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Purification of a synthetic myristylated peptide by counter-current chromatography

MARTHA KNIGHT*, SARA GLUCH, KAZUYUKI TAKAHASHI and TRUC T. DANG
Peptide Technologies Corporation, 125 Michigan Avenue N.E., Washington, DC 20017-1004 (U.S.A.)
and
RICHARD A. KAHN
Laboratory of Biological Chemistry, National Cancer Institute, Building 37, Room 5D02, National Institutes of Health, Bethesda, MD 20892 (U.S.A.)

ABSTRACT

A preparative purification of myristyl-Gly-Asn-Ile-Phe-Ala-Asn-Leu-Phe-Lys-Gly-Leu-Phe-Gly-Lys-Lys-Glu-NH_2 was accomplished using the multi-coil counter-current chromatograph. A partition coefficient was determined in the *n*-butanol–acetic acid–water (4:1:5) system. Chromatographic runs were made in this system and one modified with ethyl acetate. The peptide material showed anomalous elution behavior due to its surfactant properties. It was found that by loading the sample exclusively in the stationary phase, satisfactory retention of the compound occurred. Finally, conditions utilizing the upper phase as the mobile phase successfully separated the impurities.

INTRODUCTION

Modification of the amino terminal glycine by myristic acid has been shown to occur in a number of the protein components of signal transduction pathways (for a review see ref. 1). The role of the myristate moiety has been shown to be important in the reversible membrane association of these regulatory proteins [2]. The amino acid composition of peptides synthesized for their study are usually quite polar because of the presence of multiple lysine residues. However, the fatty acyl chain attached to the amino terminus renders the compound highly surfactant in solubility characteristics. The peptides, while being water soluble, are quite hydrophobic and are highly retained on reversed-phase columns. Over the years we have used semi-preparative reversed-phase chromatography for purification of synthetic myristylated peptides, but occasionaly we have had to resort to counter-current chromatography to effect final purification.

The peptide $CH_3(CH_2)_{12}CO$-Gly-Asn-Ile-Phe-Ala-Asn-Leu-Phe-Lys-Gly-Leu-Phe-Gly-Lys-Glu-NH_2, corresponding to the amino terminus of the ADP ribosylation factor [3], was synthesized for use in biochemical studies aimed at elucidating the role of the amino terminus in the activity and subcellular distribution of the 21 000-dalton GTP-binding protein. This sequence has a strong cationic charge, re-

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sulting from the three lysine side chains. The tetradecanoyl group and three phenylalanine residues make the peptide very hydrophobic, as well, Thus the peptide is only eluted from C_{18} columns in a high concentration of acetonitrile. When a preparative purification of 177 mg of this peptide was attempted on a 1-in reversed-phase column, less than 10 mg of material was recovered (unpublished results). The peptide, having been loaded in water on to the column, may have precipitated on the column in the conditions of the chromatographic run [4]. Therefore, we turned to counter-current chromatography in an effort to purify the compound with higher yield.

EXPERIMENTAL

Materials

The solvents used in the synthesis and chromatography are reagent grade or high-performance liquid chromatographic (HPLC) grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Water is purified through a Nanopure II system (Barnstead, Boston, MA, U.S.A.). Myristyl-Gly-Asn-Ile-Phe-Ala-Asn-Leu-Phe-Lys-Gly-Leu-Phe-Gly-Lys-Lys-Glu-NH_2 was synthesized by manual solid-phase synthesis [5] starting with 0.8 mmole Boc γ-benzyl-L-Glu *p*-methylbenzhydrylamine resin to which the *tert.*-butyloxycarbonyl (Boc) amino acid derivatives (Peninsula Labs., Belmont, CA, U.S.A. and Bachem, Torrance, CA, U.S.A.) were coupled for 2 h in 2.5 × molar excess over the first amino acid with equimolar dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt) which generate active esters *in situ*. The amino-protecting Boc groups are removed by 25% trifluoroacetic acid in dichloromethane (DCM) with indole, 1 mg/ml, for 30 min to prevent oxidation followed by DCM washes and neutralization with 10% triethylamine in DCM for 10 min. The steps are repreated as necessary to couple all the amino acids in sequence. 2-Chloro-carbobenzoxy-L-Lys was the Boc derivative used for Lys and Boc-L-Asn was used without side chain protection. After coupling of the last amino acid and deprotection, one half of the resin was removed to isolate the unmyristylated peptide and the rest was reacted with 1 mmole of myristic acid (Sigma, St. Louis, MI, U.S.A.) with DCC and HOBt in dimethyformamide (Burdick & Jackson, Muskegon, MI, U.S.A.). The couplings were monitored by the qualitative ninhydrin test [6]. The peptide resin was submitted to hydrogen fluoride, 10 ml/g resin, in the presence of anisole, 1 ml/g, for 45 min at 0°C. This reaction cleaved the peptide and simultaneously removed the side-chain-protecting groups. After evaporation of the hydrogen fluoride the peptide resin was washed with diethyl ether and dried. The peptide was extracted in water and lyophilized to give 1.15 g powder. The product and purified peptide were analyzed by HPLC as described below and amino acid analysis using a dedicated HPLC for ion-exchange chromatography and post-column fluorescence detection (St. Johns Assoc., Beltsville, MD, U.S.A.) [7]. The molar ratios found for the peptide are Asp, 1.75; Glu, 1.12; Gly, 2.99; Ala, 0.94; Ile, 1.04; Leu, 2.18; Phe, 3.30; Lys, 2.67.

Analytical chromatography

Analytical chromatography was conducted in Waters/Millipore equipment (Milford, MA, U.S.A.) including a U6K injector, dual extended flow 510 pumps, Model 481 variable-wavelength detector, Model 680 gradient controller and a SE 120 recorder. Analytical columns used were YMC-Pack 5-μm spherical, ODS, 200 Å, 150

× 6 mm I.D. (YMC, Morris Plains, NJ, U.S.A.) or μBondapak C_{18}, 250 × 3.9 mm I.D. (Waters, Milford, MA, U.S.A.). The separations of 10–50-μg sample were made in 0.1% aqueous phopshoric acid and gradients of acetonitrile at a flow-rate of 0.8 ml/min with detection at 215 nm or 254 nm according to previously published procedures [8].

Counter-current chromatography

Preparative chromatography was carried out in the multi-coil counter-current chromatograph (Peptide Technologies Corp., Washington, DC, U.S.A.) equipped with eight multi-layer coils in series on two column holders each carrying four coils mounted opposite each other across the axis of rotation. The apparatus has been described previously [9,10] and is shown in Fig. 1. The coils of continuous 1.6 mm I.D. PTFE tubing are filled with the stationary phase of a two-phase solvent system previously equilibrated in a separatory funnel and separated. The solvents used in these experiments are *n*-butanol–acetic acid–water (BAW) (4:1:5, v/v and ethyl acetate–*n*-butanol–acetic acid–water (3:1:1:5, v/v). The sample dissolved in equal volumes of the upper and lower phases is loaded into the tubing, then the rotation is started at 500 rpm and the mobile phase is pumped using a minipump (LDC/Milton

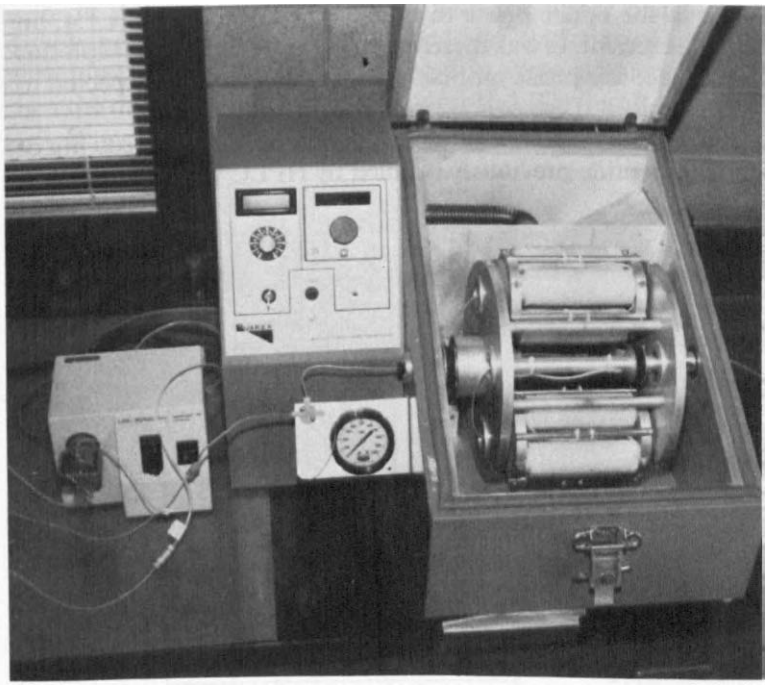

Fig. 1. The multi-coil countercurrent chromatograph that is an Ito coil planet centrifuge with eight multi-layer coils connected in series with four mounted on one column holder counterbalanced by the other four on the opposite side. Tubing enters left side with a "T" connection to a pressure gauge through the center shaft to coiling system and exits at the right side. The tubing is connected to a fraction collector. Solvent delivery is by a minipump (LDC/Milton Roy). The table-top apparatus was built at Varex Corporation, Burtonsville, MD, U.S.A.

Roy, Riviera Beach, FL, U.S.A.) at 1 ml/min. Fractions of 15 min are collected in a fraction collector (LKB, Gaithersburg, MD, U.S.A.) and presence of peptide is determined by reading the absorbance at 254 nm manually in a diode array spectrophotometer (Hewlett-Packard, Mountain View, CA, U.S.A.). After elution of a few column volumes the contents are pushed out with helium or nitrogen pressure, 20 p.s.i., and fractions collected. A small amount of acetone, approximately 50 ml, is pumped in and finally the tubing is dried by a stream of helium or nitrogen. Fractions containing peptide are analyzed by HPLC to determine purity. Those collected are evaporated in a rotary evaporator and lyophilized from water to yield a white powder.

RESULTS AND DISCUSSION

The product from the solid-phase synthesis of the myristylated peptide appeared heterogeneous by analytical HPLC due to difficulty in the coupling of some amino acids. Steric hindrance in the coupling was caused by the combination of the presence of particular amino acids that are difficult to couple, such as Asn, and the overall structure being hydrophobic with the tendency to interact with the resin or become involved in β bends. Nevertheless, a major component was present in the synthetic product as seen in the analytical chromatogram (Fig. 2). Fig. 2 shows the analysis of the sample in the two phases of the BAW solvent system. The ratio of the heights of the major peak in the upper phase to that of the lower phase is 1.4, an estimation of the partition coefficient. It was therefore decided to chromatograph this peptide in this system with the lower phase mobile. The peptide would be expected to elute well after the solvent front in these conditions.

The result of the counter-current chromatography of approximately 200 mg of crude peptide combined with peptide previously purified by HPLC was that the sam-

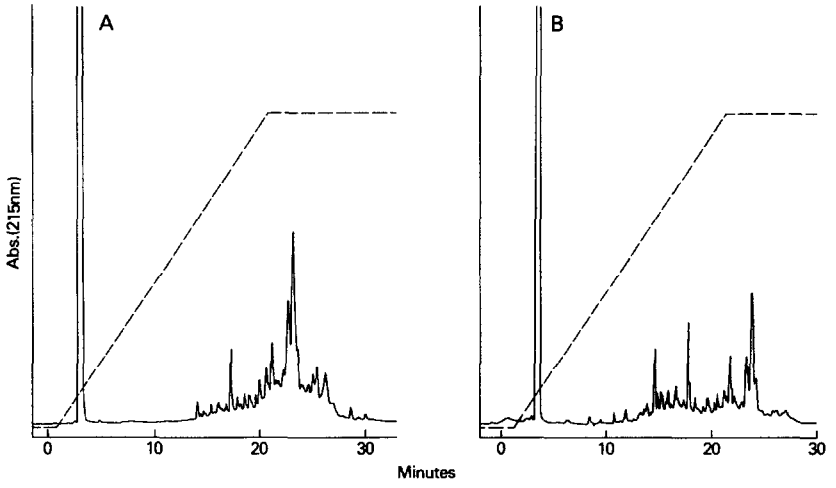

Fig. 2. Analytical HPLC of unpurified peptide distributed in upper phase (A) and lower phase (B) of the *n*-butanol–acetic acid–water (4:1:5) two-phase solvent system. Approximately 20 μg sample applied to the YMC-Pack 5-μm ODS column in 0.1% aqueous phosphoric acid and gradient of acetonitrile (broken line) from 10% to 70% in 20 min at 0.8 ml/min. Detection is at 215 nm at 0.5 a.u.f.s.

ple was eluted with the solvent front. There was some fractionation of the material. Some fractions contained more hydrophilic impurities, but no fraction contained the completely purified peptide (not shown). Also a lot of mass that was very heterogeneous was separated from the peptide and remained in the coil. Apparently, the peptide comprises a minor part of the total crude mass. Thus, this may be the reason for the observed low mass recovery of the desired peptide from the column chromatography. Another solvent system was tried, one modified with ethyl acetate to lower the partitioning of the compound into the upper phase with the lower phase being run again as the mobile phase. Poor fractionation resulted again with the sample at the solvent front. This solvent system was tried again with the upper phase mobile. The compound eluted very late as shown in Fig. 3. Since the absorbance values were low, the presence of peptide was verified by HPLC. Peptide was distributed from fractions 100 to 115 but with a major impurity as shown in Fig. 4. From this run there was 10 mg of 86% pure peptide and 13.2 mg of less pure peptide.

It was hypothesized that the elution of the peptide was affected by how it was dissolved prior to loading. In the previous runs the sample was dissolved in both phases and introduced into the tubing. Since the peptide is both highly cationic and hydrophobic it is probably lowering the surface tension of the solvent system and traveling at the interface. Therefore, it was decided to re-chromatograph 26 mg of a previous fraction 36 of the first counter-current chromatographic run. The conditions were in the original BAW system with the upper phase as the mobile phase and dissolving the compound only in the lower stationary phase. Thus, the coil was filled with lower phase, the sample charged in approximately 5 ml of the aqueous phase, then the upper phase was chromatographed. The result is shown in Fig. 5. A peak was

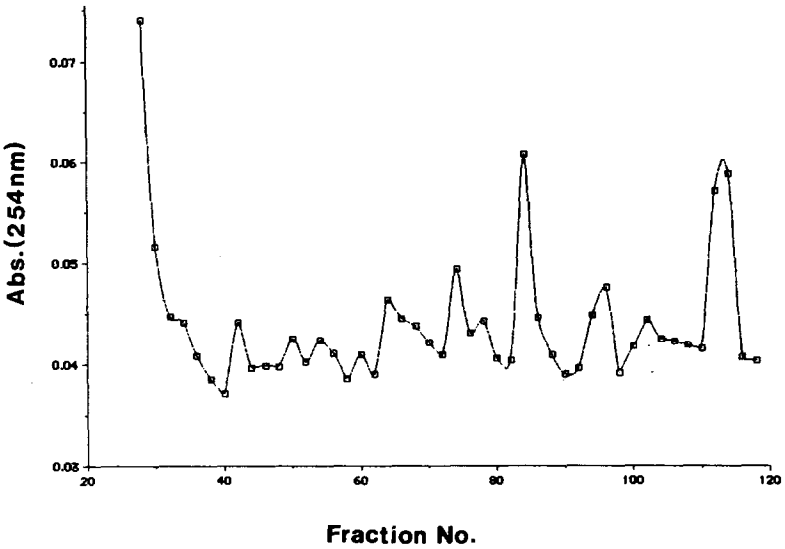

Fig. 3. Counter-current chromatography of sample recovered from a previous separation in the solvent system ethyl acetate–*n*-butanol–acetic acid–water (3:1:1:5) with the upper phase mobile. Aliquots of the fractions collected were diluted in 50% aqueous methanol and absorbance at 254 nm was read. Peptide was contained in fractions 100 to 115.

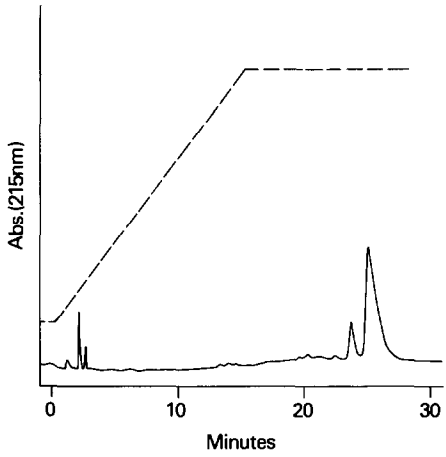

Fig. 4. Analytical chromatography of fraction 109 which contained 10 mg peptide. Separation is on a μBondapak C_{18} column in the same solvent system but with a gradient of 20% to 60% acetonitrile at 0.8 ml/min in 15 min. This was the purest fraction which appeared 86% pure.

eluted at fractions 32–40, after the solvent front which occurred at fraction 27. The material in the major part of the peak had far less contaminant and most of the material was recovered. Pure peptide was 18 mg out of 24.7 mg recovered in all the fractions. In Fig. 6 is shown the analytical HPLC of the recovered peptide compared with the sample loaded. The partition coefficient calculated from the elution volumes of the run is 1.05. From this experience it may be important to consider loading the sample in the stationary phase instead of both phases if there is a possibility that the compound has surfactant properties.

Since there is no more crude peptide left it cannot be determined whether these final conditions would have purified the compound in one step. But it is evident from this work that solid-phase synthesis products of hydrophobic peptides have a significant amount of heterogeneous side-product mass that is conveniently removed by

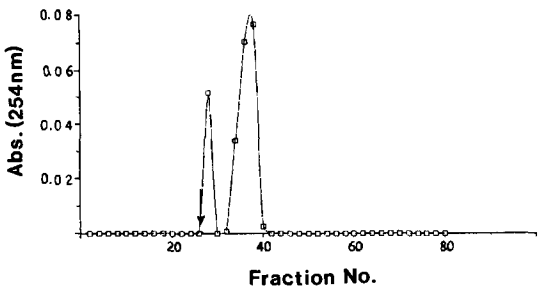

Fig. 5. Counter-current chromatography of 26 mg of a semi-purified sample (shown in Fig. 6) in the BAW system with the upper phase mobile. The solvent front emerged at fraction 27, indicated by arrow, and purified peptide was contained in fractions 33–37. The remainder of the peak contained more hydrophillic impurities.

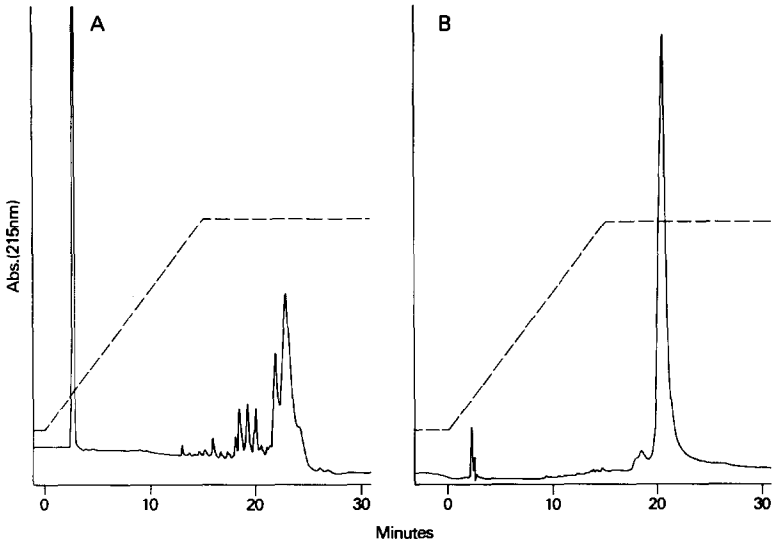

Fig. 6. (A) Analytical HPLC of sample loaded in the counter-current chromatography of Fig. 5. (B) Analysis of peptide recovered from the chromatography of Fig. 5. Conditions are as described in the legend to Fig. 4, except that the flow-rate in B was 1 ml/min.

counter-current chromatography instead of being deposited on expensive column packing.

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