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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF A NOVEL PROLINE-RICH TRYPTOPHYLLIN

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SUMMARY

A tryptophan-containing tridecapeptide of amphibian origin (tryptophyllin-13, TPH-13), having the sequence <Glu-Glu-Lys-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met-OH, was found to give peak splitting in isocratic and gradient reversed-phase high-performance liquid chromatography. The influence of many experimental conditions (column temperature, type of bonded phase, buffer pH, type of organic solvent, flow-rate, on-column incubation of the peptide, presence of a denaturant in the mobile phase, oxidation state of the peptide) on the chromatographic pattern of TPH-13 under selected gradient conditions, was studied. The raising of column temperature to 45°C was always found to suppress peak splitting, whereas the other modifications affected the resolution and/or relative intensity of the two peaks of the doublet. Using a mobile phase buffer with a pH near neutrality, the two peaks could be partially separated by preparative high-performance liquid chromatography, and were shown to be in equilibrium with each other. A pronounced conformational isomerism of the peptide chain, due to the presence of five proline residues, three of which are consecutively linked, is suggested to give rise to two major conformers of comparable stability and different hydrophobicity, detectable as a doublet by reversed-phase high-performance liquid chromatography.

INTRODUCTION

During the isolation of dermorphins[1] from skin extracts of *Phyllomedusa rohdei*, a South American frog, another set of new peptides was identified, characterized by the presence of a tryptophan residue in all their sequences. This common feature, together with their amphibian origin, suggested the collective name "tryptophyllins" (TPHs) for these peptides[2]. TPHs with different chain lengths have been isolated, ranging from some tetrapeptides to a tridecapeptide, and their primary structures have been determined[3–5]. They have been replicated by conventional synthetic methods together with some analogues, and investigated in several biological systems[6–8].

The heaviest tryptophyllin characterized so far, TPH-13, has the following amino acid sequence:
<Glu-Glu-Lys-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met-OH

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An interesting feature of its structure is the abundance of proline. Five residues are present in the molecule, and three of them are consecutively linked.

The natural peptide and its synthetic replicate were checked for purity and identity by different techniques, including reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis. The products were initially analysed on a cyanopropylsilyl column under isocratic conditions (60% acetonitrile in 0.1 *M* formic acid)[5], yielding a single sharp peak both individually and also when injected together. Nevertheless, only methionine sulphoxide was released as a C-terminal amino acid from the analysed sample of natural TPH-13 by carboxypeptidase Y (CP-Y). However, methionine, instead of methionine sulphoxide, appeared when the synthetic replicate was subjected to digestion with CP-Y[2]. This suggested that the natural product had been oxidized *via* the purification procedure, as already found for other methionine-containing peptides[9–11]. We have consequently developed a gradient elution method for separating the reduced peptide from its oxidized counterpart, and we have indeed confirmed that the preparation of natural TPH-13 examined contained methionine sulphoxide. While testing different RP-HPLC conditions, we also observed a seemingly anomalous retention behaviour of the natural and synthetic sample, namely, splitting of the chromatographic peak into an approximately symmetric doublet.

There have been various reports on single protein species yielding multiple and/or irregularly shaped peaks using RP-HPLC[12–16], as well as in high-performance size-exclusion[17] and ion-exchange chromatography[18,19]. A variety of properties of macromolecular substances can potentially play a role in this behaviour (*e.g.* conformational changes in protein structure) and the above-cited observations have in general been attributed to changes in the tridimensional structure of the biopolymer, which are caused by denaturation.

The finding that pure TPH-13 yields double peaks under some RP-HPLC conditions prompted us to investigate its chromatographic behaviour more thoroughly, with the goal of ascertaining whether peak splitting of this medium-sized peptide could also be related to conformational isomerism. As already pointed out, this tryptophyllin spans a proline-rich sequence, which might give rise to a series of conformers of comparable stability, due to the small difference in energy content between *cis*- and *trans*-conformations of proline residues[20]. The full characterization of this proline-rich peptide by RP-HPLC, described in the present paper, provides indications that two major conformational isomers of TPH-13 may be responsible for peak splitting.

EXPERIMENTAL

Chromatography

All chromatographic experiments were performed on a Hewlett-Packard 1084 B apparatus, equipped with an HP 1040 diode array detector, controlled through an HP 85 computer.

A μBondapak C_{18} and a μBondapak CN column (300 × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.) were used throughout the investigation. A flow-rate of 1 ml/min was always employed, except when the effects of flow-rate changes on the chromatographic pattern were studied. The temperature of the column was kept

at 0°C by immersing it in an ice-water bath, or controlled through the 1084 B thermostated oven when temperatures above ambient (*ca.* 25°C) were desired. The detection wavelength was fixed at 280 nm.

Materials

Natural TPH-13 and its tryptic fragments were obtained as already described[5]; the synthetic replicate was synthesized by conventional methods[8]. All other materials were of analytical grade.

The samples for chromatography were made up in 50% ethanol (*ca.* 1 mg/ml), unless otherwise indicated. Solutions and buffers containing urea were freshly prepared daily.

Chemical methods

Synthetic TPH-13 was oxidized to [Met(O)^{13}]TPH-13 by treatment with 0.18% hydrogen peroxide in 0.05 *M* acetic acid (peptide concentration, *ca.* 0.5 mg/ml) for 15 min at room temperature. Reduction of extractive TPH-13 was achieved with 2-mercaptoethanol according to the method described by Floor and Leeman for the reduction of substance P sulphoxide[9].

RESULTS

Preliminary investigation

Synthetic and natural TPH-13 were found to be unresolved by isocratic RP-HPLC on a cyanopropyl(CN)-bonded phase[5]. They were still eluted together in a sharp peak when the mobile phase (60% acetonitrile in 0.1 *M* formic acid) previously used on the CN column was employed on an octadecyl(C_{18})-bonded phase. Eluents containing the same buffer and lower percentages (from 50 to 30%) of acetonitrile were also tested on both columns, but the two peptides were not resolved. Moreover, poorly shaped or anomalous peaks were obtained as the acetonitrile content of the mobile phase was reduced. In particular, a clear doublet with a low retention time was observed when the samples were analysed on the C_{18} column with 40% acetonitrile in 0.1 *M* formic acid as eluent (see Fig. 1), both individually and in a mixture. The split peak coalesced reversibly as column temperature was raised to 45°C.

Conditions were then changed from isocratic to gradient elution. The amount of acetonitrile, always in the same aqueous buffer, was linearly increased from 16 to 64% in 15 min from injection. The elution profile of a mixture from synthetic and natural TPH-13 is shown in Fig. 2. Two comparable doublets are observed, each with clearly different retention times. After mild oxidation of the mixture with peracetic acid, only the early-eluted doublet was still present, whereas reduction with 2-mercaptoethanol[9] left only the more retained doublet. This confirmed the occurrence of methionine as the sulphoxide in the natural peptide analysed. Synthetic TPH-13 was always employed in the subsequent chromatographic characterization.

Studies at different temperatures

The above-described gradient method was used in the investigation of the influence of temperature on the elution pattern of TPH-13. As already pointed out for isocratic elution with 40% acetonitrile from the C_{18} column, the increase of temper-

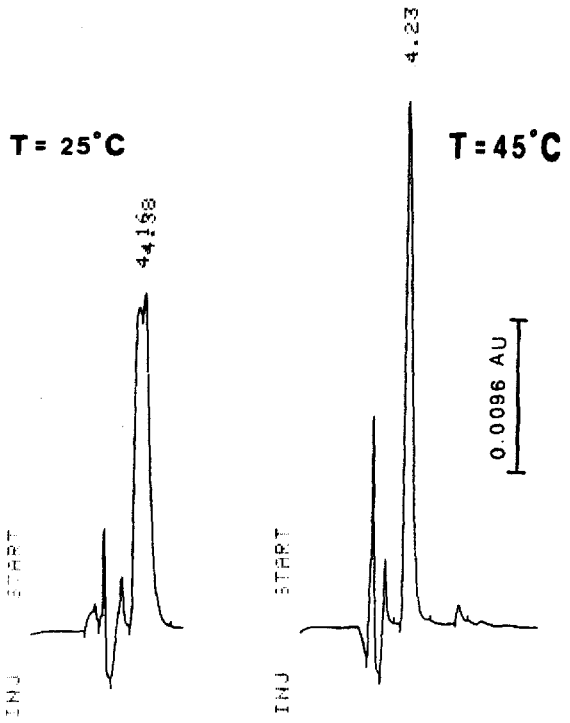

Fig. 1. Elution profile of TPH-13 under isocratic RP-HPLC conditions as a function of column temperature. Conditions: column, μBondapak C_{18}; eluent, 40% acetonitrile in 0.1 *M* formic acid; sample size, *ca.* 10 μg.

ature to 45°C caused coalescence of the double peak into a single peak with the retention time of the earlier-eluted component of the doublet (see Fig. 3). On the other hand, at 0°C the resolution of the split peak was better than at room temperature. Furthermore, the more or less 50:50 ratio between the two "horns" of the doublet was not dramatically changed by lowering the temperature. In addition, this ratio was roughly the same as that observed with isocratic elution (see Fig. 1).

TPH-13 behaved similarly when analysed on the CN column. In this case, a slightly slower gradient (from 16 to 64% acetonitrile in 20 min) than that employed on the C_{18} column was used; otherwise, a strongly asymmetrical single peak was observed at room temperature, instead of a doublet. The ratio between the two components was still about 50:50.

pH studies

These experiments, as well as those described further below, were performed on the C_{18} column, always with a linear gradient from 16 to 64% acetonitrile in 15 min. The behaviour of TPH-13 with a 0.05 *M* ammonium acetate buffer at two pH values (buffer A, pH 6.7; and buffer B, pH 7.8 with ammonia) was examined. The elution profiles of TPH-13 obtained with buffers A and B were quite similar: both showed a very clear, approximately symmetric doublet, much better resolved than at acidic pH. The retention time of TPH-13 decreased as the pH value of the buffer

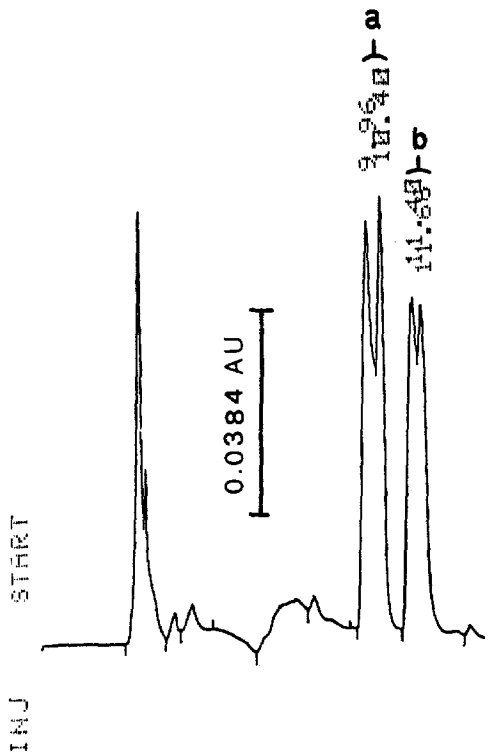

Fig. 2. Elution profile of a mixture of synthetic and natural TPH-13 under gradient RP-HPLC conditions at room temperature. Conditions: column, μBondapak C_{18}; eluent A, 0.1 *M* formic acid; eluent B, acetonitrile; gradient, from 16 to 64% B in 15 min; sample size, *ca.* 35 μg of synthetic peptide and *ca.* 45 μg of natural peptide. a = natural TPH-13, containing methionine in the sulphoxide form; b = synthetic [Met^{13}] analogue.

increased, in agreement with the acidic isoelectric point of the peptide (p*I* = 4.6)[2]. The variations of column temperature had similar effects on the chromatographic pattern to those already observed at acidic pH: in both cases, the doublet coalesced at 45°C, whereas at 0°C, peak splitting was still clearer than at room temperature. In particular, when buffer B was used, the two components of the doublet were resolved virtually at the baseline on the refrigerated column (see Fig. 4).

Interconversion inside the doublet

Preliminary experiments with 0.1 *M* formic acid as the aqueous buffer were performed, in order to investigate the equilibration between the two peaks of the doublet once they had been separated by preparative HPLC. These tests gave negative results, since the same elution pattern was always observed, even upon immediate reinjection of the pools corresponding to each of the two peaks. This finding might also suggest that peak splitting of TPH-13 was an artifact.

A pH value close to neutrality for the buffer seemed more suitable for this separation: both acidic and basic catalyses are negligible, and the interconversion

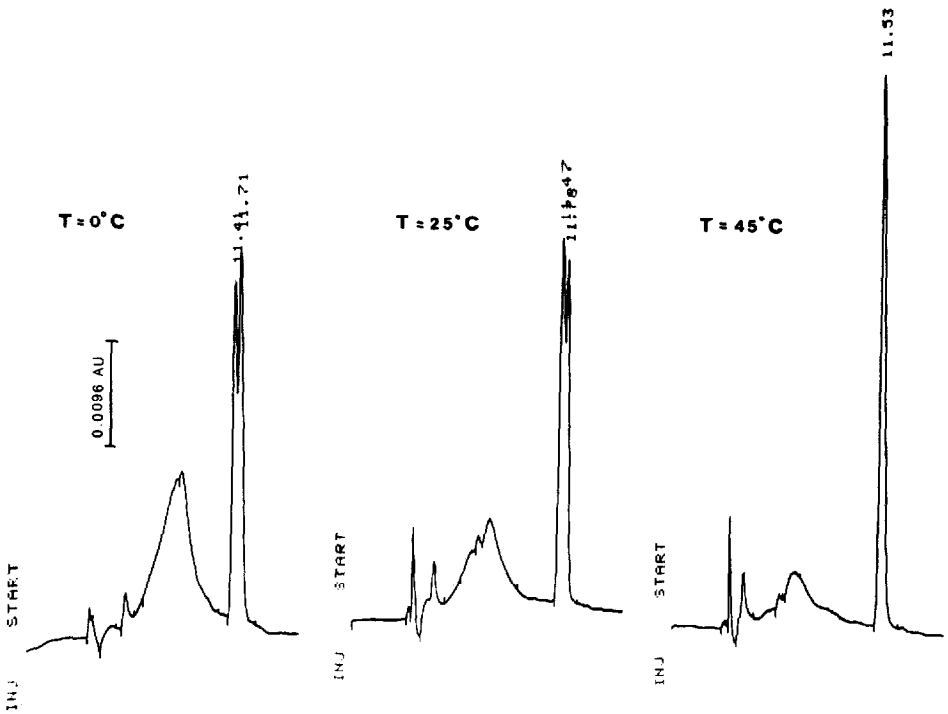

Fig. 3. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. Conditions are as in Fig. 2, except for sample size (*ca.* 10 μg).

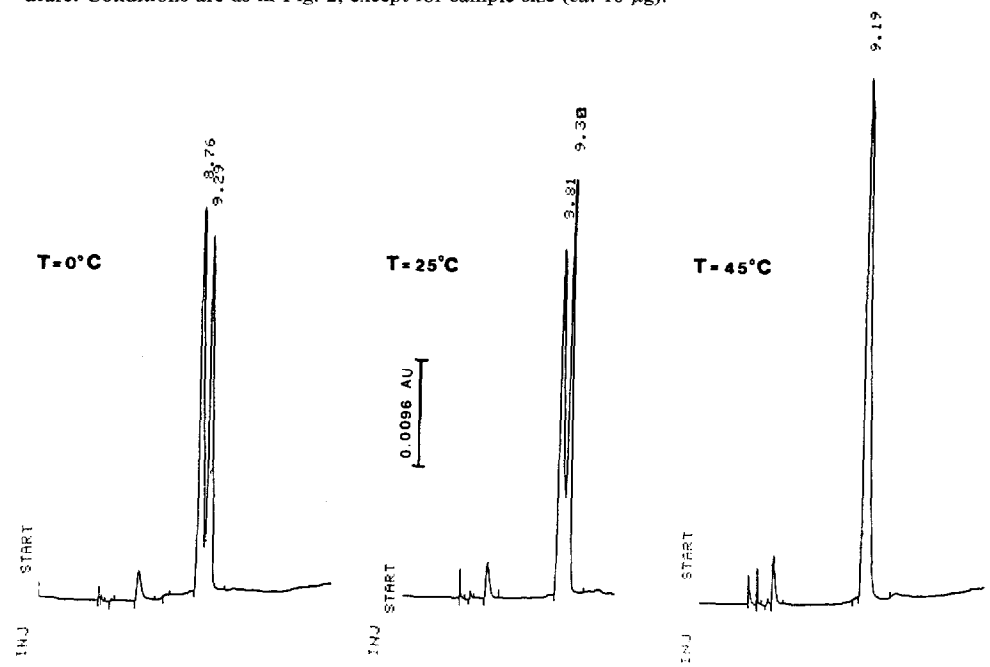

Fig. 4. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. Conditions: eluent A, 0.05 *M* ammonium acetate (pH 7.8 with ammonia); sample size, *ca.* 10 μg; other conditions as in Fig. 2.

between different conformations of the peptide chain should consequently be slow. Buffer A was then used. Two pools (pools 1 and 2) were collected after injection of TPH-13 into the refrigerated column; their elution patterns are shown in Fig. 5. Upon immediate reinjection (fig. 5a and c), each pool appeared largely enriched in the expected component, although contaminated with the other. The two pools were left at room temperature for 24 h while equilibrating. They were then reinjected, and yielded two superimposable chromatograms (see Fig. 5b and d). It was consequently confirmed that the two horns of the doublet are actually different species, whose interconversion rate depends on the pH of the buffer in the mobile phase.

Influence of the organic modifier

Acetonitrile is the most frequently used organic solvent in RP-HPLC of peptides and proteins[21]. Nevertheless, in many cases it was indicated as being responsible for denaturation of these biopolymers during chromatography, since high concentrations of this component are often required to obtain elution. The use of a more hydrophobic solvent, such as 2-propanol, results in the elution of hydrophobic peptides at lower concentrations of organic solvent[22]. The modifications induced in the chromatographic behaviour of TPH-13 by the replacement of acetonitrile with 2-propanol were consequently investigated.

The elution pattern obtained with 0.1 *M* formic acid as the aqueous buffer was not substantially modified, compared to the profile resulting from the use of acetonitrile (see Fig. 3): a virtually symmetric doublet was still present at room temperature, coalescing into a single peak at 45°C. The lower temperature was not investigated, because the high viscosity of 2-propanol led to excessive column pressure. The use of this solvent actually reduced the retention time of TPH-13, and it also significantly improved separation between the two peaks of the doublet.

Effects of contact time

The interaction of peptides and proteins with the hydrophobic surface of the column matrix in RP-HPLC certainly modifies the tridimensional structure of the polymer, and it was found to induce reversible or irreversible denaturation[12,14–16,23]. The unfolding kinetics of some proteins in RP-HPLC were recently studied by Benedek *et al.*[15]. From their work, it appears that the contact time between protein and chromatographic matrix exerts a great influence on the extent of denaturation.

The flow-rate affects the retention time of a product and, consequently, the time during which it remains in contact with the column. Therefore, the behaviour of TPH-13 in gradient elution at acidic pH was examined with both lower (0.7 ml/min) and higher (2 ml/min) flow-rates than are otherwise used (1 ml/min). Doubling the flow-rate did not significantly change the elution pattern: a doublet was observed at room temperature (better resolved than with a flow-rate of 1 ml/min), and a sharp singlet at 45°C. The lower temperature could not be investigated, because the operating pressure limit of the system was exceeded. With a flow-rate of 0.7 ml/min, the chromatographic behaviour of TPH-13 was not remarkably modified at room temperature or at 45°C, whereas at 0°C the amount of the later-eluted component of the doublet was apparently increased.

In order to increase the contact time without changing the flow-rate and the slope of the gradient, an on-column incubation period was added to the elution time.

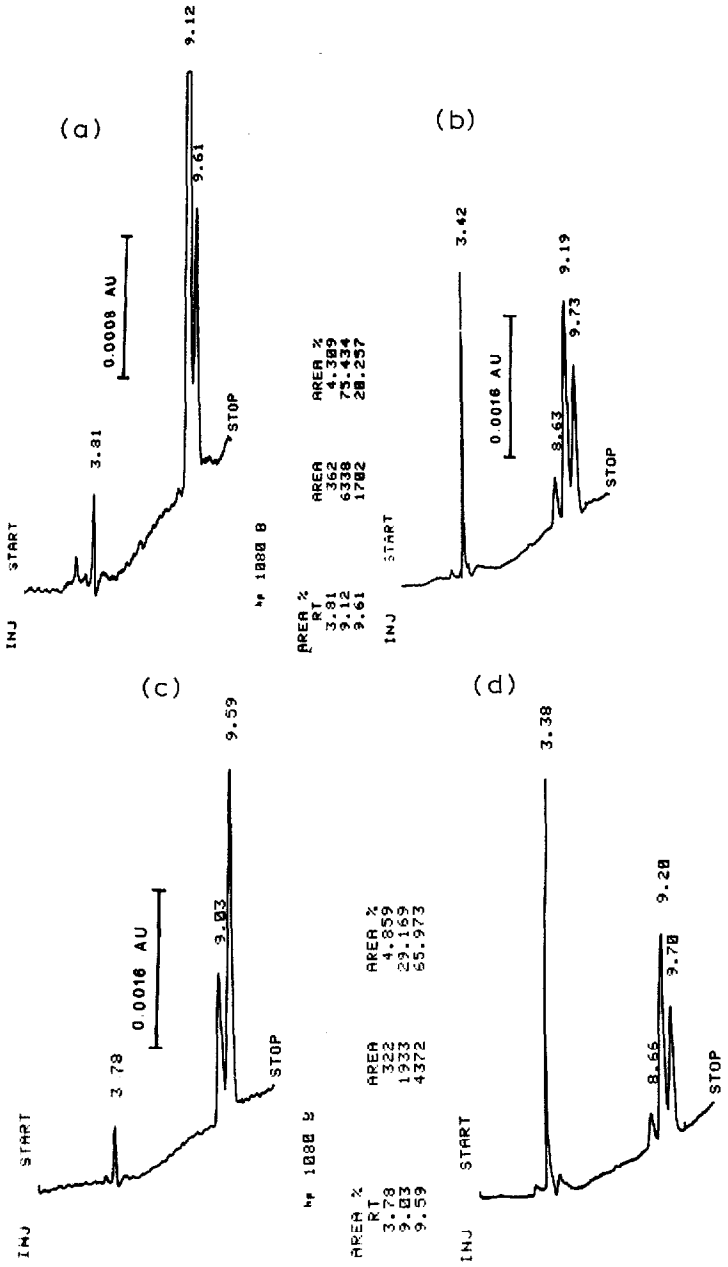

Fig. 5. Elution profiles of the two pools collected after injection of TPH-13 (*ca.* 50 μg) into the refrigerated column. Conditions are as in Fig. 2, except for eluent A (0.05 *M* ammonium acetate, pH 6.7). Pools 1 and 2 correspond to the early- and late-eluted peak of the TPH-13 doublet, respectively: pool 1 reinjected (50 μl) immediately (a) and after 24 h (b); pool 2 reinjected (50 μl) immediately (c) and after 24 h (d). The small peak with a retention time of *ca.* 8.6 min was, in both cases, due to the oxidized peptide. Reinjection conditions as above.

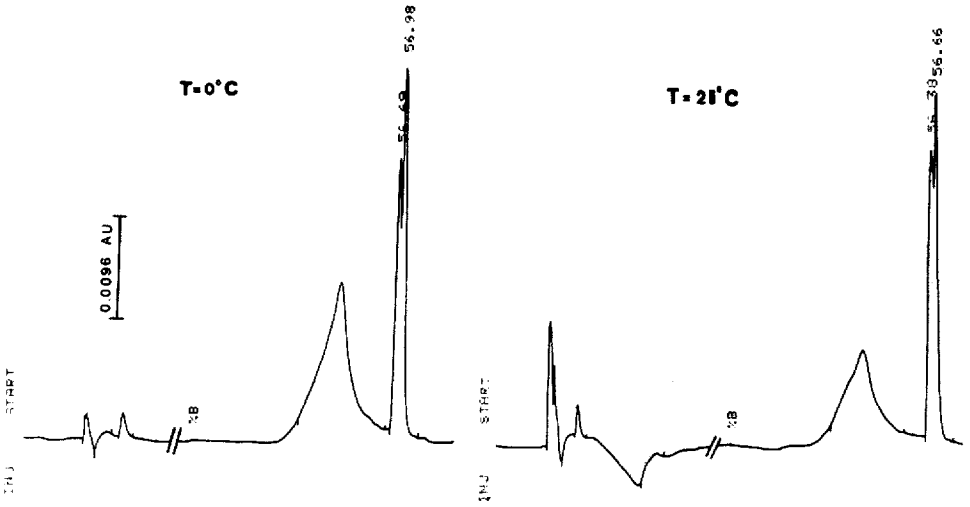

Fig. 6. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. The injected peptide was incubated on the column by keeping the mobile phase isocratic (16% B) for 45 min before the start of the gradient, which is marked by "% B" on the chromatogram. Conditions are as in Fig. 2, except for sample size (*ca.* 10 μg).

In this case, the mobile phase was kept isocratic for 45 min before the start of the gradient. The resulting elution profiles at room temperature and 0°C are shown in Fig. 6: the typical doublet of TPH-13 appears asymmetric, as the more retained horn prevails, and asymmetry is very remarkable in the chromatogram obtained at the lower temperature.

Effects of a denaturing agent

The behaviour of TPH-13 in the presence of a commonly used denaturing agent, such as urea, was also investigated. The acidic buffer employed so far (0.1 *M* formic acid) was used, plus 6 *M* urea, and the peptide sample was prepared in the starting mobile phase (*ca.* 1 mg/ml). These modifications strongly affected the elution pattern of TPH-13 at room temperature, as shown in Fig. 7. Again, a clearly asymmetric doublet was observed, but, in contrast to what was found when the contact time between peptide and column was increased, the earlier-eluted component prevailed. The double peak still coalesced as the column temperature was raised to 45°C. The lower temperature could not be considered because of the high viscosity of the mobile phase.

Since the presence of urea in the buffer also resulted in a higher pH value (3.5) than that of the unmodified buffer (2.5), a control analysis with 0.1 *M* formic acid, adjusted to pH 3.5 with sodium hydroxide, was performed. The elution pattern under these conditions was similar to that obtained with the unmodified buffer, also when the peptide sample was dissolved in the starting mobile phase of the previous experiment. The retention time at pH 3.5 was slightly lower than that at pH 2.5, in agreement with the observed trend toward decreased retention as pH was increased (see above). In contrast, when the urea-containing buffer at pH 3.5 was used, a stronger

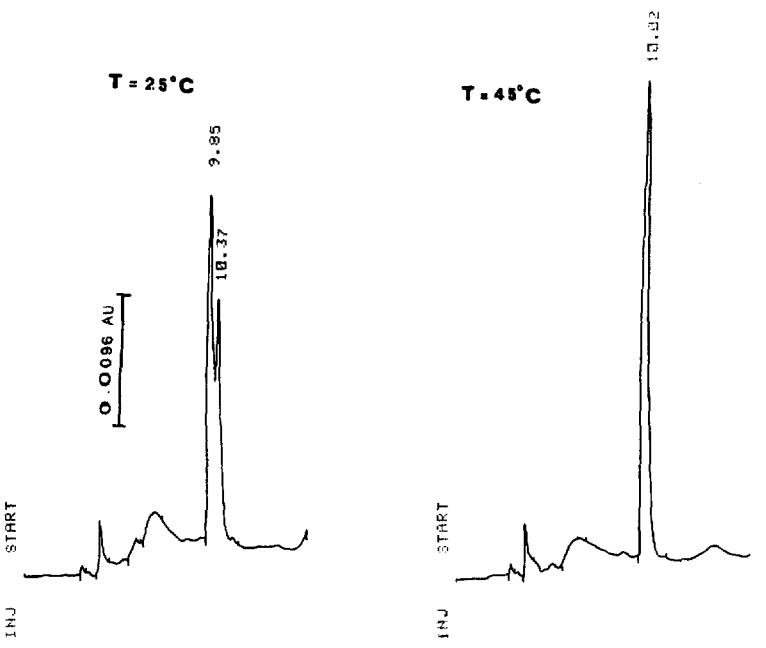

Fig. 7. Elution profile of TPH-13 under gradient RP-HPLC conditions as a function of column temperature. Conditions: eluent A, 0.1 *M* formic acid–6 *M* urea (pH 3.5); sample size, *ca.* 10 μmol; other conditions as in Fig. 2.

reduction of the retention time of TPH-13 was observed (see Fig. 7), suggesting a specific effect of the denaturing agent.

Chromatographic behaviour of the proline-rich fragment of TPH-13

Results quite similar to those previously illustrated, concerning the influence of column temperature and pH of the mobile phase on the elution pattern of TPH-13, as well as interconversion inside its typical doublet, were obtained with the (4–13) tryptic fragment (H-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met-OH). In agreement with its enhanced hydrophobic character, the decapeptide always had an elution volume slightly larger than that of TPH-13.

The results obtained in the investigation on the chromatographic behaviour of TPH-13 have been described qualitatively so far. They could also be expressed in a semiquantitative manner by measurement of resolution (R_s) and intensity ratio (A_2/A_1) between the two peaks of the TPH-13 doublet under various experimental conditions. The variations of R_s and (A_2/A_1) were then calculated with respect to the characteristic values under reference conditions, *i.e.*: column temperature, 0 and 25°C; bonded phase, C_{18}; buffer pH, 2.5 (0.1 *M* formic acid); organic solvent, acetonitrile; flow-rate, 1 ml/min; on-column incubation time, none; urea concentration in the buffer, none; TPH-13, in the reduced state. The results obtained are shown in Table I.

TABLE I

EFFECT OF THE MODIFICATION OF EXPERIMENTAL CHROMATOGRAPHIC CONDITIONS ON R_s AND A_2/A_1 IN RP-HPLC OF TPH-13

R_s, resolution of the two peaks of the TPH-13 doublet; A_2/A_1, ratio of the areas of the late- and early-eluted peak, respectively.

$$\Delta R_s\ (\%) = \frac{R_s - R_{s,ref}}{R_{s,ref}} \times 100 \qquad \Delta(A_2/A_1)\ (\%) = \frac{(A_2/A_1) - (A_2/A_1)_{ref}}{(A_2/A_1)_{ref}} \times 100$$

$R_{s,ref}$ and $(A_2/A_1)_{ref}$, values of R_s and A_2/A_1, respectively, measured under reference conditions (see text). The effects of the experimental condition modifications have been evaluated according to the following ranking factors: for ΔR_s (%): $1 = \Delta R_s \leqslant 20\%$, $2 = 20\% < \Delta R_s \leqslant 70\%$, $3 = \Delta R_s > 70\%$, $0 = \Delta R_s = 0\%$; and for $\Delta(A_2/A_1)$ (%): $1 = \Delta(A_2/A_1) \leqslant 10\%$, $2 = 10\% < \Delta(A_2/A_1) < 25\%$, $3 = \Delta(A_2/A_1) \geqslant 25\%$. + and − signs after the factors mean positive and negative variations, respectively.

Modified experimental conditions		*T = 25°C*		*T = 0°C*	
		ΔR_s (%)	*$\Delta(A_2/A_1)$ (%)*	*ΔR_s (%)*	*$\Delta(A_2/A_1)$ (%)*
Column temperature	0°C	1 +	3 +	−	−
Bonded phase*	CN	−	3 −**	1 +	3 −
Buffer pH	3.5	1 +	1 +	−	−
	6.7	2 +	1 −	3 +	3 −
	7.8	3 +	1 +	3 +	3 −
Organic solvent	2-propanol	2 +	2 +	−	−
Flow-rate	2 ml/min	2 +	1 −	−	−
	0.7 ml/min	1 −	2 +	1 −	3 +
Incubation time	45 min	0	2 +	0	3 +
Urea concentration (in the buffer)	6 mol/l	3 +	3 −	−	−
Oxidation state of TPH-13	[Met(O)^{13}]TPH-13	2 +	2 −	3 +	2 −

* In addition to the bonded phase, the gradient time was also changed, from 15 min (reference condition) to 20 min.

** The variation of (h_2/h_1) (ratio of the heights of the late- and early-eluted peak of the doublet, respectively) with respect to the reference condition was calculated instead of the variation of (A_2/A_1), because the poor resolution obtained at room temperature did not allow the two peaks to be separately integrated (see Fig. 5).

DISCUSSION AND CONCLUSIONS

Analysis of the chromatographic pattern of TPH-13 under different RP-HPLC conditions (see also Table I) suggests the following generalizations:

(1) Raising the column temperature to 45°C had the most drastic effect on the elution profile, since it changed the pattern from a doublet to a single peak under all the conditions investigated. In particular, under conditions in which the elution volumes were hardly affected by temperature, the resulting peak had a retention time close to that of the horn that decreased in intensity as column temperature was lowered from 25 to 0°C (see pH studies), or that was already prevalent at room temperature (see analysis in the presence of 6 *M* urea).

(2) The contact with the more hydrophobic bonded phase (C_{18} compared to CN) favoured the shifting of equilibrium inside the doublet towards the later-eluted

peak. This effect cannot simply be due to a "denaturation" of the peptide, caused by its interaction with the stationary phase, since the addition of a known denaturant like urea to the mobile phase had just the opposite effect. On the other hand, the replacement of acetonitrile by 2-propanol as the organic solvent also increased the relative intensity of the later-eluted peak.

(3) The presence of methionine sulphoxide in the peptide significantly improved separation between the two peaks. Thus, the bulky, polar sulphoxide function seems to accentuate the difference in hydrophobicity between the species yielding the two horns and/or seems to reduce their interconversion rate; moreover, it also remarkably affects the position of equilibrium between them. The increase of buffer pH to 6.7 and 7.8 had similar effects on resolution, but it showed a strong influence on the relative intensity of the two peaks only at low temperature.

In addition to the parameters whose influence on the chromatographic pattern of TPH-13 was purposely studied, other factors might contribute to peak splitting: (a) formation of dimeric (or polymeric) complexes due to ionic or hydrogen bonds and (b) dissociation of the ionizable groups in the side chains, occurring at pH values higher than that of the reference buffer.

The formation of non-monomeric structures was suggested by Lundanes and Greibrokk[24] to be responsible for double peaks observed in the chromatograms of pure peptides. The contribution of this phenomenon was already minimized in the case of TPH-13 by the use of sufficiently concentrated buffers, and it could be completely excluded by addition of urea to the buffer, which affected the relative intensity of the two peaks of the doublet but did not suppress it. With respect to the second factor, Horváth *et al.*[25] showed that ampholytic solutes may be eluted with different retention times, depending on the distribution of charge. Although the equilibrium forms of ampholytes are not likely to be separated by chromatography, the hydrochlorides of basic amino acids were found to give one early-eluted peak, containing chloride, and one retained peak without chloride[24]. A significant contribution of dissociation to the peak splitting of TPH-13 was actually excluded because of the close resemblance between the chromatographic behaviour of TPH-13 and that of its (4-13) fragment, which has no ionizable group in the side chains.

On the other hand, the investigation of interconversion inside the doublets characteristic of TPH-13 and of the tryptic decapeptide showed that, with a mobile phase containing a virtually neutral buffer, the two species yielding the double peak both have a slow rate of equilibration. This finding, together with the influence of different parameters on resolution and the relative intensity between the two peaks of the doublet, suggests that these horns might be attributable to conformational isomers, although each of them is not likely to correspond to exactly the same isomeric species under all the conditions investigated, which differed widely from each other. Moreover, changing the pH of the buffer might also have reversed the elution order of the supposed conformers.

With respect to the actual nature of these isomers, the proline-rich sequence that characterizes both intact TPH-13 and its tryptic fragment might give rise to two major conformers, with comparable stability and different hydrophobicity, whose relative intensity and rate of isomerization would be affected by the chromatographic conditions used to resolve them. In this connection, the triprolyl sequence appears to be a very interesting feature; in fact, the onset of a helical (polyproline-II-like)

structure* was shown to occur in oligopeptides containing three or more consecutively linked proline residues[27].

On the other hand, even simple proline-containing dipeptides were found, by Melander *et al.*[28], to yield peak splitting in RP-HPLC when proline was not at the N-terminus, and this phenomenon was ascribed to the slow kinetics of isomerization of the X–Pro imidic bond, which is on the same time-scale as the chromatographic separation of these peptides. The dynamic effect of such secondary equilibria in RP-HPLC was recently studied by the same authors[29], who also applied their theoretical work to the kinetic study of *cis–trans* proline isomerization in some dipeptides by RP-HPLC[30]. Also, the isolated proline residues present in the TPH-13 sequence at positions 4 and 12 might consequently be responsible for the peak splitting of this peptide. Engaging of the proline imino group in a peptide bond is needed for an appreciable *cis–trans* equilibrium, therefore a significant contribution of the [Pro^4] residue can be readily excluded, since the (4–13) fragment, which has this proline as an N-terminus, still yields double peaks under the same HPLC conditions as the intact molecule. On the contrary, the strong improvement of chromatographic resolution observed following methionine oxidation as well as carboxyl dissociation (see Table I), which both affect the C-terminal moiety of TPH-13, suggests that the [Pro^{12}] residue might be a determinant for peak splitting. In this regard, the heptapeptide dermorphin[1], which has a C-terminal sequence (Tyr-Pro-Ser-NH_2) similar to that of TPH-13 (Tyr-Pro-Met-OH), was also found to give double peaks under some RP-HPLC conditions, while its analogue, [Gly^6]-dermorphin, yielded a single sharp peak[31]. Moreover, in the case of dermorphin, the relative intensity of the two resulting peaks agrees well with NMR data concerning the presence of *cis*-proline in the peptide chain[32].

Since the RP-HPLC technique cannot give definitive information on the conformational isomerism hypothesized for TPH-13, studies by other techniques (NMR and circular dichroism) are in progress to confirm the indications provided by this work. In addition, isomer specific proteolysis (ISP) with proline-specific endopeptidase and other proteolytic enzymes, suggested as an indirect method for distinguishing *cis*- and *trans*-proline residues[33,34], is now being tested on TPH-13 and its proline-rich fragment in order to provide further information on the conformational equilibrium of these peptides.

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* Polyproline is known to exist either as a compact right-handed helix (designated form I) that contains *cis*-peptide bonds and has close intramolecular contacts, or as a left-handed, highly extended helix (form II) with *trans*-peptide bonds[26].

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