Journal of Chromatography, 459 (1988) 361–367
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1461

SCALE-UP METHODOLOGY FOR THE PREPARATIVE PURIFICATION OF PEPTIDE M

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SUMMARY

An octadecapeptide, peptide M, the epitope of a retinal protein that induces experimental autoimmune uveitis, was synthesized and purified by preparative reversed-phase chromatography. The flow-rate and gradient conditions for maximum separation of impurities were determined on a 30 × 0.39 cm I.D. column of Delta Pak (15-μm spherical C_{18}-bonded silica with 300-Å pores). The maximum amount of peptide that was resolved under these conditions was then determined experimentally. Using a scale factor dependent on the square of the column diameters, the flow-rate and amount loaded were increased 164 times on a 30 × 5 cm I.D. column of the same packing. The same resolution was achieved. Batches of 200–342 mg were chromatographed with reproducible results, providing a total yield of 394 mg of pure peptide.

INTRODUCTION

S-antigen, a photoreceptor cell protein involved in the visual process, can produce autoimmune inflammatory disease[1]. An octadecapeptide sequence of the S-antigen is the pathogenic region of the protein which contains the epitope that induces experimental autoimmune uveitis, a T-cell mediated inflammation of the retinal cells and pineal gland. A synthetic peptide corresponding to this region of S-antigen has been confirmed to induce this disorder and is designated peptide M[2].

To make peptide M available for many studies, a large-scale synthesis was undertaken, and procedures for purifying relatively large amounts of the synthetic product had to be developed for one of the recently available preparative columns of 5 cm I.D. Applying proposed scale factors for sample load and flow-rate, the conditions determined on analytical columns of the same chromatographic material were systematically applied to a larger diameter column of the same length. Solid-phase

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synthesized peptides usually yield relatively heterogeneous products. Impurities can result from N to O acyl shifts, lack of removal of side-chain protecting groups or isomerization. The complexity of the task increases with increasing length of the synthesized peptide. Therefore, usually the major product of the synthesis needs to be isolated and characterized.

EXPERIMENTAL

Materials

Solvents and reagents used were of high-performance liquid chromatographic (HPLC) or analytical-reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Dimethylformamide was obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Water was purified through the Millipore reverse-osmosis and cartridge filtration system (Millipore, Bedford, MA, U.S.A.). Peptide M (Asp–Thr–Asn–Leu–Ala–Ser–Ser–Thr–Ile–Ile–Lys–Glu–Gly–Ile–Asp–Arg–Thr–Val) was synthesized manually[3] starting with 7 g of Boc-Val phenylacetamidomethyl resin, 0.4 mequiv./g (Omni Biochem, National City, CA, U.S.A). The Boc-amino acid derivatives, including 2-Cl-carbobenzoxy (CBZ)-Lys, tosyl-Arg (Bachem, Torrance, CA, U.S.A.), benzyl ethers of Thr and Ser and the benzyl ester of Asp (U.S. Biochemical, Cleveland, OH, U.S.A.) were coupled as *in situ* generated active esters with equimolar amounts of dicyclohexylcarbodiimide and hydroxybenzotriazole. Deprotection was carried out by a 30-min reaction with 25% trifluoroacetic acid (TFA) in dichloromethane with 1 mg/ml indole and neutralization was effected with 10% triethylamine in dichloromethane. The peptide resin was washed between steps with 7 ml/g dichloromethane. After the assembly of the amino acids in the above sequence, the resulting peptide resin weighed 13.2 g. The peptide was liberated from the resin and side-groups were removed by treatment of the resin in two batches of 6.6 g, with 60 ml of hydrogen fluoride and 6 ml of anisole for 45 min at 0°C. After washing with ethyl acetate, extraction with water and lyophilization, 2.6 g of white powder resulted. Amino acid analysis using ion-exchange HPLC and *o*-phthalaldehyde post-column detection (St. John's Associates, Beltsville, MD, U.S.A.) of a 22-h hydrolysate gave the following molar ratios: Asp 2.95 : Thr 2.86 : Ser 1.99 : Glu 0.96 : Gly 0.89 : Ala 0.84 : Val 1.10 : Ile 2.56 : Leu 0.88 : Lys 1.05 : Arg 1.21.

Analytical chromatography

The peptide was chromatographed analytically to assess its purity and to develop the conditions for preparative chromatography. Solutions of 1 mg/ml of peptide in water were prepared and filtered, if necessary, through a 0.45-μm Durapore filter (Millipore). Crude peptide and the final purified peptide were chromatographed on μBondapak C_{18} (15 × 0.39 cm I.D. column) in 0.1% TFA or phosphoric acid in acetonitrile gradients at 1 ml/min for analytical characterization (not shown). The optimization of the separation of the crude peptide was carried out at a flow-rate of 0.5 ml/min on a 30 × 0.39 cm I.D. column of Delta Pak (C_{18}, 15 μm, 300 Å) (Waters, Milford, MA, U.S.A.) with two different volatile solvent systems: 0.02 *M* ammonium acetate (pH 6.8) and 0.1% acetic acid in acetonitrile or 0.1% aqueous TFA and 0.1% TFA in acetonitrile. The equipment used for analytical chromatography consisted of two Model 510 pumps, a WISP multiple-sample injector and a Model 481 variable-

wavelength detector, set at 214 nm, and data analysis was provided by a Model 840 Data Station. Methods development was performed on a Model 600 HPLC instrument, with a Model 490 multiwavelength detector, set at 214 nm, and the 840 data station (all from Waters). The solvents were sparged with helium.

Preparative chromatography

For preparative chromatography a 30 × 5 cm I.D. column of Delta Pak was used. The peptide was dissolved in water at a concentration of approximately 2 mg/ml and filtered through 0.45-μm Durapore filters. The solution of *ca.* 200–342 mg peptide was pumped into the column through a port on the solvent delivery system of a Delta-Prep instrument (Waters). The solvents were pumped at 80 ml/min with helium sparging. The effluent was passed through a preparative flow cell in a Model 481 detector, set at 214 nm, and connected to an 840 data station. Fractions were collected manually every 0.5 min. The fractions were analyzed by HPLC, and those containing pure peptide were lyophilized and combined. Some impure fractions were combined and rechromatographed to recover more pure peptide.

RESULTS AND DISCUSSION

The crude peptide was chromatographed on the 30 × 0.39 cm I.D. Delta-Pak column with two volatile solvent systems under various gradient conditions, as presented in Figs. 1 and 2. As all the components of the solvent systems are removable by lyophilization, no further steps in the purification were necessary. Maximum separation of impurities from the major peak was achieved with 0.1% TFA in water–acetonitrile eluents. In Fig. 1 are shown a series of linear gradients all starting at

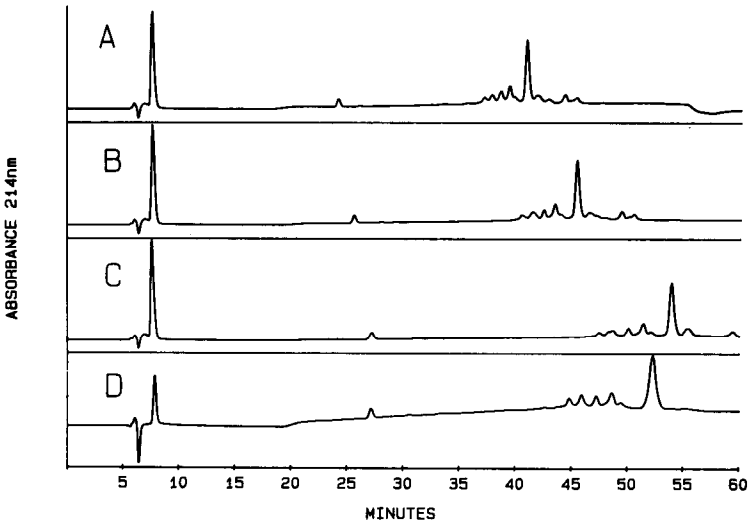

Fig. 1. Optimization of chromatographic conditions for the purification of synthetic peptide M on a 30 × 0.39 cm I.D. column of Delta Pak (C_{18}, 300 Å, 15 μm) with 0.1% TFA and acetonitrile gradients. Eluent A was 0.1% aqueous TFA and eluent B was 0.1% TFA in acetonitrile. The linear gradient for each chromatogram was as follows: A, 0–50% B in 40 min; B, 0–40% B in 40 min; C, 0–30% B in 40 min; D, 0–25% B in 30 min. Other conditions: flow-rate, 0.5 ml/min; detection, 214 nm. A.u.f.s.: A–C, 0.7; D, 0.6.

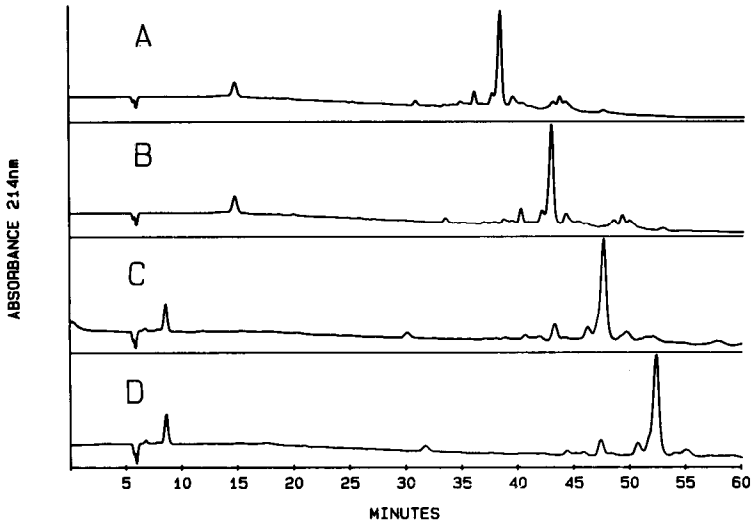

Fig. 2. Optimization of chromatographic conditions for the purification of synthetic peptide M on a 30 × 0.39 cm I.D. Delta Pak column. Eluent A was 0.02 *M* ammonium acetate (pH 6.8) and eluent B was 0.1% acetic acid in acetonitrile. The gradient conditions for each chromatogram were as follows: A, 0–30% B in 40 min; B, 0–25% B in 40 min; C, 0–20% B in 40 min; D, 5–18% B in 40 min. Flow-rate, 0.5 ml/min; detection, 214 nm. A.u.f.s.: A and B, 0.5; C and D, 0.3.

100% aqueous TFA and run with decreasing percentages of acetonitrile (50 to 25%). The 30-min gradient to 25% acetonitrile separates the hydrophilic impurities from the major peak while retaining the hydrophobic impurities on the column. Another volatile solvent system, 0.02 *M* ammonium acetate (pH 6.8), was tried to determine if a different selectivity would improve the resolution. As shown in Fig. 2, the impurities were not as well resolved from the major peak. At neutral pH the major peak is retained less than at acidic pH (compare Fig. 2A with Fig. 1C). The four acidic groups of the peptide are protonated at the low pH of TFA, making the peptide more hydrophobic, thus increasing the retention time. The increased retention time of the major peak more likely results from ion pairing of the TFA with the positive groups of the peptide[4,5].

The next step in methods development was to determine the capacity of the system without deterioration of resolution. Sample loads above 2.1 mg of crude peptide resulted in loss of resolution of the major peak from the hydrophilic impurities (Fig. 3). Also at loadings above 2.1 mg, the amount of peptide M recovered did not increase proportionately with the amount of crude material injected. Above a certain load the amount of soluble sample adsorbed on the bonded phase may be exceeded and the excess precipitates from the mobile phase. This precipitation of sample may or may not be irreversible. This is a possible explanation for the loss of material on reversed-phase columns and for the increase in column back-pressure that has been observed[6,7]. The flow-rate for a fixed linear velocity and mass for a fixed resolution applied to the preparative column are functions of cross-sectional area, and these can be increased as the squares of the diameters of the columns[8]. Using the

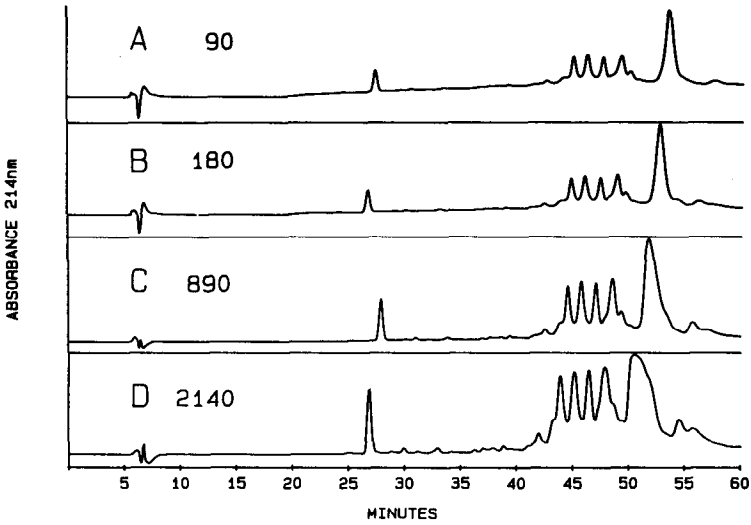

Fig. 3. Determination of load capacity. Chromatographic conditions: column, 30 × 0.39 cm I.D. Delta Pak; eluent A, 0.1% aqueous TFA; eluent B, 0.1% TFA in acetonitrile; linear gradient, 0–25% B in 30 min at a flow-rate of 0.5 ml/min. Amount (μg) of sample shown in each panel. Detection, 214 nm. A.u.f.s.: A, 0.25; B, 0.5; C, 1.5; D, 2.0.

scale factor of 164 increases the preparative flow-rate to 82 ml/min and the sample load to 345 mg.

The gradient duration was increased to compensate for the difference between the delay volumes of the analytical and preparative chromatographs. At analytical

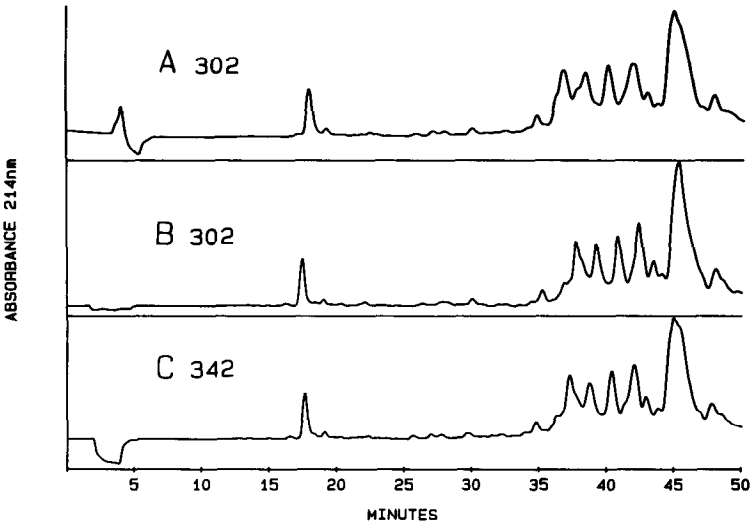

Fig. 4. Preparative chromatography of synthetic peptide M. Chromatographic conditions: column, 30 × 5 cm I.D. Delta Pak; eluent A, 0.1% aqueous TFA; eluent B, 0.1% TFA in acetonitrile. The sample was prepared and loaded as described under Experimental (sample load, mg, indicated in each panel). A 40-min linear gradient from 0 to 25% B was run at a flow-rate of 80 ml/min. Detection, 214 nm; sensitivity, 0.4 a.u.f.s.

flow-rates the time difference between the percentage of organic solvent delivered by the pumps and the percentage of organic solvent reaching the column is significant, whereas at preparative flow-rates it is almost negligible. In our experience using gradient elution, the retention time of a compound eluted from a preparative column has been observed to be less than its retention time from an analytical column even though the linear velocity was constant[9]. This and other factors, such as differences in the efficiency of the column packing, may be reponsible for the loss of resolution or decreased number of theoretical plates observed for wide-diameter columns of small particle (<20 μm) packing[10]. Therefore, to compensate for the gradient delay and to increase the resolution further, a gradient from 0 to 25% acetonitrile in 40 min was applied, 10 min longer than the analytical gradient duration. The results of three preparative separations of >300 mg each under these conditions are shown in Fig. 4. In these runs and others not shown, the major peak was eluted at 44 min. The mass of peptide recovered from 44 to 47 min averaged 24% of the mass of the crude sample loaded. The multiple preparative runs under these conditions were reproducible with respect to recovery and resolution, as shown in Fig. 4. At approximately 50 min, 50% acetonitrile was pumped to elute the hydrophobic impurities (not shown) followed by re-equilibration with 0.1% aqueous TFA for another injection. Each half-minute fraction during peak elution was analyzed by analytical HPLC, as shown in Fig. 5. The 45-min fraction was pure in this example (>99% relative peak area). The other fractions were combined with neighboring fractions of other runs and chromatographed to recover more of the pure peptide. By chromatography of most of the synthetic peptide, the final yield of biologically active peptide M was 394 mg or 24% (w/w) of crude peptide injected.

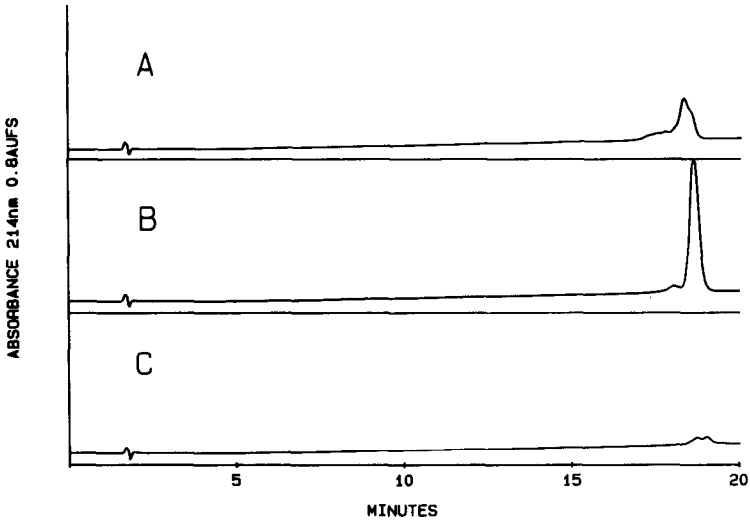

Fig. 5. Analytical rechromatography of fractions from the preparative separation (Fig. 4C) of 342 mg of synthetic peptide M on the 30 × 0.39 cm I.D. Delta Pak column. Eluent A, 0.1% aqueous TFA; eluent B, 0.1% TFA in acetonitrile. A 15-min linear gradient to 25% B was run at a flow-rate of 1 ml/min. Sample, 50-μl aliquots of the preparative fractions; detection, 214 nm; sensitivity, 0.8 a.u.f.s. A, Chromatogram of the fraction collected at 44.5 min; B, 45 min; C, 47 min. Peptide M was eluted in fractions 45–46.5.

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