00	0.0000
Leu	9.75	9.79	0.36	0.0041
Phe	9.89	9.97	0.56	0.0081
Glu	13.34	13.47	0.79	0.0097
Asp	13.74	13.88	0.71	0.0101

[a] Experimental conditions were the same as in Figure 1. [b] t_D and t_L were the migration times of Dns-D- and Dns-L-enantiomers, respectively. [c] $Rs = 2(t_L - t_D)/W_L - W_D$, where W_L and W_D are band width of Dns-D- and Dns-L-enantiomers at the baseline, respectively. [d] $\Delta t = 2(t_L - t_D)/(t_L + t_D)$.

values for the 10 DL-amino acids are presented. The difference in migration times between the DL-enantiomers of neutral amino acids or of aliphatic amino acids can be seen to be very narrow, and all of the Rs values that are used to express the separation results of the enantiomers in capillary electrophoretograms (Wan et al., 1995) were found to be <1.0. Therefore, it was necessary to modify the electrophoretic conditions.

By increasing the pH value from 8.3 to 9.5, the enantiomeric separations of Dns-DL-serine and Dns-DL-alanine could be only slightly improved, but Dns-DL-valine, Dns-DL-leucine, Dns-DL-phenylalanine, Dns-DL-glutamic acid, and Dns-DL-aspartic acid were well separated, displaying two completely independent peaks (Figure 2). In Table 2, the migration times and Rs values of the Dns-DL-amino acids, analyzed at pH 9.5, are presented, which are obviously higher than those in Table 1. In general, increase in resolution with increasing pH was the result of the corresponding increase in the migration time window in the micellar system under investigation. Increase in the pH of BGE results in increasing electrophoretic mobility of the micelle in the direction opposite to that of the electroosmotic flow (EOF), which in turn leads to increase in the migration time. The enantiomers with higher affinity to the SDS micelles migrate more slowly than the ones having less affinity to the micelles, and consequently the migration order of the enantiomers is inverted. A further advantage of using a high pH was the resultant dynamic deactivation of the capillary walls, which are known to serve to decrease the adsorption of solutes on the capillary surface.

Gassmann et al. (1985) and Gozel et al. (1987) have indicated that when Δt value exceeded 0.01, the DL-enantiomers would meet the resolution requirement at the baseline. The Δt values for Dns-DL-serine, Dns-DL-threonine, Dns-DL-alanine, and Dns-DL-methionine are <0.01, whereas the other Dns-DL-amino acids exceeded the minimum resolution requirement (Table 2), implying that the latter Dns-DL-amino acids were completely separated at the baseline. The calculated Δt values were consistent with the separation results of the enantiomers, as plotted in Figures 1 and 2. Thus, Δt values were found to be effective in evaluating the separation result of the enantiomers in capillary electrophoresis.

Effect of SDS Concentration in the BGE. SDS concentration in the BGE was increased to 150 mM (Figure 3), and the separations of Dns-DL-serine, Dns-DL-threonine, Dns-DL-alanine, and Dns-DL-methionine were obviously improved but not yet satisfactory, except only for Dns-DL-threonine, as compared with the results in Figure 2. Moreover, the enantiomers exhibited better Rs and Δt values (Table 3). The electrophoretic migration time for the Dns-DL-amino acids in Table 3 increased in the following order: serine< threonine < alanine < valine < methionine < isoleucine < leucine < phenylalanine < glutamic acid < aspartic acid. Thus, the migration sequence for Dns-DL-amino acids is directly correlated with the increase in hydrophobicity of the amino acid side chain. The more hydrophobic species tend to be strongly associated with the micelles, which migrate toward the cathode during electrophoretic operation as a result of the much stronger EOF, and thus the migration time depends on the magnitude of the interaction between SDS micelles and the amino acid side chains. Dns-DL-serine and Dns-DL-threonine possess at least one polar substituent group, which makes them hydrophilic and would tend to be dissolved by the aqueous phase, thus resulting in shorter migration times than those of the other amino acids.

The migration times of Dns-DL-glutamic acid and Dns-DL-aspartic acid were observed to be much longer than those of the other Dns-DL-amino acids (Tables 2 and 3), owing to the extra carboxyl groups which completely ionized in the BGE and were attracted toward the anode. Thus, the migration velocity of the acidic amino acids toward the cathode was affected.

Ong et al. (1991) have reported that the migration sequence of amino acid is closely related to the partition coefficient of amino acid to micelles. The migration times for the amino acids derivatized with Marfey's reagent and (+)- or (−)-1-(9-fluorenyl)ethyl chloroformate have also been found to increase with the increasing hydrophobicity of the amino acid side chains (Tran and Blanc, 1990; Wan et al., 1995). Similar migration sequence was also observed when Terabe et al. (1989) used sodium taurocholate to conduct the enantiomeric separation of Dns-DL-amino acids at pH 7.0.

The effect of SDS concentration on migration times and Rs values is found to be significant. In CD-MEKC, the retention time of a solute, tR, is determined by its apparent capacity factor, k'_{app}, which is described (Terabe et al., 1993) as

$$k'_{app} = n_{mc}/(n_{non-mc}) = n_{mc}/(n_{CD} + n_{aq})$$

where n_{mc} and n_{non-mc} are the numbers of moles of the solute incorporated into the micelle and in the nonmicellar phase, respectively. n_{non-mc} is equal to $n_{CD} + n_{aq}$, where n_{CD} and n_{aq} are the numbers of moles of the solute included by β-CD and in the aqueous phase, respectively. Thus, the increase in the SDS concentration which prolonged the migration time is due to the increasing amounts of solute incorporated into the SDS micelles.

Increase in the SDS concentration also enhanced the dynamic deactivation effect by reducing the interaction between the solutes and inner surface of the tubing, thus resulting in the increase in Rs value. The Rs values for all of the amino acid enantiomers were improved and >1.0 (Table 3), except those of DL-serine, DL-alanine, and DL-methionine. The Dns-D-amino acids were found to be eluted initially, as compared to the corresponding Dns-L-amino acids, indicating a relatively stronger interaction between Dns-D-amino acids and

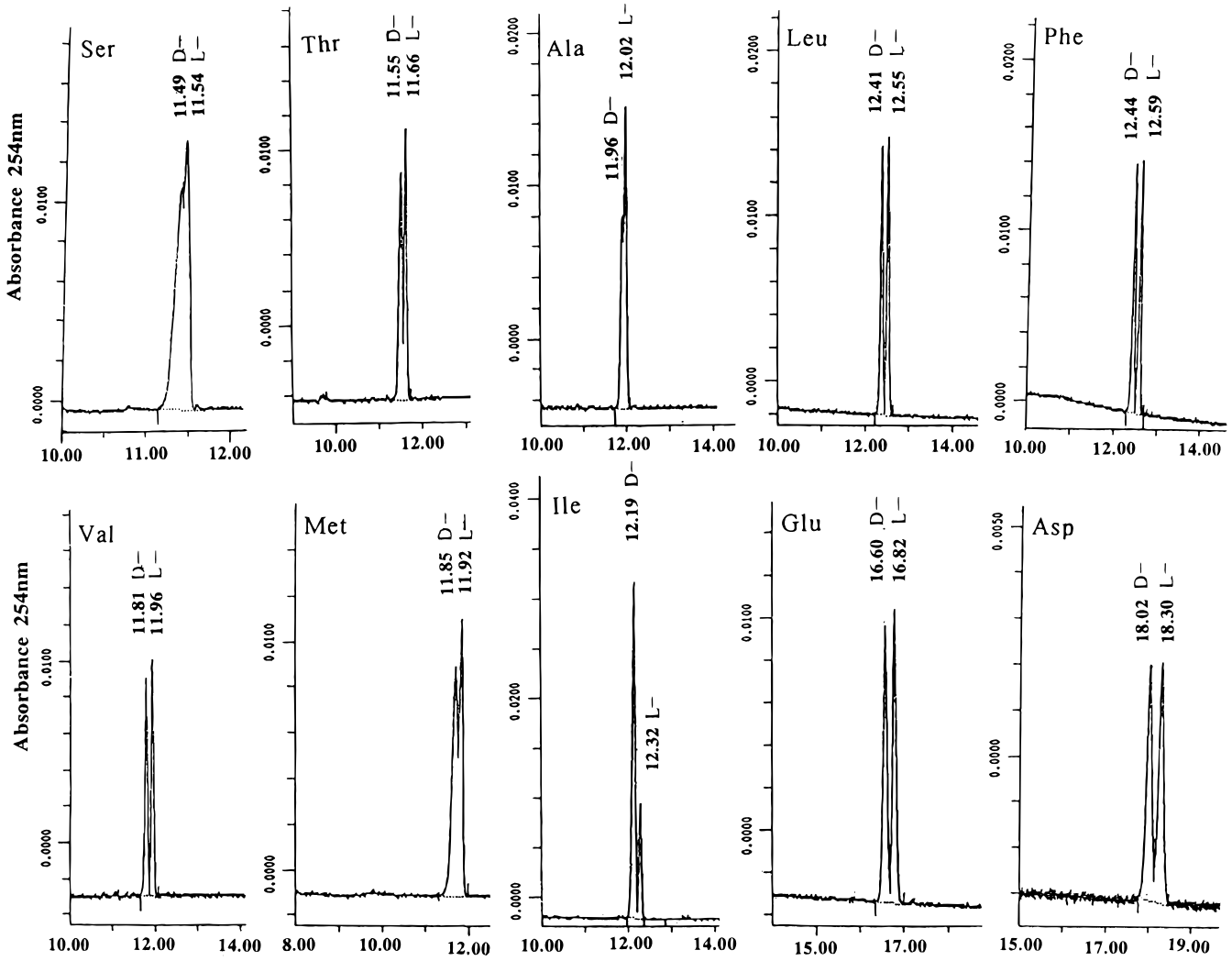

Figure 2. Enantiomeric separation of Dns-DL-amino acids by MECC with β-CD. Experimental conditions were the same as in Figure 1, except the pH of the separation solution was adjusted to 9.5.

Table 2. Migration Time (t_D, t_L), Resolution (Rs), and Δt Value of Dns-DL-Amino Acid at pH 9.5[a]

	migration time (min)			
Dns-DL-AA	t_D[b]	t_L[b]	Rs[c]	Δt[d]
Ser	11.49	11.54	0.30	0.0043
Thr	11.55	11.66	0.78	0.0095
Ala	11.96	12.02	0.71	0.0050
Val	11.81	11.96	1.03	0.0126
Met	11.85	11.92	0.63	0.0059
Ile	12.19	12.32	0.80	0.0106
Leu	12.41	12.55	0.95	0.0112
Phe	12.44	12.59	1.00	0.0120
Glu	16.60	16.82	0.94	0.0132
Asp	18.02	18.30	1.00	0.0154

[a] Experimental conditions were the same as in Figure 1, excpet the pH was raised to 9.5. [b–d] Correspond to those in Table 1.

β-CD (Tables 2 and 3). Similar results have also been reported by Yoshinaga and Tanaka (1995) and Ueda et al. (1992).

The effects of SDS concentration on the separation efficiency and theoretical plate height were also studied (Table 4). The presence of 150 mM SDS in the BGE apparently reduced the separation efficiency ($N/m \times 10^5$) and increased the theoretical plate height (H) of most of the Dns-DL-enantiomers. Sepaniak and Cole (1987) have reported that the separation efficiency increased in the low micellar concentration between 1 and 20 mM, owing to the increased mass transfer of the mobile phase resulting from the reduced distance between the micelles. On the other hand, as the SDS concentration increased to 50 mM, the separation efficiency was reduced because of the increase in thermal-related dispersion effects as well as the decreasing EOF. Similar results can also be noted from Table 4, as well as the study by Foley (1990).

Figure 4 presents the electropherogram of the racemic mixtures of seven Dns-DL-amino acids separated with 200 mM SDS/75 mM β-CD/250 mM borate buffer (pH 9.5). As may be seen, all of the Dan-DL-amino acids were completely separated and displayed individual sharp peaks. However, the separation of the enantiomer mixture of serine, threonine, and alanine failed and only four peaks were observed (data not shown). The migration sequence for the Dns-DL-amino acids was found to be different from those shown in the previous figures (Figures 2 and 3). The migration times of the Dns-DL-glutamic acid and Dns-DL-aspartic acid were shorter than those of the Dns-DL-isoleucine, Dns-DL-

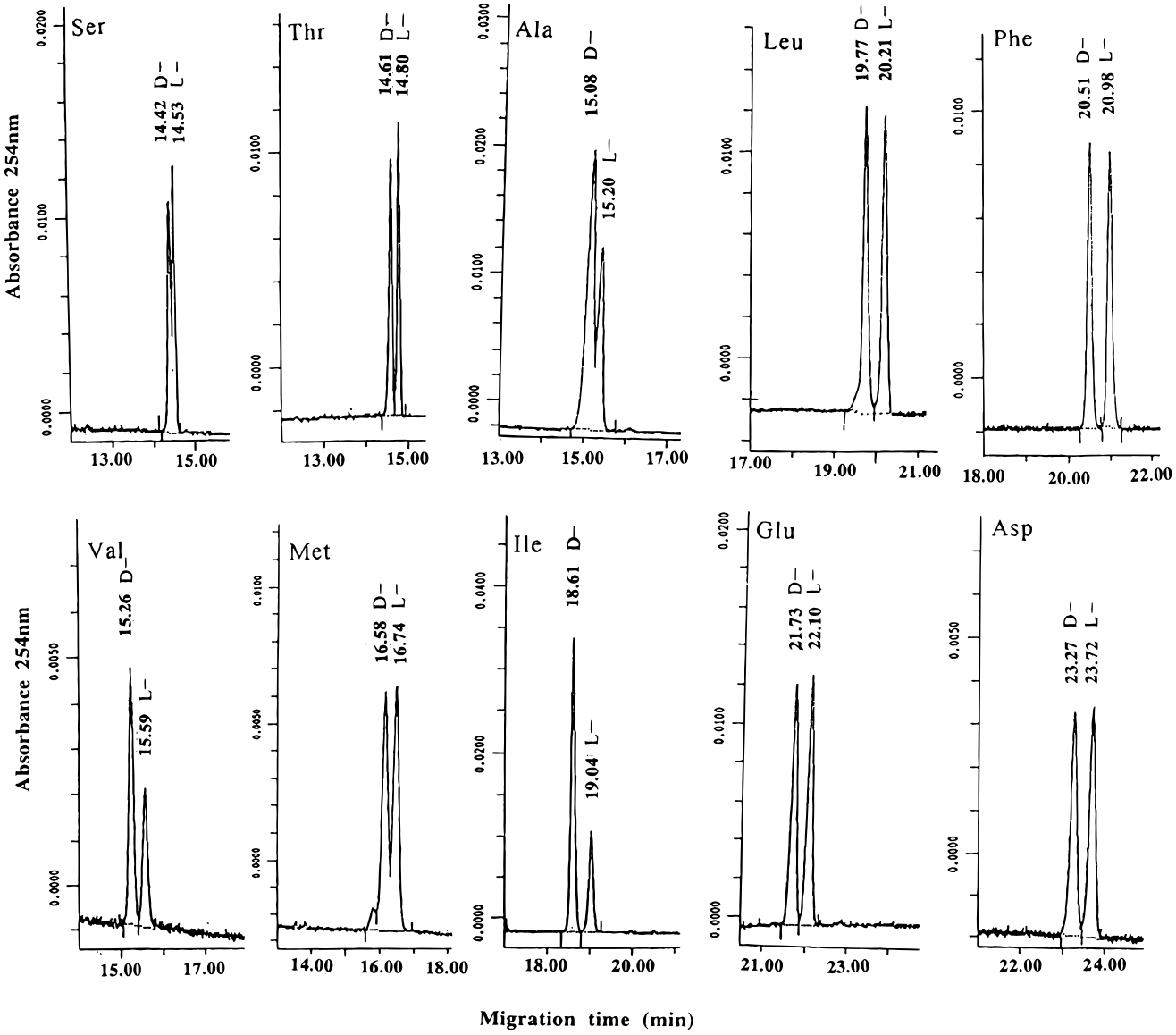

Figure 3. Enantiomeric separation of Dns-DL-amino acids by MECC with β-CD. Experimental conditions were the same as in Figure 1, except the SDS concentration was raised to 150 mM.

Table 3. Migration Time (t_D, t_L), Resolution (Rs), and Δt Value of Dns-DL-Amino Acid[a]

Dns-DL-AA	migration time (min)			
	t_D[b]	t_L[b]	Rs[c]	Δt[d]
Ser	14.42	14.53	0.67	0.0076
Thr	14.61	14.80	1.09	0.0129
Ala	15.08	15.20	0.68	0.0079
Val	15.26	15.59	1.27	0.0214
Met	16.58	16.74	0.82	0.0096
Ile	18.61	19.04	1.67	0.0228
Leu	19.77	20.21	1.43	0.0220
Phe	20.51	20.98	1.50	0.0227
Glu	21.73	22.10	1.04	0.0169
Asp	23.27	23.72	1.16	0.0192

[a] Experimental conditions were the same as in Figure 1, except the separation solution was 150 mM SDS/75 mM β-CD/250 mM borate buffer (pH 8.3) [b–d] Correspond to those in Table 1.

Table 4. Effect of SDS Concentration on Separation Efficiency ($N/m \times 10^5$) and Plate Height (H) of Dns-DL-Amino Acid

	100 mM SDS[a]				150 mM SDS[b]			
	N/m ($\times 10^5$)		H (μm)[d]		N/m ($\times 10^5$)[c]		H (μm)[d]	
Dns-DL-AA	D	L	D	L	D	L	D	L
Ser					2.662	4.934	3.756	2.026
Thr	2.815	4.458	1.421	0.897	4.192	5.393	3.552	2.243
Ala	2.993	3.466	3.410	2.886	3.089	5.262	3.236	1.900
Val	3.404	5.519	2.937	1.812	3.201	4.468	3.124	2.238
Met	4.335	5.292	2.307	1.889	3.733	3.806	2.679	2.628
Ile	2.985	3.942	3.350	2.536	3.893	3.266	2.569	3.062
Leu	5.920	6.268	1.689	1.596	3.059	2.654	3.268	3.766
Phe	5.761	5.528	1.736	1.809	3.728	3.781	2.683	2.646
Glu	3.398	4.252	2.943	2.352	2.794	3.403	3.404	2.939
Asp	2.294	2.583	4.359	3.872	0.839	0.945	4.766	4.232

[a] and [b] are the SDS concentrations in Tables 2 and 3, respectively. [c] $N/m \times 10^5$, where $N = 5.54(t/W_{1/2})^2$; t = migration time (min); $W_{1/2}$ = peak width at half-height of peak; m = capillary length. [d] $H = (L_d/N)$, where L_d is the capillary effective length (m).

leucine, and Dns-DL-phenylalanine, probably owing to the stronger repulsive effect between the negatively charged SDS micelles and the glutamic acid or aspartic acid at higher SDS concentration. However, Rs values for the Dns-DL-amino acid enantiomers were remarkably improved (for example, Rs values of methionine and threonine are 1.05 and 1.11, respectively) by increasing the SDS concentration to 200 mM (Table 5), but enantiomeric separation of Dns-DL-serine and Dns-DL-alanine

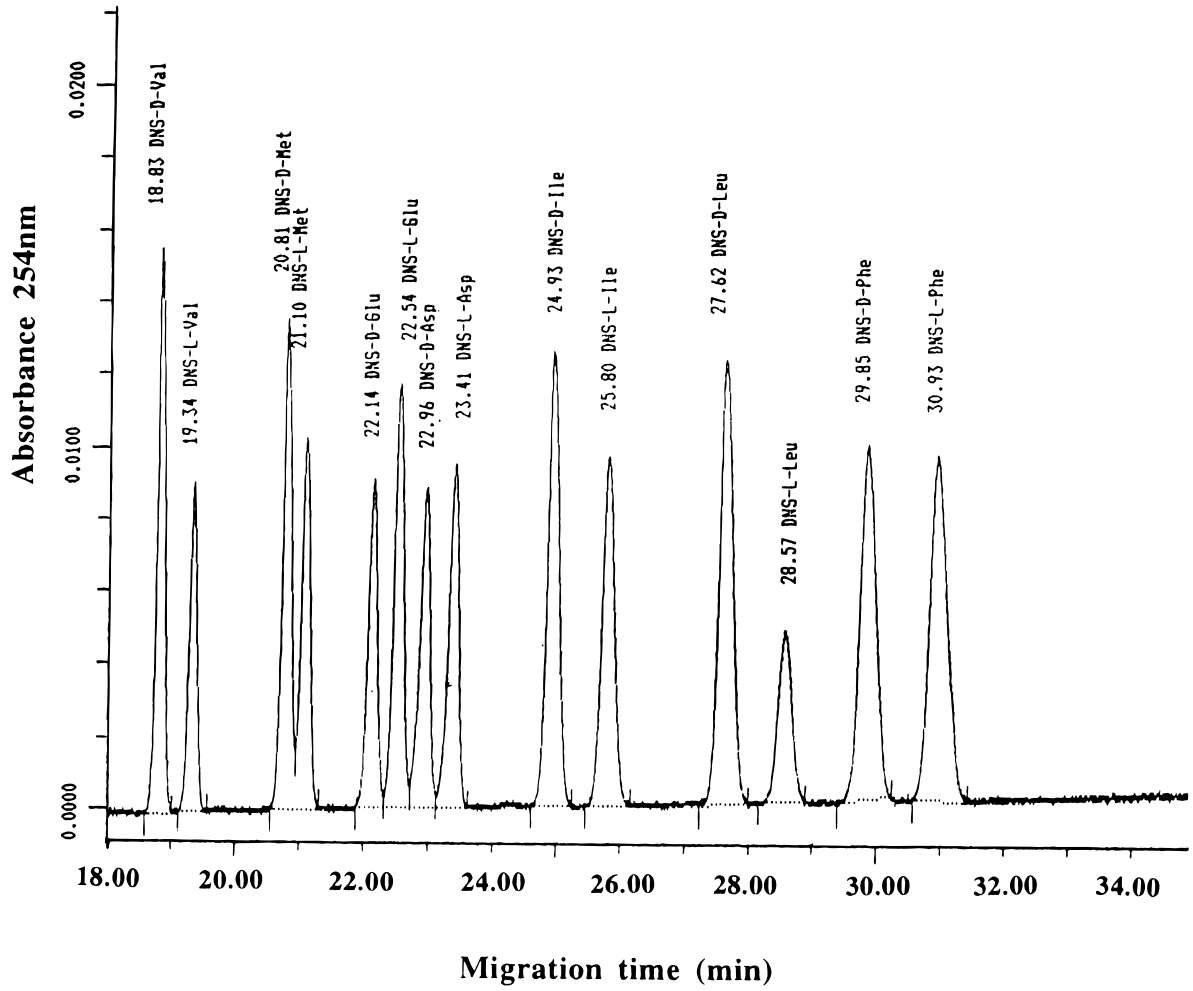

Figure 4. Enantiomeric separation of Dns-DL-amino acids by MECC with β-CD. Experimental conditions were the same as in Figure 1, except the separation solution was 200 mM SDS/75 mM β-CD/250 mM borate buffer (pH 9.5).

Table 5. Resolution (Rs) and Separation Efficiency ($N/m \times 10^5$) of Dns-DL-Amino Acid[a]

		N/m ($\times 10^5$)	
Dns-DL-AA	Rs	D	L
Ser	0.70	3.068	4.708
Thr	1.11	4.028	4.760
Ala	0.85	2.215	2.745
Val	1.45	1.590	1.559
Met	1.05	2.346	2.276
Glu	1.15	2.267	2.353
Asp	1.33	1.861	2.014
Ile	2.36	1.753	1.685
Leu	1.91	1.558	1.671
Phe	2.26	1.684	1.452

[a] Separation solution was 200 mM SDS/75 mM β-CD/250 mM borate buffer (pH 9.5). Rs and separation efficiency correspond to those in Tables 1 and 4, respectively.

still appeared to be unsatisfactory (Rs < 1.0). Unsuccessful separations of Dns-DL-methionine and Dns-DL-threonine were conducted by using 100 mM SDS/60 mM β-CD/0.1 M borate buffer (pH 8.6) as BGE, but those enantiomers were separated well through the use of γ-CD or methanol (Terabe et al., 1993). Yoshinaga and Tanaka (1995) separated Dns-DL-amino acids by adding 7 M urea to BGE (pH 9.0), which was considered to affect the complex formation of the enantiomers with β-CD and improve some of the Rs values of Dns-DL-amino acids including DL-serine.

Conclusion. β-CD was utilized as chiral selector to conduct the enantiomeric separation of amino acids which were liable to cause racemization during alkali treatment. The migration times, Rs values, and Δt values were apparently influenced by the pH value of the BGE and the SDS concentration. When 100 mM SDS/75 mM β-CD/250 mM borate buffer (pH 8.3) was used as BGE, the enantiomeric separation results were less than satisfactory (Rs < 1.0). By increasing the pH value to 9.5 and the SDS concentration to 200 mM, the separation results were remarkably improved and the Rs values and Δt values of all of the Dns-DL-amino acids, except serine and alanine, were higher than 1.0 and 0.01, respectively. Migration times and migration sequence of the Dns-DL-amino acids were also apparently affected by the pH value and SDS concentration of the BGE during the electrophoretic operation.

The separation of the enantiomer mixture of serine, threonine, and alanine was unsuccessful and further trial is needed. Complete separation of the enantiomers of all 20 amino acids in protein foods appeared to be impossible to be conducted under only one experimental condition. In the present study, amino acids, except alanine, which are liable to cause racemization during alkali treatment were separated well (Figure 4) and the optimized 200 mM SDS/75 mM β-CD/250 mM borate (pH 9.5) system appeared to be available for the quantitative determination of amino acid racemization

in alkali-treated protein foods. Further studies are necessary to establish the experimental conditions for analyzing the racemic mixtures of basic DL-amino acids and specific amino acids such as lysinoalanine and lanthionine, since complicated reactions between proteins occur in the alkali-treated foods.

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