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PURIFICATION OF SYNTHETIC PEPTIDES ON A HIGH-RESOLUTION PREPARATIVE REVERSED-PHASE COLUMN

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SUMMARY

A 2-in. I.D. column filled with 10-μm spherical C_{18} bonded silica with 120 Å pores was used for the preparative purification of various synthetic peptides in one step. The small-sized silica packing afforded high resolution and the spherical shape helped maintain a relatively low back-pressure during the chromatography. Conditions for performing the separations were derived from the analytical chromatography of samples on a column of similar 5-μm material. The same amount of organic modifier, but with the gradient duration increased, achieved very similar separations on the preparative column.

INTRODUCTION

The purification of solid-phase synthesized peptides has been performed using a recently available preparative column filled with small-diameter spherical C_{18} bonded silica support material. To achieve the one-step purification of laboratory-scale preparative amounts of peptides, a high-resolution column is advantageous over a column containing larger diameter particles (>20 μm) for the highly heterogeneous products of automatic or manual solid-phase synthesis. Although these columns are expensive, a one-step purification procedure effects significant cost savings as it is more rapid. Moreover, the use of precolumns can extend their lifetimes. The analytical chromatography was carried out on very high-resolution columns of 5-μm ODS silica with 120- or 200-Å pores, which are suitable for the size of peptides synthesized here. After some experience the conditions and results of analytical-scale chromatography suggested preparative-scale chromatographic conditions that were satisfactory without the need for extended chromatographic studies. We describe here the conditions and columns used for both analytical and preparative chromatography that achieved the preparative purification of many linear peptides. This strategy seems to be suitable for the peptides that we have studied, which are up to twenty residues in length.

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EXPERIMENTAL

Solvents were of high-performance liquid chromatographic (HPLC) or analytical-reagent grade from Fisher Scientific (Pittsburgh, PA, U.S.A.) and trifluoroacetic acid (TFA) was obtained from Halocarbon (Hackensack, NJ, U.S.A.). Water was distilled and passed through a Nanopure cartridge filtration system (Barnstead, Boston, MA, U.S.A.). Peptides were synthesized by standard solid-phase techniques[1] using Boc-amino acid derivatives, either manually or in a Biosearch (Novato, CA, U.S.A.) 9500 AT peptide synthesizer. The syntheses were made on phenylacetamidomethyl (PAM) or chloromethyl resin. After the assembly of amino acids, the peptide–resin was treated with anhydrous hydrogen fluoride, which cleaved the peptides and removed side-chain protecting groups. After lyophilization, the product was analyzed by HPLC for the determination of impurities and the amino acid composition was determined by amino acid analysis of a 22-h mercaptoethanesulfonic acid hydrolysis using ion-exchange chromatography with post-column *o*-phthalaldehyde fluorescence detection[2] (St. Johns Assoc., Beltsville, MD, U.S.A.).

Analytical chromatography was carried out in Waters/Millipore equipment consisting of a U6K injector, two Model 510 pumps with extended flow heads, a Model 681 variable-wavelength UV detector, a Model 680 gradient controller and an SE120 recorder (all from Waters/Millipore, Milford, MA, U.S.A.). A YMC-Pack, 5-μm spherical ODS, 200 Å, 150 × 6 mm I.D. column was used (AMP-312-5; YMC, Morris Plains, NJ, U.S.A.). The solvent system was 0.1% aqueous phosphoric acid and acetonitrile gradients at a flow-rate of 1 ml/min. Preparative chromatography was conducted with a Waters/Millipore LC3000 solvent-delivery system with helium sparging of the mobile phase consisting of 0.1% aqueous TFA and gradients of 0.1% TFA in acetonitrile at 50 ml/min. A 300 × 50 mm I.D. column of spherical, 10-μm ODS, 120 Å, silica (R-354-S10; YMC) was used with a 50 × 50 mm I.D. guard column of 50 μm ODS, 120 Å, silica (YMC). The effluent was passed through a Model 680 UV detector equipped with a preparative flow cell. An SE120 recorder was used and fractions were collected manually.

The sample was dissolved in water at a concentration of 1–5 mg/ml and filtered through 0.45-μm filters. The sample was pumped at 25 ml/min onto the column, which had been equilibrated in 0.1% aqueous TFA. Subsequently, one column volume (600 ml) of 0.1% aqueous TFA was pumped at 50 ml/min, then the gradient was started. The peak fractions were analyzed by HPLC and those containing the peptide were directly lyophilized. The purified peptide was analyzed by analytical HPLC and amino acid analysis. These procedures were modified from previously published methods[3,4].

RESULTS AND DISCUSSION

An analog of kallidin, designed for radiolabeling, dehydrokallidin (Lys–Arg–ΔPro–ΔPro–Gly–Phe–Ser–ΔPro–Phe–Arg; ΔPro = dehydroproline), was synthesized manually on 4 g of Boc-tosyl-Arg-chloromethyl resin. After cleavage with hydrogen fluoride, 890 mg of powder resulted. Dehydrokallidin was initially separated on an analytical column to determine the complexity of the compound, optimize the separation and develop gradient conditions for the large-scale column. The lower

part of Fig. 1 shows the analytical separation with detection at 254 nm, indicating a major peak eluting at 24 min and an earlier eluting component well separated in the gradient to 20% acetonitrile. The peptide was purified in two batches of 400 and 490 mg on the 2-in. diameter column (300 × 50 mm I.D.) packed with 10-μm spherical ODS, 120 Å, silica (Fig. 2). The sample was loaded as described under Experimental. After passing 600 ml of 0.1% aqueous TFA, a gradient to 20% acetonitrile was run in 20 min, *i.e.*, the same final conditions as in the analytical chromatography. The first peak that was eluted contained very little mass (43 min, Fig. 2). The analytical chromatography of this material is shown in Fig. 1 (15-min peak, upper panel). This

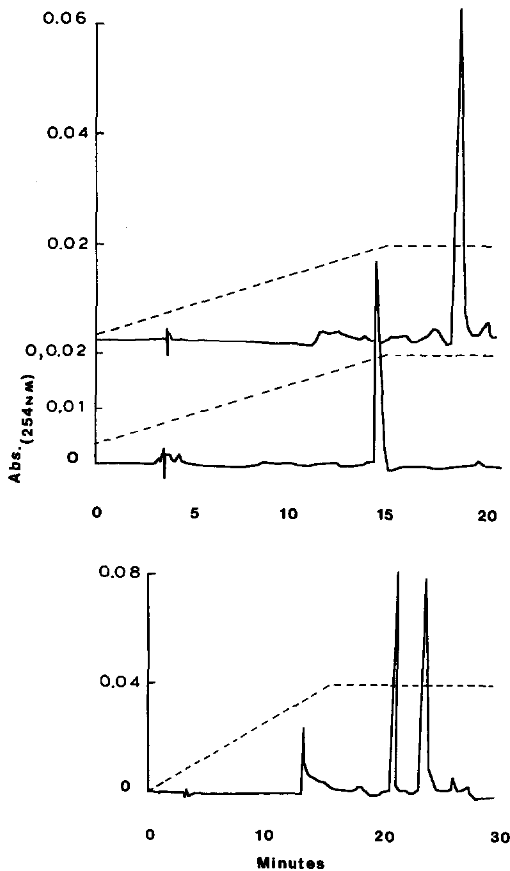

Fig. 1. Analytical chromatography of unpurified dehydrokallidin (lower chromatogram) and of the two peaks separated in the preparative chromatography (top two traces). The lower panel represents *ca.* 100 μg of the synthetic product chromatographed on the YMC 6-mm I.D. column as described under Experimental using 0.1% aqueous phosphoric acid as A and acetonitrile as B at a flow-rate of 1 ml/min; the gradient used was 0–20% B in 15 min. Peptide was eluted at 24 min and an impurity at 20.5 min. Upper panel: samples of 20 μl of the peak fractions from the preparative run in Fig. 2 were chromatographed with a gradient from 3 to 20% B in 15 min. Detection at 254 nm. The lower trace in the upper panel represents the 43-min preparative peak or side-fraction eluting at 14.5 min and the upper trace the peak fraction of the second peak containing the purified peptide which was eluted at 19 min under these conditions.

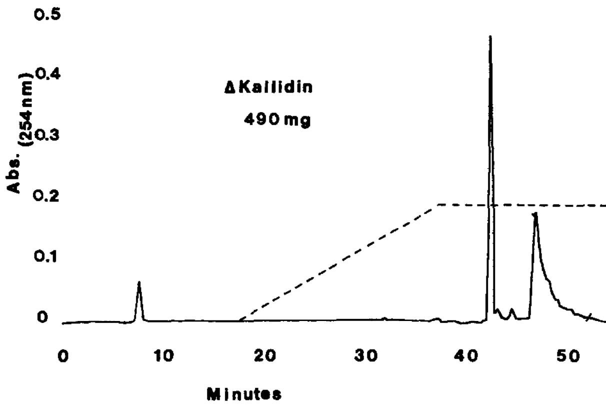

Fig. 2. Chromatogram of half of the dehydrokallidin synthesis product on the YMC 50-mm I.D. column as described under Experimental using 0.1% aqueous TFA as A and 0.1% TFA–acetonitrile as B at a flow-rate of 50 ml/min. The end of sample loading was 0 min and, because some material was eluted at the void volume, the gradient was started later and went from 0 to 20% B in 20 min. The peak at 43 min was collected as one fraction and the following peak (46.5 min) was collected in nine fractions and contained the pure peptide. The fractions between the marks on the peak trace were pure and were combined. Detection at 254 nm with 0.5 a.u.f.s.

corresponded to the first component in the crude peptide. The second peak was collected in many fractions. Analytical HPLC of these appeared as shown in Fig. 1 (18-min peak, uppermost trace). The fractions were dried and fractions 4–10 were pure, containing 276 mg of white crystals. The other run gave similar results, 219 mg

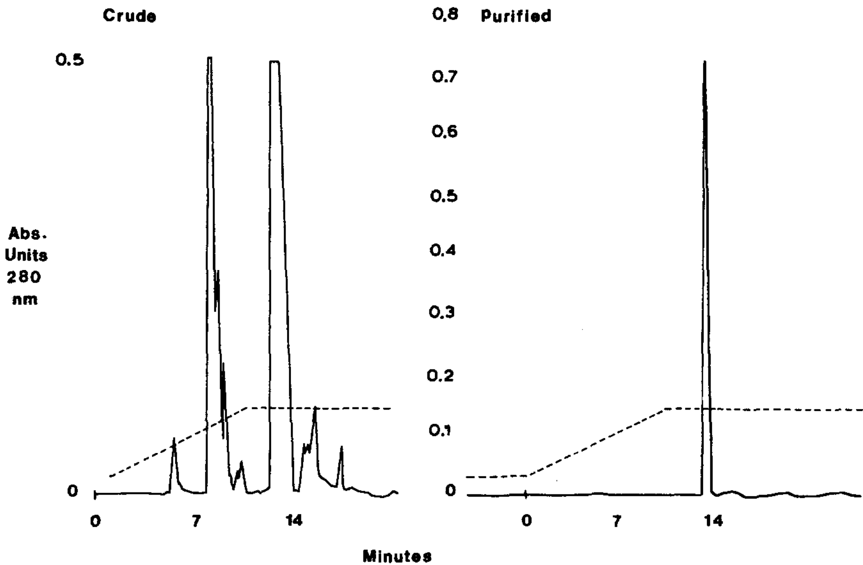

Fig. 3. Analytical chromatography of *ca.* 50 μg of crude (left) and 25 μg of final purified hexapeptide Lys–Ala–Met–Tyr–Ala–Pro (right). Conditions similar to those in Fig. 1 except that the gradient was from 3 to 15% B in 10 min and detection was at 280 nm. The peptide was eluted at *ca.* 14 min (both chromatograms) and an impurity was eluted at *ca.* 8 min (left).

of crystals being recovered. The amino acid analysis showed the expected molar ratios of the constituent amino acids. The chromatographic result on the 5-μm column was similar to that of the preparative chromatography. A gradient to the same percentage of acetonitrile with only a 5-min increase in the duration resulted in similar elution of the material. The back-pressure under these conditions was *ca.* 400 p.s.i.

A hexapeptide was synthesized on a Boc-Pro-PAM resin. On analytical chromatography a significant amount of earlier eluting impurities was evident (Fig. 3, left chromatogram). Originally we used a 5–10% lower acetonitrile concentration in the final conditions for preparative elution, but for a series of small hexapeptides elution did not occur until the level of acetonitrile was increased. Therefore, to maintain the maximum separation and establish the level of acetonitrile that would effect elution, we increased the gradient time so as to reach a percentage of organic modifier that would definitely elute the compound. Thus, for Lys–Ala–Met–Tyr–Ala–Pro, the analytical gradient conditions were 3–15% acetonitrile in 15 min and a large heterogeneous impurity was eluted before the major product, as seen in Fig. 3 (left chromatogram). The sample was chromatographed with a long gradient, the compound being expected to elute before the end of the gradient. However, as shown in Fig. 4, the components were eluted at about 15% acetonitrile. The appearance of the minor components is very similar to that in the analytical chromatography of the crude peptide (Fig. 3). The second peak in the chromatogram contained the major product that was purified, as seen in the chromatogram of Fig. 3, with a recovery of 65% of the total mass loaded. Hence long gradient times are advantageous for maximizing separations especially when there is uncertainty about the percentage of acetonitrile required to elute the sample. The earlier eluting impurities could be due to oxidized methionine or carbonates of lysine, which would create more polar structures. Nevertheless, pure peptide was obtained in one step.

Another peptide, Ala–His–Ser–Asn–Arg–Lys–Leu–Met–Glu–Ile–Ile, was synthesized on Boc-Ile-PAM resin. The tosyl protecting group on histidine was removed from the imidazole ring after the coupling by treatment with hydroxybenzotriazole.

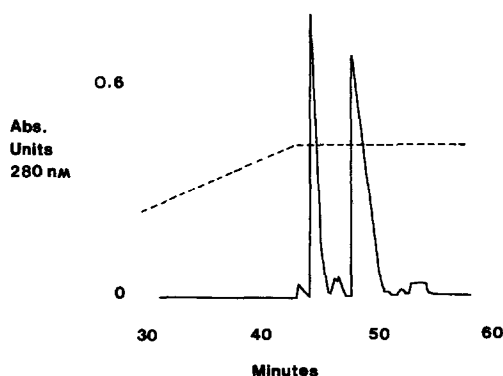

Fig. 4. Preparative chromatography of the hexapeptide Lys–Ala–Met–Tyr–Ala–Pro (158 mg) on the YMC 50-mm I.D. column in the solvent system as described in Fig. 2. The gradient was started 13 min after loading and went from 0 to 25% B in 30 min. The part of the run from 30 to 60 min is shown. The fractions across the whole of the second peak (48–50 min) were pure by analytical HPLC (not shown). The 45-min peak contained impurities.

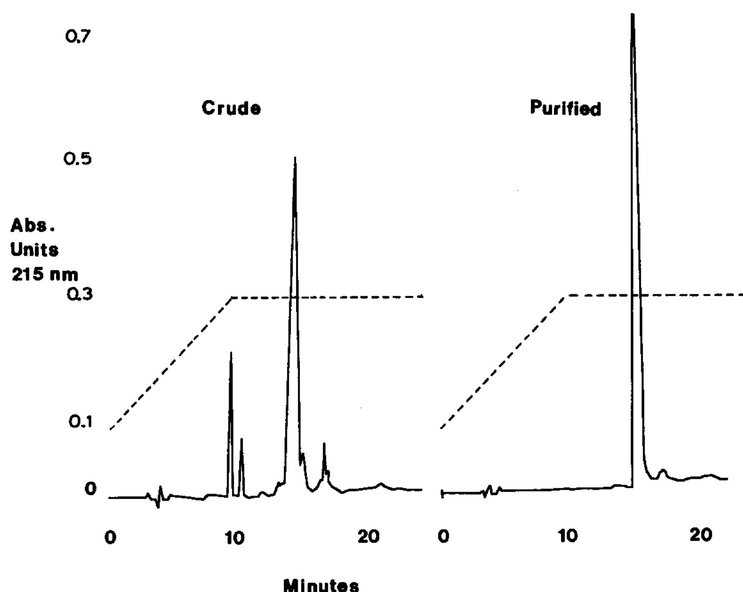

Fig. 5. Chromatographic analysis of the undecapeptide Ala–His–Ser–Asn–Arg–Lys–Leu–Met–Glu–Ile–Ile unpurified (left) and purified (right), 10 μg each, in a gradient from 10–30% B (same mobile phase as in Fig. 1) in 10 min with detection at 215 nm with 1 a.u.f.s. The peptide was eluted at *ca.* 15 min.

The resulting peptide had the expected molar ratios and the analytical chromatography showed the presence of multiple but minor impurities (Fig. 5). Most of the peptide (330 mg) was loaded and a gradient to 30% acetonitrile was run in 30 min. Fig. 6 shows the preparative chromatogram; the composition is similar to the analyt-

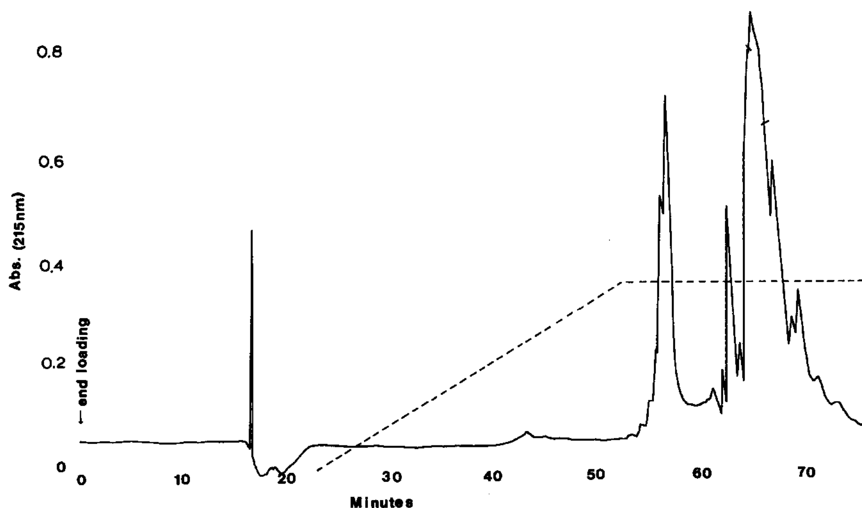

Fig. 6. Preparative chromatography of the undecapeptide Ala–His–Ser–Asn–Arg–Lys–Leu–Met–Glu–Ile–Ile (330 mg) on the YMC 50-mm I.D. column. The gradient was started after 20 min and was run from 0 to 30% B (same solvent system as in Fig. 2) in 30 min. The part of the major peak (65 min) between the marks was taken as the pure peptide. Detection was at 215 nm at 1 a.u.f.s.

ical separation. To increase the separation of the components, it was important to increase the duration of the gradient. The fractions collected between the marks on the large peak were pure and used for experiments. The fractions on either side were 80–90% pure and not pooled with the purest fractions. The recovery of the pure peptide was approximately 80 mg. The remainder of the mass in the whole peak was undetermined. To recover more pure peptide, it is possible to rechromatograph the impure fractions, but we derived sufficient material for the experiments.

The column of 10-μm spherical ODS bonded-phase particles gave results of high resolution for preparative-scale chromatography. The issue of whether the pore size should be larger, *i.e.*, 300 Å, at least for peptides of MW <2300, does not appear to be important with respect to capacity or resolution. We found the kind of elution that can be scaled up successfully is one in which the peak of interest is eluted 3–6 min following the completion of the gradient reaching the final solvent percentage. The gradient duration is usually 10–15 min. The same percentage of acetonitrile can be used as the final conditions in the 2-in. column, but always starting from 0% and making the gradient duration two to four times that in the analytical run, or 30–60 min. The longer gradient duration can be used to elute the more polar impurities. The linear velocities of the analytical and preparative columns are comparable but not identical, being 3.9 and 2.9 cm/min, respectively. Hence the final percentage of acetonitrile, which is reached *ca.* 5 min before the peptide elutes, can be used as the final conditions with appropriate lengthening of the time to reach that level, as was done in the examples of dehydrokallidin and the undecapeptide. For convenience in detection, phosphoric acid is used for the analytical chromatography owing to its transparency at low wavelengths. Although the elution may be different using TFA, especially for basic peptides, this has rarely occurred in our experience (unpublished results).

The examples shown here involve more heterogeneous synthetic products of relatively small peptides because of the presence of certain amino acids known to give more impurities owing to the methods of synthesis. However, these were removed expeditiously by these procedures. The large column used in these purifications has been used continuously for more than 6 months for the purification of over 25 different peptides.

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