ELSEVIER

Journal of Chromatography A 822 (1998) 137–145

JOURNAL OF CHROMATOGRAPHY A

Electrophoretic stereoisomer separation of aspartyl dipeptides and tripeptides in untreated fused-silica and polyacrylamide-coated capillaries using charged cyclodextrins

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Received 16 April 1998; received in revised form 29 June 1998; accepted 16 July 1998

Abstract

The separation of diastereomeric and enantiomeric aspartyl model dipeptides and tripeptides including the isomeric iso-Asp containing β-peptides was studied in untreated fused-silica capillaries and polyacrylamide-coated capillaries by capillary electrophoresis using negatively charged cyclodextrins as chiral selectors. While most diastereomers could be separated in the acidic pH range two dipeptide diastereomers were also resolved at alkaline pH. The separation of the enantiomers using carboxymethyl-β-cyclodextrin or sulfobutyl-β-cyclodextrins could be performed either between pH 2 and 3 when the peptides are positively charged or at pH 5.25 where the peptides migrate as uncharged compounds with the electroosmotic flow. Exploiting the self-mobility of the negatively charged cyclodextrins and reversing the polarity of the applied voltage, a reversal of the migration order of the enantiomers was achieved in polyacrylamide-coated capillaries.

Keywords: Peptides; Cyclodextrins; Diastereomer separation; Enantiomer separation; Buffer composition

1. Introduction

Capillary electrophoresis (CE) has become a powerful tool for the analysis of polar compounds such as peptides and proteins [1,2]. The chiral separation of small peptides has been reported using cyclodextrin (CD) derivatives [3–6], a chiral crown ether [7–9], or the macrocyclic glycopeptide antibiotics vancomycin [10–12] and teicoplanin [12,13] as buffer additives. Additionally, CD-modified micellar electrokinetic chromatography (MEKC) [12,14,15], as well as indirect separation upon derivatization with chiral reagents [12,14,16], have been applied for the resolution of peptide enantiomers.

CDs are the most abundant chiral selectors used in CE, with the charged derivatives possessing advantages compared to neutral CDs [17,18]. First, the electrophoretic self-mobility of charged CDs allows the resolution of charged as well as neutral chiral solutes. Second, charged CDs can bind oppositely charged analytes more strongly due to higher electrostatic interactions which can sometimes favor a separation. Third, the self-electrophoretic mobility allows to reverse the migration order, which cannot be achieved with neutral chiral selectors. Fourth, the self-electrophoretic mobility of charged CDs allows their use as carriers for analytes [18]. Some of these

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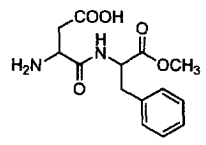
0021-9673/98/$19.00
PII: S0021-9673(98)00602-5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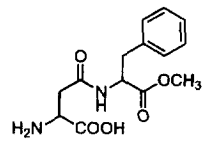
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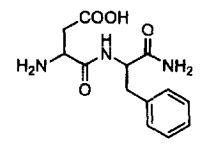
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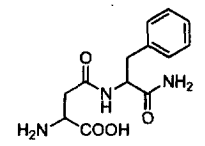
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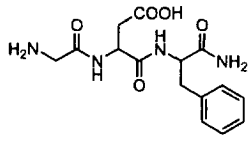
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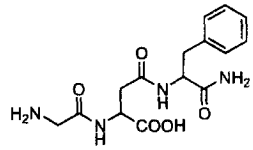
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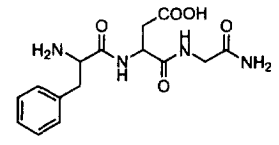
Phe-α-Asp-GlyNH$_2$

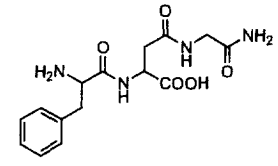
Phe-β-Asp-GlyNH$_2$

Fig. 1. Structures of the Asp peptides.

advantages will be illustrated in this paper for the enantioseparation of di- and tripeptides.

The stereoisomer resolution of aspartyl peptides is of special interest due to the easy racemization and isomerization of the aspartyl residue [19,20]. Aspartyl model peptide stereoisomers have been separated using neutral CDs or carboxymethyl-β-cyclodextrin (CM-β-CD) [6] as well as sulfobutyl-β-cyclodextrin (SBE-β-CD) in the CZE mode and the CD-modified MEKC mode [4]. The present study describes the enantiomeric separation of the stereoisomers of the aspartyl peptides including their β-isomers Asp–PheOMe, β-Asp–PheOMe, Asp–PheNH$_2$, β-Asp–PheNH$_2$, Gly–Asp–PheNH$_2$, Gly–β-Asp–PheNH$_2$, Phe–Asp–GlyNH$_2$, Phe–β-Asp–GlyNH$_2$ (Fig. 1) by CE using CM-β-CD and two SBE-β-CDs with different substitution degrees as chiral selectors in untreated fused-silica capillaries and polyacrylamide-coated capillaries with suppressed electroosmotic flow. The potential of the charged CDs in untreated and coated capillaries for the separation of the model peptides as charged and uncharged compounds will be compared.

2. Experimental

2.1. Chemicals

L-Asp–L-PheOMe was a gift from NutraSweet (Zug, Switzerland), β-cyclodextrin (β-CD), CM-β-CD (average substitution degree, 3.5) and sulfobutyl-β-cyclodextrin [SBE(7)-β-CD, average substitution degree, 7.0] were from Wacker Chemie (Munich, Germany). Sulfobutyl-β-cyclodextrin [SBE(4)-β-CD, average substitution degree, 3.9] was a gift from Dr J.F. Stobaugh (University of Kansas, Lawrence, KS, USA). β-L-Asp–L-PheOMe, L-Asp–L-PheNH$_2$ and protected amino acid derivatives were obtained from Bachem (Heidelberg, Germany). All other chemicals were of analytical grade. Diastereomeric mixtures as well as enantiomerically pure dipeptides and tripeptides were prepared in solution using *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 benzyl esters were deprotected by hydrogenolysis using a palladium on carbon catalyst.

2.2. Instrumentation

All experiments were performed on a Beckman P/ACE 5510 (Beckman Instruments, Waldbronn, Germany) equipped with a diode array detector at 20°C using 50-μm fused-silica capillaries (Beckman Instruments) or polyacrylamide-coated capillaries prepared as described [21]. The effective length of the capillaries was 40 cm. UV detection at 215 nm

was performed at the cathodic end or at the anodic end when the polarity of the applied voltage was reversed. Sample solutions were introduced into the capillary at a pressure of 0.5 p.s.i. for 3 s (1 p.s.i.= 6894.76 Pa). Separations were performed in 50 m*M* sodium phosphate buffer. The pH was adjusted using 100 m*M* phosphoric acid or 100 m*M* sodium hydroxide after the addition of the CDs. Between the analyses the capillaries were washed 2 min with 50 m*M* phosphate buffer without CDs and 2 min with the run buffer. Buffers and sample solutions were prepared in double-distilled, deionized water, filtered (0.47 μm), and degassed by sonication. The migration order of the peptide enantiomers was confirmed by spiking with the enantiomerically pure individual peptides.

3. Results and discussion

The dependence of the achiral separation of the diastereomers of α/β-Asp–PheOMe, α/β-Asp–$PheNH_2$ and Gly–α/β-Asp–$PheNH_2$ on the pH of the run buffer is shown in Fig. 2. Below pH 4 the amino group of the peptides is fully protonated. Small differences in the pK_a values of the aspartyl carboxylic acid groups of the diastereomers result in a different effective mobility of the diastereomeric pairs of both α- and β-isomers. The optimum region for the achiral separation was between pH 2.5 and 3.5, which is similar to the pH region observed for the diastereomeric resolution of related Asp peptides [4,6,22]. Between pH 7 and 9, the region of the pK_a of the amino group [23,24], the separation of the diastereomers of the α-isomers of Asp–PheOMe and Asp–$PheNH_2$ was also observed. Between pH 4 and 6 the amino groups are still protonated, while the carboxyl groups are deprotonated resulting in a very low electrophoretic mobility. The isoelectric point of the peptides was found between pH 5.1 and 5.3 as they migrated as uncharged compounds with the electroosmotic flow (EOF).

The resolution of the diastereomers of Phe–α/β-Asp–$GlyNH_2$ and Gly–α/β-Asp–$PheNH_2$ is shown in Fig. 3A and Fig. 3B, respectively. In contrast to Phe–α/β-Asp–$GlyNH_2$, the α-isomers of Gly–α/β-Asp–$PheNH_2$ were not resolved illustrating the effect of the amino acid sequence of peptides on diastereomer separations. However, the stereoisomers could easily be resolved using β-CD as additive to the run buffer (Fig. 3C).

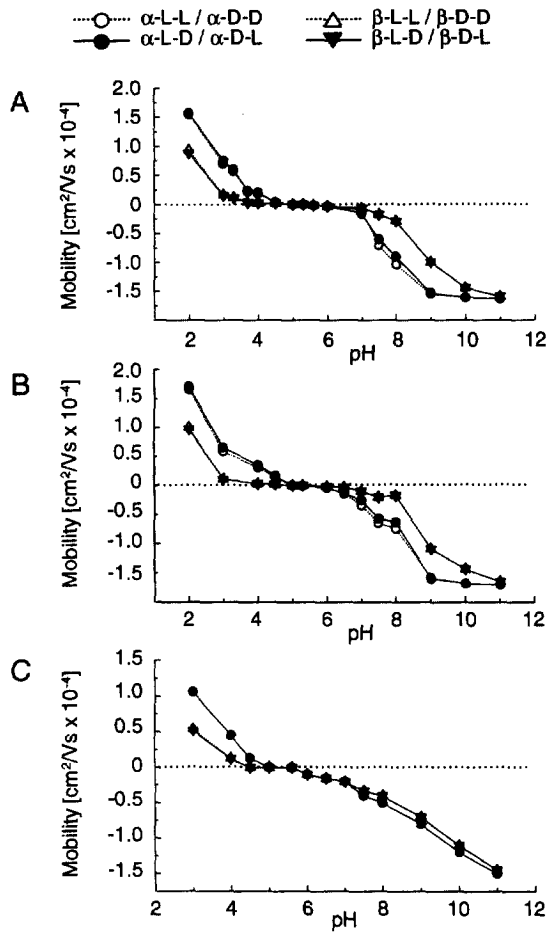

Fig. 2. Dependence of the effective mobility, μ_{eff}, of Asp peptides on the pH of the run buffer. Conditions: 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, +23 kV. (A) α/β-Asp–PheOMe, (B) α/β-Asp–$PheNH_2$, (C) Gly–α/β-Asp–$PheNH_2$.

The negatively charged cyclodextrins CM-β-CD, as well as two SBE-β-CDs varying in their substitution degree, SBE(4)-β-CD and SBE(7)-β-CD, were evaluated as chiral selectors for the separation of the peptides. The experiments were performed in untreated fused-silica capillaries and polyacrylamide-coated capillaries with suppressed EOF. The electropherograms obtained for the dipeptides α/β-Asp–PheOMe and α/β-Asp–$PheNH_2$ and the tripeptide Gly–α/β-Asp–$PheNH_2$ at pH 5.25 using CM-β-CD are shown in Fig. 4 and Fig. 5, respectively. In a

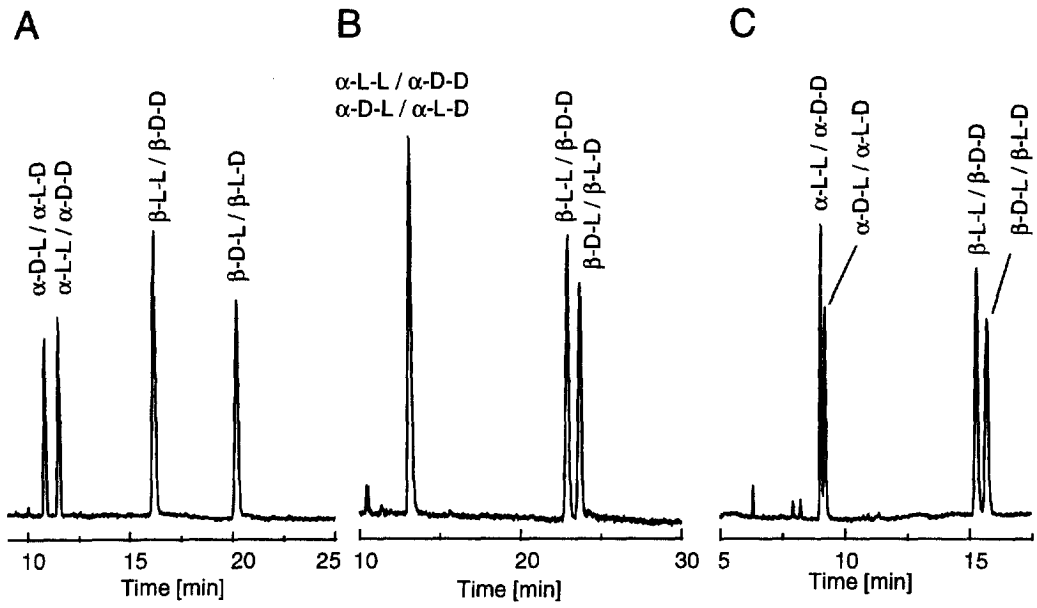

Fig. 3. Separation of the diastereomers of isomeric Asp tripeptides. (A) Phe–α/β-Asp–$GlyNH_2$, conditions: 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 3.0, +23 kV. (B) Gly–α/β-Asp–$PheNH_2$, conditions: 47/40-cm capillary, 50 m*M* sodium phosphate buffer, pH 3.0, +23 kV. (C) Gly–α/β-Asp–$PheNH_2$, conditions: 37/30-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 3.0, 17.5 mg/ml β-CD, +20 kV.

fused-silica capillary (Fig. 4A,C and Fig. 5A) the peptides migrate as uncharged compounds with the EOF towards the cathode at this pH while CM-β-CD is deprotonated [25] and possesses an electrophoretic self-mobility towards the anode. When the EOF is suppressed by coating the capillary with polyacrylamide, CM-β-CD can be exploited as a carrier due to its self-mobility at pH 5.25, reversing the polarity of the applied voltage and detecting at the anode. The electropherograms of the separation of the peptides using 60 mg/ml CM-β-CD in the run buffer are shown in Fig. 4B,D and Fig. 5B, respectively. A reversal of the migration order of the peptide enantiomers compared to the order obtained in untreated fused-silica capillaries is observed.

Differences in the binding constants between the peptide enantiomers and the CD result in a chiral separation. In fused-silica capillaries the enantiomer which forms stronger complexes with CM-β-CD migrates slower than the weaker bound enantiomer. In coated capillaries the EOF is suppressed and the charged CD is employed as a carrier for the neutral analyte due to the self-mobility of the CD reversing the polarity of the applied voltage and detecting at the anodic end of the capillary. Under these conditions the preferentially complexed enantiomer migrates faster than the less complexed enantiomer. This results in a reversal of the migration order of the peptide enantiomers in polyacrylamide-coated capillaries. This option of a reversal of the migration order of enantiomers is important because it is often desirable to detect minor components in front of major components. This can be especially useful for peak identification in complex electropherograms.

The pH-dependence of the resolution of the enantiomers of α/β-Asp–PheOMe and Gly–α/β-Asp–$PheNH_2$ in a coated capillary using 60 mg/ml CM-β-CD as buffer additive between pH 4 and 7 is shown in Fig. 6. In order to avoid any deterioration of the polyacrylamide coating the experiments were only performed up to pH 7. While the resolution of α-L-Asp–D-PheOMe/α-D-Asp–L-PheOMe decreased with increasing pH due to a loss of selectivity, the resolution of α-L-Asp–L-PheOMe/α-D-Asp–D-PheOMe displayed a maximum at pH 5.25. The separation of the enantiomers of the β-Asp stereoisomers was not significantly affected by the pH. Although the enantiomeric pairs of all isomers of

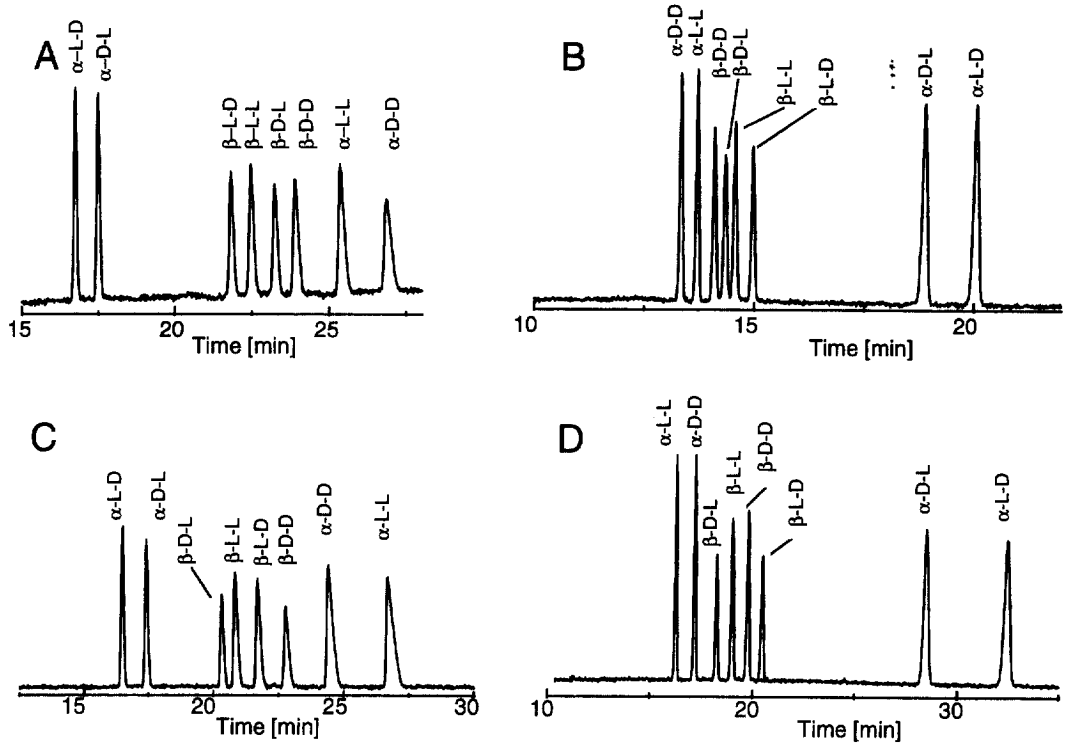

Fig. 4. Chiral separation of the isomeric dipeptides α/β-Asp–PheOMe and α/β-Asp–$PheNH_2$ in (A,C) fused-silica and (B,D) polyacrylamide-coated capillaries. α/β-Asp–PheOMe: (A) 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 5.25, 15 mg/ml CM-β-CD, +20 kV; (B) 47/40-cm polyacrylamide-coated capillary, 50 m*M* sodium phosphate buffer, pH 5.25, 60 mg/ml CM-β-CD, −20 kV. α/β-Asp–$PheNH_2$: (C) 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 5.25, 15 mg/ml CM-β-CD, +20 kV; (D) 47/40-cm polyacrylamide-coated capillary, 50 m*M* sodium phosphate buffer, pH 5.25, 60 mg/ml CM-β-CD, −20 kV.

α/β-Asp–PheOMe were resolved at pH 4.0 overlap of several isomers was observed. Only slightly decreasing R_S values were found for the enantiomeric tripeptides Gly–α-L-Asp–L-$PheNH_2$/Gly–α-D-Asp–D-$PheNH_2$ and Gly–β-L-Asp–L-$PheNH_2$/Gly–β-D-Asp–D-$PheNH_2$ with increasing pH. In contrast, Gly–α-L-Asp–D-$PheNH_2$/Gly–α-D-Asp–L-$PheNH_2$ and Gly–β-L-Asp–D-$PheNH_2$/Gly–β-D-Asp–L-$PheNH_2$ were not separated at pH 4.0. Maximum resolution was observed at pH 6.5 and 5.25, respectively. The simultaneous separation of all stereoisomers of the di- and tripeptides also occurred at pH 7. At this pH the peptides have a slight negative charge as they display a negative electrophoretic mobility (Fig. 2) and CM-β-CD is also negatively charged.

The dependence of the resolution of the enantiomers of α/β-Asp–PheOMe and Gly–α/β-Asp–$PheNH_2$ in polyacrylamide-coated capillaries on the concentration of CM-β-CD at pH 5.25 is summarized in Fig. 7. While acceptable migration times of about 60 min for the last migrating enantiomer of α/β-Asp–PheOMe were observed at CM-β-CD concentrations as low as 10 mg/ml, extremely long analysis times for the tripeptide (>60 min for the first migrating enantiomer) were noted at concentrations below 30 mg/ml. Thus, the stereoisomer separation of Gly–α/β-Asp–$PheNH_2$ was not studied below 30 mg/ml CM-β-CD. Generally, increasing concentrations of CM-β-CD led to a decrease of the resolution except for the enantiomeric tripeptides Gly–α-L-Asp–D-$PheNH_2$/Gly–α-D-Asp–L-$PheNH_2$ and Gly–β-L-Asp–D-$PheNH_2$/Gly–β-D-Asp–L-$PheNH_2$ which displayed a slight

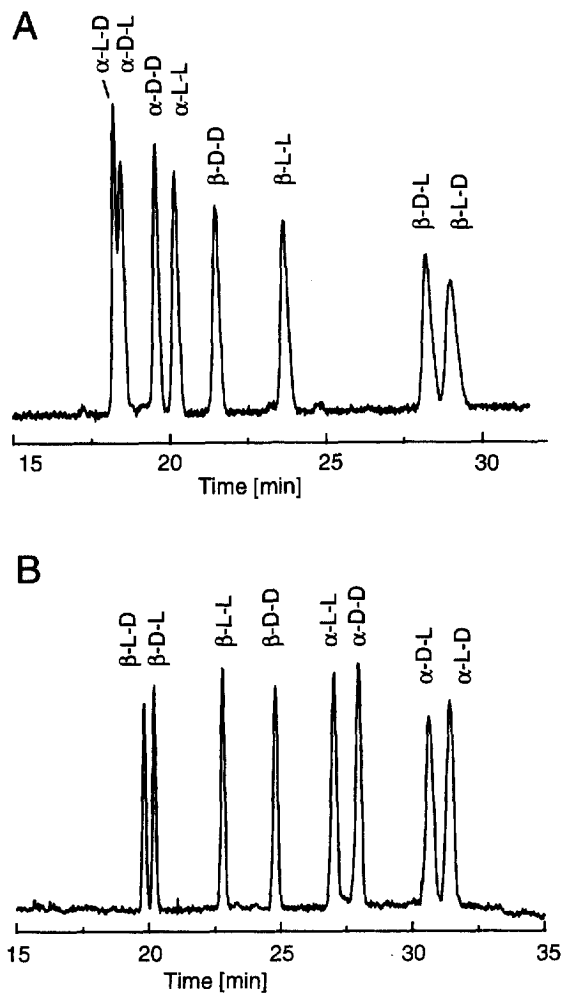

Fig. 5. Chiral separation of the isomeric tripeptides Gly–α-Asp–$PheNH_2$ and Gly–β-Asp–$PheNH_2$ in (A) fused-silica and (B) polyacrylamide-coated capillaries. Conditions: (A) 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 5.25, 25 mg/ml CM-β-CD, +25 kV; (B) 47/40-cm polyacrylamide-coated capillary, 50 m*M* sodium phosphate buffer, pH 5.25, 60 mg/ml CM-β-CD, −20 kV.

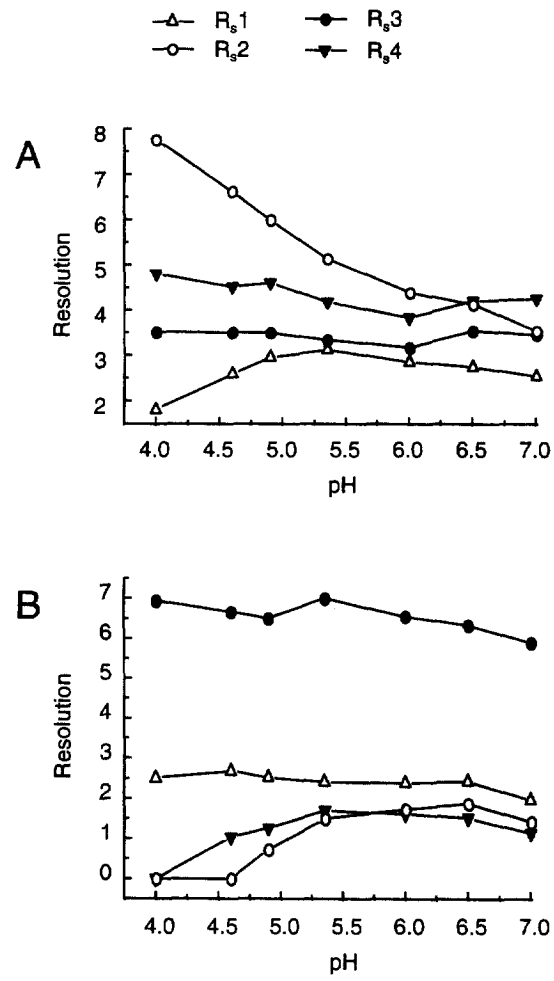

Fig. 6. Plot of the resolution (R_S) of the enantiomeric pairs of (A) α/β-Asp–PheOMe and (B) Gly–α/β-Asp–$PheNH_2$ between pH 4 and 7 in polyacrylamide-coated capillaries using 60 mg/ml CM-β-CD. Experimental conditions same as Fig. 5B. R_S 1, α-L-L and α-D-D; R_S 2, α-L-D and α-D-L; R_S 3, β-L-L and β-D-D; R_S 4, β-L-D and β-D-L.

increase. Analysis of the electrophoretic mobility, the separation selectivity, calculated as the ratio of the observed mobilities, and the number of theoretical plates, revealed a general decrease of the selectivities while the mobilities and the plate numbers increased initially and leveled off at approximately 60–70 mg/ml CM-β-CD. As net result a decrease of the resolution is observed except for the enantiomeric pairs of the tripeptide mentioned above. This is in contrast to the CD concentration dependence of the resolution observed previously for α/β-Asp–PheOMe in untreated fused-silica capillaries [6]. An initial increase of the resolution of the enantiomers upon increasing concentrations of CM-β-CD with a maximum has been observed in this study as a result of increasing selectivities, decreasing mobilities and largely unchanged plate numbers [6].

The simultaneous separation of all stereoisomers of Phe–α/β-Asp–$GlyNH_2$ was not achieved at pH

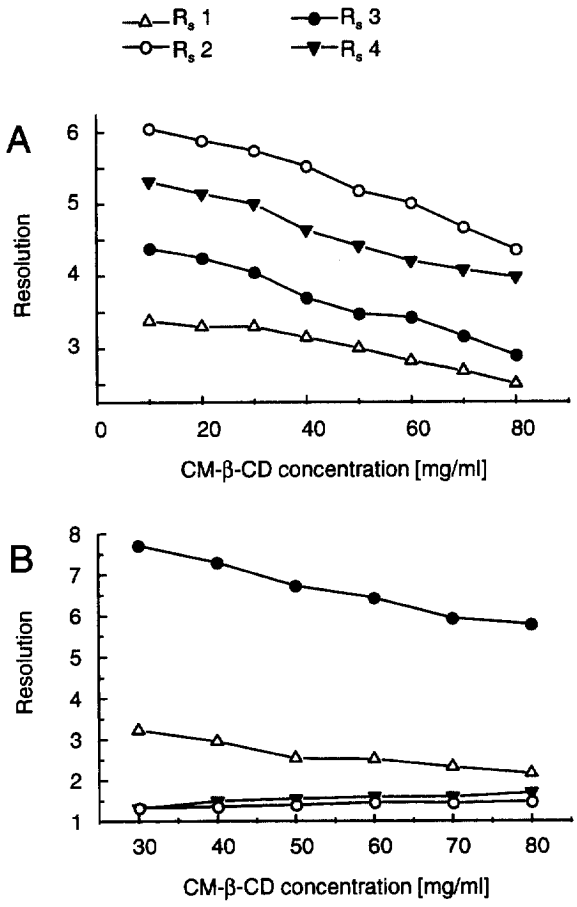

Fig. 7. Plot of the resolution (R_S) of the enantiomeric pairs of (A) α/β-Asp–PheOMe and (B) Gly–α/β-Asp–$PheNH_2$ versus the concentration of CM-β-CD in polyacrylamide-coated capillaries. Experimental conditions same as Fig. 5B. R_S 1, α-L-L and α-D-D; R_S 2, α-L-D and α-D-L; R_S 3, β-L-L and β-D-D; R_S 4, β-L-D and β-D-L.

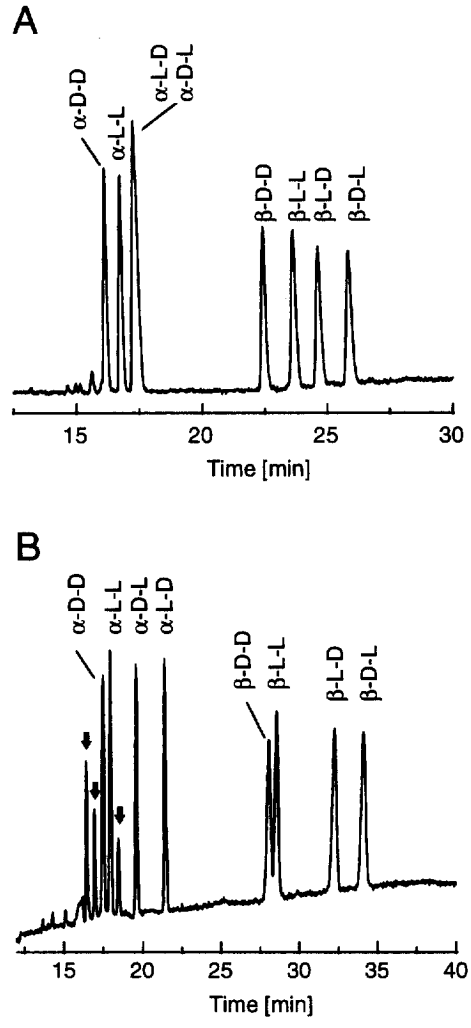

Fig. 8. Chiral separation of the tripeptides (A) Gly–α/β-Asp–$PheNH_2$ and (B) Phe–α/β-Asp–$GlyNH_2$. The arrows indicate synthetic impurities. Conditions: (A) 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 2.6, 25 mg/ml CM-β-CD, +25 kV; (B) 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 2.9, 32 mg/ml CM-β-CD, +23 kV.

5.25 either in an uncoated capillary or a coated capillary using CM-β-CD. Only the diastereomers were resolved (data not shown). Thus, reversing the amino acid sequence from Gly–α/β-Asp–$PheNH_2$ to Phe–α/β-Asp–$GlyNH_2$ changed the chiral recognition ability of CM-β-CD towards the enantiomers of the two peptides. However, except for the enantiomeric pair Gly–α-L-Asp–D-$PheNH_2$/Gly–α-D-Asp–L-$PheNH_2$ all stereoisomers of both tripeptides could be resolved at pH 2.6 and pH 2.9, respectively, when the peptides bear an overall positive charge and, thus, a positive electrophoretic mobility (Fig. 8). At these pH values CM-β-CD is slightly negatively charged [26] and migrates slowly towards the anode. It is most interesting to note that, compared to the electropherogram at pH 5.25 (Fig. 5A), the migration order of the enantiomeric pair Gly–β-L-Asp–D-$PheNH_2$/Gly–β-D-Asp–L-$PheNH_2$ is not reversed at pH 2.6 (Fig. 8A). The weaker complexed enantiomer at pH 2.6 when CM-β-CD bears only a slight negative charge while the peptide is positively charged appears to be complexed stronger at pH

5.25, the isoelectric point of the peptide, where CM-β-CD is negatively charged. Further studies of this phenomenon are currently in progress.

All stereoisomers of Gly–α/β-Asp–$PheNH_2$ could also be resolved at pH 2.7 using either SBE(4)-β-CD or SBE(7)-β-CD at a concentration of 4.5 mg/ml (Fig. 9A,C). Due to the high negative charge of both sulfobutylated CDs, even in the low pH range resulting in a high electrophoretic mobility in the other direction than the positively charged peptides, lower concentrations of these CDs are required compared to CM-β-CD. The high self-mobility of the CDs towards the anode is also the reason that a separation of the peptide enantiomers was not achieved at pH 5.25 in fused-silica capillaries within a reasonable period of time for the analysis. Using coated capillaries and exploiting SBE(4)-β-CD or SBE(7)-β-CD as carrier for the analytes towards the detector at the anodic end under reversed polarity of the applied voltage the resolution of the stereoisomers of Gly–α/β-Asp–$PheNH_2$ was obtained (Fig. 9B,D) except for the diastereomeric pair Gly–α-L-Asp–D-$PheNH_2$/Gly–α-L-Asp–L-$PheNH_2$ which comigrate under these conditions. Compared to the respective assay in untreated fused-silica capillaries, a reversal of the migration order was also observed.

4. Conclusions

The simultaneous separation of the stereoisomers of Asp dipeptides and tripeptides including the isomeric β-Asp peptides can be achieved using CM-β-CD and two SBE-β-CDs with different degrees of substitution in untreated fused-silica capillaries and polyacrylamide-coated capillaries. The negatively charged CDs offer a high flexibility concerning the pH of the run buffer and the option of reversing the migration order of the enantiomers. Moreover, these

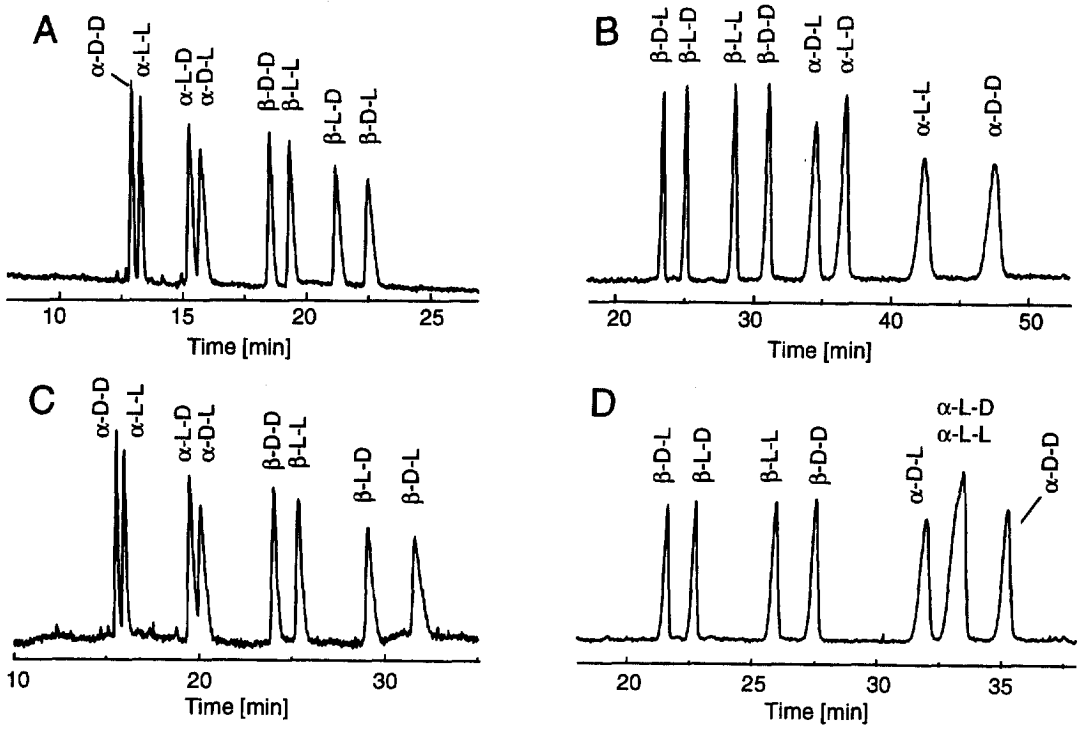

Fig. 9. Chiral separation of the isomeric tripeptides Gly–α/β-Asp–$PheNH_2$ using (A,B) SBE(4)-β-CD and (C,D) SBE(7)-β-CD as chiral selectors. Conditions: (A,C) 47/40-cm fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 2.7, 4.5 mg/ml SBE(4)-β-CD (A) or SBE(7)-β-CD (C), +23 kV; (B,D) 47/40-cm polyacrylamide-coated capillary, 50 m*M* sodium phosphate buffer, pH 5.25, 50 mg/ml SBE(4)-β-CD (B) or 50 mg/ml SBE(7)-β-CD (D), −23 kV.

CDs allow the stereoisomer separation of the peptides in their charged as well as their uncharged form. Thus, charged CDs also possess advantages for the enantioseparation of small peptides as described for other classes of compounds.

Acknowledgements

The donations of L-Asp–L-PheOMe by NutraSweet (Zug, Switzerland), β-CD, CM-β-CD and SBE(7)-β-CD by Wacker Chemie (Munich, Germany), SBE(4)-β-CD by Dr J.F. Stobaugh (University of Kansas, Lawrence, KS, USA) as well as the financial support by Fonds der Chemischen Industrie are gratefully acknowledged. S. Sabah was supported by a stipend of the Friedrich Ebert Foundation.

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