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# pH-Dependent reversal of the chiral recognition of tripeptide enantiomers by carboxymethyl-β-cyclodextrin

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**Abstract**

The pH-dependent reversal of the chiral recognition pattern of the complexation between a cyclodextrin host and analyte enantiomers is a new phenomenon which was studied by capillary electrophoresis. Using carboxymethyl-β-cyclodextrin (CM-β-CD) as selector for the chiral separation of the isomeric tripeptides Gly–α/β-D/L-Asp–D/L-PheNH$_2$, the migration order of the enantiomeric pair Gly–β-L-Asp–D-PheNH$_2$/Gly–β-D-Asp–L-PheNH$_2$ was reversed at pH 5.25 compared to pH 3.60 while the migration order of the other peptide epimers was not affected by varying the pH of the run buffer. The apparent binding constants between CM-β-CD and the enantiomers Gly–β-L-Asp–D-PheNH$_2$/Gly–β-D-Asp–L-PheNH$_2$ as determined by CE were 45.2 and 49.8 $M^{-1}$ at pH 3.60 and 38.8 and 38.4 $M^{-1}$ at pH 5.25, respectively, demonstrating the effect of the pH on the stereoselective complexation. The pH-dependent reversal of the chiral recognition is a new phenomenon in CD chemistry which might be applied in some specific cases for a reversal of the enantiomer migration order in CE. 

*Keywords:* Enantiomer separation; pH Effects; Complexation constants; Peptides; Cyclodextrins

## 1. Introduction

Chiral recognition between a chiral host and a chiral solute is an important phenomenon in enzymatic and chemical catalysis as well as analytical separation techniques, such as gas chromatography, high-performance liquid chromatography, supercritical fluid chromatography and capillary electrophoresis (CE).

Cyclodextrins (CD) are the most widely used chiral selectors in CE [1–3]. The separation principle is based on the formation of transient diastereomeric complexes between the CD host and a chiral solute. The stabilities of the complexes determine the migration order of the enantiomers in a given system. In chiral CE a reversal of the enantiomer migration order is an important issue. In contrast to chromatographic techniques, where peak tailing is usually observed, peak fronting as well as peak tailing can be observed in CE. Depending on the migration of the minor component compared to the migration of the major component it is often desirable to change the migration order for lowering the detection limit and the limit of quantitation [4,5]. This is especially true when only small mobility differences between the impurity and the major component are observed.

Several techniques which were applied for a reversal of the enantiomer migration order in CE have been summarized in a recent review [6] and a

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book chapter [7]. Reversal of the migration order can be achieved due to (1) opposite chiral recognition when using different cyclodextrins (CD) [5,8,9] or chiral micelles with opposite configuration [10,11], (2) a combination of different chiral selectors [12], and (3) the concentration of a chiral selector [13,14]. Further techniques exploit (4) the pH-dependent mobility of chiral selectors by using a charged and an uncharged CD which possess the same chiral recognition towards an analyte [9] or by varying the electrophoretic mobility of a charged CD by changing the pH of the run buffer while at the same time not affecting the charge of the analyte [5,9]. (5) The pH-dependent mobility of the chiral solute can result in a different migration order of the analyte enantiomers, when it can be analyzed as cationic and anionic species [15,16], or (6) by exploiting the carrier ability of a charged selector and reversing the polarity of the applied voltage [17]. The migration order can be altered (7) by a change, i.e. elimination or reversal, of the electroosmotic flow (EOF) [5,18]. Moreover, (8) reversal has been observed upon addition of achiral micelles or organic solvents to a chiral separation system [19–21].

Studying the simultaneous chiral resolution of isomeric α-Asp and β-Asp peptides using carboxymethyl-β-cyclodextrin (CM-β-CD) as chiral selector in fused-silica capillaries we observed a reversal of the migration order of the enantiomeric pair Gly–β-L-Asp–D-$PheNH_2$/Gly–β-D-Asp–L-$PheNH_2$ upon changing the pH of the run buffer from 2.60 to 5.25 [22]. The migration order of other enantiomeric pairs of the tripeptide Gly–α/β-D/L-Asp–D/L-$PheNH_2$ was not affected by the change of the pH. Apparently, Gly–β-D-Asp–L-$PheNH_2$ which formed weaker complexes with CM-β-CD than Gly–β-D-Asp–L-$PheNH_2$ at pH 2.60 was the preferentially complexed enantiomer at pH 5.25. This phenomenon is different from the mobility-based techniques of the reversal of the enantiomer migration order discussed in the above paragraph and represents the first example of a pH-dependent reversal of the chiral recognition ability of a CD. To the best of our knowledge, this pH-dependency has not been described in the literature to date. Therefore, the present study was conducted in order to investigate this new phenomenon in CD chemistry by CE in more detail.

## 2. Experimental

### 2.1. Chemicals

Carboxymethyl-β-cyclodextrin (CM-β-CD, average substitution degree 3.5) was from Wacker Chemie (Munich, Germany). Diastereomeric mixtures as well as the enantiomerically pure tripeptides were prepared in solution using Z- or BOC-protected amino acids and *N*-(3-dimethylaminopropyl)-*N*-ethylcarboximide as coupling reagent as described [23]. All other chemicals were of analytical grade. Buffers and sample solutions were prepared in double-distilled, deionized water, filtered (0.47 μm), and degassed by sonication.

### 2.2. Instrumentation

All experiments were performed on a Beckman P/ACE 5510 (Beckman Instruments, Waldbron, Germany) equipped with a diode array detector at 20°C using 50 μm I.D. polyacrylamide-coated capillaries prepared according to Hjerten [24]. The absence of the EOF was monitored with mesityl oxide. Only capillaries that did not display any measurable EOF at pH 7.0 during 2 h were used. The effective length of the capillaries was 40 cm, the total length was 47 cm. UV-detection at 215 nm was performed at the anodic end. Sample solutions were introduced at the cathodic end 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 CM-β-CD. The ionic strength of the buffer was $\mu=0.026$. Between the analyses the capillaries were washed for 2 min with 50 m*M* phosphate buffer without CD and for 2 min with the run buffer. The migration order of the peptide enantiomers was confirmed by spiking with the enantiomerically pure individual peptides. For the determination of the binding constants each sample was analyzed three times and the mean of the migration times was used for the calculations.

### 2.3. Buffer viscosity measurements

The viscosity of the buffers was determined using a capillary viscosimeter (Schott-Geräte, Hofheim,

Germany) which was thermostated at 20°C. Each determination was repeated six times. The time of flow ($t$, in s) was multiplied by the constant of the viscosimeter ($k$=0.0102 $mm^2/s^2$) and the pycnometrically determined density ($\rho$, in $g/cm^3$) to give the dynamic viscosity $\eta = kt\rho$ (mPa s).

## 3. Results and discussion

The separation of the isomeric tripeptides Gly–α/β-Asp–$PheNH_2$ in polyacrylamide-coated capillaries at pH 3.60 and 5.25 using CM-β-CD as chiral selector is shown in Fig. 1. As observed for the resolution of the peptides in uncoated fused-silica capillaries [22] at pH 2.60 and pH 5.25, respectively, the migration order is reversed for the enantiomeric pair Gly–β-L-Asp–D-$PheNH_2$/Gly–β-D-Asp–L-$PheNH_2$ in coated capillaries at pH 3.60 and 5.25, while the migration order of the other peptide stereoisomers is unchanged. Compared to the analysis in untreated fused-silica capillaries [22], the migration order at the respective pH values is reversed for all peptide stereoisomers except for the pair Gly–β-L-Asp–D-$PheNH_2$/Gly–β-D-Asp–L-$PheNH_2$. The migration behavior of Gly–β-L-Asp–D-$PheNH_2$ and Gly–β-D-Asp–L-$PheNH_2$ between pH 3.60 and pH 5.25 is summarized in Fig. 2. The peak resolution decreased with increasing pH leading to comigration of the peptide enantiomers at pH 4.10. Further increase of the buffer pH resulted in an increase of the resolution but with the opposite migration order of the enantiomers.

Differences in the binding constants between the peptide enantiomers and the CD result in a chiral separation. In coated capillaries the EOF is suppressed and the charged CD is employed as a carrier for the analytes. Under these conditions the preferentially complexed enantiomer migrates faster than the less complexed enantiomer. At pH 3.60, when the peptide is positively charged [22] while CM-β-CD bears a negative charge Gly–β-D-Asp–L-$PheNH_2$ is complexed stronger than Gly–β-L-Asp–D-$PheNH_2$ and, thus, migrates faster. In contrast, at pH 5.25 the isoelectric point of the peptide [22] Gly–β-D-Asp–L-$PheNH_2$ forms a weaker complex than Gly–β-L-Asp–D-$PheNH_2$. This results in a reversal of the migration order of these peptide enantiomers. The

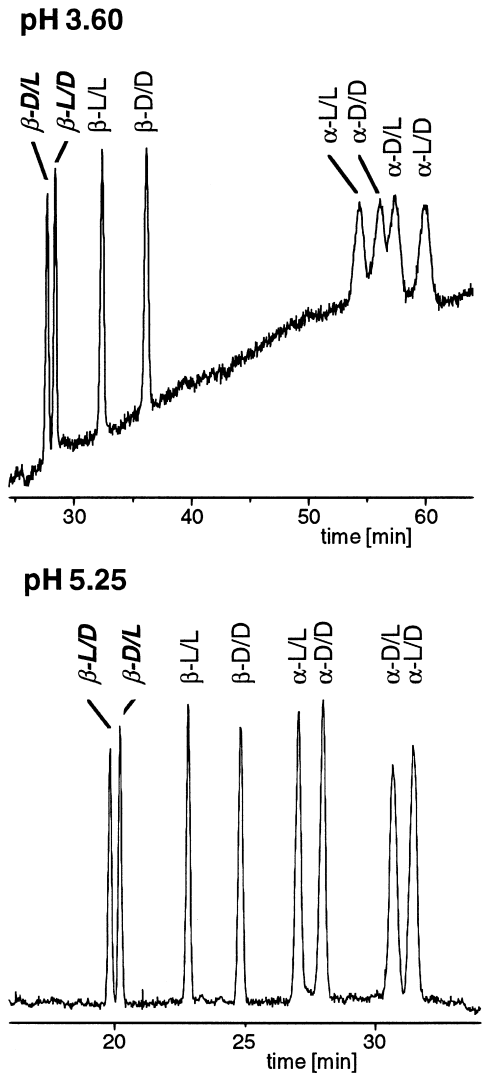


Fig. 1. Separation of the stereoisomers of the isomeric tripeptides Gly–α/β-D/L-Asp–D/L-$PheNH_2$ in polyacrylamide-coated capillaries at pH 3.60 and 5.25. Conditions: 47/40 cm capillary, 50 m*M* sodium phosphate buffer, 60 mg/ml CM-β-CD, −20 kV.

stereoselectivity of the binding between CM-β-CD and the enantiomeric pair Gly–β-L-Asp–L-$PheNH_2$/Gly–β-D-Asp–D-$PheNH_2$ is not affected by the pH of the run buffer as their migration order does not change. The same is true for the α-isomers of the tripeptide (Fig. 1). The migration order of all peptide stereoisomers in coated capillaries at pH 7.0 when they are negatively charged [22] is identical to the migration order at pH 5.25 (data not shown).

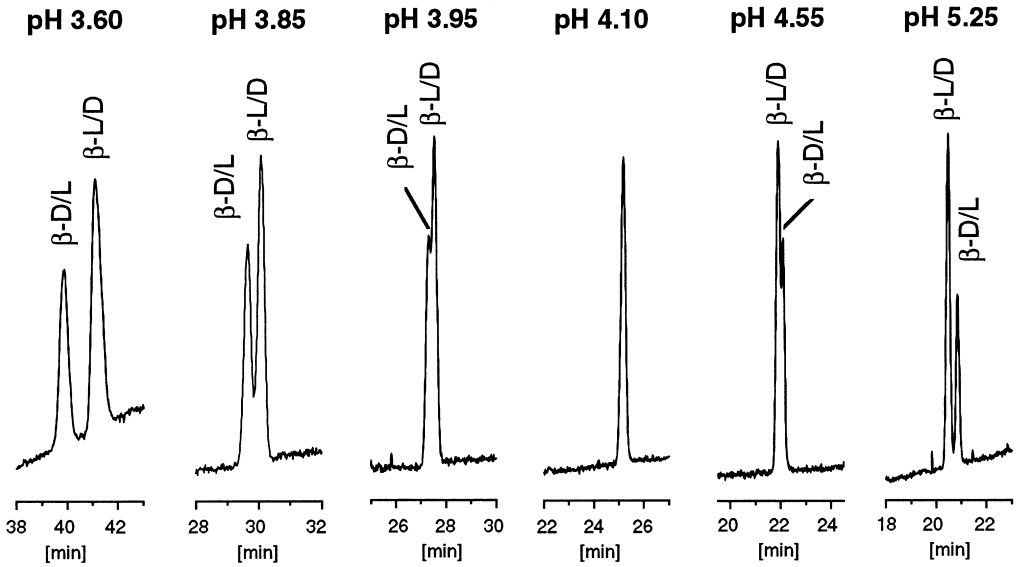


Fig. 2. pH-Dependent migration order of the tripeptide enantiomers Gly–β-L-Asp–D-$PheNH_2$ and Gly–β-D-Asp–L-$PheNH_2$. Conditions: 47/40 cm capillary, 50 m*M* sodium phosphate buffer, 60 mg/ml CM-β-CD, −20 kV.

The complexation constants, *K*, between CM-β-CD and Gly–β-D/L-Asp–D/L-$PheNH_2$ stereoisomers at pH 3.60 and 5.25 were determined by CE using Eq. (1) assuming a 1:1 complexation [25]:

$$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)K} \cdot \frac{1}{[CD]} + \frac{1}{(\mu_c - \mu_f)} \qquad (1)$$

where $\mu_i$ is the mobility of the analyte at a given CD concentration, $\mu_f$ and $\mu_c$ are the mobilities of the free and complexed analyte, respectively. [CD] is the CM-β-CD concentration in the buffer. The mobilities of the analytes were corrected for the increasing viscosity of the buffers due to the increasing concentration of CM-β-CD according to Eq. (2) [26]:

$$\mu_i = \mu_{app} \cdot \frac{\eta_c}{\eta_0} \qquad (2)$$

where $\mu_{app}$ is the observed mobility and $\eta_c$ and $\eta_0$ are the viscosity of buffer with CD and without CD, respectively. One advantage of coated capillaries for the determination of complexation constants is the fact that due to the suppressed electroosmotic flow (EOF) the mobility of the EOF does not have to be considered. At pH 5.25 the peptides are uncharged and have no electrophoretic mobility, i.e., $\mu_f = 0$. At pH 3.60, the peptides are positively charged and possess a self-mobility towards the cathode, i.e., directed oppositely to the migration of CM-β-CD. The mobility of the free analytes, $\mu_f$, was determined in CD-free buffer injecting at the anode and detecting at the cathode. $\mu_f$ of the enantiomeric pair Gly–β-L-Asp–D-$PheNH_2$/Gly–β-D-Asp–L-$PheNH_2$ was $2.7\times10^{-5}$ $cm^2/Vs$, $\mu_f$ of the pair Gly–β-L-Asp–L-$PheNH_2$/Gly–β-D-Asp–D-$PheNH_2$ was $3.1\times10^{-5}$ $cm^2/Vs$.

The plots of the reciprocal CM-β-CD concentration versus the reciprocal mobility $(\mu_i - \mu_f)$ of the enantiomers of Gly–β-D/L-Asp–D/L-$PheNH_2$ at pH 3.60 and 5.25 are shown in Fig. 3. Linear relationships with $r > 0.995$ were obtained indicating a 1:1 complexation. The apparent complexation constants are summarized in Table 1. The constants of the α-epimers were not determined. Commercial CM-β-CD is a mixture of isomers with different substitution degrees. Moreover, the exact ionization status is unknown so that the ionic strength of the buffers cannot be determined. Therefore, the numbers in Table 1 are apparent constants averaged for all CD isomers rather than thermodynamic constants. Generally, the binding constants decrease with increasing pH. At pH 3.60, Gly–β-D-Asp–L-$PheNH_2$ is complexed stronger than Gly–β-L-Asp–D-$PheNH_2$ while

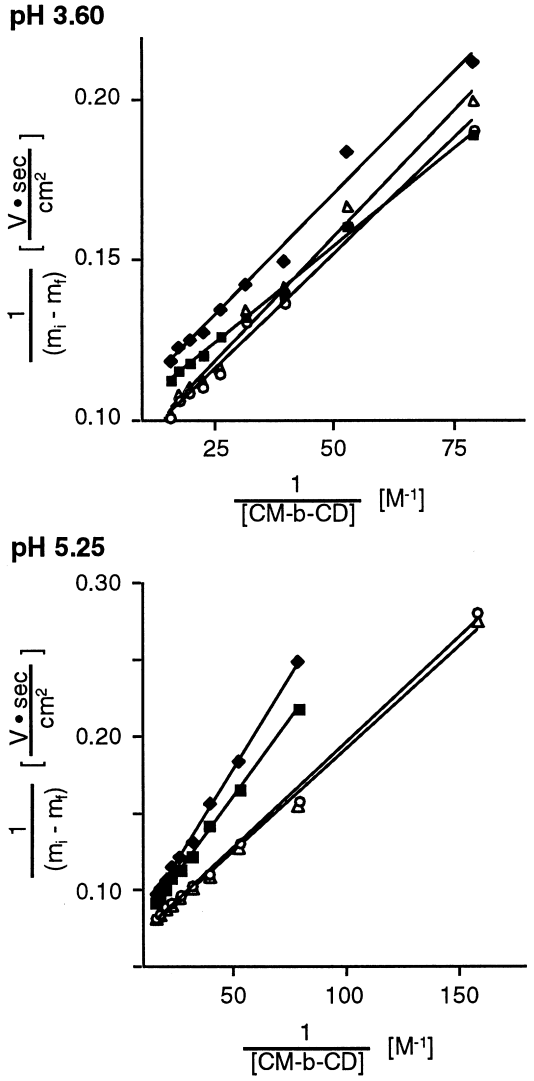


Fig. 3. Plots $1/[\text{CM-}\beta\text{-CD}]$ versus $1/(\mu_i - \mu_f)$ of Gly–β-L/D-Asp–L/D-$PheNH_2$ enantiomers at pH 3.60 and 5.25. (■) Gly–β-L-Asp–L-$PheNH_2$; (◆) Gly–β-D-Asp–D-$PheNH_2$; (△) Gly–β-L-Asp–D-$PheNH_2$; (○) Gly–β-D-Asp–L-$PheNH_2$

Table 1
Apparent complexation constants between CM-β-CD and Gly–β-D/L-Asp–D/L-$PheNH_2$ stereoisomers at pH 3.60 and 5.25

| Peptide | pH 3.60 | pH 5.25 |
|---|---|---|
| Gly–β-L-Asp–D-$PheNH_2$ | 45.2 | 38.8 |
| Gly–β-D-Asp–L-$PheNH_2$ | 49.8 | 38.4 |
| Gly–β-L-Asp–L-$PheNH_2$ | 68.4 | 27.0 |
| Gly–β-D-Asp–D-$PheNH_2$ | 55.6 | 22.2 |

The values are expressed in $M^{-1}$.

it forms weaker complexes at pH 5.25 than Gly–β-L-Asp–D-$PheNH_2$. This clearly proves a pH-dependent reversal of the chiral recognition of CM-β-CD towards both peptide enantiomers. In contrast, Gly–β-L-Asp–L-$PheNH_2$ is bound stronger than Gly–β-D-Asp–D-$PheNH_2$ at both pH values.

It is interesting to note that the β-L-L and β-D-D stereoisomers form stronger complexes but migrate after the β-D-L and β-L-D epimers. From the absolute values of the constants one would expect a different migration order as the stronger bound analytes should migrate first under the applied CE conditions. However, the higher mobility of uncomplexed Gly–β-L-Asp–L-$PheNH_2$/Gly–β-D-Asp–D-$PheNH_2$ in the opposite direction than CM-β-CD counteracts the stronger complexation. As net result the β-L-L and β-D-D stereoisomers migrate after the β-D-L and β-L-D epimers.

Three principles for a pH-dependent reversal of the enantiomer migration order have been described in the literature. The first is based on the mobility difference of complexed solutes which can be analyzed in their cationic and anionic form [15,16]. Neutral CD derivatives are used in this case. Second, the electrophoretic mobility of a chargeable CD can be varied by changing the pH of the run buffer while at the same time not affecting the charge of the analyte [5,9]. The third principle makes use of chargeable CDs in their uncharged form at low pH normal polarity of the applied voltage and exploiting their carrier ability in the charged form at higher pH and reversed polarity of the voltage [17]. However, these principles require that the chiral recognition of the CDs towards the enantiomers is unchanged. Thus, the present results are the first example of a new principle based on the pH-dependent reversal of the stereoselectivity of the complexation.

Vigh and co-workers developed theoretical models for enantiomer resolutions of acidic and basic electrolytes as well as neutral solutes using neutral and charged selectors [27–29]. The theories also predict that the migration order of enantiomers can be reversed by varying the pH of the run buffer. However, these models are primarily based on mobilities and do not predict a change of the stereoselectivity of the complexation between selectors and solutes as observed for CM-β-CD and the peptide enantiomers Gly–β-L-Asp–D-$PheNH_2$ and

Gly–β-D-Asp–L-PheNH$_2$. Generally, the reversal of the chiral recognition by a CD cannot be predicted at present. This is further illustrated by the fact that the reversal could be observed for the β-D-L and β-L-D enantiomers, but not for the enantiomeric pair β-L-L and β-D-D. Further studies on the chiral recognition of peptides by CDs are currently in progress.

In conclusion, this study describes for the first time the pH-dependent reversal of the chiral recognition pattern of the complexation between a CD and a chiral analyte. This is a new phenomenon in CD chemistry which can be applied as a further pH-dependent principle for a reversal of the migration order of enantiomers in CE.

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