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Note

Purification of a cholecystokinin fragment peptide in the horizontal flow-through coil planet centrifuge

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For the preparation of cholecystokinin (CCK) fragment peptides and their analogs which have been found to be inhibitors of its activity[1,2] we have been using a novel method of counter-current chromatography (CCC) carried out in the horizontal flow-through coil planet centrifuge for purifying the synthetic peptides. Described here are the modifications of CCC solvent systems for the fractionation of Ac-Tyr-Met-Gly-Trp-Met-Tfa Lys and the sulfated and deprotected product, Ac-Tyr-(SO_3H)-Met-Gly-Trp-Lys ([Lys^{31}]Ac-CCK 26–31). The horizontal flow-through coil planet centrifuge is an instrument designed for equilibration of a two-phase solvent system in a centrifugal force field[3]. One phase is retained while the other mobile phase is eluted and substances are separated according to their partition coefficients. This method has been previously described for the purification of endorphin peptides[4].

Ac-Asp-Tyr-Met-Gly-Trp-Tfa Lys was synthesized by solid-phase methods. The peptide was purified by CCC and subsequently sulfated[5]. The incorporation of sulfated tyrosine directly during the solid-phase synthesis would have required the avoidance of acid reagents for the deprotection and peptide-resin cleavage and thus is not compatible with the present methods. However, in solution synthesis sulfated tyrosine derivatives have been incorporated directly into CCK[6] and analogs of caerulein[7]. To protect amino groups from sulfamation during the sulfur trioxide reaction, acid labile amino protection has been used previously[5]. However, trifluoroacetic acid treatment to remove the Boc group resulted in low yields averaging 30%[7]. Therefore, protection of the amino group in this peptide was attempted here with the trifluoroacetyl group because it is stable to the conditions of the synthesis

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and its removal does not require acid conditions. After sulfation the peptide was rechromatographed by CCC and the biologic activity was determined.

EXPERIMENTAL

CCC was conducted in a flow-through coil planet centrifuge. One instrument utilized was built by the Laboratory of Technical Development (NHLBI, MD, U.S.A.) with coiled 2.6 mm I.D. PTFE tubing with a total volume of 260 ml[4]. A Kontes prototype instrument with 1.5 mm I.D. PTFE tubing with a total volume of 115 ml was also used. Solvents were reagent or HPLC grade.

Solid-phase synthesis[8] of Acetyl-Asp-Tyr-Met-Gly-Trp-ε-Tfa-Lys was started with 1 mmol of ε-Tfa-Boc-Lys resin which had been prepared by esterification of the cesium salt to chloromethyl divinyl benzene polystyrene resin (Bio-Rad)[9]. Boc amino acids were coupled according to the conditions described[2]. After coupling of the amino terminal Boc-β-Asp, the peptide resin was acetylated and treated with anhy-

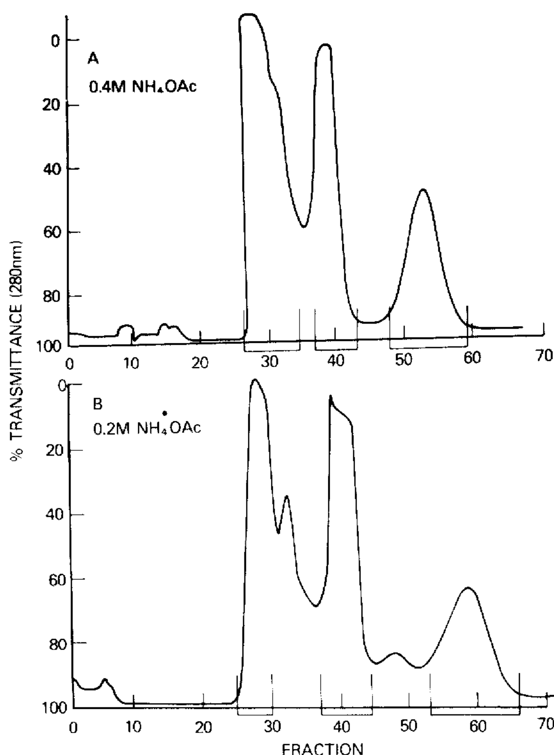

Fig. 1. CCC of Ac-Asp-Tyr-Met-Gly-Trp-Tfa Lys. Percent transmittance of the 6-ml fractions detected by a LKB Uvicord II monitor is shown. (A) Chromatography of 265 mg peptide on the planet centrifuge in 0.4 *M* ammonium acetate–*n*-butanol (1:1, v/v) with upper phase mobile. Conditions are described in the Experimental section. The solvent front where upper phase emerged is at tube 27. Fractions (27–31) peak I, (37–42) peak II and (48–58) peak III contained 91, 94 and 36 mg respectively after lyophilization. (B) Chromatography of 188 mg of the same synthesis in 0.2 *M* ammonium acetate–*n*-butanol (1:1, v/v). Solvent front emerged at fraction 25. Fractions (37–44) containing 100 mg and peak II of the 0.4 *M* ammonium acetate run contained the purified Tfa peptide.

drous hydrogen fluoride at 0°C for 45 min in two batches (2 g each). The peptide was extracted from the resin with glacial acetic acid, then lyophilized; the yield of white powder in each batch was 265 mg (66%) and 188 mg (43%), respectively. Each product was submitted to CCC on the flow-through coil planet centrifuge in the solvent system, 0.4 *M* (Fig. 1A) or 0.2 *M* (Fig. 1B) ammonium acetate–*n*-butanol (1:1, v/v) with the upper phase mobile at a rotational speed of 400 rpm and flow-rate of 24 ml/h with 15 min fractions collected. The UV absorbance of the effluent was monitored at 280 nm. Fractions containing peptide were pooled, evaporated and lyophilized in glacial acetic acid. The contents of the three peptide peaks were analyzed by thin-layer chromatography (TLC) on reversed-phase plates (Analtech) in *n*-butanol–acetic acid–water (4:1:1, v/v/v) and developed by the Ehrlich spray[8]. Fraction (27–31) had three spots with R_F values of 0.81, 0.71 and 0.63; fraction (37–42) had an R_F of 0.68 and fraction (48–58) had two spots with R_F values of 0.68 and 0.15. The contents of the second peak (fraction 37–42) chromatographed in TLC as one major component and amino acid analysis showed the expected molar ratios: Asp, 1.02; Gly, 1.01; Met, 1.00; Tyr, 1.02; Lys, 1.04. The yield of pure peptide was 94 mg. The other two peaks were non-homogeneous. The other batch of peptide was purified in 0.2 *M* ammonium acetate–*n*-butanol (1:1, v/v). The second CCC peak contained the major product, 100 mg. Elemental analysis of the peptide gave C, 51.06%; H, 5.65%; N, 11.49%; expected C, 52.49%; H, 5.60%; N, 11.95%. Purified Tfa peptide, 39 mg, was sulfated with 147 mg pyridine sulfur trioxide complex in 5 ml DMF–pyridine (1:2, v/v)[10]. The reaction in a Pyrex culture tube was bubbled with nitrogen for 1 min then capped with a PTFE-lined screw cap and stirred for 36 h. After drying, 2 ml of 1 *M* $NaHCO_3$ was added and the reaction stirred for 1 h; then 1-ml amounts of the solution were chromatographed on a 45 × 1.5 cm I.D. P-2 column (Bio-Rad) in 0.1 *M* ammonium acetate in 50% ethanol. The peptide recovered was lyophilized and stirred in 2 ml of 1 *M* NaOH for 1 h at room temperature to remove the trifluoroacetyl group. The pH was reduced to 4.0 with conc. HCl and the solution was treated with 10 μl ethyl methyl sulfide for 30 min to prevent oxidation. At this point the mixture was chromatographed on the P-2 column in two batches. Since the peptide material eluted with tailing, the major peak and the ma-

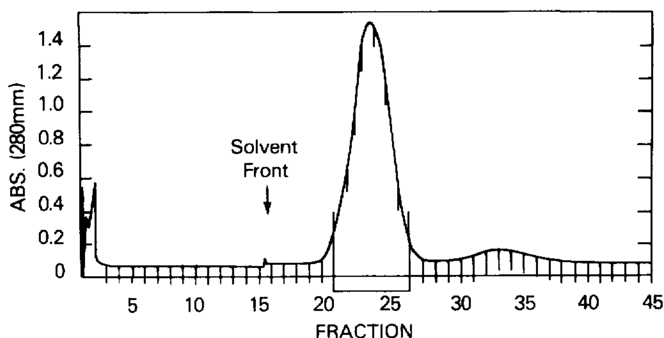

Fig. 2. Purification of gel-filtered sulfated and deprotected peptide Ac-Asp-Tyr(SO_3H)-Met-Gly-Trp-Lys by CCC in the Kontes prototype instrument. Recorder tracing of 280 nm absorbance of the effluent is shown. The fractions are marked on the absorbance tracing (I.S.C.O. Model UA-5 monitor). The amount of peptide in the peak (21–26) is 20 mg. The CCC of the remainder of the gel-filtration product resulted also in one peak containing 10 mg.

terial eluting later were pooled separately. Each fraction was submitted to CCC in the *n*-butanol–acetic acid–water (BAW) system (4:1:5, v/v/v) (Fig. 2). Both fractions chromatographed with similar results. The combined yield was 30 mg white powder. The TLC on silica gel in BAW showed one homogeneous spot, with an R_F of 0.16 that was Ehrlich- and ninhydrin-positive, indicating a free epsilon-amino group. Amino acid analysis of the product, Acetyl-Asp-Tyr(SO_3H)-Met-Gly-Trp-Lys gave the following molar ratios: Asp, 1.00; Gly, 1.02; Met, 0.98; Tyr, 1.04; Lys, 0.91, for a mercaptoethanesulfonic acid hydrolysis. Spectral analysis showed the presence of equimolar Trp. A much lower ratio of Tyr in base hydrolysis and the UV spectra showing no shift upon alkalinization indicated the presence of sulfated Tyr. The UV spectrum also failed to show a peak at 350 nm which, if present, would have indicated the presence of ring sulfonation. The infrared spectrum was read and the characteristic peak of the sulfate group at 1040 cm^{-1} was observed. Elemental analysis gave C, 45.64%; H, 6.2%; N, 12.58%; S, 6.32%; expected C, 50.85%; H, 5.70%; N, 12.17%; S, 6.96%.

Biologic activity

Binding of the peptide to central nervous system CCK receptors on guinea pig cortical membranes was determined[11]. The ability of [Lys^{31}]Ac-CCK 26–31 to inhibit CCK-stimulated amylase secretion from dispersed acini of guinea pig pancreas was measured using the phadebas reagent[12]. The effect of the peptide on 0.3 n*M* CCK-induced amylase secretion from acini incubated at 37°C for 30 min was determined as the percent of the amylase activity in the cells at the beginning of the incubation that was released into the extracellular medium during the incubation.

RESULTS AND DISCUSSION

The substitution of Boc-ε-Tfa Lys on the resin was 0.25 mm/g, an amount enough for synthesis. Thus the yield of pure peptide in this synthesis was possible using the Boc-ε-Tfa Lys resin. The Tfa group for epsilon-amino protection has been preferred by some chemists for solid-phase synthesis because it is more stable than the CBZ group[13]. Another protecting group that can be used in a similar fashion is the 2-(methylsulfonyl)ethyloxycarbonyl (Msc) group, which is removed by beta-elimination conditions[10]. The sulfating procedure utilized in this synthesis is essentially that of Rajh *et al.*[10], a modification of Ondetti *et al.*[5]. Modification of the solvent composition in the sulfating step improved the yields, and the resulting sodium salt is stable. The yield of pure sulfated peptide from the sulfation step in this synthesis was 75%. Because no starting material was detected, the sulfation reaction was quantitative.

The CCC purification steps showed that a lower ionic strenght ammonium acetate system could decrease the partitioning of the Tfa peptide into the upper phase and consequently improve the resolution. The *K* of the second peak was 1.33 in the 0.4 *M* ammonium acetate–*n*-butanol system (Fig. 1A) and 1.22 in the 0.2 *M* ammonium acetate–*n*-butanol system (Fig. 1B); the latter showed better resolution. The R_F of these peptides on TLC correlates with the order of elution in the CCC. Thus, the fractionating potential of a solvent system can be assessed by TLC. The sulfated peptide eluted with a *K* of 0.91 in the BAW system with the upper phase mobile (Fig. 2). This peptide is more hydrophilic than the trifluoroacetylated unsulfated peptide

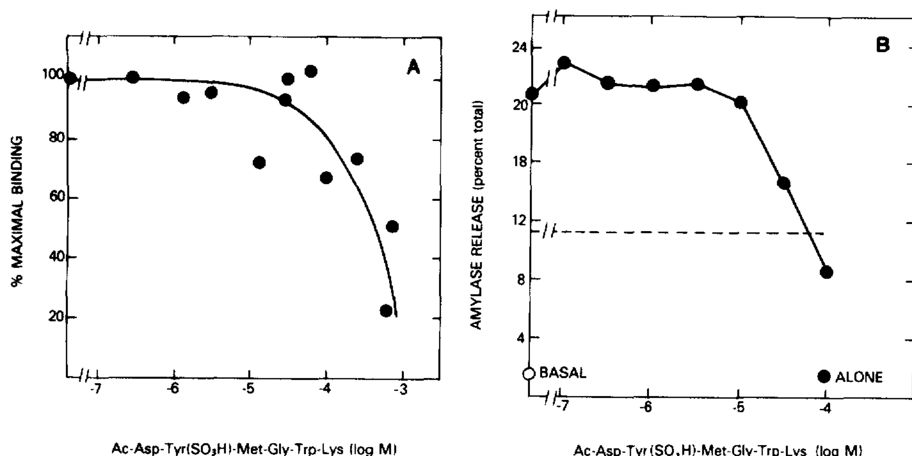

Fig. 3. (A) Inhibition of the binding of [^{125}I]CCK to guinea pig brain cortical membranes by varying concentrations of [Lys^{31}]Ac-CCK 26–31. Mean values from two experiments are presented. (B) Inhibition by [Lys^{31}]Ac-CCK 26–31 of amylase secretion from pancreatic acini in the presence of 0.3 n*M* CCK 26–33. Results given are means of triplicate determinations. Basal amylase release is indicated by the open symbol. Amylase release with 100 μM [Lys^{31}]Ac-CCK 26–31 alone is indicated by the symbol labeled "alone". Half-maximal inhibition of stimulated amylase release is indicated by the dashed line.

thus the BAW system is a faster solvent system than the ammonium acetate system for these peptides. The peptide demonstrated binding to CCK receptors with an IC_{50} of $4.4 \cdot 10^{-4}$ *M* (Fig. 3A) and showed antagonism in the guinea pig acinar cell assay with an IC_{50} of $5.0 \cdot 10^{-5}$ *M* (Fig. 3B). These potencies are equivalent to that of dibutyryl cyclic guanosine 5′-monophosphate, a non-peptide CCK receptor antagonist[14]. Thus [Lys^{31}]Ac-CCK 26–31 is another member of the class of CCK antagonists comprised of N-terminal fragments of CCK 26–33[1,2]. Thus these experiments have shown that the flow-through coil planet centrifuge, a compact apparatus compared to the large counter-current distribution instrument, is useful in the preparative purification of biologically active synthetic peptides.

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