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SLOW ISOMERIZATION OF SOME PROLINE-CONTAINING PEPTIDES INDUCING PEAK SPLITTING DURING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A slow conformational equilibrium, commensurable with the retention times, was shown to induce peak broadening or peak splitting during reversed-phase high-performance liquid chromatography of several medium-sized peptides. Elution at 50°C resulted in sharp unique peaks, while at sub-ambient temperature well resolved peaks were observed. Linear peptides which show this phenomenon had a Pro–Pro bond, but the phenomenon was also observed in the case of a cyclic peptide containing two non-vicinal proline residues.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) is the most appropriate method to assess the purity of synthetic peptides. The observation of more than a single peak is usually attributed to the presence of impurities which have occurred as a result of side reactions during the different steps of the synthesis, or to misfunctioning of the HPLC apparatus such as uneven sample distribution or non-uniform eluent flow in the column. However, when a molecule exists in several conformations having different retention factors and when the relaxation times are commensurable with the time-scale of the chromatographic process, peak broadening, distortion of peak shape and eventually peak splitting can occur and be misinterpreted. We report in this communication several cases of medium-sized synthetic peptides (Table I) for which such unusual chromatographic behaviours were observed during usual reversed-phase gradient HPLC as a consequence of a slow conformational equilibrium[1].

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TABLE I

AMINO ACID SEQUENCES OF PEPTIDES I–XII

<Glu = Pyroglutamic acid.

No.	*Amino acid sequence*
I	Met–Ser–Ile–Pro–Pro–Glu–Lys
II	Ile–Pro–Met–Ser–Ile–Pro–Pro–Glu–Lys
III	Leu–Ala–Ile–Pro–Pro–Lys–Arg–Leu–Asn
IV	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg (bradykinin)
V	His–Asp–Leu–Pro–Lys–Ala–Val–Val–Lys–Leu–Glu–Pro–Pro–Trp–Ile–Gln
VI	Thr–Pro–Lys–Lys–Ile–Lys–Pro–Pro–Leu–Pro–Ser–Val–Thr–Lys
VII	Pro–Asp–Pro–Pro–Gln–Pro–Asp–Phe–Pro–Gln–Leu–Asn–Ser–Asp
VIII	<Glu–Glu–Lys–Pro–Tyr–Trp–Pro–Pro–Pro–Ile–Tyr–Pro–Met
IX	<Glu–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro
X	Trp–Arg–Arg–Ala–Tyr–Asp–Ile–Pro–Pro–Pro–Pro–Val–Asp–Ile–Ser–Asp–Pro–Arg–Phe–Pro–Gly–Asn–Glu–Pro–Lys
XI	Cys—Leu—Pro—Arg—Glu—Pro—Gly—Leu—Cys (S—Acm; S—Acm)
XII	Cys—Leu—Pro—Arg—Glu—Pro—Gly—Leu—Cys (S—S)

MATERIALS AND METHODS

Chromatography

The apparatus consisted of two Model 302 pumps, a Model 704 system manager, a Model 116 UV detector and a Model 231 automatic sample injector (Gilson medical electronics). The 100-5 Nucleosil C_{18} Macherey-Nagel) column (300 mm × 4 mm I.D.) was immersed in a constant temperature bath.

Except where stated to the contrary, peptides were eluted at a flow-rate of 0.7 ml/min using a 15-min linear gradient from 3 to 50% acetonitrile in aqueous 0.01 *M* phosphate buffer, pH 3.5. The column effluent was monitored at 215 nm. For preparative purposes, 1-mg samples were injected and fractions were collected in tubes immersed in a refrigerated bath; the tubing in and out of the detector was made as short as possible.

Peptide synthesis

Peptides IV (bradykinin) and IX (bradykinin potentiator C) were obtained from Bachem. All other peptides were prepared in our laboratory using classical solid-phase methodology[2].

Briefly, peptides were synthesized on chloromethylpolystyrene 1% divinylbenzene resin, using N^{α}-*tert.*-butyloxycarbonyl (Boc) and benzyl side-chain protection as follows : Ser (benzyl, Bzl), Glu (OBzl), Lys (2-Cl-Z), Arg (tosyl, Tos), Cys (acetamidomethyl, Acm), Tyr(2-Br-Z). The first amino acids were anchored as their caesium

salts. Syntheses were performed on an Applied 430 (Applied Biosystems, Foster City, CA, U.S.A.) apparatus, starting with 0.5 mmol of aminoacyl-resins (average loading : 0.6 mmol/g).

Each synthetic cycle consisted of: (i) a 20-min deprotection with 50% trifluoroacetic acid–dichloromethane (after incorporation of Met or Trp residues, 2% dithioethane was added until the end of synthesis); (ii) neutralization with 10% diisopropylethyl amine–dichloromethane and (iii) coupling with preformed symmetrical anhydride (1 mmol) for 24 min in dimethylformamide (DMF). Boc-Arg (Tos), Boc-Asn and Boc-Gln were coupled as their preformed 1-hydroxybenzotriazole (HOBT) esters (2 mmol) for 40 min in DMF and recoupled for 40 min in dichloromethane.

Peptidyl-resins were cleaved by treatment with HF using *p*-cresol as a scavenger, and dimethyl sulphide was added during cleavage of peptides containing Met or Trp residues[3]. Peptides were purified by gel filtration on TSK HW40 Trisacryl® and their purity was checked by amino acid analysis after acid hydrolysis (5.6 *M* HCl, 24 h).

Cyclization

After removal of acetamidomethyl groups by mercuric acetate treatment at pH 4, peptide XI (10^{-5} *M* solution) was oxidized by air bubbling at pH 8 to give XII.

RESULTS AND DISCUSSION

Linear peptides containing one Pro–Pro bond

A typical HPLC profile for these compounds is shown in Fig. 1. Two peaks in a 4:1 ratio were observed and collected separately, and can be attributed to the presence of two different molecular species in the sample. Both peaks were subjected to amino acid analysis and fast atom bombardment (FAB) mass spectroscopy, yielding identical amino acid compositions and molecular weights. Each peak was collected and stored at room temperature during several hours. When reinjected under the same conditions, both gave the same profiles as shown in Fig. 1, suggesting the occurrence of a conformational equilibrium. Moreover, examination of the profiles showed that the two peaks had coalesced, suggesting that the region located between them contains molecules which have experienced a conformational inversion during elution.

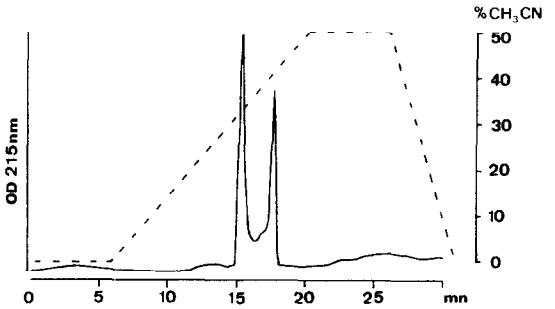

Fig. 1. HPLC profile of peptide I. Column: Nucleosil 100-5 C_{18}. Eluents: A = phosphate buffer pH 3.5 containing 3% acetonitrile; B = phosphate buffer pH 3.5 containing 50% acetonitrile. Flow-rate: 0.7 ml/min. Temperature: 20°C.

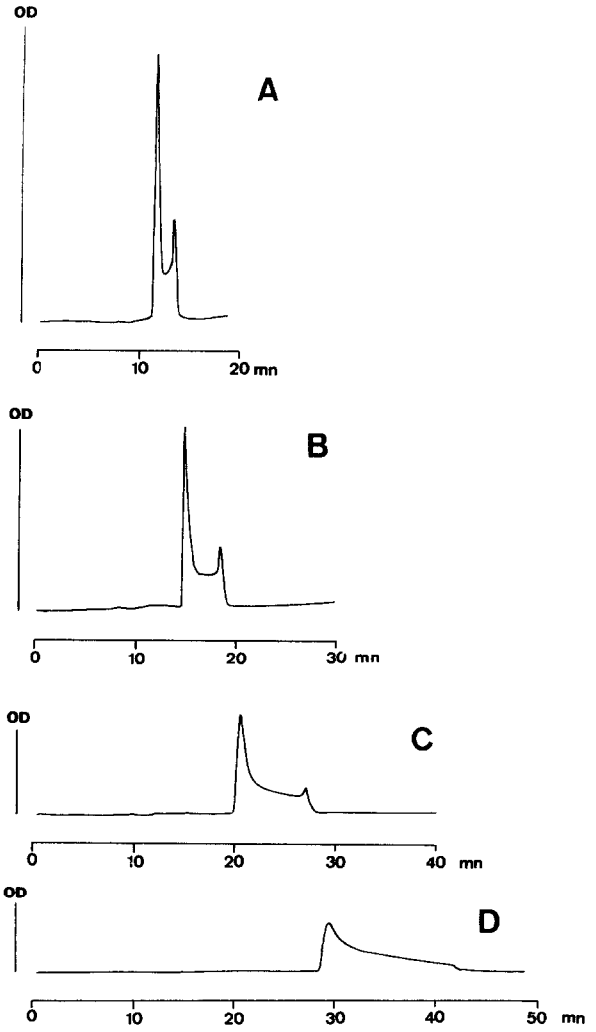

Fig. 2. HPLC profiles of peptide I as a function of gradient slope. Eluents and flow-rate as in Fig. 1. Gradient slope: times from 100% solvent A to 100% B, (A) 15, (B) 30, (C) 60 and (D) 120 min.

and that significant on-column isomerization of conformers occurred at room temperature during the chromatographic process.

There are several possible ways of influencing this phenomenon.

Effect of gradient slope. A change in the retention time of the peptide can be achieved by modification of the flow-rate under isocratic conditions, or, as was done here at constant flow-rate, by changing the slope of the gradient. Results are shown in Fig. 2, examplified with peptide I. Predictably, as the retention time increased, the probability that a molecule would undergo a conformational inversion increased simultaneously, resulting in broadening of the region between the peaks.

Effect of temperature. Increasing or lowering the column temperature had a dramatic effect on elution profiles as is seen in Fig. 3 with peptide I. Lowering the

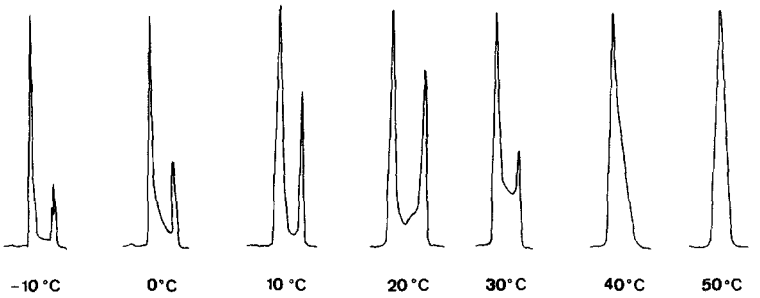

Fig. 3. HPLC profiles of peptide I as a function of temperature. Eluents and flow-rate as in Fig. 1. Gradient: 100% solvent A to 100% B in 15 min.

temperature of the column decreased the rate of isomer interconversion, whereas the retention times were only slightly affected. As a consequence, the separation between the two peaks improved. At 0°C the rate of on-column interconversion was slow enough to allow an almost baseline separation of the two conformers. As expected, increasing the column temperature above room temperature had the opposite effect, and at 50°C the rate of interconversion was high enough to yield a single symmetrical

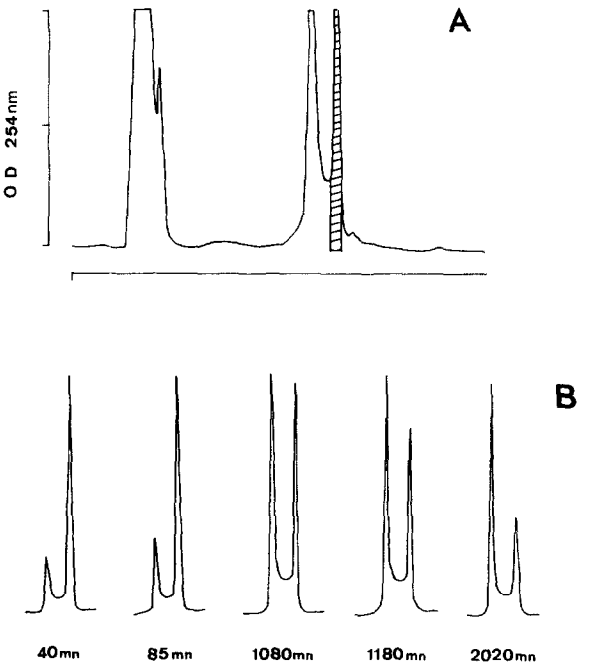

Fig. 4. Preparative low-temperature HPLC separation of peptide I conformers and reisomerization. (A) Isolation of the minor isomer. Column: Nucleosil 100-5 C_{18}. Eluent: phosphate buffer pH 3.5 containing 3% acetonitrile for solvent (A) and 50% acetonitrile for solvent (B). Flow-rate: 0.7 ml/min. Temperature: −10°C. Gradient: 5 min A then to 100% B in 15 min. Amount injected: 1 mg peptide in 20 μl 5% acetic acid. The peak collected, corresponding to the minor isomer, is shaded. (B) Reisomerization of the minor conformer as a function of time.

peak. However, this peak remained broad, indicating that, even at this temperature, the conformational equilibrium was still interfering with the chromatographic process.

Isolation of conformers. The almost complete separation of the two conformers which was observed at 0 and 10°C under analytical conditions prompted us to attempt their separation on a semipreparative scale. A solution of 1 mg of peptide I in 20 μl of 5% acetic acid was injected onto the column which was maintained at −10°C. As seen in Fig. 4A, two separated peaks were obtained which were collected and maintained at −10°C. Aliquots of the slower eluting peak, which corresponds to the minor isomer, were reinjected over a period of 48 h to observe the return to the equilibrium. As shown in Fig. 4B, a mixture containing equal quantities of both isomers was obtained after 18 h and after 48 h the profile was almost identical to that observed under equilibrium conditions.

Other linear, Pro–Pro containing peptides

Following these observations, we tested ten different linear peptides containing at least one Pro–Pro bond and found several types of behaviours.

Two peptides (II and III) had HPLC profiles very similar to that observed with peptide I; the two peaks were also in the same ratio of 4:1, the slower eluting being in each case the minor conformer.

Four peptides containing Pro–Pro bonds (IV–VII) showed no peak splitting even at low temperature. Among them is bradykinin (IV), a well known peptide with a Pro–Pro bond. A first explanation is that both conformers had similar retention times. However, we were not able to separate any conformers of IV, when using different eluting buffers or organic modifiers, even at temperatures as low as −10°C.

It is thus more likely in the case of these peptides that a single conformer is thermodynamically favoured or that certain Pro–Pro bonds are prone to rapid isomerization.

Three peptides had more than one Pro-Pro bond : VIII (Pro–Pro–Pro), IX (Pro–Pro–*X*–Pro–Pro–*X*–Pro–Pro) and X (Pro–Pro–Pro–Pro). In these cases, the HPLC profiles at 0°C were more complex as four or eight different conformers are likely to coexist and some, at least, will display different chromatographic behaviours. However, elution at 50°C resulted in single sharp peaks for these three peptides.

Cyclic peptides

A second type of slow conformational equilibrium was observed in the case of the cyclic peptides XII. During cyclization of XI, which was performed by air oxidation in dilute solution after removal of Acm protecting groups, a very broad peak was observed by HPLC analysis of the reaction medium. At this point, a slow conformational equilibrium was suspected. This was confirmed by performing HPLC at different temperatures (Fig. 5): a single sharp peak was obtained at 50°C while a well resolved doublet containing two isomers in a 1:1 ratio was observed at 0°C. When the linear peptide XI was examined under the same conditions, no alteration of peak shape was detected.

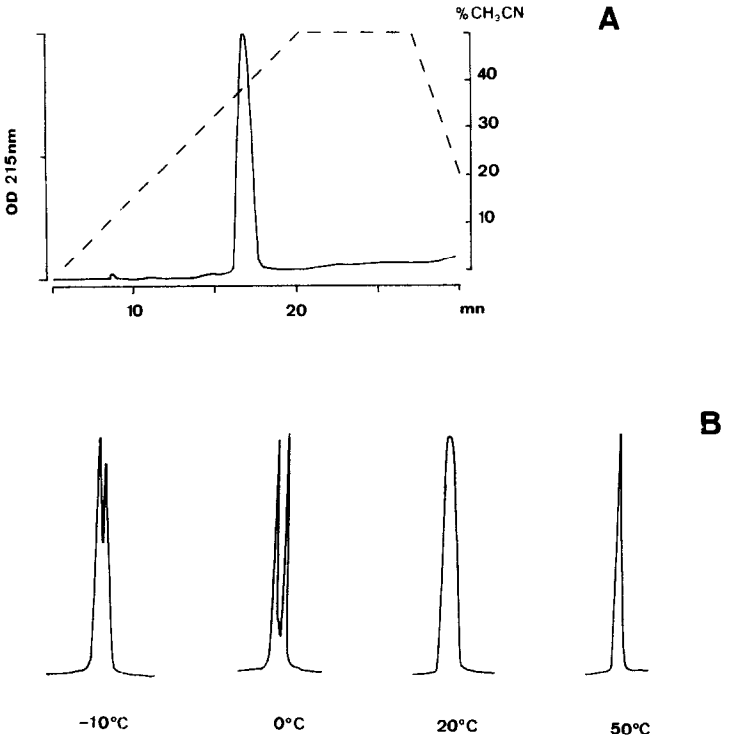

Fig. 5. HPLC of peptide XII. Eluents and flow-rate as in Fig. 1. (A) Profile at 20°C. (B) Profiles as a function of temperature.

DISCUSSION

The development of rapid methods of chromatography such as HPLC has dramatically decreased the time of chromatographic processes. Not surprisingly, slow isomerization of molecules can thus interfere more frequently with their chromatographic behaviours.

We have searched the relevant literature for similar observations. Melander *et al.*[4] have studied in detail the effect of *cis–trans* isomerization of proline dipeptides in reversed-phase chromatography. Their results clearly show that the slow isomerization of the imido peptide bond is responsible for peak splitting under isocratic conditions. They also provided a theoretical framework for the treatment of on-column reactions and introduced the use of low-temperature HPLC to characterize such equilibria.

However, to our knowledge, only one paper by Rusconi *et al.*[5] describes in detail a similar phenomenon in the case of a medium sized linear peptide (VIII), tryptophyllin (<Glu–Glu–Lys–Pro–Tyr–Trp–Pro–Pro–Pro–Ile–Tyr–Pro–Met).

Except for the proline-containing dipeptides which were analysed under isocratic conditions with very short retention times, all the longer peptides giving rise to this phenomenon have at least one Pro–Pro bond in their sequence.

Among proteogenic amino acids, proline is known to play an unique rôle be-

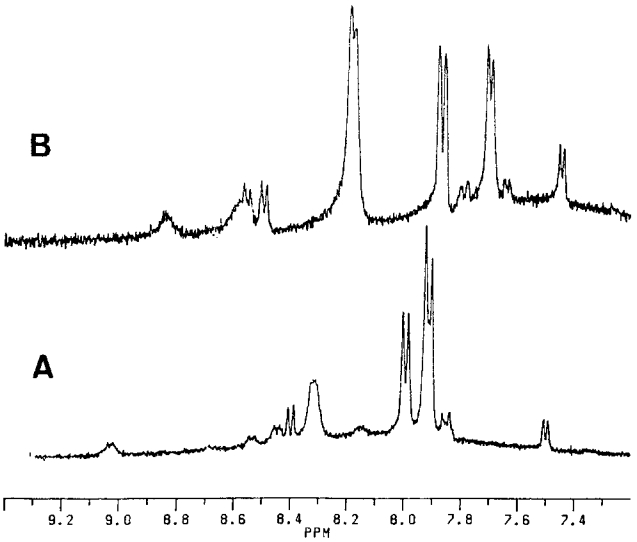

Fig. 6. NH region of the NMR spectra of peptide I (solution in [$^2H_6$]dimethyl sulphoxide). (a) Just after dissolution (preponderance of *cis*-isomer); (B) four days after dissolution (preponderance of the all-*trans*-isomer).

cause its side chain is linked to the α-nitrogen atom. As a result, *cis* isomers of these peptide bonds are only slightly less stable than *trans* isomers and both isomers are generally observed during NMR studies of small proline-containing peptides. Thus when the NMR spectrum of the NH region of peptide I was examined (Fig. 6), the presence of *cis-trans* isomers was observed, corresponding respectively to Ile–Pro and Pro–Pro bonds. This observation is consistent with the fact that the isomerization rates of both structures are slow when compared to the NMR time-scale. However, among the different possible *X*–Pro peptide bonds, only the Pro–Pro bond appears to be endowed with an exceptionally slow *cis–trans* isomerization rate comparable with the chromatographic time-scale. As an example, this slow *cis–trans* isomerization rate was recently proposed[6] to explain why the folding of porcine RNase differs significantly from the general folding pattern of other RNases. In porcine RNase, the change $Tyr^{115} \rightarrow Pro^{115}$ leads to a Pro^{114}–Pro^{115} bond which is found only in the porcine enzyme.

Determination of the kinetics of unfolding and refolding made it possible to determine an activation enthalpy of 22 kcal/mol (92 kJ/mol) and a half-life of 900 s at 10°C. This value is compatible with our chromatographic observations.

However, it should not be considered inevitable that the sole presence of a Pro–Pro bond in a peptide will induce a peak distortion, given that four peptides, including bradykinin (IV) gave rise to no peak distortion.

In the cyclic peptide XII, no Pro–Pro bond is present. The observations that the chromatographic properties of the linear peptide XI were not affected by temperature variations indicate that steric constraints upon cyclization are responsible for the decrease in the isomerization rate. A similar observation involving a proline-containing cyclic peptide has been reported[7] in the case of conotoxin MI (Gly–Arg–Cys–Cys–His–Pro–Ala–Cys–Gly–Lys–Asn–Tyr–Ser–Cys–NH_2) but not with closely re-

lated α-conotoxins. In this case also, HPLC at low temperature (0°C) gave a complete separation of the two possible forms, while a single sharp peak was observed at 60°C.

CONCLUSIONS

Slow *cis–trans* isomerization of particular peptide bonds such as Pro–Pro bonds or steric constraints induced by cyclization may be responsible for alteration of the chromatographic behaviour of medium-sized peptides.

Increasing the column temperature allows the peptide to be eluted as a single sharp peak and thus its purity can be assessed while still performing the elution at a reasonable speed. Lowering the temperature of the column and using the most rapid elution conditions compatible with isomer separation allows a baseline separation of the conformers. It might be of great interest to use this method to study separately the physicochemical properties of each conformer. Moreover, such peptides may prove to be valuable substrates in a study of the properties of the enzyme peptidyl-prolyl *cis–trans* isomerase discovered and purified from pig kidney, as this enzyme, which catalyses the *cis–trans* isomerization of proline imidic peptide bonds during refolding of proteins[8], accepts Pro–Pro-containing chain segments as substrates.

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