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Evaluation of the enantioselective possibilities of sulfated cyclodextrins for the separation of aspartyl di- and tripeptides in capillary electrophoresis

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Abstract

Highly sulfated α-, β- and γ-cyclodextrins were used to investigate the simultaneous separation of isomeric α- and β-aspartyl containing di- and tripeptides as well as their enantiomers in capillary electrophoresis. Separation of the enantiomers was accomplished with the different sulfated cyclodextrin types under acidic conditions (pH 2–3). In some cases, comigration of diastereomerically related isomers or epimers resulted in incomplete separations. The results obtained with the highly sulfated cyclodextrins, having an average degree of substitution 12, were compared to those obtained with sulfated cyclodextrins with degree of substitution 4.

Keywords: Enantiomer separation; Peptides; Cyclodextrins

1. Introduction

Capillary electrophoresis (CE) has become a powerful tool for the analysis of polar compounds such as peptides and proteins [1,2]. Presently, there is an increasing interest in the application of CE for chiral separation of small peptides. The chiral separation of some di- and tripeptides has been reported using β-cyclodextrin derivatives [3–5], chiral crown ethers [6–8], or vancomycin [9,10] as buffer additives. Additionally, micellar electrokinetic chromatography (MEKC) [11] as well as cyclodextrin (CD)-modified MEKC [3,12] have been applied for the resolution of peptide enantiomers.

The peptides used in this study were previously separated with other chiral selectors namely carboxymethyl-β-CD [13], sulfobutylether-β-CD [14], neutral CD derivatives [13,14] and the chiral crown ether 18-crown-6 tetracarboxylic acid [15].

Only a few reports appeared with sulfated-β-CD (S-β-CD) as chiral selector. This CD derivative is negatively charged over the entire pH range and moves in the opposite direction of the electroosmotic flow (EOF). The degree of substitution (DS) is determined to be around 7 to 11. A large number of pharmaceutical compounds were enantioseparated with S-β-CD with detection at the cathodic end of an untreated fused-silica capillary, under neutral or basic conditions [16]. When the pH was decreased to a value of 5, no peaks were observed for any of the

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analytes within 120 min. This is probably due to a migration of the solutes towards the anode. When working under limited EOF conditions (pH 3.8), 56 racemic drugs were analyzed and detected at the anodic side of the capillary [17]. This paper proved that S-β-CD is capable of separating a large number of structurally diverse neutral as well as cationic analytes.

Wang and Khaledi [18] did some experiments using non-aqueous media (formamide, *N*-methylformamide and *N*,*N*-dimethylformamide) for the enantioseparation of some pharmaceutical racemic acids. Much lower concentrations of the charged CD derivative were needed compared with the neutral CD derivatives.

Scientists at the Beckman Company have recently developed a new chiral reagent, namely well characterized highly sulfated CDs (HS-CDs) with a degree of substitution 12. These CDs will be available in the near future in α, β and γ formats. The new HS-CDs have been evaluated for the separation of aspartyl containing di- and tripeptides (Fig. 1). The results are compared to those obtained with a S-β-CD with degree of substitution 4.

2. Experimental

2.1. Instrumentation

CE experiments were performed on a Beckman P/ACE System MDQ (Beckman Instruments, Fullerton, CA, USA). Uncoated fused-silica capillaries of 57 cm (effective length of 50 cm)×50 μm I.D. were used. Samples were hydrodynamically introduced by applying pressure during 1 s, analyzed with a voltage of −20 kV (reversed polarity mode, injection at the cathodic end of the capillary), if not stated otherwise, and detected with UV detection at 200 nm. In all cases, the temperature remained constant at 25°C. Between analysis, the capillary was rinsed with deionized water (1 min) followed with buffer (1 min).

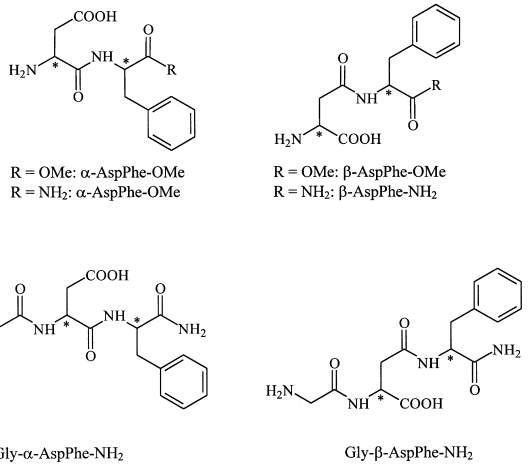

Fig. 1. Chemical structures of the analysed di- and tripeptides.

2.2. Chemicals

Highly sulfated CDs were synthesized by Dr. A. Chen and are available in a kit from Beckman Instruments. The kit contains aqueous solutions of 20% (w/v) HS-α-CD, 20% (w/v) HS-β-CD and 20% (w/v) HS-γ-CD; 50 m*M* triethylammonium phosphate buffer pH 2.5; 1,3,6,8-pyrenetetrasulfonate migration time reference marker; pseudoephedrine test mix; glutethimide test mix and a capillary treatment solution.

Another sulfated cyclodextrin (S-β-CD), with a substitution degree of 4, was obtained from Bas-Technicol (Stockport, UK) and triethylammonium phosphate buffer, pH 3 was obtained from Fluka (Bornem, Belgium). Diastereomeric mixtures as well as enantiomerically pure di- and tripeptides were prepared in solution using commercial benzyloxycarbonyl (Z-) or *tert*.-butoxycarbonyl (Boc)-protected amino acids and *N*-(3-dimethylaminopropyl)-*N*-ethylcarboximide as coupling reagent. Boc deprotection was performed using 20% trifluoroacetic acid in methylene chloride while Z-groups and the benzyl esters were deprotected by hydrogenolysis using a palladium on carbon catalyst.

3. Results and discussion

Separation of the diastereomeric pairs of the dipeptides Asp–Phe–OMe and Asp–Phe–NH_2 is relatively easy and was obtained using a buffer solution of pH 2–3 without chiral selector [14]. At this pH the amino groups of the peptides are fully protonated. Small differences in the pK_a of the

carboxylic acid groups resulted in a different electrophoretic mobility of the diastereomers.

The highly sulfated α-, β- and γ-CDs (DS=12) and S-β-CD (DS=4), which are negatively charged over the entire pH range, were evaluated as chiral selectors for the enantiomeric part of the separation of the peptides. In first instance, the highly sulfated CDs (HS-CDs), provided by Beckman as a chiral separation kit for a P/ACE CE system, were used applying the conditions mentioned on the Beckman application sheet. A 37.5 m*M* triethylammonium phosphate (TEA phos) buffer (pH 2.5) containing 5% (w/v) chiral selector (HS-α-, HS-β- or HS-γ-CD), was used in the reversed polarity mode (detection at the anodic end) for the separation of the dipeptides Asp–Phe–OMe and Asp–Phe–NH_2. Under these conditions, HS-β-CD was the best suited chiral selector (Fig. 2).

In both cases comigration of two diastereomers (β-D-Asp–D-Phe–NH_2 and β-L-Asp–D-Phe–NH_2) or two epimers (α-D-Asp–L-Phe–OMe and β-D-Asp–L-Phe–OMe) occurred. All the enantiomeric pairs are well resolved. The relatively short migration times indicate strong complexation with the CD derivative. Normally, the weaker the host–guest interaction between positively charged compounds and negatively charged chiral selectors in the normal polarity mode, the faster the migration times. In case the polarity is reversed (detection at the anode), the weaker the interactions between the chiral selector and the solutes, the longer the migration times. Interesting to note is that the migration order of the α-epimers of Asp–Phe–NH_2 is reversed compared to the migration order obtained in Asp–Phe–OMe. The use of HS-α-CD at pH 2.5 gave incomplete and less efficient separations of both peptides. The interactions of the analytes with HS-γ-CD were too low to obtain separation within short analysis times. Only four peaks of both peptides were detected within 60 min.

The utilization of TEA phos at pH 3 resulted in partial separation of the previous unseparated epimers α-D-Asp–L-Phe–OMe and β-D-Asp–L-Phe–OMe (Fig. 3A). Baseline separation of all diastereo-

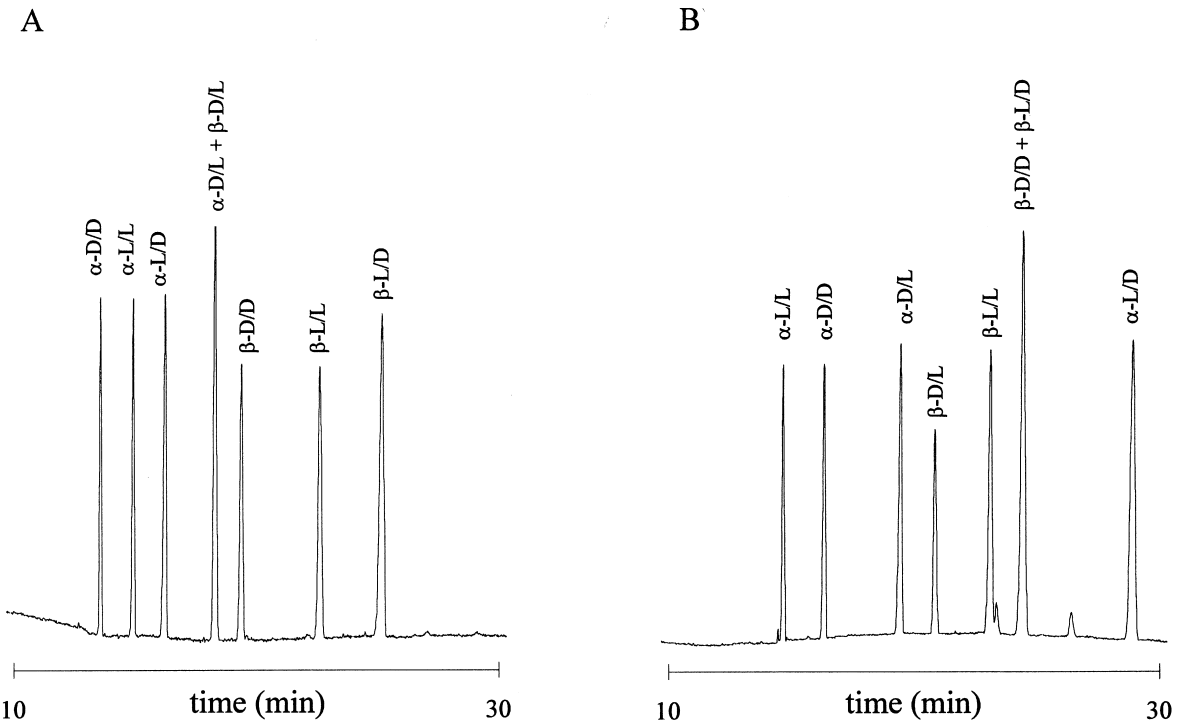

Fig. 2. Separation of Asp–Phe–OMe (A) and Asp–Phe–NH_2 (B) with HS-β-CD as chiral selector. Buffer composition: 37.5 m*M* TEA phos, pH 2.2, 5% (w/v) HS-β-CD. Conditions: 200 nm, 25°C, −20 kV, fused-silica 50 cm effective length×50 μm I.D.

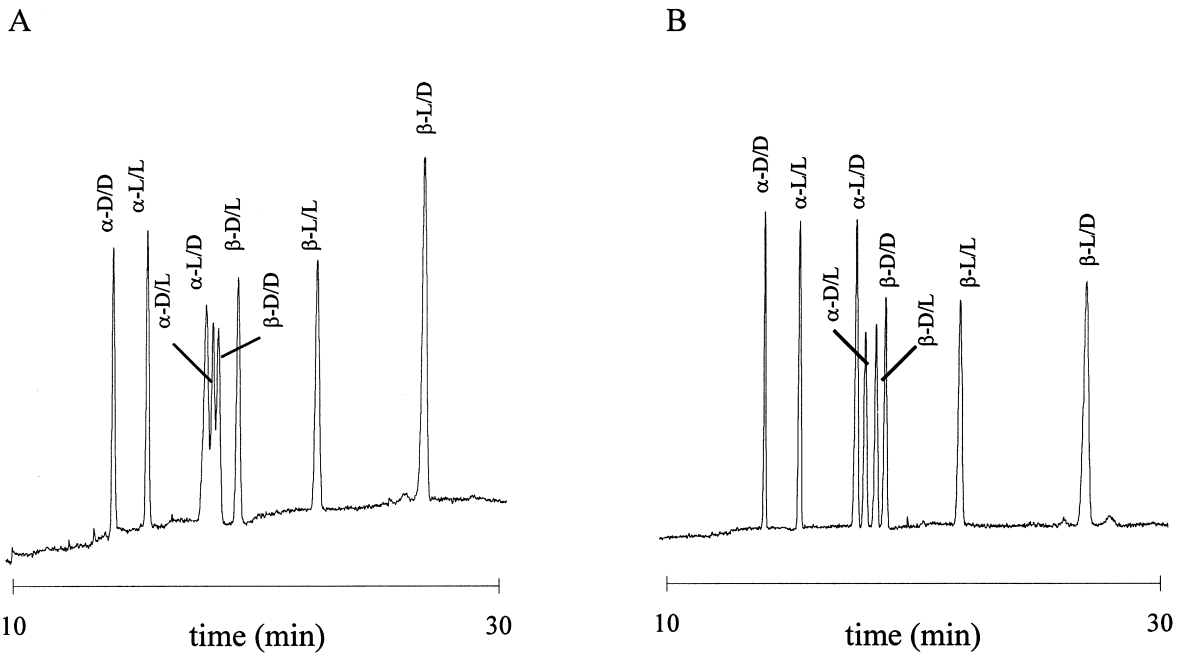

Fig. 3. Separation of Asp–Phe–OMe with HS-β-CD as chiral selector. (A) Buffer composition: 37.5 m*M* TEA phos, pH 3, 5% (w/v) chiral selector. Conditions: 200 nm, 25°C, −20 kV, fused-silica 50 cm effective length×50 μm I.D. (B) Buffer composition: 37.5 m*M* phosphate, pH 3, 5% (w/v) chiral selector. Conditions: 200 nm, 25°C, −20 kV, fused-silica 50 cm effective length×50 μm I.D.

mers and enantiomers of Asp–Phe–OMe was obtained with a phosphate buffer at pH 3 (Fig. 3B).

The same optimized conditions were used for the dipeptide Asp–Phe–NH_2. The separation was not improved and a small change in selectivity, applying a phosphate buffer at pH 3, was noticed (Fig. 4). Higher pH values (pH 4.5) proved unuseful for the separation of those kind of peptides, probably due to a decrease in mobility differences of the diastereomers as mentioned above.

Baseline separation of both Asp–Phe–OMe and Asp–Phe–NH_2 was achieved when 5% (w/v) HS-α-CD was added to a phosphate buffer (pH 3) or triethylammonium phosphate buffer (pH 3), respectively (Fig. 5). Under the same conditions, only four peaks for both dipeptides were detected within 60 min, when HS-γ-CD was used as chiral discriminator.

From Figs. 3–5, it is obvious that the pH is of utmost importance.

S-β-CD with a much lower degree of substitution (DS 4) was tested for the separation of the two dipeptides. In general less stable systems were observed because the CD structure is not so well defined. The resolution of Asp–Phe-OMe decreased (Fig. 6B), but surprisingly almost baseline separation of all the isomers of Asp–Phe–NH_2 was obtained (Fig. 6A). In both cases, the migration times were reduced, indicating that the complexation with S-β-CD with DS 4 is higher for the tested compounds, compared to S-β-CD with DS 12.

The same chiral selectors were evaluated for the separation of the isomeric tripeptide Gly–Asp–Phe–NH_2. Fig. 7 shows the separation of the tripeptide Gly–Asp–Phe–NH_2, with the different HS-CDs, using the optimal conditions found for the dipeptides.

The sulfated CD derivative with the largest cavity namely HS-γ-CD, performed best. More stable systems were obtained with a TEA phos buffer at pH 2.5 (Fig. 8). Nevertheless, the separation with HS-γ-CD resulted in seven peaks instead of eight peaks (Fig. 8B). Combining both electropherograms shows that complete elucidation of the enantiomers is

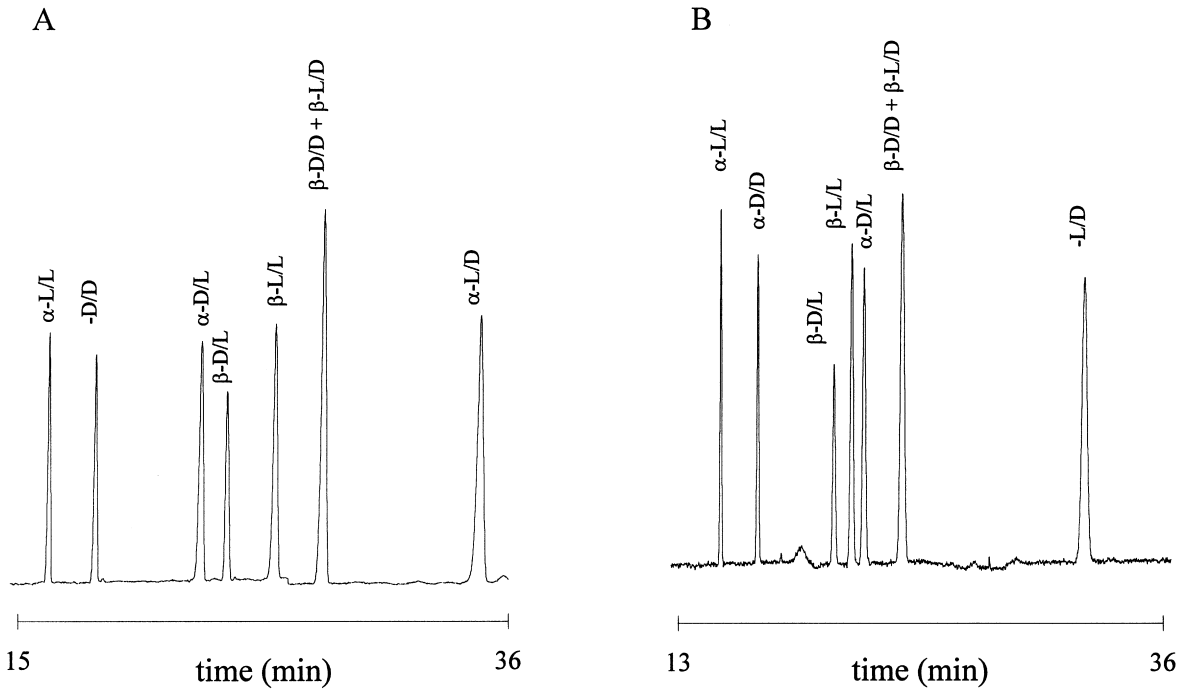

Fig. 4. Separation of Asp–Phe–NH_2 with HS-β-CD as chiral selector. (A) Buffer composition: 37.5 m*M* TEA phos, pH 3, 5% (w/v) chiral selector. Conditions: 200 nm, 25°C, −20 kV, fused-silica 50 cm effective length×50 μm I.D. (B) Buffer composition: 37.5 m*M* phosphate, pH 3, 5% (w/v) chiral selector. Conditions: 200 nm, 25°C, −20 kV, fused-silica 50 cm effective length×50 μm I.D.

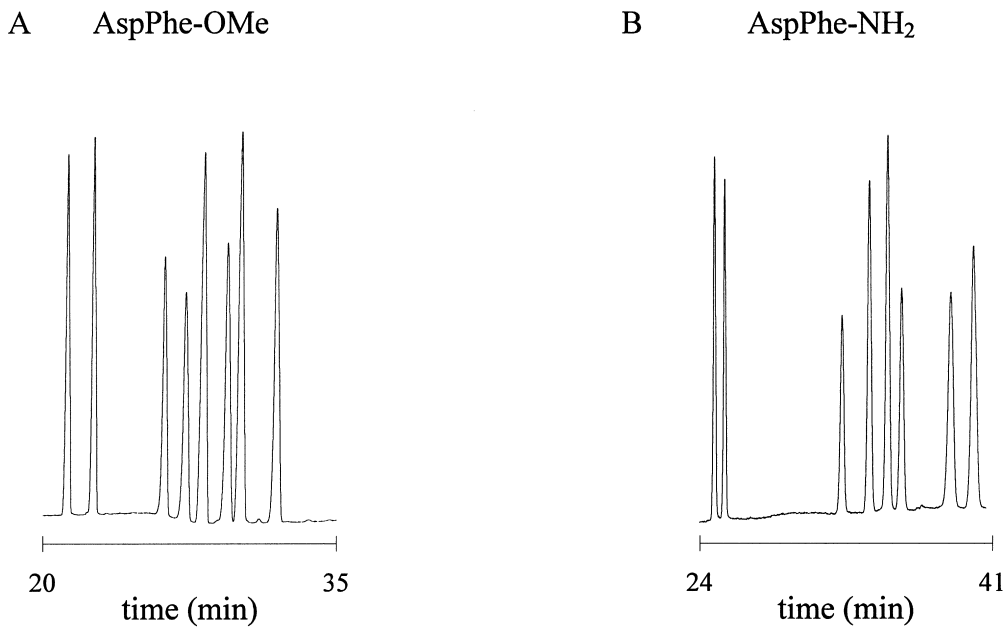

Fig. 5. Separation of the dipeptides (A) Asp–Phe–OMe and (B) Asp–Phe–NH_2 with HS-α-CD as chiral selector, conditions as in Fig. 4. (A) Buffer composition: 37.5 m*M* phosphate, pH 3, 5% (w/v) HS-α-CD. (B) Buffer composition: 37.5 m*M* TEA phos, pH 3, 5% (w/v) HS-α-CD.

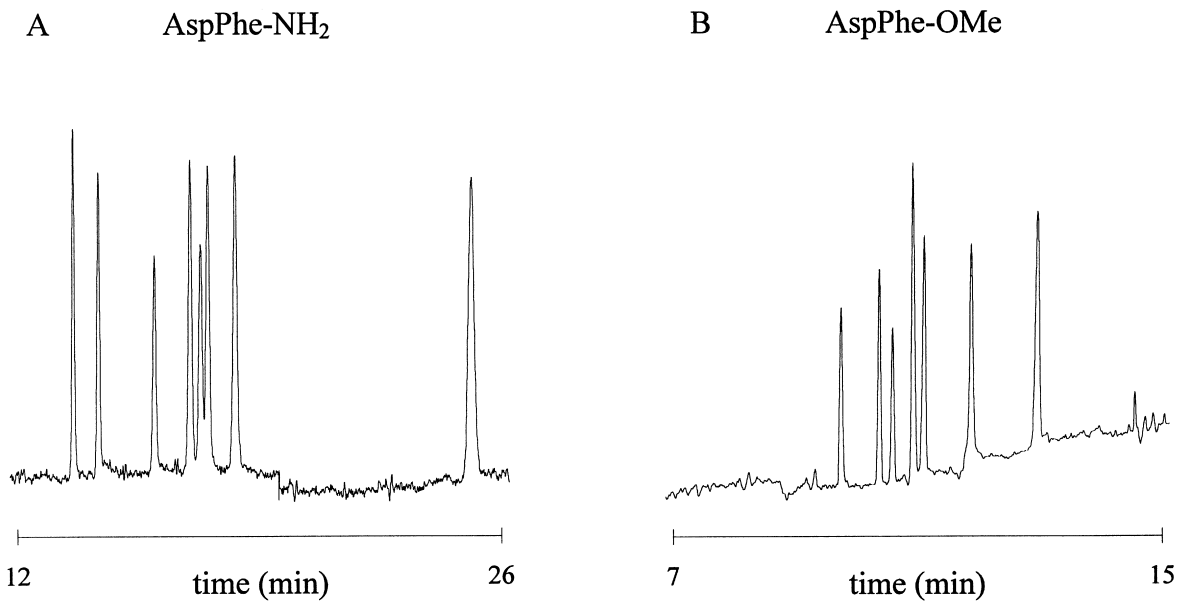

Fig. 6. Separation of (A) Asp–Phe–NH_2 and (B) Asp–Phe–OMe with sulfated-β-CD (DS=4). Buffer composition: 30 m*M* TEA phos, pH 2.5, 5% (w/v) chiral selector. Conditions: 200 nm, 25°C, −20 kV, fused-silica 50 cm effective length×50 μm I.D.

feasible. Comparison of the migration order of the tripeptide Gly–Asp–Phe–NH_2 and Asp–Phe–NH_2 shows that the addition of a glycyl group to Asp–Phe–NH_2 has an influence on the enantiomeric migration order. The migration order of the enantiomeric pair α-D/L and α-L/D is reversed.

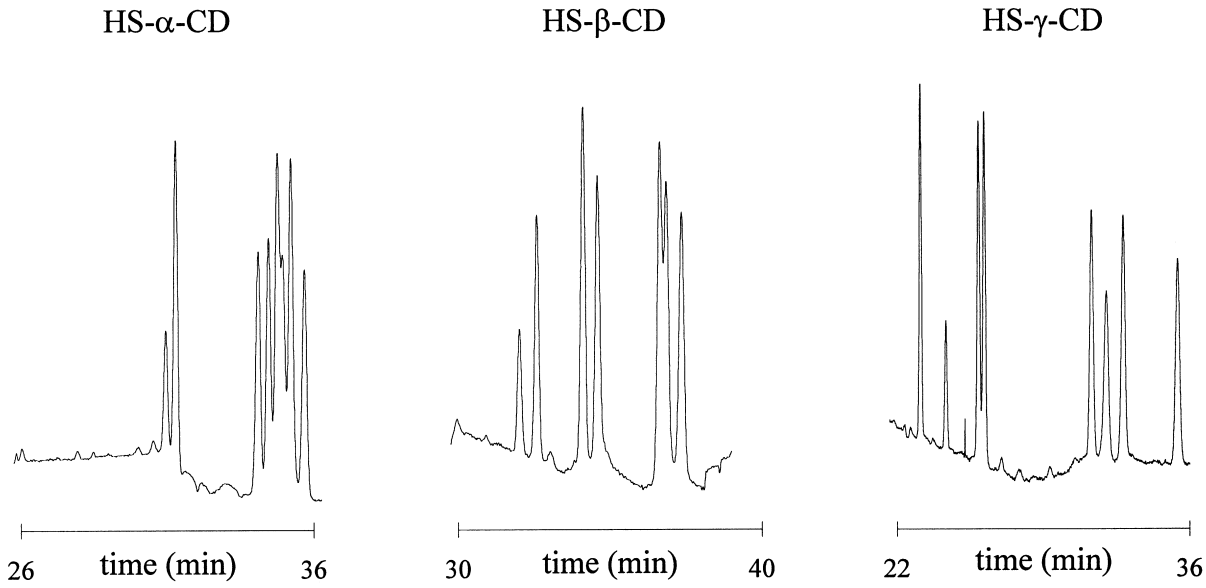

Fig. 7. Separation of Gly–Asp–Phe–NH_2 with HS-α-, HS-β- and HS-γ-CD as chiral agents. (A) Buffer composition: 37.5 m*M* phosphate, pH 3, 5% (w/v) chiral selector. Conditions: 200 nm, 25°C, −20 kV, fused-silica 50 cm effective length×50 μm I.D.

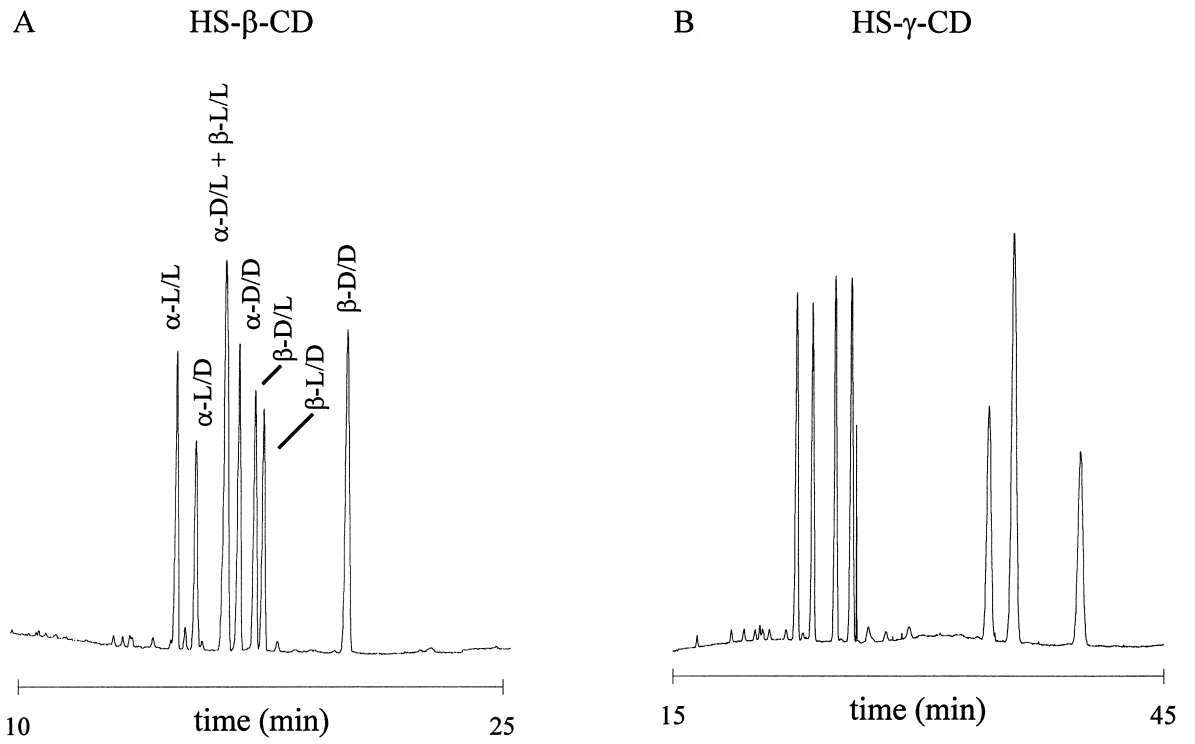

Fig. 8. Separation of Gly–Asp–Phe–NH_2 with (A) HS-β- and (B) HS-γ-CD as chiral selectors, conditions as in Fig. 4. (A) Buffer composition: 30 m*M* TEA phos, pH 2.5, 4% (w/v) HS-β-CD. (B) Buffer composition: 30 m*M* TEA phos, pH 2.5, 4% (w/v) HS-γ-CD.

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