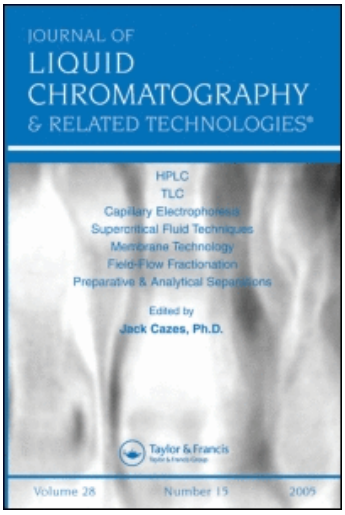

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Purification of Acidic Synthetic Peptides by High Performance Liquid Chromatography Using Ammonium Acetate Buffer

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PURIFICATION OF ACIDIC SYNTHETIC PEPTIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING AMMONIUM ACETATE BUFFER

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ABSTRACT

The purification of acidic synthetic peptides was achieved by using a linear gradient of ammonium acetate and methanol with reverse phase liquid chromatography.

INTRODUCTION

HPLC purification of peptides requires a choice of suitable solvent conditions as no ideal solvent exists for all performances.

HPLC-separation of synthetic peptides is commonly performed by linear gradients of trifluoroacetic acid

(TFA) and methanol, using reverse phase C18 columns (1).

However strongly acidic peptides are usually insoluble at low pH and need a neutral or alcaline solvent for solubilization.

In the purification of acidic synthetic peptides common separation techniques utilising TFA and methanol may cause some problems (for example precipitation) which can impair further analysis.

A satisfactory separation of acidic peptides can be obtained using ammonium acetate(2) in place of TFA, utilizing usual reverse phase C18 column.

As has been well establisher is well known, the silica phase of this column does not withstand pH values higher than 8, while an ammonium acetate buffer may have a pH of about 7 by simply adding acetic acid .

This report describes a simple method for semi-preparative separation for acidic synthetic peptides under neutral conditions.

MATERIALS

Reagents:

Methanol (chromatography grade) and ammonium acetate were from Merck, Darmstadt, Germany.

Water (12 MegaOhms) was prepared by reverse osmosis (MilliQ ,Millipore Bedford, Ma, U.S.A.).

Automatic Peptide Synthesizer mod.430A (Applied Biosystems, Foster City, CA., USA).

Protein Sequencer mod.470A (Applied Biosystems, Foster City CA., USA).

Pico-Tag™ Work Station (Millipore Waters, Bedford MA., USA).

Chromatography equipment :

A Perkin Elmer 3B Series HPLC Gradient System equipped with a LC 75 Detector and a Sigma 10B Chromatography Data Station was used.

μBondapack™ C_{18} cartridge (7.8x300 mm; 10 micron particle size) Water assoc., Mildford, MA, U.S.A.

For optimal column life the column was protected with a Guard-pack precolumn insert from Waters Associates.

METHODS

Synthesis

The F-W-E-E-D-E-Y-E-V peptide was synthesized by the solid-phase method (3).

At the end of the synthesis, in order to prevent side-reactions(4), the peptide was deprotected and detached from the resin by treatment with liquid hydrofluoric acid in the presence of 7.5% paracresol and 2.5% paratiocresol.

After cleavage from the resin with hydrogen fluoride

the peptide was passed over a 2.5x90 cm. Sephadex G-10 column (Pharmacia,Uppsala,Sweden) in 0.5M acetic acid.

Chromatographic procedure

The ammonium acetate buffer 0.1M pH 7,was prepared freshly each day, carefully degassed by sonication and was used as the initial solvent (B) in the gradient separation.

The second solvent (A) consisted of methanol.

A linear gradient from 0 to 100% of A over 60 minutes was used throughout the study.

The separation was carried out at room temperature and at a flow -rate of 5 ml/min; the pressure varied during the gradient between 2200 and 3000 psi.

The eluted peptides were detected by UV absorbance at 220 nm or at 280 nm.

RESULTS

When our synthetic peptide was chromatographed using the conditions already described, we obtained the chromatograms reported in Fig.1a and 1b which were recordered at 220 nm and 280 nm, respectively.

Five peaks were detected in both cases but peak C was considerably reduced when the detector was set at 280nm.

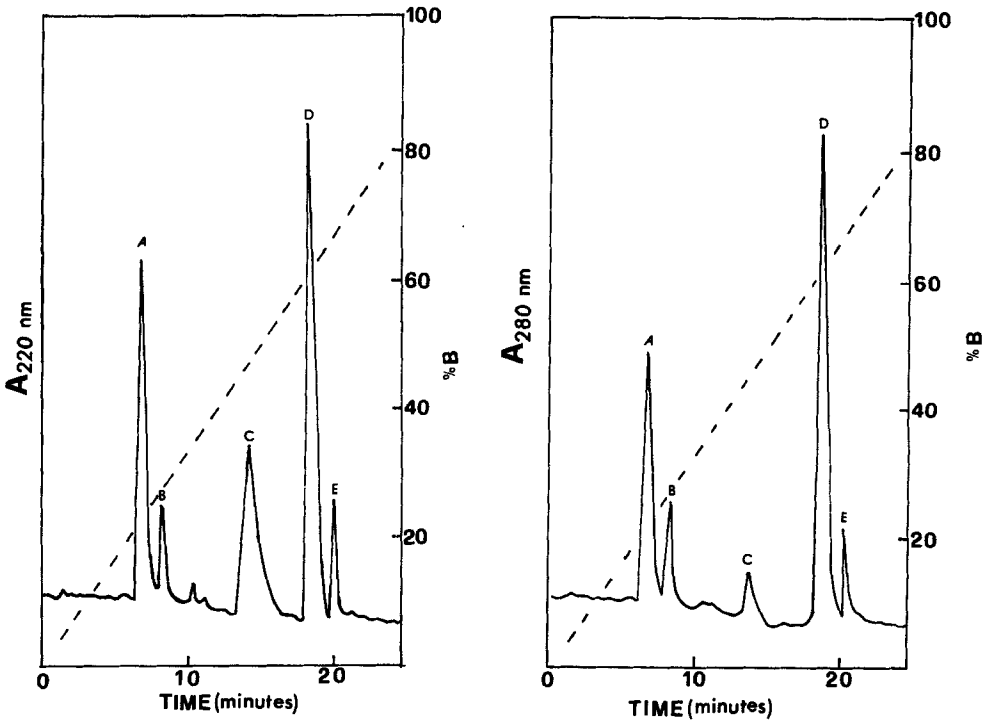

FIGURE 1 -Purification of F-W-E-E-D-Q-E-Y-E-V peptide on a C_{18} µBondapack column was achieved with a linear gradient from 0 to 100% of methanol and from 100% to 0% of 0.1M ammonium acetate at a flow-rate of 5ml/min. Peaks were monitored at 220 nm (a) and at 280 nm (b). Load 2ml (c.a. 2mg).

We deduced that peak C does not contain any aromatic rings as was expected from the structure of our peptide.

Moreover, amino acid analysis of peak C as well of peak B and E showed no correspondance with the expected amino acid composition: from the analysis data we can

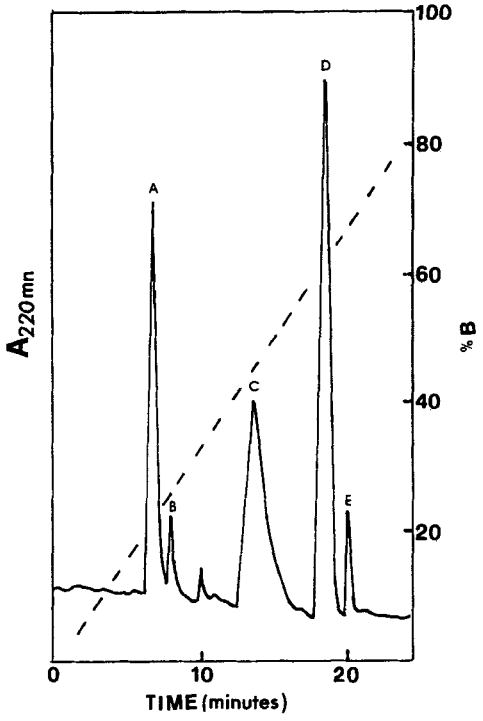

FIGURE 2-Purification of F-W-E-E-D-Q-E-Y-E-V peptide on a C_{18} µBondapack column using a linear gradient from 0 to 100% of methanol and from 100% to 0% of 0.1M ammonium bicarbonate at a flow-rate of 5 ml/min.Peaks were monitored at 280 nm. Load 2 ml (c.a. 2 mg).

deduce that these peaks were due to incomplete peptides. (Amino acid composition was determined by using the PITC derivatization methodology and PICO-TAG Work Station(4), after collection, concentration, filtration through 0.22 µm membranes and freeze-drying).

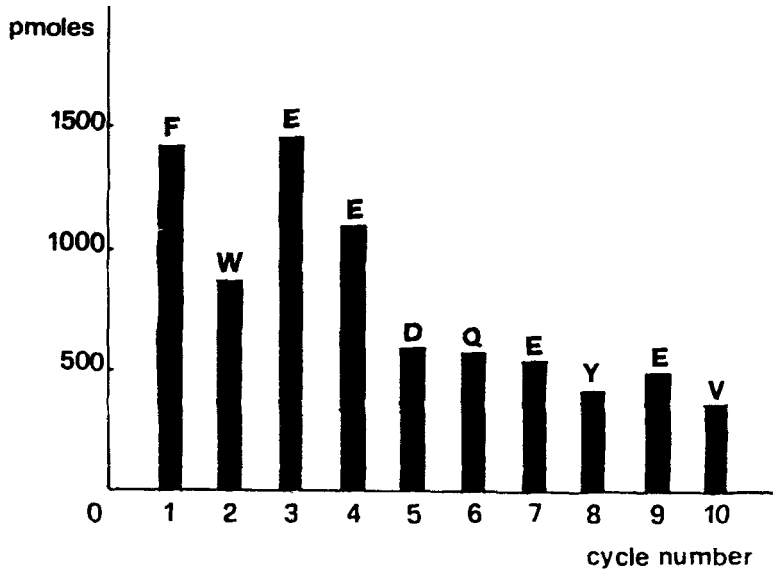

FIGURE 3- Yields in aminoacidic sequence of 2 nmoles of synthetic peptide F-W-E-E-D-Q-E-Y-E-V.

On the contrary peak D composition fully agreed with the expected amino acid composition F(1) 1.0, E(4) 3.8, D(1) 0.9, Y(1) 1.0, V(1) 1.0.

From its retention time and our experience of other peptides we deduced peak A to be in consequence of the presence scavenger and side-products of the synthetic reactions.

The correspondence of peak D to the expected peptide was confirmed by the determination of the amino acid sequence control (Fig.3) (6-9).

DISCUSSION

It is known that trifluoroacetic acid can be used as a volatile ionic modifier for the preparative separation of peptides of medium or low polarity (10).

Synthetic peptides containing many acidic amino acids such as, in our case, F-W-E-E-D-E-Y-E-V can be insoluble in strong acids and cannot be purified with the standard strategy, using trifluoroacetic acid .

In these cases solutions of ammonium salts (ammonium bicarbonate (11) or ammonium acetate (1)) can be used in place of TFA: both of them are extremely volatile and can be removed from the synthetic peptides by rotary evaporation and freeze-drying following water dilution. However, the use of ammonium bicarbonate poses some problems.

As first it tends to produce CO_2 bubbles (also after very careful degasing), especially when one deals with a pH around 7.

Moreover pH adjusting by mineral acids can lead to formation of amine salts which cannot be removed by liofilization .

However, when CO_2 is used to reach the required pH, bubbling is obviously increased.

This inconvenience can be avoided by using ammonium acetate in place of ammonium bicarbonate (Fig.2), obtaining an equally good separation.

In conclusion the use of ammonium acetate buffer instead of ammonium bicarbonate or TFA allows on effective preparative separation of synthetic acidic peptides.

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