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# Short Communication

# High-performance liquid chromatographic separation of *cis–trans* isomers of proline-containing peptides

## I. Separation on cyclodextrin-bonded silica

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ABSTRACT

β-Cyclodextrin-bonded silica is shown to be a suitable stationary phase for high-performance liquid chromatography of conformational isomers of proline-containing peptides. A variety of selective interactions may be used to separate *cis–trans* conformers by steric discrimination. The formation of an inclusion complex seems to be particularly effective if an aromatic amino acid N-terminal-bonded to proline is enclosed in the analyte. Unusually high resolution values for such separations under low-temperature conditions suggest a steric hindrance of isomer conversion owing to the formation of inclusion complexes.

## INTRODUCTION

*cis–trans* Isomerism of the prolyl peptide bond seems to be of central importance for the folding and biological activity of various oligopeptides and small proteins [1]. Enzymes exhibiting peptidyl prolyl *cis–trans* isomerase (PPIase) activity efficiently catalyse the *cis–trans* isomerization of proline imidic peptide bonds [2,3]. In order to investigate the enzymatic catalysis of conformational changes applying pure conformers instead of mixtures of *cis–trans* short peptides with C-terminal proline or N-alkylamino acid can be utilized to study the separation of conformers. Such oligopeptides show particularly high barriers of rotation of the peptide bond [4,5]. In the equilibrium the *trans* conformation is favoured in most peptides, but the *cis* conformers occur in detectable amounts. Quantitative data about this type of conformational interconversion could be obtained by different spectroscopic and kinetic methods [3,6–10].

Reversed-phase high-performance liquid chromatographic (HPLC) studies of di- and oligopeptides with C-terminal proline were published by Melander *et al.* [11] and others [12,13]. Chromatographic resolution of conformers by solvophobic interaction

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with the reversed-phase was described as a possible method for separating pure peptide bond isomers, but the relaxation times of conformational changes have to be comparable to the time scale of the HPLC run used.

It was assumed that the elution behaviour of *cis* and *trans* isomers is determined by the larger solvophobic surface area of the *cis* isomer, interacting with hydrocarbons bonded to silica gel. In relation to aqueous solution, the isomer distribution and the relaxation times of the isomerization may be seriously affected by the chromatographic conditions. Therefore, experimental selection of the optimum eluent composition, pH, ionic strength, system temperature and flow-rate is necessary for the successful separation of conformational isomers.

In this paper, we present an HPLC method which allows the separation of *cis* and *trans* isomers of proline-containing di- and oligopeptides by steric discrimination on cyclodextrin-bonded silica.

## EXPERIMENTAL

### *Materials*

Optically pure dipeptides were purchased from Bachem Biochemica (Heidelberg, Germany). Biologically active oligopeptides were synthesized by K. Neubert and co-workers at the Department of Biochemistry/Biotechnology, Martin-Luther University Halle, Germany. Chiral $\beta$-cyclodextrin Si 100 (10 $\mu$m) columns were obtained from Serva (Heidelberg, Germany). All other solvents and chemicals were of analytical-reagent grade.

### *Apparatus*

HPLC measurements were performed with a Merck–Hitachi LiChroGraph system using an L-6200 low-gradient pump, an L-3000 photodiode-array detector and an HM computing integrator. The columns and the eluents were immersed in a Lauda RM 6 constant-temperature bath.

### *HPLC conditions*

The columns, both 125 mm × 4.6 mm I.D., were connected. Chromatographic experiments were performed isocratically using various 0.02 *M* ammonium dihydrogenphosphate–acetonitrile mixtures. The analyte absorptions were monitored at 210 nm. The flow-rate was 2 ml/min. Before elution, the columns were equilibrated with the mobile phase at 5°C for 60 min. Peak splitting as a result of isomerization kinetics was demonstrated by peak collection and rechromatography of the fractions.

## RESULTS AND DISCUSSION

Cyclodextrin (CD)-bonded silicas as stationary phases in HPLC were developed for separating enantiomers by forming diastereomers with chiral centres of cyclodextrins. In addition to chiral applications, CD-bonded phases have been used as packings for the separation of polynuclear aromatics, substituted aromatics, stilbenes and structural isomers [14], and also as stationary phases for the separation of selected groups of di- and tripeptides [15,16].

The introduction of CD-bonded silicas for separating conformational isomers offers the opportunity to exploit a variety of selective interactions between peptides and cyclodextrins, such as host–guest and hydrophobic interactions, hydrogen bonding and dipole–dipole interactions. Using phosphate buffer of low ionic strength and pH values that force peptides into their zwitterionic form, a number of conformers of di-, tri- and tetrapeptides were resolved. To separate both conformers of Ala–Pro, Ile–Pro, Leu–Pro, Phe–Pro, Phe–D-Pro, Ile–Pro–Ile, Val–Pro–Leu, Tyr–Pro–Phe and Pro–D-Phe–Pro–Gly by inclusion complexation and hydrophobic interaction for each individual peptide, the organic solvent content has to be adjusted.

Lowering the temperature below ambient decreases the interconversion rates and can improve the resolution of isomer peaks. As demonstrated in Fig. 1, peptides bearing aromatic amino acids N-terminal to proline (phenylalanine or tyrosine) show baseline separations between the two conformers with relative resolution ($R_s$) values up to 5. The unusually high $R_s$ values and the absence of the typical plateau between the two isomer peaks, in contrast to reversed-phase chromatographic runs, suggest a steric hindrance of isomer conversion owing to the formation of inclusion complexes. In contrast to these results, peptides consisting of aliphatic amino acids N-terminal-bonded to proline show well resolved peaks without baseline resolution.

It seems that aromatic amino acids are essential

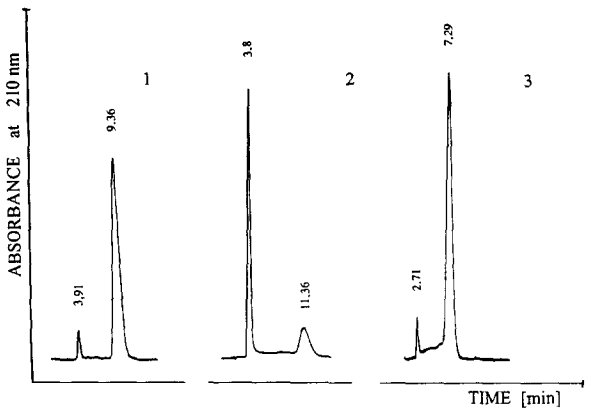


Fig. 1. Elution profiles of (1) Phe–Pro, (2) Tyr–Pro–Phe and (3) Pro–D-Phe–Pro–Gly on chiral β-cyclodextrin Si-100 at 5°C. For chromatographic conditions, see Experimental. Mobile phase: (1) 0.02 *M* ammonium dihydrogenphosphate (pH 6.2)–acetonitrile (90:10); (2) 0.02 *M* ammonium dihydrogenphosphate (pH 6.2)–acetonitrile (80:20); (3) 0.02 *M* ammonium dihydrogenphosphate (pH 6.2)–acetonitrile (70:30). Detector: 0.0128 a.u.f.s.

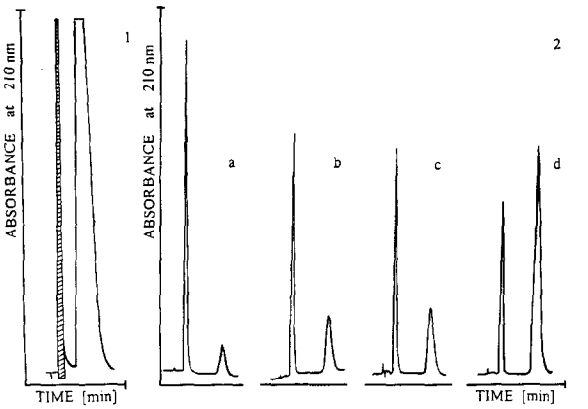


Fig. 2. Semi-preparative low-temperature fractionation of Phe–Pro on chiral β-cyclodextrin Si-100 (see Experimental). (1) Isolation of the minor isomer (shaded peak); injection 0.5 mg per 100 μl. (2) Rechromatography of the first-eluting peak from (1), corresponding to the minor isomer; reisomerization of this fraction as a function of incubation time and temperature. (a) 15 min, −10°C; (b) 45 min, −10°C; (c) 60 min, −10°C; (d) 90 min, 25°C. Detector: (1) 1.0 a.u.f.s.; (2) 0.01 a.u.f.s.

parts in the sequence to distinguish conformers by inclusion complexation. These results are in accordance with those of other workers [15,17,18], which suggested that the formation of an inclusion complex for exploiting the chiral recognition principle and a separation system for geometric isomers is particularly effective if an aromatic ring system is enclosed in the analyte.

In contrast to reversed-phase techniques, the elution pattern of conformer separations on CD-bonded silica may be affected by additional different interactions. Starting from the hypothesis that *cis* or *trans* isomers are selectively included in the CD cavity by steric discrimination, some of the observed phenomena, *e.g.*, elution order of *cis* and *trans* isomers and the *cis–trans* ratio of conformers, could be explained. Enzymatic and NMR spectroscopic studies of fractionated single isomers are in progress to establish the elution order.

In order to demonstrate that peak splitting was a result of dynamic equilibrium during the chromatographic run, fractions of effluents were collected. Using rechromatography in each instance the original chromatogram was obtained after relaxation of the conformer distribution, as demonstrated for Phe–Pro in Fig. 2. Refrigeration of the fractionated conformers offers the opportunity to delay conformer relaxation for a substantial period and allows the acquisition of nearly pure *cis* and *trans* isomers for further studies, *e.g.*, structural and enzymatic investigations.

In conclusion, low-temperature HPLC on CD-based silicas has been found to be applicable to the determination of conformational changes in proline-containing peptides and for the fractionation of *cis* and *trans* conformers as valuable substrates for advanced studies. Cyclodextrin additives should provide versatile chromatographic systems for the investigation of conformational changes in prolyl peptides. These applications are under investigation.

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