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Chiral separation of amino acids derivatized with fluoresceine-5-isothiocyanate by capillary electrophoresis and laser-induced fluorescence detection using mixed selectors of β-cyclodextrin and sodium taurocholate

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Abstract

Chiral separation of 20 pairs of amino acids derivatized with fluoresceine-5-isothiocyanate (FITC) by capillary electrophoresis and laser-induced fluorescence detection was studied using the mixture of β-cyclodextrin (β-CD) and sodium taurocholate (STC) as selector. Resolution was considerably superior to that obtained by using either β-CD or STC alone. The molar ratio of β-CD to STC of about 2:3 was found to be critical to achieve maximum separation. At this β-CD-to-STC ratio, chiral separation occurred at really low total concentration of β-CD and STC (<0.1 m*M*). Other impacting factors were investigated including the total concentration of β-CD and STC, pH, and capillary conditioning procedure between two successive runs. Using a running buffer of 80 m*M* borate containing 20 m*M* β-CD and 30 m*M* STC at pH 9.3, all of the 20 pairs of FITC-amino acid enantiomers were baseline resolved. The resolutions of the most pairs of the amino acid enantiomers (17 of 20) were higher than 3.0, only three pairs gave a resolution lower than 3.0 but higher than 1.90 (β-phenylserine, pSer). The highest resolution reached 14.58 (Glu). Two derivatives of β-CD, 2-hydroxypropyl-β-CD (HP-β-CD) and heptakis(2,6-di-*O*-methyl)-β-CD (DM-β-CD) were also explored. HP-β-CD showed similar cooperative effect with STC, while DM-β-CD together with STC led to poorer chiral separation.

Keywords: Enantiomer separation; Derivitisation, electrophoresis; Amino acids; β-Cyclodextrin; Sodium taurocholate

1. Introduction

Chiral separation of amino acids is of great importance in biology, pharmaceutics and agriculture. This has been performed traditionally with Chromatographic methods such as gas chromatography (GC) [1] and high-performance liquid chromatography (HPLC) [2]. In recent years, chiral capillary electrophoresis (CE) [3] has grown rapidly and has been proven to be a useful tool for the resolution of amino acid enantiomers. Chiral CE offers significant advantages over GC or HPLC in terms of higher separation efficiency and lower sample and reagent consumption.

In general, the derivatization of amino acids is performed prior to CE separation in order to achieve

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good detection sensitivity of UV absorbance or fluorescence. A variety of derivatization reagents have been explored for the enantioseparation of amino acids such as 2-(9-anthryl)ethyl chloroformate (AEOC) [4], cyanine (Cy5) [5], 5-dimethylamino-naphthalene-1-sulphonyl chloride (Dns) [6–9], 2,4-dinitrophenyl fluoride (DNP) [10], 9-fluorenylmethyl chloroformate (FMOC) [11,12] and *o*-phthaladehyde (OPA) [13,14] (a recent specific review on chiral separation of amino acids and their derivatives is also listed in Ref. [15]). Among these derivatization reagents, fluoresceine-5-isothiocyanate (FITC) is an excellent one for the good electrophoretic property and very low detection limit of its derivatives achieved when laser-induced fluorescence detection (LIF) is used [16]. The applications of CE–LIF to chiral separation of FITC-amino acids are found in a few instances [17–21]. Nouadje et al. studied the racemization of L-Ser labeled with FITC by micellar electrokinetic chromatography (MEKC) using β-cyclodextrin (β-CD) as selector and sodium dodecyl sulfate (SDS) as surfactant [17]. Similarly, Liu et al. employed the technique of γ-CD-MEKC to analyze FITC-derivatized Ser and Val enantiomers [18]. More recently, Jin et al. have systematically investigated the chiral separation of 21 FITC-amino acids by MEKC using β-CD or γ-CD as a selector [19]. They found that γ-CD was superior to β-CD for the chiral resolution of FITC-amino acids. Baseline resolutions of 20 amino acids were achieved when the buffer contained 10 m*M* γ-CD, 30 m*M* SDS and 100 m*M* borate at pH 9.5. When γ-CD was replaced with β-CD, however, most of the tested amino acids were poorly resolved. This is clearly an excellent work because it demonstrates that many amino acids with a broad range of physical and chemical properties can be optically resolved using a single selector. Rodriguez et al. and Hutt et al. also demonstrated the possibility of chiral separations of FITC-amino acids by γ-CD-mediated MEKC on a chip platform [20,21]. As expected, the analysis time was dramatically reduced. But the resolutions were also reduced greatly due to the much shorter separation channel. To suit chip technology, a further improvement of the chiral resolution is required.

It has been well recognized that the use of the binary selectors of CDs such as the mixture of a neutral CD and an ionic CD can enhance the selectivity and resolution in chiral CE [22,23]. The combination of a CD and chiral surfactant like a bile salt is also an effective way [24]. This was clearly illustrated in the work of Okafo et al. in which the chiral separation of Dns-amino acids was greatly improved when the mixture of β-CD and taurodeoxycholic acid was used instead of either β-CD alone or taurodeoxycholic acid alone [25]. The application of the mixed selectors of a CD and bile acid surfactant has been also extended to the chiral separation of the amino acid derivatives of naphthalene-2,3-dicarboxaldehyde [26] and OPA [14], and some drugs [24].

In the present work, we attempted to improve the chiral resolution of 20 pairs of FITC-amino acids by using binary selectors or mixed selectors. The combination of β-CD and sodium taurocholate (STC, a natural chiral surfactant) has been tried and found effective. Two β-CD derivatives, namely 2-hydroxypropyl-β-CD (HP-β-CD) and heptakis(2,6-di-*O*-methyl)-β-CD (DM-β-CD), have also been investigated and compared with β-CD. Impacting factors such as molar ratio of β-CD to STC, total concentration of β-CD and STC and pH were studied and optimized.

2. Experimental

2.1. Chemicals

D,L-β-phenylserine (pSer) was of biochemical reagent grade from the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). Other D- and L-amino acids of TLC grade were purchased from Sigma Co. (St. Louis, MO, USA). Sodium taurocholate (STC, TLC grade) and fluoresceine-5-isothiocyanate (FITC, HPLC grade) were also obtained from Sigma. β-Cyclodextrin (β-CD), 2-hydroxypropyl-β-CD (HP-β-CD) and heptakis (2,6-di-*O*-methyl)-β-CD (DM-β-CD) were all of TLC grade from Aldrich (Milwaukee, WI, USA). Other reagents were all of analytical reagent grade obtained from Beijing Chemical Work (Beijing, China). Running buffer was composed of 80 m*M* borate and selectors. All solutions were prepared in double-distilled water.

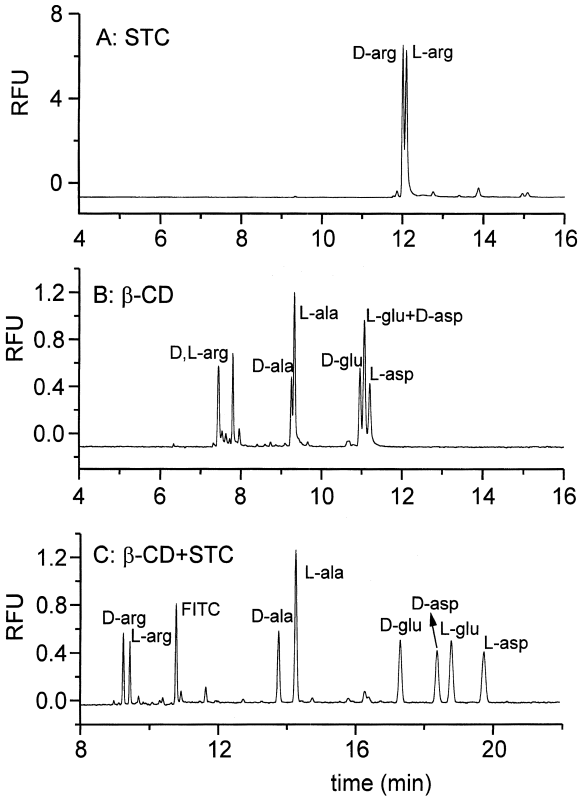

Fig. 1. Chiral separation of FITC-amino acids in the presence of (A) sodium taurocholate alone (STC 30 m*M*); (B) β-CD alone (15 m*M*) and (C) STC (12 m*M*) and β-CD (8 m*M*). Buffer, 80 m*M* borate at pH 9.3; injection, 0.5 p.s.i.×1 s; separation voltage, 20 kV; capillary, 57 cm×50 μm (I.D.); capillary temperature, 20 °C.

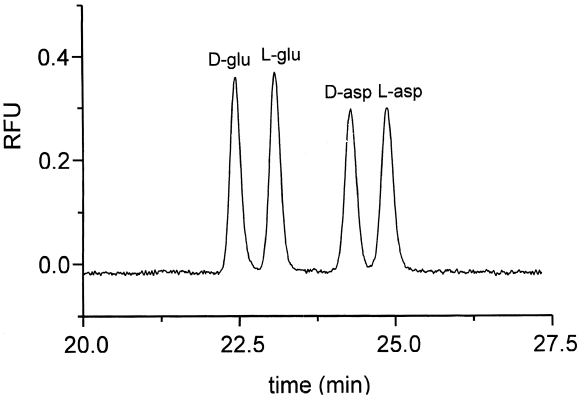

Fig. 2. Chiral separation of Asp and Glu at low concentration of β-CD and STC. Buffer, 0.10 m*M* β-CD and 0.15 m*M* STC in 80 m*M* borate at pH 9.3; other conditions are the same as in Fig. 1.

2.2. Instrumentation

The CE separations were performed with a P/ACE 5500 system equipped with an argon ion laser and a laser-induced fluorescence detector (Beckman Instruments, Fullerton, CA, USA). The laser-induced fluorescence detection was carried out with excitation at 488 nm and emission at 520 nm. The excitation power at 488 nm was set at 5 mW. An uncoated fused-silica capillary of 50 μm I.D and 57 cm (effective length 50 cm) was used throughout (Yongnian Optic Fiber Work, Heibei, China). The capillary temperature was maintained at 20 °C and the room temperature at 20±2 °C by the cooling system of the CE instrument and an air-condition, respectively. The capillary was conditioned before each analysis by flushing successively with 0.1 *M* NaOH, H_2O, and buffer each for 2 min, until noted otherwise. Samples were injected with pressure at 0.5 p.s.i. for 1 s and separated at 20 kV (1 p.s.i.≡ 6894.76 Pa). Data were acquired and processed through the P/ACE station version 1.2.

2.3. Sample preparation

Amino acids were individually dissolved in 80 m*M* borate at pH 9.3 to a concentration level of 10^{-3} *M*. The solution of FITC was prepared by dissolving 3 mg FITC in 25 ml acetone (ca. 3.0×10^{-4} *M*) and stored at −20 °C. Derivatization of individual amino acid was carried out by mixing 100 μl amino acid solution with 100 μl FITC solution and keeping the mixture in darkness for overnight. Before analysis, each of the amino acids derivatized with FITC were diluted by 100–1000 times with running buffer and the mixed samples were prepared by mixing an identical volume of individual FITC-amino acids.

3. Results and discussion

3.1. Cooperative effect of β-CD and STC

Chiral separation of FITC-amino acids using either β-CD or STC alone was not satisfactory. In a buffer of 30 m*M* STC and 80 m*M* borate at pH 9.3, only Arg of the 20 pairs of amino acids was resolved (Fig. 1A), and three pairs of amino acids were resolved

(Fig. 1B) when the STC was replaced by 15 m*M* β-CD. But when β-CD and STC were simultaneously present in the same buffer, good chiral separations were achieved (Fig. 1C). This shows clearly that β-CD and STC cooperate in chiral separation of FITC-amino acids. Further study showed that such a cooperative effect also took place at really low concentration of β-CD and STC. Fig. 2 shows that baseline chiral separation of Asp and Glu occurs even at 0.1 m*M* β-CD and 0.15 m*M* STC. It should be noted that no chiral separation of Asp or Glu was observed when either 0.1 m*M* β-CD alone or 0.15 m*M* STC alone was present in the buffer. At such a low concentration, STC molecules exit predominantly as monomers for its critical micellar concentration is much higher (10–15 m*M* at 25 °C [27]). Thus, not only a mixture of β-CD and STC monomers but also a mixture of β-CD and STC micelles shows a cooperative effect on the chiral resolution of FITC-amino acids.

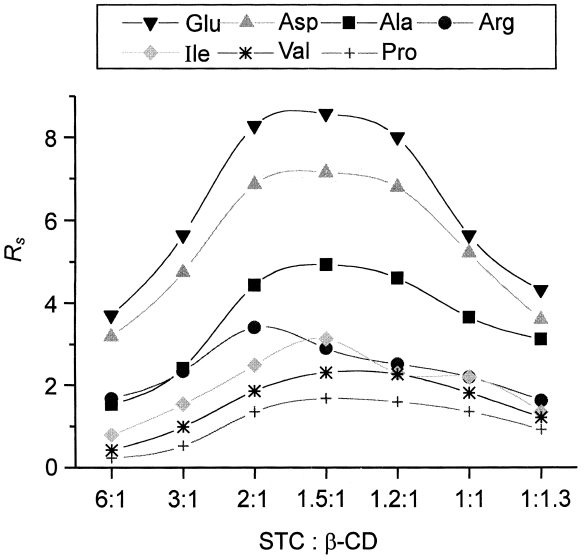

Fig. 3. Influence of molar ratio of β-CD to STC on chiral resolution (R_s). Buffer, 80 m*M* borate with 20 m*M* β-CD and STC at pH 9.3; other conditions as in Fig. 1.

3.2. Optimization of the separation

The separation conditions with the binary selectors of β-CD and STC were then optimized including the molar ratio of β-CD to STC, their total concentration, buffer pH and capillary rinse procedure.

3.2.1. Molar ratio of STC to β-CD

The influence of the STC-to-β-CD ratio was studied by the addition of different amounts of STC to a running buffer containing 20 m*M* β-CD. Fig. 3 shows that optimum chiral separations occur at an STC-to-β-CD ratio of 1.5:1 (or 3:2) for most of the tested amino acids except Arg at 2:1. The exception of Arg may result from its basic residue. Generally, better separations of the tested amino acids can be obtained as the ratio of STC to β-CD is between 1.2:1 and 2:1.

3.2.2. Total concentration of β-CD and STC

As mentioned above, low concentration of β-CD and STC is applicable, but to further increase the resolution, higher concentrations of the selectors were investigated. As expected, chiral resolutions of the tested amino acids increased rapidly with the total concentration of β-CD and STC up to 5 m*M*. The increase then slowed down. Above 20 m*M*, a plateau profile of resolution against concentration was observed (Fig. 4). It is easy to understand the increase effect but not the plateau phenomenon, which is interesting. In the case of the use of a single selector, an optimum concentration of selector is predicated and usually observed [28]. In this work,

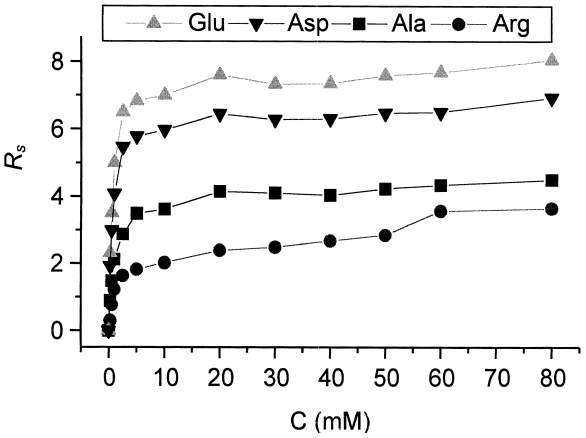

Fig. 4. Influence of total concentration of β-CD and STC on chiral resolution (R_s). Buffer, 80 m*M* borate with β-CD and STC (β-CD/STC=2:3) at pH 9.3; other conditions are the same as in Fig. 1.

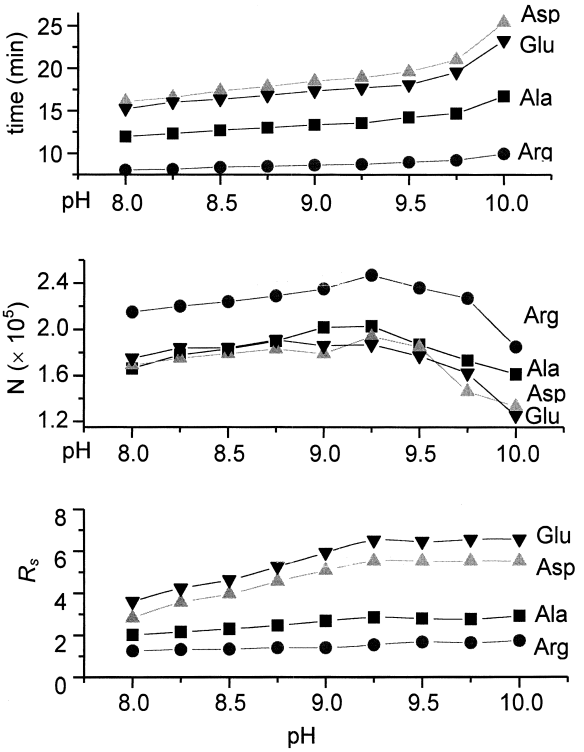

Fig. 5. Influence of pH on chiral separation. Buffer, 1.0 m*M* β-CD and 1.5 m*M* STC in 80 m*M* borate; other conditions as in Fig. 1.

the plateau phenomenon may be explained as follows: the FITC-amino acids distribute between the aqueous phase and the two pseudo-phases of β-CD and STC micelles in the buffer solution. As the concentrations of β-CD and STC increase, more FITC-amino acids would bind to β-CD and STC micelles, leaving less free FITC-amino acids in the aqueous phase. It is thus possible that, above a certain total concentration of β-CD and STC (about 20 m*M* in this work), the FITC-amino acids partition predominantly between β-CD and STC micelles and the left free FITC-amino acids in aqueous phase is negligible. In this case, the resolution of a pair of FITC-amino acid enantiomers is only dependent on the different partition ratios of the two enantiomers between β-CD and STC and is independent on the total concentration of β-CD and STC. Sequentially, at a fixed ratio of β-CD to STC, a plateau phenomenon is to be observed.

3.2.3. Buffer pH

The influence of buffer pH was investigated from 8.0 to 10.0 at an increment of 0.25 because FITC-amino acids emit fluorescence strongly only under alkali conditions. Fig. 5 shows that the buffer pH has a complex influence on chiral separation. The migration time rises with pH seemingly due to the more negative charges produced at solute from the FITC moiety. Both the chiral resolution and separation efficiency increase with pH up to 9.25. Above this pH, the chiral resolution maintains while the plate numbers decrease rapidly. pH 9.25 was thus considered to be the best.

3.2.4. Capillary rinse

A rinse with 1% HNO_3 for 3 min instead of 0.1 *M* NaOH for 2 min between two successive runs could improve the resolution to some extent at the expense of longer migration time (Table 1). This is clearly due to the suppression of the dissociation of the silanol group by H^+. Sequentially, the electroosmotic flow is reduced and the migration time increases.

The optimized conditions thus include the use of

Table 1
Influence of the capillary rinse procedure used between two successive runs

Rinse	Arg			Ala			Glu			Asp		
	t (min)	$N(\times 10^5)$	R_s	t (min)	$N(\times 10^5)$	R_s	t (min)	$N(\times 10^5)$	R_s	t (min)	$N(\times 10^5)$	R_s
A	10.01	2.32	2.91	14.51	2.62	1.94	18.18	1.94	10.12	19.34	1.57	8.43
B	15.10	3.44	4.45	26.66	2.71	6.53	39.51	1.99	15.60	44.19	1.86	14.31

t, migration time of the first peak; N, plate numbers of the first peak; R_s, resolution calculated by $R_s = 1.18 \times (t_2 - t_1)/(W_{1/2} + W_{1/2})$. Rinse procedure (A) 0.1 *M* NaOH, H_2O, and buffer each for 2 min; (B) H_2O for 1 min, 1% HNO_3 for 3 min, H_2O for 1 min, and buffer for 2 min. Conditions: buffer, 20 m*M* β-CD and 30 m*M* STC in 80 m*M* borate at pH 9.3; injection, 0.5 p.s.i.×1 s (1 p.s.i.≡6894.76 Pa); separation voltage, 20 kV; capillary, 57 cm×50 μm (I.D.); capillary temperature, 20 °C.

running buffer composed of 20 m*M* β-CD, 30 m*M* STC and 80 m*M* borate at pH 9.3, and capillary rinse procedure with 1% HNO_3 between two successive runs.

Under these conditions, all of the 20 pairs of amino acids derivatized with FITC were baseline resolved (Fig. 6), with better resolution than the reported data [19]. The resolution of most amino acids (17 of 20) is higher than 3.0, only three pairs of amino acids enantiomers give a resolution lower

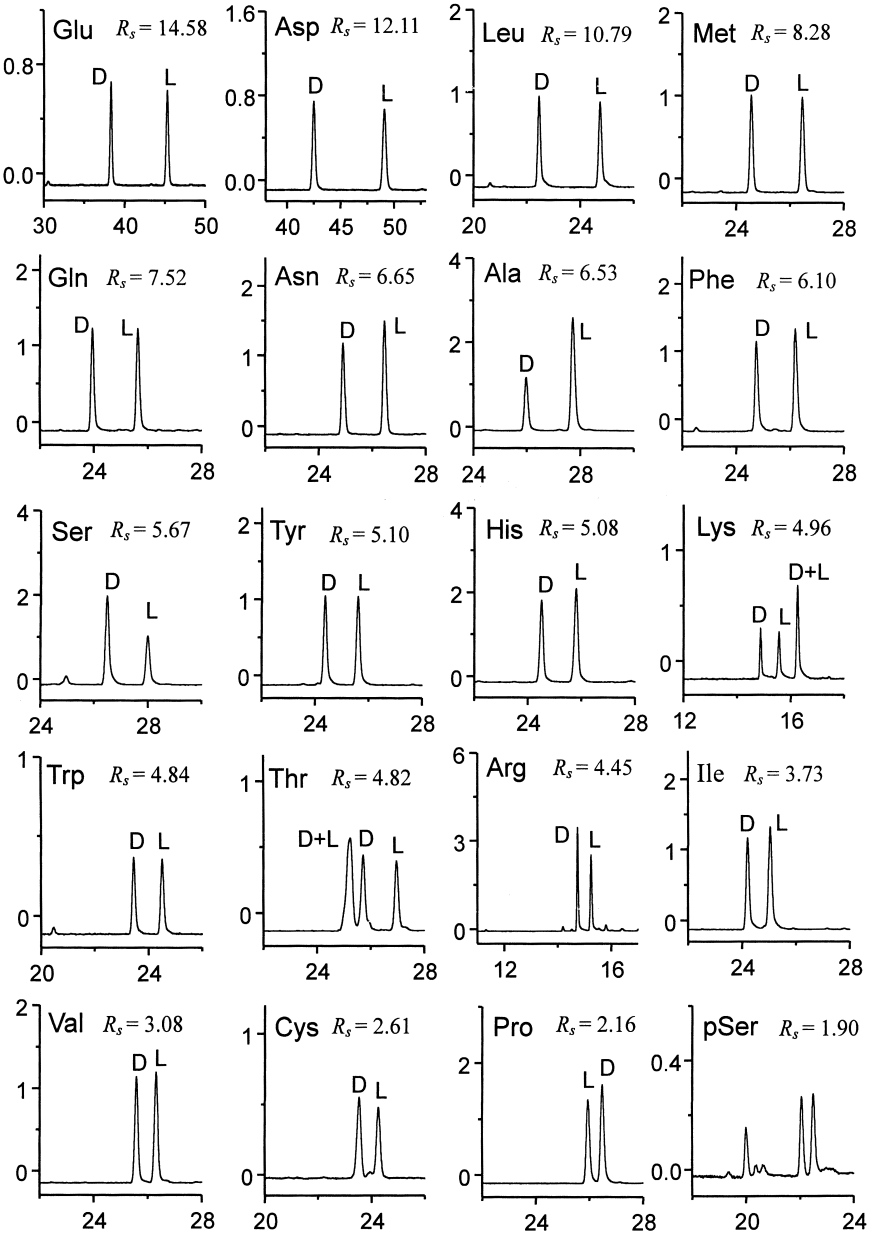

Fig. 6. Chiral separation of 21 pairs of FITC-amino acids under optimized conditions. Buffer, 20 m*M* β-CD and 30 m*M* STC in 80 m*M* borate at pH 9.3; capillary, washed between two injections successively with H_2O for 1 min, 1% HNO_3 for 3 min, H_2O for 1 min, and buffer for 2 min; other conditions are the same as in Fig. 1.

than 3.0 but higher than 1.90 (β-phenylserine, pSer). The highest resolution reaches 14.58 (Glu). Interestingly, the D-form normally migrates faster than the L-form except Pro (L-form faster). Fig. 7 shows also the chiral separation of six pairs of mixed amino acids.

3.3. Chiral separation using STC with β-CD derivatives

2-Hydroxypropyl-β-CD (HP-β-CD) also showed cooperative chiral selectivity with STC. For example, the chiral separation of Phe was observed when 10 m*M* HP-β-CD and 30 m*M* STC were used simultaneously but no chiral separation when either of them was employed. The elution order was also the D-form migrating faster as in the chiral separation with β-CD and STC. In contrast, DM-β-CD together with STC led to worse separation. For instance, Val was resolved in the presence of 20 m*M* DM-β-CD with L-Val migrating faster. But the chiral separation was lost when 30 m*M* STC was added to 20 m*M* DM-β-CD.

3.4. Brief discussion on the chiral separation mechanism

In theory, the chiral separation mechanism in this study involves the inclusion of FITC-amino acids in β-CD cavity and the interaction of FITC-amino acids with STC monomers and/or micelles. As can be seen from Fig. 1, β-CD selectively complexes with the D-form of FITC-Ala, Asp, and Glu, while STC selectively interact with L-form of FITC-Arg. At the pH investigated in this work (>7), FITC-amino acids eluted before STC but behind β-CD. A cooperative effect thus occurs that β-CD selectively accelerates the D-form while STC selectively decelerates the L-form of these amino acids. A cooperative effect also takes place in the chiral separation of Pro, but contrarily L-Pro is now selectively accelerated by β-CD while D-Pro is decelerated by STC. So generally in the chiral separation of the FITC-amino acids, a concerted effect that β-CD selectively accelerates one enantiomer while STC selectively decelerates the corresponding opposite enantiomer appears to take place. Sequentially, the binary selectors of β-CD and STC are much more effective than either β-CD alone or STC alone. Differently, DM-β-CD and STC seem to selectively interact with the same enantiomer at different direction (for example, L-form of Val). Thus, a negative effect is generated.

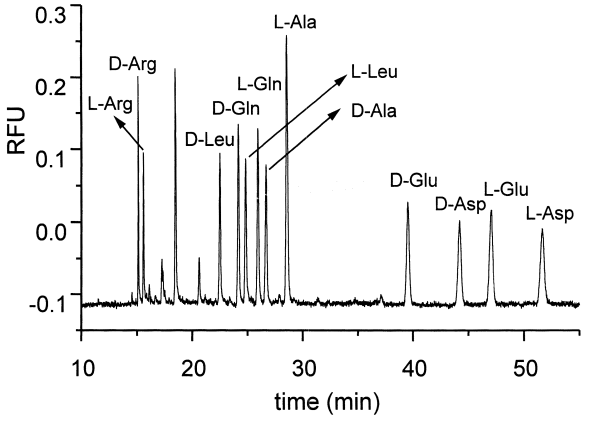

Fig. 7. Chiral separation of 6 pairs of FITC-amino acids in one run. Conditions are the same as in Fig. 6.

Jin et al. have proposed that the benzene ring with amino acid moiety might enter the cavity of β-CD [16]. They found that the chiral carbon atoms with $-CH_2$ neighbors can be resolved at a higher resolution than with other functional groups such as $-CH(CH_3)_2$ by CD-mediated MEKC. Similar results were also observed in this work. For instance, the resolutions of Ala, Leu and Ser are much higher than those of Val, Ile and pSer. The basic amino acids such as Arg and His, whose resolutions were lower than those of other FITC-amino acids in Jin's work, were well resolved in our work. This suggests that the electrostatic interactions of amino acids with anionic STC micelles also favor the chiral resolution.

4. Conclusion

β-CD and STC cooperate in chiral separation of FITC-amino acids because they selectively interact with different enantiomers and have different migration behaviors. Based on such a cooperative effect, a method of CE–LIF has been developed to resolve 20 pairs of FITC-amino acids enantiomers. These results suggest that the combination of two selectors is a simple but effective way to achieve or improve chiral separation.

Acknowledgements

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