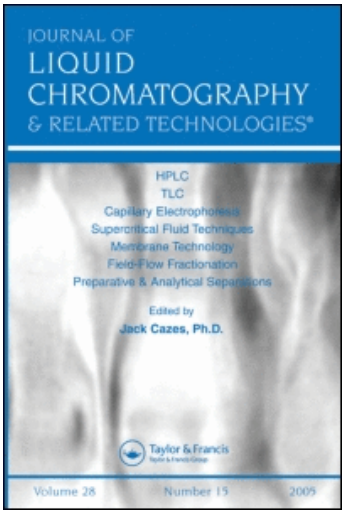

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Single Step Purification of a Synthetic Peptide Fragment of Neurofilaments by Preparative High Performance Liquid Chromatography

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SINGLE STEP PURIFICATION OF A SYNTHETIC PEPTIDE FRAGMENT OF NEUROFILAMENTS BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

It was recently shown that fragment Lys-Ser-Pro-Val (1,2) is the major site in vivo neurofilament phosforilation. A suitable method for the preparation of this peptide was therefore studied paying particular attention to the purification step. Even thought solubility of this peptide in methyl t-butil ether does not allow the standard purification. It is possible achieve a good separation in a single step by HPLC, after a simple extraction with acidic water.

INTRODUCTION

Neurofilaments are the most abudant cytoskeletal structures in large diameter axons. The carboxy-terminal region of the proteins contains 13 amino acid proline- and serine- rich sequence reapeted six times in succession. The sequence Lys-Ser-Pro-Val is reapeted more times. The four amino acids may represent a kinase recognition site in a region of the protein that is known to be highly phosphorylated (1). In order to study the conformation and the activity of such peptide, we have synthesized it by the solid-phase method of Erickson and Merrifield (3) and the peptide was detached by trifluoromethane sulfonic acid (4). Usually the peptide is collected in methyl t-butyl ether, so the peptide precipitates and the acidic scavengers materials remain in solution. In our case the peptide remained in solution. The purpose of this work is to show the possibility of purification of the peptide also in the case of a non ideal system, obtaining furthemore, high purity.

EXPERIMENTAL

Synthesis of tetrapeptide

The peptide Lys-Ser-Pro-Val was synthesized by Erickson and Merrifield's solid-phase method. The polystyrene

resin contains the first (carboxy terminal) amino acid with a degree of substitution of 0.63 mmol of amino acid per gram of dry resin. The coupling reaction was carried out with pre-formed Boc amino acid symmetrical anhydrides. The Boc-aminoacids were obtained from Applied Biosystem (Foster City, CA., USA). Each amino acid coupling step was monitored for completeness of reaction by the ninhydrine test (5). A coupling percentage of about 99.0 was obtained at each step.

At the end of the synthesis, in order to prevent unwanted modifications in the peptide structure, the peptide was deprotected and detached from 0.25g of resin (corresponding to about 50 mg of peptide) by treatment with trifluormethane sulfonic acid (TMSA) in the presence of thioanisole/dimercaptoethan.

Reverse-phase HPLC purification of peptide

The purification was then carried out on a Perkin Elmer 3B Series HPLC Gradient System equipped with a LC 75 Detector and a Sigma 10B Chromatography Data Station. We use a Waters uBondapack C_{18} cartridge (7.8X300 mm; 10 micron particle size, Water assoc., Mildford, MA, USA) provided with a pre-column filter Guard Pack C18(Waters. Assoc.). The eluents were trifluoroacetic acid (3) (TFA, HPLC grade) (Merck Darmstadt,

Germany) 0.1% solution water, Milli Q grade, and Methanol (HPLC grade) (Merk, Darmstadt, Germany)

Amino acid composition and sequence analyses

The amino acid composition was determined after acid hydrolysis in 6M hydrochloric acid 110^{0}C for 24hrs in the Pico-Tag^{TM} Work Station (Millipore Waters, Bedford MA., USA) (7). Derivatization of sample with phenylisothiocyanate was performed by using a pre-column procedure. Separation of PTC-derivatives was carried out by reverse-phase HPLC using two mod. 510 pumps equipped with a Pico-Tag column, an absorbance detector mod. 441, an automated gradient controller and a Wisp 710B automatic sampler (all from Millipore Waters).

Primary structure control was performed by Edman degradation using a protein and peptide automatic sequencer (8,9) mod. 470A equipped with a PTH derivatives analyzer mod. 120A and a Data module station mod. 900A (all from Applied Biosystems).

RESULTS AND DISCUSSION

After the cleavage of the peptide from the resin with TMSA, the resin and the peptide were pipetted to a sintered glass funnel that had been fitted with a

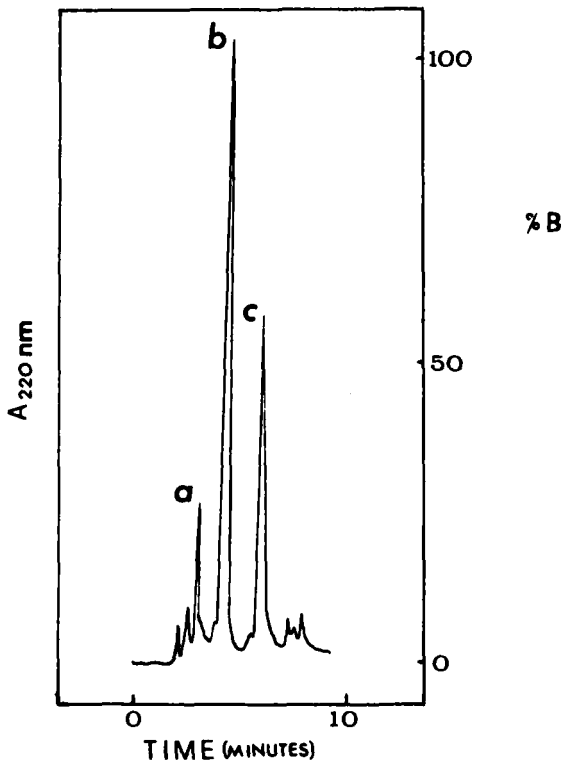

Figure 1 -Analytical chromatogram of the extract in water of the crude tetrapeptide on a C18 uBondapack column was achieved with a linear gradient from 0% to 100% of methanol (B) and from 100% to 0% of 0.1M trifluoracetic acid (A) 0.1% at flow-rate 5ml/min. Peaks were monotired at 220 nm; chart speed 5 mm/min. Load 100µl. Peak b contains the desired peptide.

collection flask containing methyl t-butil ether. The methyl t-butil ether is utilised in order to obtain a primary separation of the peptides from the scavangers(4). In fact peptides are generally insoluble in ether while the scavangers are soluble. So it is possible to separate the peptide by a simple

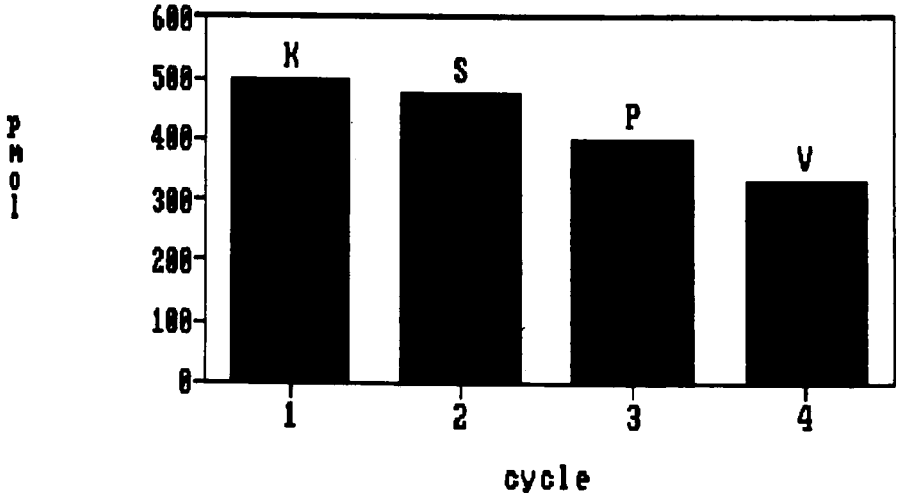

Figure 2 -Yields in amino acid sequence of 1 nmole of synthetic peptide K-S-P-V.

filtration. In our case also the peptide was soluble in methyl t-butil ether, but however we could achieve a good separation by extracting the peptide from the water. In this case we evaporated the methyl t-butyl ether by the rotavapor and then we have added milli-Q water to the mixture. In this case, just utilising the strong solubility of the peptide in the acidic water, it was possible to inject the water solution containing the peptide in the reverse phase HPLC column.

Fig1 shows the purification of about 1 mg of the synthetic peptide, after injection of 100 ul, using the chromatographic system described in the Experimental Section. This separation was repeated three times

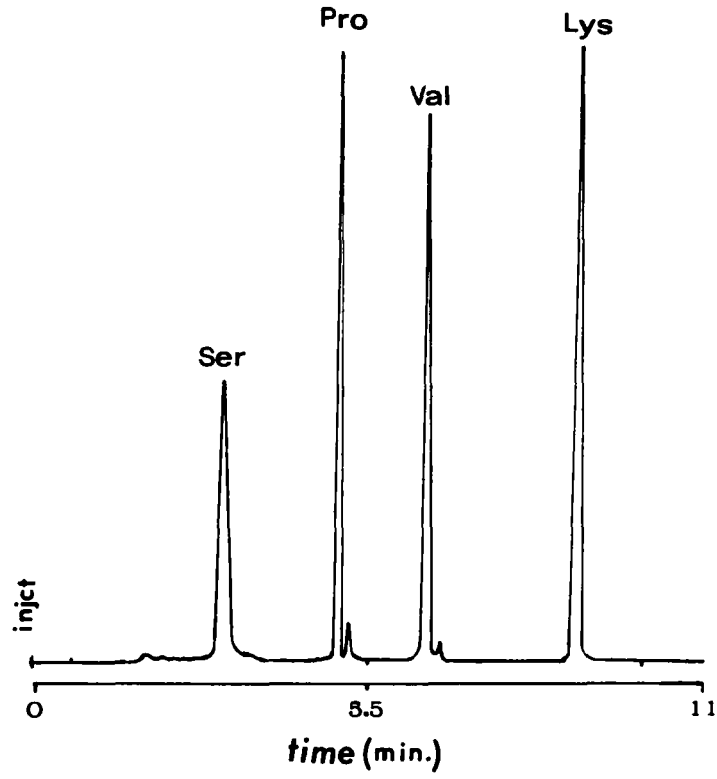

Figure 3 -High-performance liquid chromatography separation of PTC amino acids on Pico-Tag column (10-µm silica beads, 3.9X150mm, Waters) . The initial solvent (A) was 0.14M sodium acetate buffer, pH 6.4 triethylamine 0.05%, acetonitrile 6%, and the secondary solvent (B) was acetonitrile/water (60:40). Separation of PTC was carried out at 38° C by a 10-min convex gradient (curve 5 of solvent programmer) from 0 to 46% of solvent B with flow rate of 1 ml/min. Eluted PTC was detected by their UV absorption at 254nm. The sample consisted of 15µl of buffer A containing about 250 picomoles of each of the PTC amino acids.

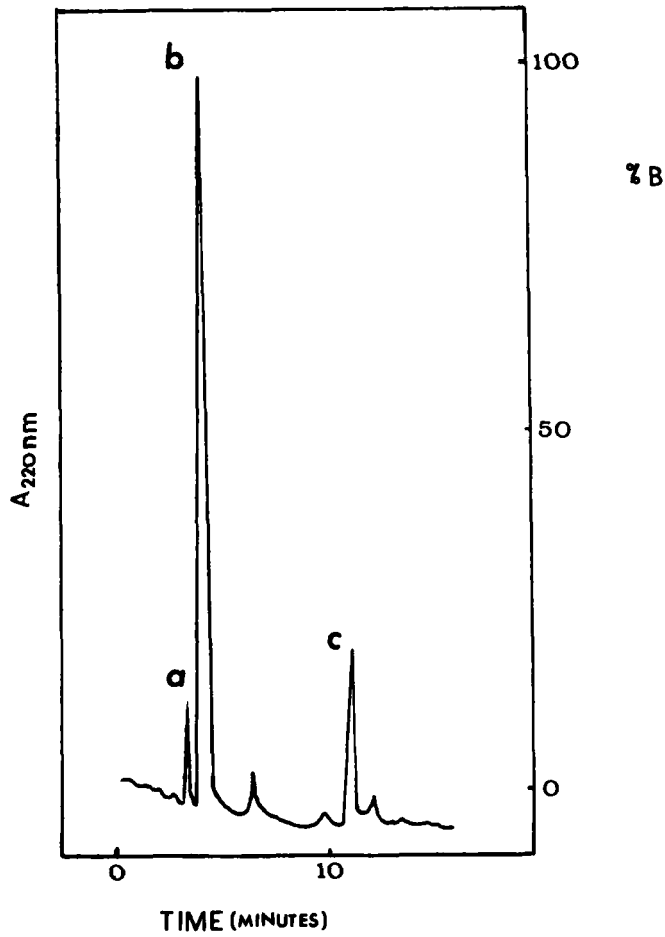

Figure 4 -Analitycal chromatogram of tetrapeptide, after G10 sephadex on a 90x2.5 column, on a C18 uBondapack column was achieved with a linear gradient from 0% to 100% of methanol (B) and from 100% to 0% of 0.1M trifluoroacetic acid (A) 0.1% at flow-rate 5ml/min. Peaks were monotired at 220 nm; chart speed 5 mm/min. Load 100µl. Peak b contains the desired peptide.

injecting about 1.3 ml of the peptide solution. The fractions were collected, concentrated, and lyophilized. The material of fraction 2, peak b, (about 37mg) shows the correct amino acid sequence (Fig2). Moreover, this fraction shows the correct amino acid composition: Lys(1) 0.8, Ser(1) 1.0, Pro(1) 1.1, Val(1) 0.9. (Fig.3).

In alternative, it was also possible to obtain a purification utilising the sephadex column step: this gel filtration step is commonly utilized to remove the reaction side-products and scavengers. After a strong agitation, the mixture was passed directly through the column. The collected fractions were assaied with trinitrobenzen sulfonic acid. The positive fraction was freeze-dried and then passed through the HPLC column. The resulted chromatogram (Fig.4) is analogous to the one referred to the peptide purified without the sephadex column step (Fig.1). In both cases a good purification can be achieved, even though the first method we have described is certainly faster.

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REFERENCES

1) Lee V.M.Y., Otvos L., Carden M.J., Hollosi M., Dietzschold B. and Lazzarini R.A. Identification of the major multiphosphorylation site in mammalian neurofilaments. Proc. Natl. Acad. Sci 85 1998, 1988

2) Myers M.W., Lazzarini R.A., Lee V.M.-Y., Schlaepfer W.W. and Nelson D.L. The human mid-size neurofilament subunit: a repeated protein sequence and the relationship of its gene to the intermediate filament gene family. EMBO 6 1617 1987

3) Stewart J.M. and Young J.D. Solid phase peptide synthesis, 2nd edition, 88, Pierce, Rockford, Illinois, 1984.

4) Bergot B.J., Noble L.R. and Geiser T. Utility of trifluoremethane sulfonic acid as a cleavage reagent in solid-phas peptide synthesis User Bulletin N_0 16 1986

5) Kaiser E., Colescott R.L., Bossinger C.D. Cook, P.J. Anal. Biochem 34 595 1970

7) Cohen S.A., Bidlingmeyer B.A. and Tarvin T.L. PTC derivates in amino acid analysis, Nature, 320 769 1986

8)Hunkapiller M.W. et al.,Meth. in Enzym.,Hirs C.H.W., Timasheff S.N., 91, 399, 1983.

9)Strickler J.E.,Hunkapiller M.W.,and Wilson K.J., Utility of the Gas-Phase Sequencer for Both Liquid- and Solid-Phase Degradation of Proteins and Peptides at Low Picomole Levels, Anal. Biochem., 140, 553, 1984.