PURIFICATION OF SYNTHETIC LIPID ASSOCIATING PEPTIDES AND THE MONITORING OF THE DEFORMYLATION OF N^{in}-FORMYLTRYPTOPHAN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

D. R. KNIGHTON*, D. R. K. HARDING, J. R. NAPIER and W. S. HANCOCK

Department of Chemistry, and Biophysics, Massey University, Palmerston North (New Zealand)

SUMMARY

A series of five synthetic lipid assocating peptides have been purified by reversed-phase high-performance liquid chromatography (HPLC). Volatile mobile phases were used to allow facile isolation of the purified peptide. These consisted of either 0.1 M ammonium bicarbonate, pH 7.9, or 0.5 M ammonium formate, pH 6.3, with gradients of 2-propanol and acetonitrile. The columns used were either Radial-Pak C_{18} or Radial-Pak CN. The recovery of the purified peptides was usually in excess of 95%. In addition, this chromatographic system allowed the facile purification of the product isolated from the removal of the N^{in}-formyl protecting group from tryptophan. The use of reversed-phase HPLC has allowed an improvement in both the purity and yield of purified material when compared with the results of low-pressure gel filtration and ion-exchange chromatography. The use of guanidine hydrochloride in the injection mixture and the presence of organic solvents in the mobile phase have allowed the minimization of aggregation of the lipid associating peptides with a consequent improvement in the chromatographic properties of these peptides.

INTRODUCTION

The purification of lipophilic peptides has been shown to be difficult[1] and often characterised by low yields. Recently we have investigated a variety of mobile phases that are designed to facilitate the preparative separation of peptide samples, such as trifluoroacetic acid[2], heptafluorobutyric acid[3] and ammonium bicarbonate[4]. The low yields previously observed with the purification of these lipophilic peptides can be partly attributed to the low solubility of these peptides in aqueous solvents and their tendency to aggregate. As will be shown in this report, semi-preparative reversed-phase high-performance liquid chromatography (HPLC), with a mobile phase which contained ammonium bicarbonate or formate as an ionic modifier, allowed the rapid and high yield purification of lipid associating peptides.

MATERIALS AND METHODS

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC gradient system was used for the separations (see refs. 4 and 5 for details). Sample injections were made using a microliter 802 syringe or a 10-ml gas-tight 1010 W syringe (Hamilton, Reno, NV, U.S.A.). The Radial-Pak CN and C_{18} cartridges (8 mm I.D.) were also purchased from Waters Assoc. For optimal column life the column was protected with a guard column and an in-line prefilter (Waters Assoc.).

UV spectra were determined using a Shimadzu MPS-5000 instrument.

Chemicals and separation conditions

The solvents and chemicals used in this study were identical to those described in a previous paper[4]. The peptide solutions were prepared as described previously[2], except that all solutions contained 6 M urea or 3 M guanidine–HCl to prevent sample aggregation.

The ammonium bicarbonate buffer (0.1 M, pH 7.7) was prepared freshly each day. The separation was achieved at ambient temperatures and a flow-rate of 1 ml/min was used. The eluted peptides were detected by UV absorbance (generally at 280 nm).

Synthesis procedure

The process used to synthesise the peptides is based on procedures described previously and used the generation of an *in situ* symmetrical anhydride[5]. Syntheses were performed on a scale of 2 g Boc-amino acid resin for all peptides except the synthesis of peptide 199 which was performed on a scale of 3 g Boc-amino acid resin. Each oil obtained from the HF cleavage reaction was dissolved in a minimum volume of 65% acetic acid and loaded onto a Sephadex G-10 gel filtration column (32 cm × 5.5 cm I.D.) at approximately 10°C. The eluted fractions containing the bulk of absorbance at 280 nm were pooled, diluted to approximately 1 l in volume with water and freeze dried.

Deformylation procedure and semi-preparative separations

A sample of 79.1 mg of Trp(CHO)-peptide 208 was dissolved in 100 ml of water and 57.3 g of guanidine hydrochloride. This solution was cooled to 4°C and 14 ml of ethanolamine were added. The solution was stirred for 5 min while the pH was monitored (apparent pH 11.8–11.9). After this time the solution was titrated to pH 7.8 with 6 M hydrochloric acid. UV spectra before and after the deformylation showed that the deformylation had been completed (see Fig. 1).

The semipreparative separations used in this study will be illustrated by the following example:

A 60-ml portion of the neutralised deformylation mixture containing approximately 26 mg of peptide 208 was diluted 3-fold with 0.1 M ammonium bicarbonate and loaded directly onto a Radial-Pak-C_{18} column via Pump A of the HPLC system. The sample was eluted with a gradient of 2-propanol. The recovery of peptide was 17% of the theoretical yield derived from the initial substitution on the resin. However, the recovery of a sample of the purified peptide rechromatographed on the same
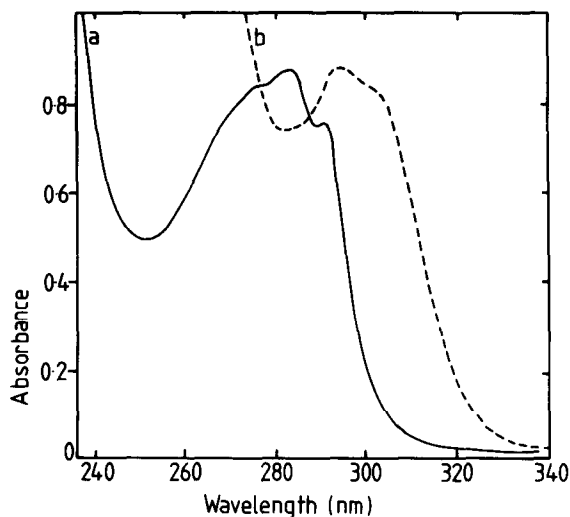
Fig. 1. A comparison of the UV spectra of peptide 208 (curve a) and Trp(CHO)-peptide 208 (curve b).

column was 96% (based on absorbance measurements of the isolated material, $\lambda_{max.}$ = 280 nm; molar absorptivity, ε_M (molar extinction coefficient) = 5200 1 mol^{-1} cm^{-1}; mol.wt. 2278). This yield, which was determined here by spectrophotometric techniques, correlated well with corresponding dry weights and amino acid analyses obtained for the samples.

RESULTS AND DISCUSSION

It is now widely appreciated that reversed-phase HPLC offers an excellent procedure for the purification of synthetic peptides[5-7]. Preparative procedures are facilitated by the use of volatile mobile phases such as 0.1% trifluoroacetic acid[8,9] or 0.1 M ammonium bicarbonate[4,10]. This paper will demonstrate that reversed phase HPLC with a mobile phase that contains 0.1 M ammonium bicarbonate allows the chromatography of lipid associating peptides without the normal problems of aggregation, insolubility, incomplete resolution and low recoveries, often observed during low-pressure gel filtration and ion-exchange chromatography[1]. This system also avoids the tedious work-up of reaction mixtures normally encountered after removing the N^{in}-formyl group used for the protection of tryptophan during solid phase peptide synthesis[5]. The lipid associating peptides used in this study are listed in Table I.

The N^{in}-formyl group was removed with the procedure developed by Sparrow[11,12], which used ethanolamine as the nucleophile to displace the formyl group from the indole ring of tryptophan. Lipid associating peptides are designed to bind to phospholipid micelles and in the absence of lipid tend to self-associate[13]. Since aggregation can inhibit the deformylation reaction, a protein denaturant, guanidine hydrochloride, was added to the reaction mixture. It was previously shown that a peptide could be readily desalted by reversed-phase chromatography, since ionic material such as guanidine hydrochloride is not retarded by the reversed-phase col-

TABLE I

SEQUENCE OF LIPID ASSOCIATING PEPTIDES PREPARED BY THE SOLID PHASE METHOD

Synthesis number	Peptide sequence
199	H-Arg-Ala-Leu-Ala-Ser$_5$-Ser-Leu-Lys-Glu-Tyr$_{10}$-Trp-Ser-Ser-Leu-Lys$_{15}$ Glu-Ser-Phe-Ser$_{19}$-OH
202*	H-Leu-Glu-Ser-Phe-Leu$_5$-Lys-Ser-Trp-Leu-Ser$_{10}$-Ala-Leu-Glu-Gln-Ala$_{15}$ Leu-Lys-Ala$_{18}$-OH
203	H-Leu-Glu-Ser-Phe-Lys$_5$-Val-Ser-Trp-Leu-Ser$_{10}$-Ala-Leu-Glu-Glu-Tyr$_{15}$ Thr-Lys-Ala$_{18}$-OH
208	H-Val-Ser-Ser-Leu-Leu$_5$-Ser-Ser-Leu-Lys-Glu$_{10}$-Tyr-Trp-Ser-Ser-Leu$_{15}$-Lys Glu-Ser-Phe-Ser$_{20}$-OH
209	H-Leu-Glu-Ser-Phe-Leu$_5$-Leu-Ser-Trp-Leu-Ser$_{10}$-Ala-Lys-Glu-Gln-Ala$_{15}$ Leu-Lys-Ala$_{18}$-OH

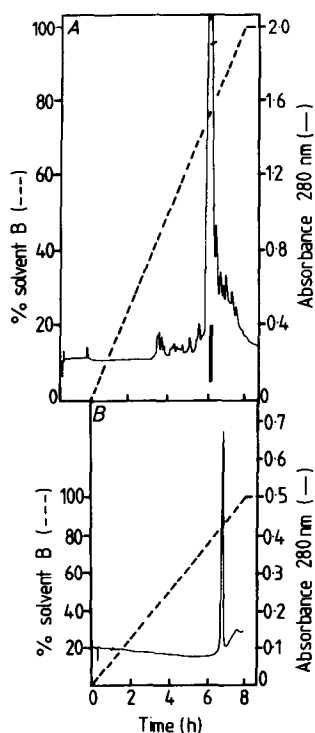* This peptide was previously synthesised and named LAP-20 (ref. 13).

Fig. 2. The purification of peptide 209 on a Radial-Pak C$_{18}$ column with a guard column of μBondapak C$_{18}$/Porasil B. In this separation solvent A consisted of 0.1 M ammonium bicarbonate and solvent B of 2-propanol–solvent A (80:20). A linear gradient from 0–100% solvent B over 8 h was used. The flow-rate was 1 ml/min and 180 ml of the deformylation mix, which contained 24 mg of peptide 209 and had been diluted with solvent A, 1:2 (v:v), was loaded through pump A onto the reversed-phase column. In part B 79 μg of purified peptide was analysed.

umn[10]. Therefore, as is shown in Fig. 2, the reaction mixture from the deformylation reaction could be both purified and desalted by chromatography on a Radial-Pak C_{18} column. However, it was necessary to use an 8-h gradient and include only the back of the main peak in the collected fraction due to a contaminant which eluted immediately before the main peak. The yield of peptide 209 was 16%; however, the recovery of a reinjected sample was 81%.

Geiger and Konig[14] have shown that transformylation from the nitrogen atom of the indole ring to the α-amino group of the peptide or the ε-amino group of lysine can be a significant side reaction in the deformylation procedure. Since the closely eluting contaminant in the separation of peptide 209 could arise from this side reaction, it was decided to study the deformylation reaction on another lipid associating peptide in which the mixture of products was more completely separated. Therefore the deformylation reaction was carried out with a sample of peptide 208 that had been purified by reversed-phase HPLC. Fig. 3A shows the purification of a sample of the N^{in}-formyl derivative of peptide 208. The material that eluted in the center of the peak was collected and analysed for purity in the same chromatographic system (Fig. 3B). In addition, the peptide was shown to be homogeneous by amino acid analysis and further chromatographic analysis (data not shown). The purified peptide was then subjected to the deformylation procedure and the product was again analysed (Fig. 3C). This time the deformylation procedure gave two products, one of
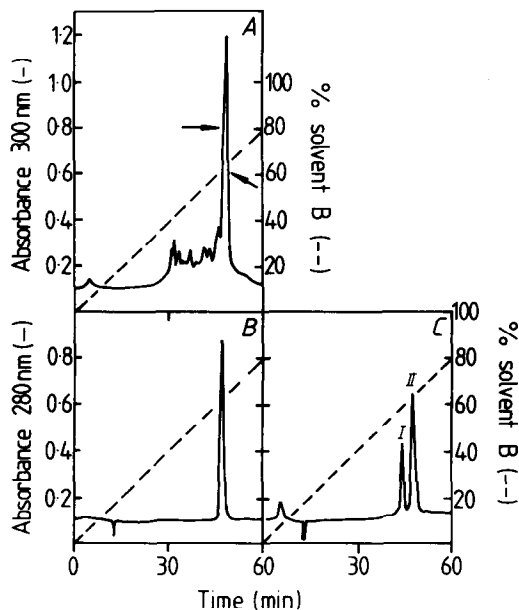

Fig. 3. The detection of a transformylation side reaction during the removal of the N^{in}-formyl group from peptide 208. The chromatographic conditions were as in Fig. 2, except that a linear gradient from 0–80% solvent B over 1 h was used. In part A, 2.5 mg of the peptide dissolved in 10 ml of 6 M guanidine hydrochloride was loaded through the solvent manifold of pump A. The purity of the peptide was analysed in the same system (part B). For the deformylation reaction the collected peak from part A (contained in 5 ml, denoted by arrows) was left at 4°C for 5 min after the addition of 2.87 mg of guanidine hydrochloride and 0.7 ml of ethanolamine. After the reaction was complete and the pH of the sample was adjusted, the sample was diluted with buffer A and the sample was loaded following the procedures described in the Materials and methods section. The elution profile is shown in Part C.
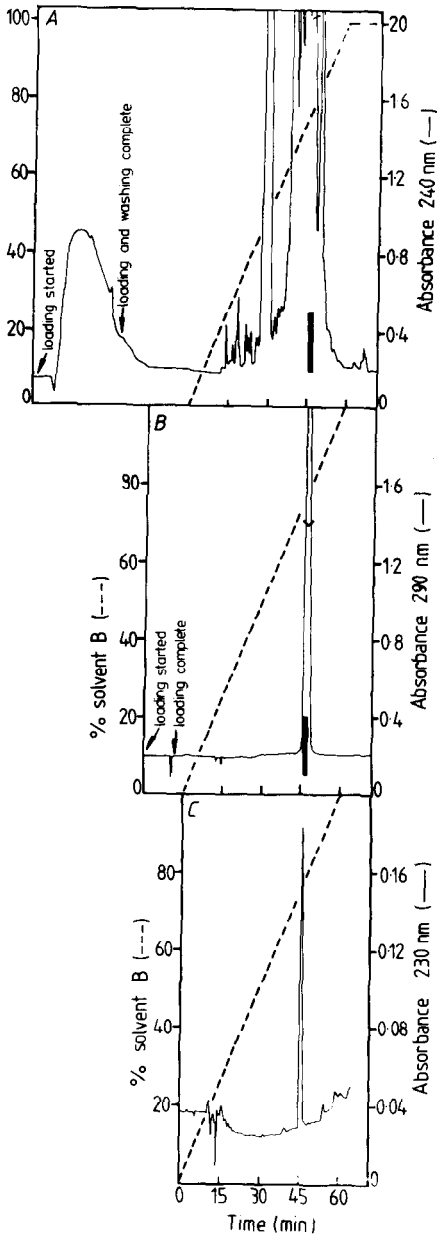
Fig. 4. The semi-preparative reversed-phase HPLC purification of peptide 203. A linear gradient from 0–100% solvent B over 1 h was used to elute the peptide. Solvent B was 2-propanol–acetonitrile–solvent A (3:3:4). A flow-rate of 1 ml/min was used. In part A 11.5 mg of peptide 203 dissolved in 18 ml of eluent was loaded on the Radial-Pak C_{18} column. In part B, 6.1 mg of peptide 203 from part A in 2 ml of eluent was diluted with 8 ml of 0.1 M ammonium bicarbonate and then purified. In part C, 5.6 μg of purified peptide was analysed.

which could be attributed to the formation of the α- or ε-amino formyl derivative of the desired peptide. Peaks I and II were found to have identical amino acid analyses, but peak I was identified as a formylated derivative because of its reduced cationic charge at acidic pH values as measured by ion exchange chromatography. Thus these results demonstrated that reversed-phase HPLC could be used in the deformylation procedure both to detect the presence of side reactions and to purify the product in a simple and high yield procedure.

In view of the excellent separations for a number of peptides that have been reported using mobile phases that contained 0.1 M ammonium bicarbonate[4,10], it was decided further to test the general applicability of this mobile phase by studying the purification of the peptides listed in Table I. Fig. 4A shows that the complex mixture present in an impure sample of peptide 203 was well resolved. The collected
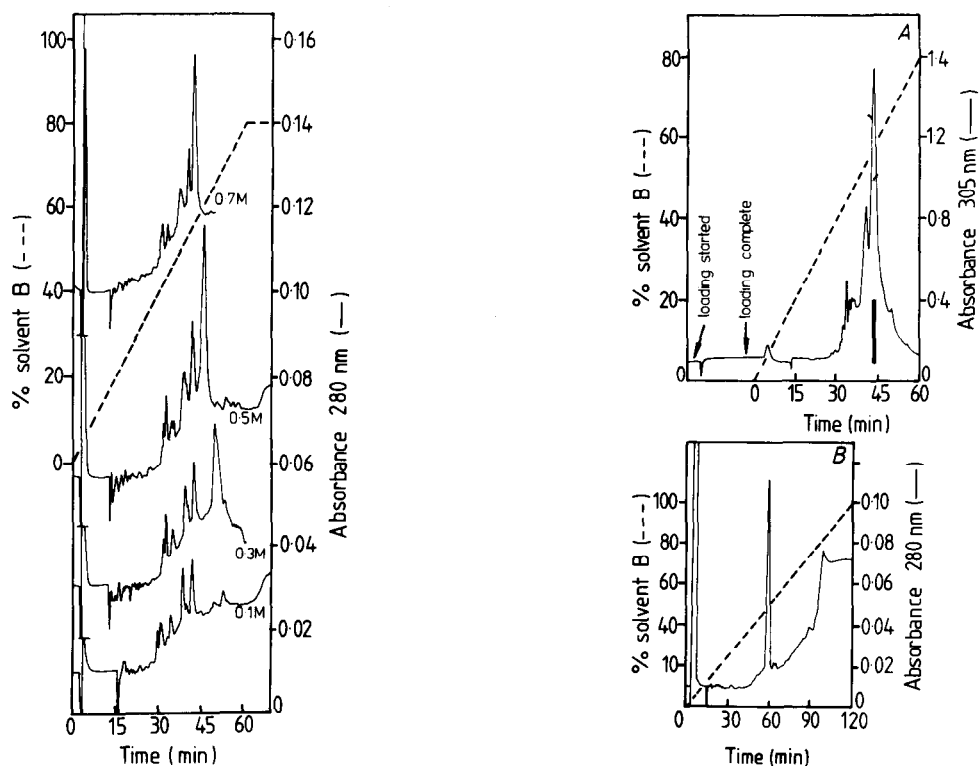

Fig. 5. The effect of increasing the concentration of ammonium formate in solvent A on the retention of Trp(CHO)-peptide 199 in reversed-phase HPLC. The separation was achieved on a Radial-Pak C₁₈ column at a flow-rate of 1 ml/min. The concentration of ammonium formate used in solvent A is shown above each trace (pH 6.3). Solvent B (see dotted line) consisted of 2-propanol–solvent A (80:20). The sample loading for each analysis was 120 µg of Trp(CHO)-peptide 199 in 250 µl of 6 M guanidine hydrochloride.

Fig. 6. The semi-preparative reversed-phase HPLC purification of peptide 199 using a mobile phase which contained 0.5 M ammonium formate (solvent A). The other chromatographic conditions used were as in Fig. 3, except that for part B a linear gradient from 0–100% solvent B over 2 h was used. Part A was achieved with a loading of 9.4 mg of the peptide in 12.5 ml of the neutralised deformylation mixture, diluted to 40 ml with solvent A, filtered and loaded through pump A at 2 ml/min. The loading part B was 60 µg of purified peptide in 800 µl of guanidine hydrochloride.

fraction was diluted 4-fold with solvent A and rechromatographed with the same solvent system (see Fig. 4B). The purity of the peptide after the second chromato- graphic step is shown in Fig. 4C. The total yield for the two steps was 51%; however, a recovery of 97% was recorded for the rechromatography of the purified peptide.

Peptide 199, the only peptide that contained the strongly basic arginine residue, behaved very differently from the other peptides in this chromatographic system. In the 0.1 M ammonium bicarbonate system used for purifying the other peptides a very broad peak was observed upon elution of peptide 199 from a Radial-Pak CN column. With the same solvent system and a Radial-Pak C_{18} column no peak was eluted. It was reasoned that the anomalous behaviour of this peptide was caused by the interaction of ionised silanol groups with the basic groups of the peptide, since an increase in electrolyte concentration in the mobile phase greatly improved the separation (see later). It was also thought that an increased concentration of am-
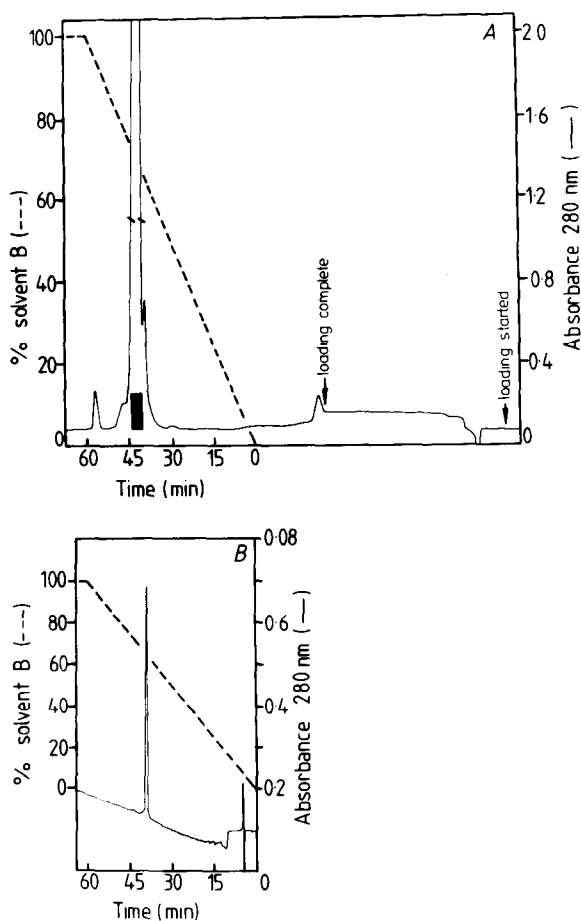

Fig. 7. The use of reversed-phase HPLC for the purification of a lipid associating peptide after purification by gel filtration on Sephadex G-10 and ion-exchange chromatography on SP-Sephadex. In this separation 6.8 mg of peptide 202 was chromatographed on a Radial-Pak CN column using the conditions described in Fig. 4. The purity of the pooled HPLC fractions is shown in part B where 16 μg of peptide was analysed.

monium ions in the mobile phase would reduce this interaction and therefore 0.5 *M* ammonium bicarbonate was added to solvent A. This mobile phase proved very difficult to work with, however, due to formation of carbon dioxide bubbles in the detector and therefore the less volatile salt, ammonium formate was tried. Peptide 199 was retained less and eluted with greater efficiency with increasing concentrations of ammonium formate in the mobile phase. Fig. 5 shows the effect of increasing concentration of ammonium formate in solvent A upon the elution profile of Trp(CHO)-peptide 199. From these results it was decided to use 0.5 *M* ammonium formate in the purification of peptide 199 and Fig. 6 shows a typical separation. The recovery of purified peptide was 27%, but rechromatography resulted in a recovery of 95% and demonstrated that the mobile phase containing ammonium formate had achieved the desired purification. The other peptides (202, 208, 209) behaved similarly to peptide 202 and could be successfully chromatographed with the ammonium bicarbonate containing mobile phase.

The semi-preparative system described in this publication can be used to purify either a partially purified lipid associating peptide (Fig. 7) or a crude preparation (Fig. 8). The general utility of a mobile phase which contains ammonium bicarbonate for the isolation of lipid associating peptides was demonstrated by the successful
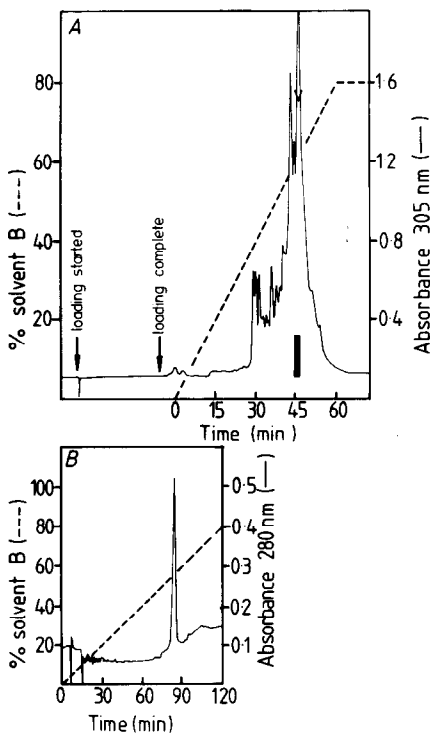

Fig. 8. The use of reversed-phase HPLC for the purification of a crude sample of lipid associating peptide. In this separation 26 mg of peptide 208 was chromatographed on a Radial-Pak C_{18} column using the conditions described in Fig. 3. Part B shows the corresponding analytical chromatogram that was obtained for 48 μg of the purified peptide.

purification of peptides 202 to 209 (see Table I). However, lipid associating peptide 199, which was the only peptide that contained an arginine residue, required a higher concentration of electrolyte in the mobile phase before a satisfactory chromatographic separation was achieved. This result is consistent with the observation of others[15,16] that strongly basic solutes interact significantly with residual silanol groups present on the stationary phase and that the coulombic interaction can be minimised by the addition of electrolyte to the mobile phase. As is shown in Fig. 9
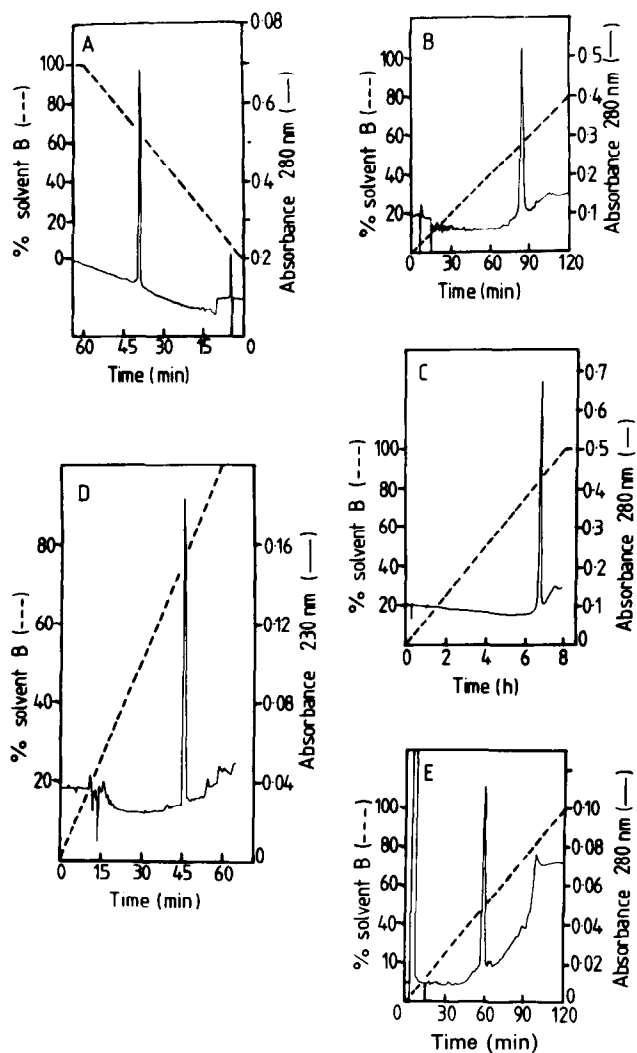

Fig. 9. Analysis of purified peptides by reversed-phase HPLC on a Radial-Pak CN column (A) and a Radial-Pak C_{18} column (B–E) at a flow-rate of 1 ml/min. The following mobile phases were used: A (peptide 202), solvent A: 0.1 M ammonium bicarbonate, solvent B: 2-propanol–acetonitrile–solvent A (3:3:4); B (peptide 208), solvent A: 0.1 M ammonium bicarbonate, solvent B: 2-propanol–solvent A (80:20); C (peptide 209), same as 208; D (peptide 203), same as 202; E (peptide 199), solvent A: 0.5 M ammonium formate, pH 6.3, solvent B: 2-propanol–solvent A (4:1).

TABLE II

AMINO ACID ANALYSIS VALUES FOR PURIFIED LIPID ASSOCIATING PEPTIDES

Conditions of hydrolysis: 6 M hydrochloric acid, 22 h, 110°C in evacuated tubes. Values in parentheses are the theoretical ratios of amino acids in the peptides.

Amino acid	Peptide 202	Peptide 203	Peptide 208	Peptide 209	Peptide 199
Thr*	—	0.8 (1)	—	—	—
Ser*	2.5 (3)	2.3 (3)	5.6 (8)	2.2 (3)	5.9** (6)
Glu + Gln	3.0 (2 + 1)	2.8 (3)	2.0 (2)	3.0 (2 + 1)	2.0 (2)
Ala	3.1 (3)	1.9 (2)	—	3.1 (3)	1.9 (2)
Val	—	0.9 (1)	0.9 (1)	—	—
Leu	5.0 (5)	3.0 (3)	4.0 (4)	5.0 (5)	3.0 (3)
Tyr	1.1 (1)	1.0 (1)	1.2 (1)	1.1 (1)	1.0 (1)
Phe	1.1 (1)	1.0 (1)	1.2 (1)	1.1 (1)	1.0 (1)
Lys	2.0 (2)	1.8 (2)	1.9 (2)	2.0 (2)	2.0 (2)
Arg	—	—	—	—	1.0 (1)
Trp***	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)

　* Values not corrected for oxidation except where stipulated by footnote **.
　** Values corrected for oxidation by quantitation of a series of timed hydrolyses and extrapolation back to zero time.
　*** N.D. = not determined. Analysis of the UV spectrum of each peptide shows that each peptide contains approximately 1 residue of tryptophan ($\varepsilon_M = 5200\ \mathrm{lmol^{-1}\ cm^{-1}}$, $\lambda_{max.} = 280$ nm).

the peptides purified by use of either system could be shown to be homogeneous by analytical HPLC with mobile phases that contained either ammonium formate or bicarbonate (see Fig. 9) or triethylammonium phosphate (data not shown) or by amino acid analysis (see Table II). In addition the preparative system described here allows the facile isolation and purification of peptides from the reaction mixture after removal of the N^{in}-formyl group that is commonly used to protect tryptophan in solid phase peptide synthesis.

ACKNOWLEDGEMENTS

REFERENCES

1 C. Edelstein and A. M. Scanu, in W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, p. 405 and references therein.
2 C. A. Bishop, D. R. K. Harding, L. J. Meyer, W. S. Hancock and M. T. W. Hearn, *J. Chromatogr.*, 192 (1980) 222.
3 D. R. K. Harding, C. A. Bishop, M. F. Tarttelin and W. S. Hancock, *Int. J. Pept. Protein Res.*, 18 (1981) 214.
4 D. R. Knighton, D. R. K. Harding, J. R. Napier and W. S. Hancock, *J. Chromatogr.*, 249 (1982) 193.
5 D. R. K. Harding, J. E. Battersby, D. R. Husbands and W. S. Hancock, *J. Am. Chem. Soc.*, 98 (1976) 2664.

 6 W. S. Hancock, J.D. Capra, W.A. Bradley and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 59.
 7 M. T. W. Hearn, in Cs. Horváth (Editor), *HPLC—Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 88.
 8 W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino Acid, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984.
 9 M. T. W. Hearn and W. S. Hancock, *Trends Biochem. Sci.*, 4 (1979) N58.
10 C. Olieman, E. Sedlick and D. Voskamp, *J. Chromatogr.*, 207 (1981) 421.
11 D. R. Knighton, *Ph.D. Thesis*, Massey University, Palmerston North, New Zealand, 1984.
12 J. T. Sparrow, Baylor College of Medicine, Houston, TX, personal communication.
13 D. H. Rich and E. Gross (Editors), *Peptides*, Pierce Chemical Company, Rockford, IL, 1981, p. 253.
14 R. Geiger and W. Konig, in E. Gross and J. Meienhofer (Editors), *The Peptides*, Vol. 3, Academic Press, New York, 1981, p. 82.
15 K. Sugden, G. B. Cox and C. R. Loscombe, *J. Chromatogr.*, 149 (1978) 377.
16 A. Sokolowski and K-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299.