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Short Communication

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

II. Fractionation in different cyclodextrin systems

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ABSTRACT

β-Cyclodextrin-bonded silica is demonstrated to be a suitable stationary phase for high-performance liquid chromatography of conformational isomers of proline-containing peptides. In contrast to reversed-phase chromatography, the principle of inclusion complexation shows significant selectivities in conformer resolution based on a variety of interactions. New results of inclusion HPLC of biologically active oligopeptides related to β-casomorphin on stationary phases containing bonded cyclodextrins of different internal diameters indicate a steric discrimination process during the conformer separation. β-Cyclodextrin used as a mobile phase additive in reversed-phase systems is shown to offer the opportunity to investigate conformational changes using commercially available reversed-phase columns.

INTRODUCTION

Recently, we described the HPLC separation of *cis–trans* isomers of proline-containing oligopeptides on β-cyclodextrin (β-CD)-bonded silica [1].

Proline-containing peptides are unique in terms of their capacities to form peptide bond conformers. Xaa–Pro peptide bonds can adopt two different conformations (*cis* and *trans*), which can be simultaneously present in solution because of the energy barriers of rotation about the peptidyl–proline imidic bond [2,3]. Quantitative data describing this type of conformational interconversion can be obtained by different spectroscopic and kinetic methods [4–8].

Reversed-phase high-performance liquid chromatographic studies of di- and oligopeptides containing proline have been published by Melander *et al.* [9] and others [10,11]. Conformers were resolved by the solvophobic interaction of different hydrophobic surface areas of the *cis* and *trans* isomers with the hydrocarbons of reversed-phase silica gel. Low-temperature chromatography was introduced to diminish interconversion rates and to improve the resolution of isomer peaks, but the relaxation times of conformational changes have to be in the same time scale as the chromatographic runs. However, the comparatively small differences in hydrophobic surface areas result in inefficient chromatographic resolution of conformers.

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In contrast to reversed-phase chromatography, the inclusion principle shows significant selectivities in conformer separation caused by a variety of selective interactions, such as host–guest and hydrophobic interactions, hydrogen bonding and dipole–dipole interactions. Peptides bearing aromatic amino acids N-terminally bonded to proline have been shown to be separated with relative chromatographic resolution (R_s) values suggesting a steric hindrance of isomer interconversion.

In this paper chromatographic data of new proline-containing peptides of the β-casomorphin type will be presented, which demonstrate that β-CD-bonded silica is applicable as a stationary phase in HPLC for conformer separation of small oligopeptides in a common manner. Using stationary phases with bonded cyclodextrins of different internal diameters we demonstrate that peak splitting is in fact a result of inclusion complexation. Moreover, β-CD added to the mobile phase also offers the opportunity to use selective interactions between peptide structure and toroid shape of cyclodextrins.

EXPERIMENTAL

Materials

Optically pure dipeptides were purchased from Bachem Biochemica (Heidelberg, Germany) and β-cyclodextrin from Merck (Darmstadt, Germany). The β-casomorphin peptides were synthesized by conventional solution methods [12]. Their purity was checked by analytical TLC, HPLC, field desorption mass spectrometry and amino acid analysis.

Chiral α-, β- and γ-cyclodextrin Si-100 (10 μm) columns were purchased from Serva Feinbiochemica (Heidelberg, Germany) and 250-4 LiChroSpher RP-8 (10 μm) from Merck. All solvents and chemicals were of high purity.

Apparatus

HPLC measurements were performed with a Merck-Hitachi LiChroGraph system using a L-6200 low-gradient pump, a 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

Two chiral β- (α-, γ-) cyclodextrin Si-100 (10 μm) columns, 125 × 4.6 mm, were connected. Chromatographic experiments were performed isocratically using various 0.02 *M* ammonium dihydrogenphosphate–acetonitrile mixtures. The analyte absorptions were monitored at 210 nm. 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

In order to evaluate inclusion HPLC on β-CD-bonded silica columns for the study of conformational changes in the peptide bond, a number of dipeptides and oligopeptides related to β-casomorphins were investigated on cyclodextrins with different internal diameters.

β-Casomorphins represent compound with opioid activities that may be released from the milk protein β-casein by proteolytic fragmentation. The insertion of D-amino acids into the sequences improves the proteolytic stability of such peptides and partially significantly enhances and prolongs opioid activity [12]. Moreover, des-Tyr^1-β-casomorphin analogues [13], show several interesting behavioural pharmacological effects.

In order to investigate the influence of the configuration of the internal amino acids Phe and Pro on the *cis–trans* isomerization about the X–Pro bond (X = Phe, D-Phe) the four diastereomers of such des-Tyr^1-β-casomorphins, Pro–Phe–Pro–Gly (labelled as LL), Pro–D-Phe–Pro–Gly (DL), Pro–D-Phe–D-Pro–Gly (DD) and Pro–Phe–D-Pro–Gly (LD), were analysed using the same chromatographic conditions described in ref. 1. As shown in Fig. 1, we achieved baseline separations with high chromatographic resolution between the two conformers for all four peptides. In the case of DL and LD the small peak (about 10%) was eluted before the large peak (about 90%), whereas surprisingly the elution order for LL and DD was reversed and, thus, the larger peak (about 70%) was eluted ahead of the smaller one (30%). On the under-

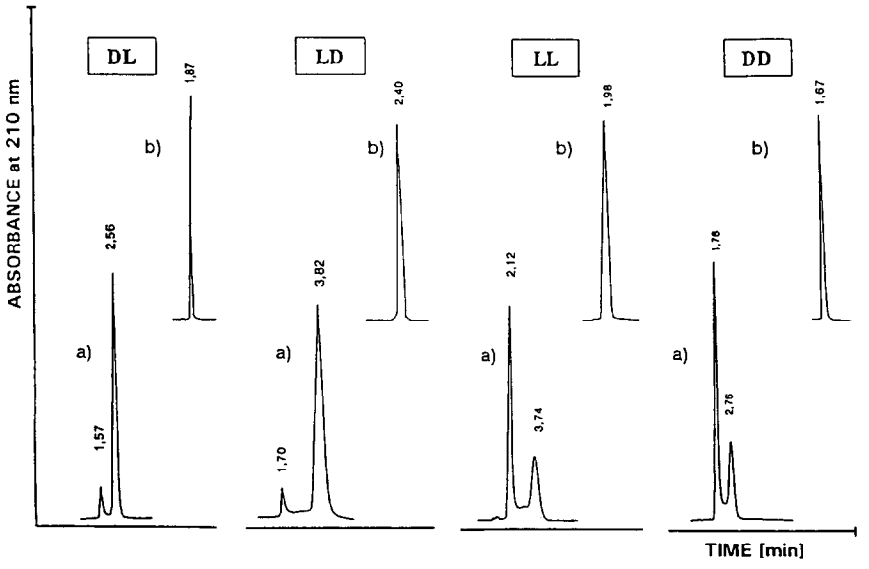

Fig. 1. Elution profiles of Pro–D-Phe–Pro–Gly (DL), Pro–Phe–D-Pro–Gly (LD), Pro–Phe–Pro–Gly (LL) and Pro–D-Phe–D-Pro–Gly (DD) on two connected 125 × 4.6 mm chiral β-cyclodextrin Si-100 (10 μm) columns: (a) at 2°C, (b) at room temperature. Mobile phase: 0.02 *M* ammonium dihydrogenphosphate (pH 6.2)–acetonitrile (70:30). Flow-rate: 3 ml/min.

standing that in each case the minor peak corresponds to the *cis* and the major one to the *trans* conformer, the calculated *cis–trans* ratio would be in agreement with data determined by NMR studies (*cis* content in aqueous solution: DL and LD about 10%, LL 35–40%, DD 25–30%) [13]. Molecular modelling studies are in progress in order to explore this phenomenon.

Furthermore, the high selectivity of β-CD silica as stationary phase for the study of conformational changes is demonstrated for β-casomorphin derivatives which contain two Xaa–Pro bonds in their sequence. In Fig. 2 the detected bonding isomers of these peptides are demonstrated. NMR spectroscopic studies of fractionated isomers are in progress to establish the elution order.

Starting from the hypothesis that the molecular dimension of bonded cyclodextrin can be adapted to the size and shape of the analyte, we tried to improve *cis–trans* isomer resolution by additional use of α- and γ-CD-bonded phases to allow isomer isolation of smaller and larger prolyl peptides.

We used an α-CD-bonded silica, formerly used for the separation of small peptide diastereomers composed of aliphatic amino acids [14], for the resolution of conformers of Xaa–Pro dipeptides (Xaa = Ala, Leu, Ile). The result was that only peak splitting without baseline separation was observed. Obviously, the cavity of the α-form is too small to include at least partially the investigated analytes.

γ-Cyclodextrin contains eight glucopyranose units, resulting in a larger internal diameter of 9.5 Å [15]. The corresponding silica was pos-

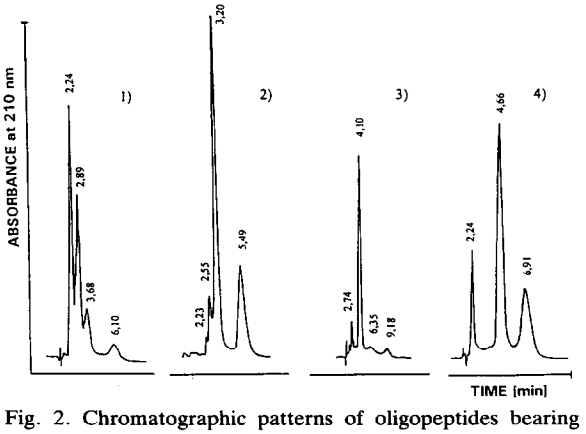

Fig. 2. Chromatographic patterns of oligopeptides bearing two Xaa–Pro bonds: Tyr–Pro–Phe–Pro–Gly (CM 5) (1), Phe–Pro–Phe–D-Pro–Gly (2), Tyr–D-Pro–Phe–Pro–NH_2 (3), Tyr–Pro–Phe–D-Pro–Gly (4). For chromatographic conditions see Fig. 1.

tulated to be suitable for enantiomer resolution of larger ring systems and cyclic peptides [16]. Using this stationary phase a number of oligopeptides of the β-casomorphin type were examined to be separated into *cis* and *trans* isomers. As shown in Fig. 3 our results demonstrate that γ-CD-bonded silica is, like β-CD, a suitable stationary phase for the study of conformational changes in proline-containing peptides, but in most cases the chromatographic resolution is poor compared with β-CD. The larger internal diameter enables analyte penetration into the hydrophobic cavity and, consequently, a solvophobic interaction, whereas additional interactions, such as hydrogen bonding and dipole–dipole interactions, seem to be less effective. Only pentapeptides, including β-casomorphin-5 (Tyr–Pro–Phe–Pro–Gly), are split into their *cis–trans* conformers with similar efficiency as on β-CD silica, in theory because the larger molecule fits better into γ-CD. However, for many oligopeptides, including cyclic peptides, the γ-CD-bonded stationary phase may represent an additional valuable tool to solve difficult separation problems.

Starting from the observed phenomenon that β-cyclodextrin-bonded silica gel shows the highest efficiency in separating peptide bond isomers, such as enantiomers, diastereomers and geometric and structural isomers, we showed that β-CD is a suitable mobile phase additive for reversed-phase systems. Several papers published recently describe the use of β-CD as a chiral selector in HPLC or capillary electrophoresis systems [17–20]. Small structural changes in analytes can be exploited by the interaction with the cyclodextrin toroid. Using reversed-phase systems published by Melander *et al.* [9] we investigated the effect of adding β-CD to the mobile phase on conformer resolution of the dipeptides Ala–Pro, Leu–Pro, Ile–Pro and Phe–Pro. The concentration of β-CD is limited by the poor solubility of the molecule. A 0.01 *M* β-CD solution proved to be the most suitable to take advantage of the β-CD–analyte interaction without interference on chromatographic devices. Fig. 4 demonstrates that *cis–trans* isomer resolution of dipeptides is noticeably improved if the mobile phase contains β-CD. The time scale of the interconversion rate decreases as a result of steric hindrance during inclusion complexation and, consequently, the flow-rate of mobile phase should not be as high

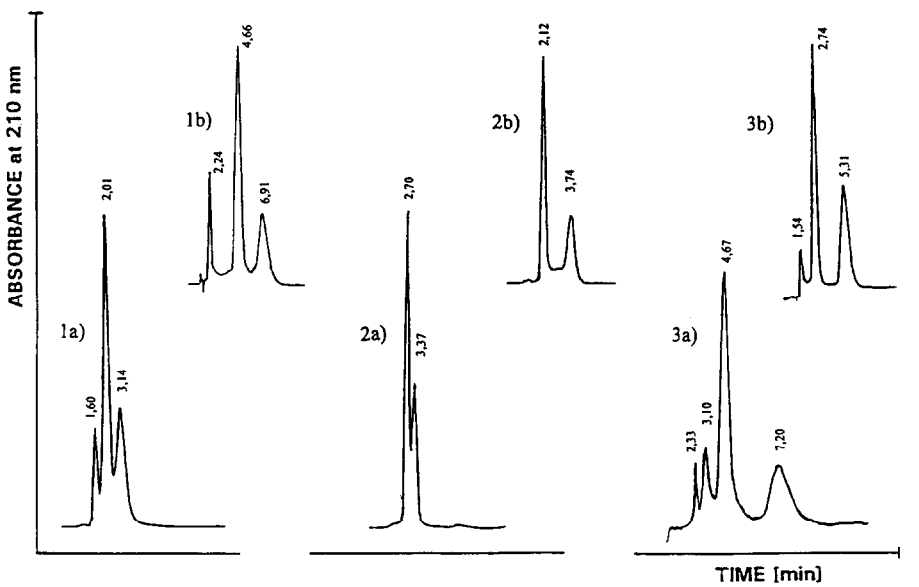

Fig. 3. Elution profiles of Tyr–Pro–Phe–Pro–Gly (1), Pro–Phe–Pro–Gly (2) and Phe–Pro–D-Phe–Pro–Gly (3). (a) On two connected 125 × 4.6 mm chiral γ-cyclodextrin Si-100 (10 μm) columns. Mobile phase: 0.02 *M* ammonium dihydrogenphosphate (pH 6.2)–acetonitrile (85:15). Flow-rate: 2 ml/min. (b) On two connected 125 × 4.6 mm chiral β-cyclodextrin Si-100 (10 μm) columns. For chromatographic conditions see Fig. 1. (1b) Flow-rate: 2 ml/min.

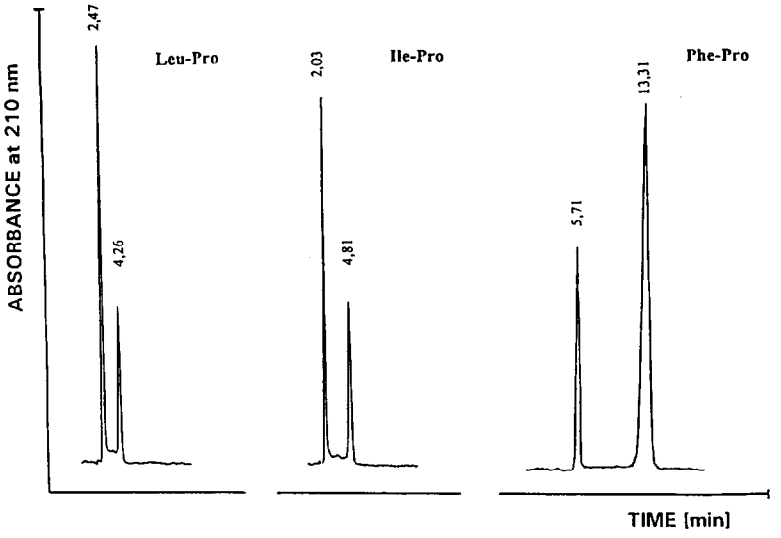

Fig. 4. Elution profiles of Leu–Pro, Ile–Pro and Phe–Pro on 250-4 LiChroChart, LiChroSpher 100 RP-8 (10 μm) at 5°C. Mobile phase: 0.02 *M* ammonium dihydrogenphosphate (pH 6.2)–methanol (95:5), 0.01 *M* β-cyclodextrin. Flow-rate: 2 ml/min.

as reported in reversed-phase systems. The typical plateaus between isomer peaks detected in reversed-phase systems disappear and the hydrophobic properties of peptides are shielded by the inclusion process. These features result in baseline separation and higher chromatographic resolution values, R_s. However, the process of inclusion of analytes in the cavity of β-CD can change seriously the conformer equilibrium in aqueous peptide solution. Thus, for example, in the case of Phe–D-Pro, we observed the complete disappearance of one isomer after several days, if the peptide was dissolved in the mobile phase containing β-CD. We separated only dipeptide conformers. Tetra- and pentapeptides were not investigated.

The application of β-CD as additive in mobile phase allows only the detection of conformational changes in prolyl peptides. The isolation of pure isomers is impossible because of the β-CD content in the fractions.

In conclusion, inclusion complexation with β-CD bonded to silica gel or dissolved in the mobile phase has been found to be a separation principle for the study of conformational changes in proline-containing peptides applicable in different HPLC systems. Separation of *cis–trans* peptide conformers on β-CD-bonded stationary phases has been established. The method is highly efficient, despite the fact that it requires a short time for the optimization of the chromatographic conditions, and allows conformer fractionation based on high chromatographic resolution. β-CD dissolved in the mobile phase of reversed-phase HPLC systems provides a versatile system for the investigation of *cis–trans* isomerism of the prolyl peptide bond using commonly available reversed-phase columns.

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