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High-performance liquid chromatographic purification of extremely hydrophobic peptides: transmembrane segments

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Abstract

Transmembrane peptides of integral membrane proteins often exhibit extremely high hydrophobicity. Therefore, the solubility of such peptides in solvents commonly used in HPLC is usually very low and the interaction with generally applied stationary phases such as silica gel or C_{18} reversed phases appears to be extremely strong, which makes the characterization and purification of these peptides difficult. The analytical characterization and preparative separation of the synthesized M1 transmembrane sequence of the inhibitory glycine receptor M_r 48 000 subunit and some of its fragments is shown. M1 and its larger fragments could be dissolved in a dichloromethane–hexafluoro-2-propanol mixture containing a trace amount of pyridine for their separation on a C_4 phase by employing linear two-component gradients of formic acid–2-propanol and formic acid–water with ratios up to 4:1 (v/v). Conditions to avoid formylation of the peptides are indicated.

1. Introduction

In contrast to their fundamental biological importance such as in biological membranes, unusually hydrophobic peptides are not only considered to be notoriously difficult regarding their synthesis but also with respect to their chromatographic separation and characterization. A major difficulty consists in preparing molecularly dispersed solutions by breaking hydrophobic and polar interactions in the absence of detergents so that HPLC techniques are useful for their separation. A systematic solubility study [1–7] has indicated that hydrophobic peptides are often considerably soluble in mixtures of dichloromethane and hexafluoro-2-propanol (HFIP). It is hoped that binary solvent mixtures of this type will be applicable for the preparation of concentrated stock solutions of such peptides for the purpose of column injection.

The standard RP-18 stationary phases interact strongly with hydrophobic peptides, preventing elution with a polar mobile phase even with extreme compositions. Chemically unmodified silica gel, on the other hand, shows a similar behaviour in the presence of non-polar mobile phases. To overcome the strong tendency of hydrophobic peptides to adsorb on stationary phases, it seems to be advisable to apply reversed stationary phases that are neither very non-polar nor very polar, provided that a suitable elution medium can be found. In addition to the analytical HPLC characterization of hydrophobic peptides, it is also important to develop preparative isolation procedures employing the same technique.

Heukeshoven and Dernick [8,9] and others [10,11] have described the application of mobile

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phases containing high concentrations of formic acid to perform analytical RP chromatography with hydrophobic proteins. However, the possible usefulness of such systems for the analytical characterization and preparative purification of synthetic peptides has not been shown so far. A significant chemical problem with formic acid-rich systems consists in the possible chemical modification due to formylation and/or oxidation (in case of Met-containing peptides) of peptides while in contact with this acid, especially under conditions where the peptides are dissolved and stored in such a medium prior to column injection or after their separation.

This paper shows, as an illustrative example, how these difficulties, which appeared in the course of a chemical synthesis of the hydrophobic putative transmembrane segment M1 of the M_r 48 000 subunit of the ligand-induced inhibitory glycine receptor [12] and some of its fragments (Fig. 1), can be solved. It is considered that the procedures described represent a major progress in the handling, characterization and purification of such peptides with unusual properties. These procedures are considered to be applicable not only to purifying and characterizing strongly hydrophobic peptides of synthetic origin but also such peptides originating from biological sources, e.g., after enzymatic digestion of integral membrane proteins or from peptide isolation from expression experiments.

M1-I: H-Leu-Ser-Trp-Ile-Ser-Phe-Trp-Ile-Asn-NH2

M1-II: H-Val-Ile-Leu-Ser-Trp-Ile-Ser-Phe-Trp-Ile-Asn-NH2

M1-III: H-Pro-Ser-Leu-Leu-Ile-Val-Ile-Leu-Ser-Trp-Ile-Ser-Phe-Trp-Ile-Asn-NH2

M1-IV: H-Tyr-Ile-Pro-Ser-Leu-Leu-Ile-Val-Ile-Leu-Ser-Trp-Ile-Ser-Phe-Trp-Ile-Asn-NH2

M1: H-Gly-Tyr-Tyr-Leu-Ile-Gln-Met-Tyr-Ile-Pro-Ser-Leu-Leu-Ile-Val-Ile-Leu-Ser-Trp-Ile-Ser-Phe-Trp-Ile-Asn-NH2

Fig. 1. Peptide fragments of the transmembrane sequence M1 of the M_r 48 000 subunit of the inhibitory glycine receptor. Hydrophobic residues are shown in bold type.

2. Experimental

2.1. Chromatography

All chromatographic experiments were performed with a Model 420 HPLC pump (Kontron Instruments, Neufahrn, Germany) in connection with a Model 425 gradient former (Kontron) and a variable-wavelength monitor (Knauer, Berlin, Germany). The system was controlled by a Model 450 data system (Kontron).

The following Vydac columns (obtained from Grom Analytical + HPLC, Herrenberg, Germany) were used: (I) 300-C_{18}, 5 μm, 250 × 4 mm I.D.; (II) 300-C_4, 10 μm, 40 × 4 mm I.D.; (III) 300-C_4, 10 μm, 100 × 4.6 mm I.D.; (IV) 300-C_4, 10 μm, 125 × 8 mm I.D. A flow-rate of 1.5 ml/min was always employed, except for the 125 × 8 mm I.D. column, with which 5 ml/min were used. All separations were carried out at ambient temperature. The detection wavelength was fixed at 280 nm.

All indicated percentages and ratios concerning media compositions are based on volumes.

2.2. Materials

The peptide sequences M1-I to M1-IV and M1 were obtained by solid-phase peptide synthesis using the Fmoc strategy as described elsewhere [13,14] and the purified peptides were characterized by fast atom bombardment (FAB) MS, amino acid analyses and in some instances by racemization determinations. Peptide M1-II was purified by LH-20 chromatography with acetic acid–methanol (2:3) and exhibited a uniform mass spectrum with an $[M + H]^+$ peak at m/z 1377. The mass spectrum of the HPLC-purified peptide M1-III contained only peaks that could be attributed to the expected structure; the $[M + H]^+$ peak was observed at m/z 1901 and is consistent with the expected peptide sequence.

HFIP (Fluka, Buchs, Switzerland) was of

synthetic grade and was distilled over K_2CO_3 (1 g per 100 ml) before use. All other materials (Merck, Darmstadt, Germany) were of analytical-reagent grade and were used without further purification. Pyridine of analytical-reagent grade was obtained from Fluka.

All peptide stock solutions for chromatography were prepared by dissolving the corresponding peptides in CH_2Cl_2–HFIP (4:1) (ca. 1 mg in 100 μl for non-purified peptides and 250 μg in 100 μl for purified peptides) and in some instances with subsequent addition of pyridine (10 μl per 100 μl of peptide solution) as indicated in the text. Volumes of 20 μl of the peptide samples were injected on to the analytical columns and 50 μl were injected on to the semi-preparative columns for each run. The elution conditions are given in the captions of the figures and in the text.

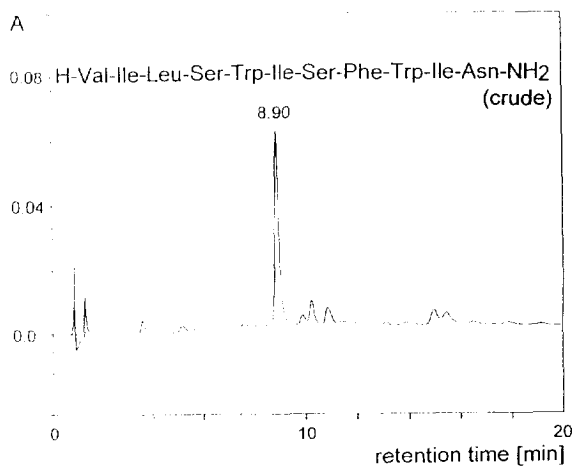

Fig. 2. Chromatogram of crude M1-II. Column, Vydac C_4, 100 × 4.6 mm I.D.; linear gradient (20 min) from formic acid–water (2:3) to formic acid–2-propanol (4:1) starting 1 min after injection.

3. Results

Peptide M1-I (containing 67% hydrophobic residues, Fig. 1) could be chromatographed under standard conditions on a C_{18} RP column employing a linear gradient between 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) (from 10% to 30% B in 13 min, then to 70% B in 10 min), the retention time being 19 min under these conditions. Prolongation of this peptide by the two additional hydrophobic amino acids, Ile and Val (M1-II), led to a situation where no elution within an acceptable time was observable by employing standard mobile phases of different compositions, as applied, for example, for M1-I on a C_{18} or a C_4 RP column. If, however, this peptide was chromatographed on a C_4 RP column by employing a linear gradient between formic acid–water (2:3) and formic acid–2-propanol (4:1), elution was possible with good resolution and the desired peptide component could be characterized by a retention time of 8.90 min (Fig. 2).

It was also possible to perform a semi-preparative HPLC purification of the even more hydrophobic, non-purified peptide M1-III (containing 75% hydrophobic residues including an extremely hydrophobic row of six successive amino acid residues) with a similar gradient as applied for M1-II, ranging from formic acid–water (4:1) to formic acid–2-propanol (4:1) (column IV). Under these conditions, the retention time of M1-III in the crude preparation originating from the synthesis (column II) was 8.92 min (Fig. 3). In the presence of formic acid as the major solvent component a much higher resolution could generally be observed than with acetic acid. Fig. 4 shows the analytical chromatogram of the collected, HPLC-purified M1-III peptide (8.92 min component of the semi-preparative run) that had been injected from a CH_2Cl_2–HFIP (4:1) stock solution containing a trace amount of pyridine (see Experimental). The result shown in Fig. 4 indicates the high purity achieved. The visible minor component, characterized by a retention time of 8.38 min, could be unambiguously assigned to a trace contaminant in the pyridine used.

The effect of the addition of pyridine to the injection solution of the peptide [CH_2Cl_2–HFIP (4:1)] on the properties of the chromatogram could also be demonstrated in a convincing manner in analytical HPLC runs performed on a longer C_4 column (100 mm) (Fig. 5). In the

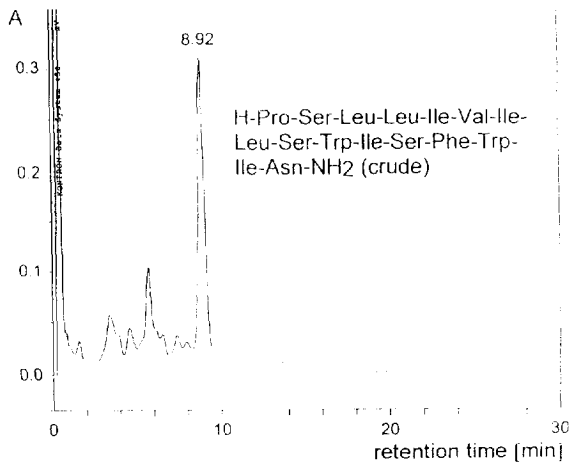

Fig. 3. Chromatogram of crude M1-III. Column, Vydac C_4, 40 × 4 mm I.D.; linear gradient (20 min) from formic acid–water (4:1) to formic acid–2-propanol (4:1) starting 1 min after injection. The sample was dissolved in CH_2Cl_2–HFIP (4:1) containing a trace of pyridine (see Experimental).

absence of pyridine the chromatogram of the crude peptide M1-III (Fig. 5A) showed very broad peaks and thus only a low resolution could be observed. In addition to these broadening effects, the peaks assigned to the desired peptide M1-III even showed a pronounced shoulder. Narrow peaks with comparatively very good resolution could be observed, however, on repe-

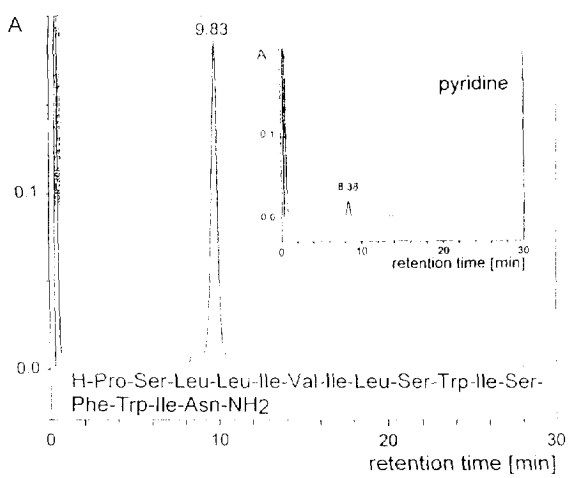

Fig. 4. Chromatogram of purified M1-III. Conditions as in Fig. 3. The small peak at 8.38 min is due to an impurity in the pyridine used (see text).

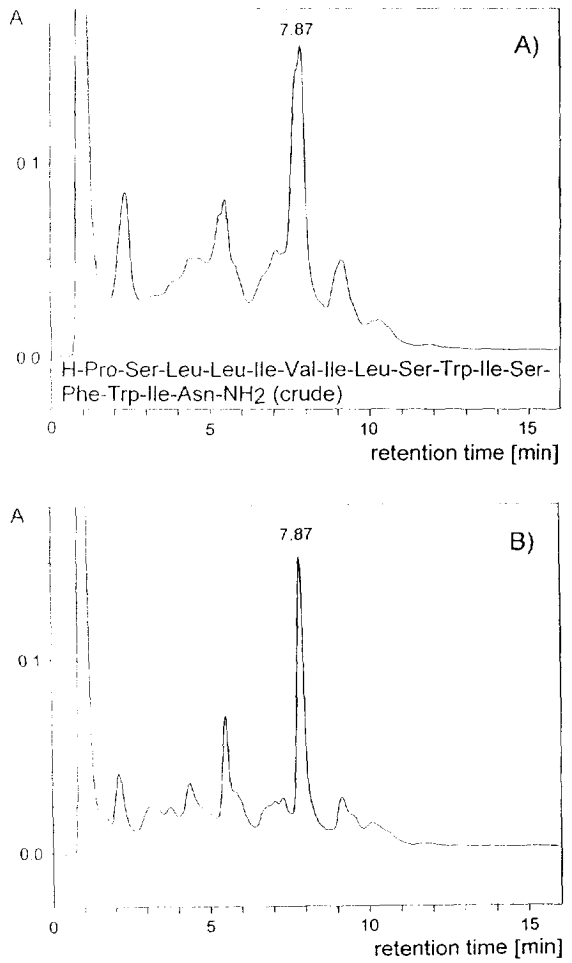

Fig. 5. Chromatogram of crude M1-III. Conditions as in Fig. 3 except that the column dimensions were 100 × 4.6 mm I.D. (A) Without addition of pyridine; (B) with addition of pyridine prior to injection (see Experimental).

tition of this chromatogram under identical conditions but now with a small addition of pyridine to the peptide stock solution prior to injection (Fig. 5B). The effect of the pyridine addition consists in the appearance of a well resolved, single, narrow and symmetrical peptide peak, attributed to M1-III, but not in a change of the corresponding retention time (Fig. 5A and B). Similar HPLC results could be achieved with the even longer hydrophobic peptides M1-IV and M1.

To characterize further the surprising effect of

pyridine that was added to stock solutions of the peptides, additional experiments were carried out. In the absence of pyridine, the intense peptide peak observed at 7.87 min seems to consist of a superposition of at least two components. These two components could be due either to two chemically different peptides or to two different states of the same peptide that are converted into a uniform state by small amounts of pyridine. In order to decide between these possibilities, two different control experiments were performed. The uniform and narrow 7.87-min component of the crude M1-III was collected after having added pyridine to the corresponding stock solution (conditions according to Fig. 5B) and isolated. If this isolated component is dissolved in the absence of pyridine and rechromatographed, the typical shoulder, as shown in Fig. 5A, appears again. In order to probe whether the broad 7.87-min peak observed in the absence of pyridine in the corresponding stock solution (conditions of Fig. 5A) exhibiting a pronounced shoulder is chemically identical, two fractions were collected between retention times of 7.5 and 8.1 min. The FAB mass spectra of these fractions were almost identical. This result suggests that shoulder and main peak consist of the same peptide, namely M1-III. In retrospect, we also have to conclude that the 9.83-min peak on the chromatogram shown in Fig. 4 and both 7.87-min peaks indicated in Fig. 5A and B are characteristic of the pure uniform peptide M1-III. The surprising improvement of the chromatographic resolution as a consequence of the addition of pyridine to the peptide stock solution has to be attributed to an effect of this base on the properties of the peptide, e.g., with regard to its conformational or protolytic features in the chosen binary solvent mixture of the stock solution.

To avoid formylation of the peptides separated on a preparative scale, the eluent was collected directly behind the detector in an ice-cooled flask containing some degassed water. If the peptides isolated by preparative HPLC employing the described formic acid-containing media were formylated, a characteristic upward shift of 28 units on the m/z scale would have to be detectable in the corresponding FAB mass spectra. As no such peaks could be observed for peptides isolated under the conditions specified here, it is concluded that no substantial formylation occurred. Another source of possible contamination during chromatography under the conditions recommended here could be due to Met oxidation in the case of M1, which again should be easily recognizable by FAB-MS. However, no evidence for the formation of such oxidation products could be found in the corresponding spectra. In summary, these additional experiments demonstrate that the investigated peptides were indeed pure.

4. Conclusions

Extremely hydrophobic peptides such as transmembrane peptide segments of synthetic or biological origin can be separated with excellent resolution on a C_4 RP column and are obtained as highly purified compounds using binary gradient elution systems containing high concentrations of formic acid. In order to prevent possible side-reactions, it is necessary to avoid long contact times of the desired peptide with this acid and it is therefore also recommended to dissolve and store the peptide in a CH_2Cl_2–HFIP mixture before injecting it on to the column. Concerning preparative-scale HPLC separation, the fractions containing the eluted peptide have to be diluted immediately with cooled water to decrease the acid concentration. Following these rules, no formylation or oxidation (e.g., in case of the Met-containing peptide M1) is observed. Peak broadening, which was observed in some instances, could be avoided by adding trace amounts of pyridine to the CH_2Cl_2–HFIP stock solution prior to injection.

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