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# RAPID IDENTIFICATION OF $NH_2$-TERMINAL MYRISTYL PEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

$NH_2$-terminal myristyl peptides in mixtures of other peptides having the $NH_2$-terminal blocked with acetyl, formyl or pyroglutamyl groups were selectively determined by high-performance liquid chromatography using a poly(vinyl alcohol) resin column eluted with 50 m*M* sodium hydrogen carbonate, pH 8.3, and a linear gradient of acetonitrile. This method was applied to the analysis of an $NH_2$-terminal blocked peptide from a peptic digest of the catalytic subunit of adenosine 3′:5′-phosphate-dependent protein kinase type II from bovine heart, showing that the peptide has a sequence myristyl-Gly-Asn-Ala.

## INTRODUCTION

A covalently bound myristate of the $NH_2$-terminal residue of a protein was first discovered in the catalytic subunit of adenosine 3′:5′-phosphate (cAMP)-dependent protein kinase type II[1,2]. This finding contributed to the characterization of an $NH_2$-terminal blocking group which could not be identified as an acetyl (Ac–), a formyl (HCO–) or a pyroglutamyl (<Glu–) group. Aitkin *et al.*[3] demonstrated subsequently that the $NH_2$-terminus of calcineurin, a component of a calmodulin-binding phosphatase, was blocked with myristic acid. Ozols *et al.*[4] also identified the $NH_2$-terminal blocking group of cytochrome $b_5$ reductase as myristic acid and discussed the function of the myristyl group followed by hydrophobic amino acid residues. Recently, a lymphoma tyrosine protein kinase[5] and protein kinase $p60^{src}$ (ref. 6) were shown to have the $NH_2$-termini blocked with myristic acid.

In all the above proteins, myristylation occurs at the $NH_2$-terminal glycine residues. Schultz *et al.*[6] suggested that myristylation of the $NH_2$-terminal glycine residue may be critical for the functioning of certain proteins related to cell transformation and growth control. Pellman *et al.*[7,8] specified the requisite $NH_2$-terminal sequence for myristylation of $p60^{src}$ and proposed that myristylation is the initial even required for membrane association, which in turn is required for cell transformation.

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Several methods have been described for the analysis of $NH_2$-terminal blocked peptides. Ninhydrin-negative peptides from proteolytic digests were identified as peptides having their $NH_2$-termini blocked with acetyl, formyl or pyroglutamyl groups by both chlorination-positive and ninhydrin-negative tests after paper electrophoresis[9], or by taking advantage of the acidic characteristics of $NH_2$-terminal blocked peptides on a column of sulphonated resin[10]. The acetyl and pyroglutamyl groups of $NH_2$-terminal blocked peptides can be determined by the methods of Narita and Ishii[10] and Lux *et al.*[11], respectively. The formyl group is easily removed from HCO-peptide by treatment with acid[12]. Recently, peptides from proteolytic digests were separated by high-performance liquid chromatography (HPLC) using a silica-based reversed-phase column under acidic conditions, and then an $NH_2$-terminal blocked peptide detected by its negative reactions with ninhydrin, fluorescamine and *o*-phthalaldehyde[1,2]. An $NH_2$-terminal long chain fatty acyl-blocked peptide could be separated from the proteolytic peptides by HPLC under acidic conditions only with difficulty because of the strong hydrophobicity of the peptides. Therefore, it was first determined by gas chromatography–mass spectrometry[1].

The purpose of the present study was to establish a method for selective identification of $NH_2$-terminal long chain fatty acyl-peptides. The hydrophilic poly(vinyl alcohol) resin Asahipack GS-310™, which is currently available, has weak hydrophobicity and can be used under both acidic and alkaline conditions in methanol, acetonitrile or other organic solvents. Taking advantage of these favourable properties, we have determined suitable HPLC conditions for the selective separation of $NH_2$-terminal long chain fatty acyl-peptides, especially the myristyl-peptide[2] from cAMP-dependent protein kinase type II.

The method includes the *o*-phthalaldehyde reaction system[13] which can be monitored simultaneously by an ultraviolet (UV) detector and by a fluorescence detector. This made it possible to determine directly $NH_2$-terminal blocked peptides under alkaline conditions and to identify the $NH_2$-terminal blocking group as a long chain fatty acid. An $NH_2$-terminal long chain fatty acyl-peptide separated from a peptic digest of cAMP-dependent protein kinase type II was thus demonstrated to be myristyl-Gly-Asn-Ala.

## EXPERIMENTAL

### *Materials*

Reagents were obtained from the following sources: Ac-Gly-Lys-OMe, Ac-Phe, Ac-Trp, HCO-Met-Leu-Phe, <Glu-Ala, <Glu-His-Gly, Gly-Gly-Tyr-Arg, Gly-Phe-$NH_2$ and dicyclohexylcarbodiimide, Protein Research Foundation (Osaka, Japan); Ac-Phe-OEt, Ac-Trp-OEt and Ac-Tyr-OEt, Sigma Chemical Co. (St. Louis, MO, U.S.A.); Gly-$NH_2$ and Gly-Gly-Gly-$NH_2$, BACHEM, Feinchemilalien AG, (Bubendorf, Switzerland); *n*-capric, *n*-lauric, *n*-myristic, *n*-palmitic and *n*-stearic acids and 2-mercaptoethanol, Nakarai Chemical Co. (Kyoto, Japan): *p*-bromophenacyl bromide (*p*-BPB), *o*-phthalaldehyde, crown ether and other solvents, Wako Chemicals Co. (Osaka, Japan). cAMP-dependent protein kinase type II was partially purified from bovine heart[14].

*Apparatus*

HPLC analysis was performed on a Waters liquid chromatograph (compact type) equipped with Model 660 and 6000A solvent-delivery systems, a Model U6K universal sample injector, a Model 441 UV (214 nm) detector and a Model 420 E and 420C fluorescence detector (excitation at 380 nm and emission at 425 nm) (Waters Assoc., Milford, MA, U.S.A.). The chromatographic separation was achieved on an Asahipack GS-310™ column (particle size 10 $\mu$m, 150 × 8 mm I.D.; Asahi Kasei, Tokyo, Japan), eluted with 50 m*M* sodium hydrogen carbonate, pH 8.3, containing acetonitrile. The concentration of acetonitrile was increased linearly from 5 to 60% in 20 min. The solvent flow-rate was set at 0.5 ml/min. The eluate was monitored simultaneously by the UV and the fluorescence detectors.

The peptides were first identified by the two-way detectors on an analytical scale; the solvent was then changed from 50 m*M* sodium hydrogen carbonate to 50 m*M* ammonium hydrogen carbonate (volatile), and the *o*-phthalaldehyde reaction system was removed from the HPLC system. Fatty acid analysis was performed by the method of Borch[15]. $NH_2$-terminal myristylation of peptides was carried out according to the method of Sheehan and Hess[16] using dicyclohexylcarbodiimide.

## RESULTS AND DISCUSSION

*Analysis of $NH_2$-terminal blocked peptides*

HPLC of <Glu-peptides (<Glu-His-Gly and <Glu-Ala), HCO-Met-Leu-Phe, Ac-Arg-OMe and Ac-Gly-Lys-Ome is shown in Fig. 1. The $NH_2$-terminal blocked peptides and Ac-Arg-OMe exhibited the same retention time (3.5 min); <Glu-Ala had a retention time of 10 min (Fig. 1A). The corresponding to <Glu-His-Gly and HCO-Met-Leu-Phe were slightly positive to *o*-phthalaldehyde. The *o*-phthalaldehyde-positive components of these peaks may have been contained originally in the samples or solvents as impurities (Fig. 1A and B).

*Analysis of Ac-Phe-OEt, Ac-Trp-OEt, and Ac-Tyr-OEt*

Fig. 2 shows that Ac-Phe, Ac-Trp and Ac-Tyr, all having a free α-carboxyl group, were eluted with slightly increased retention times (4–6 min) compared with the mobile phase hold-up time (3.5 min), whereas their ethyl esters, all strongly hydrophobic, were bound tightly to the resin. $NH_2$-blocked hydrophobic amino acid esters, therefore, will not interfere with the resolution of $NH_2$-terminal long chain fatty acyl-peptides, because peptides produced by proteolytic digestion always have a free terminal α-carboxyl group.

*Analysis of myristyl-Gly-Gly-Tyr-Arg, -Gly-Phe-$NH_2$, -Gly-$NH_2$ and -Gly-Gly-Gly-$NH_2$*

The myristyl-peptides were chromatographed with retention times between 20 and 30 min, whereas the materials which were not myristylated passed through the column unretained (Fig. 3). Fig. 3A shows that the myristyl-peptide contained in the major peak was identified as myristyl-Gly-Gly-Tyr-Arg on the basis of its amino acid composition (Gly 2.0, Tyr 1.0 and Arg 1.0). The second major peak in Fig. 3B was determined to be myristyl-Gly-Phe-$NH_2$ from the results of amino acid analysis (Gly 1.0 and Phe 1.0). Several other peaks in Fig. 3B, however, could not be identified

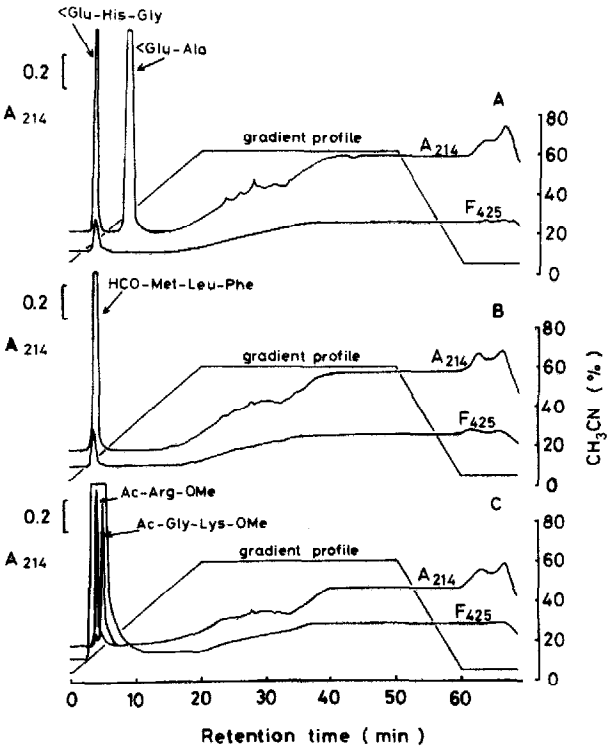


Fig. 1. HPLC of $NH_2$-terminal blocked peptides and Ac-Arg-OMe. $NH_2$-terminal blocked peptides (< Glu-His-Gly, < Glu-Ala, HCO-Met-Leu-Phe and Ac-Gly-Lys-OMe) and Ac-Arg-OMe, each 330 nmol, were separately injected into the HPLC system as described in Experimental. Column: Asahipack GS-310™, 150 × 8 mm I.D. Eluents: A, 50 m*M* sodium hydrogencarbonate, pH 8.3, containing 5% acetonitrile; B, 50 m*M* sodium hydrogen carbonate, pH 8.3, containing 60% acetonitrile. Gradient: 0–100% B in 20 min, linear (number 6), and then 100% B for 30 min. Detector: UV (214 nm) at 0.2 a.u.f.s.; fluorescence ($F_{254}$), excitation at 380 nm and emission at 425 nm, gain 4. Flow-rate: 0.5 ml/min.

because no amino acid was detected in their hydrolysates. Two major peaks, having retention times of 26 and 36 min, were obtained on the chromatogram in Fig. 3C. The faster moving peak was determined to be myristyl-Gly-Gly-Gly-$NH_2$ from its glycine content (3.0), but the slower moving peak could not be identified because of the lack of amino acids in its hydrolysate. The myristic acid in the acid hydrolysates of these peptides was determined separately by HPLC as described below.

*Analysis of standard fatty acids after derivatization with p-bromophenacyl bromide*

The standard fatty acids, each 100 pmol, were derivatized with *p*-bromophenacyl bromide according to the method of Borch[15]. As shown in Fig. 4, capric acid ($C_{10}$, peak 1), lauric acid ($C_{12}$, peak 2), myristic acid ($C_{14}$, peak 3), palmitic acid ($C_{16}$, peak 4) and stearic acid ($C_{18}$, peak 5) were eluted at retention times of 16, 20, 25, 29 and 33 min, respectively. The peaks chromatographed before capric acid (peak 1) were not identified. They are probably due to impurities contained in the solvents of reagents.

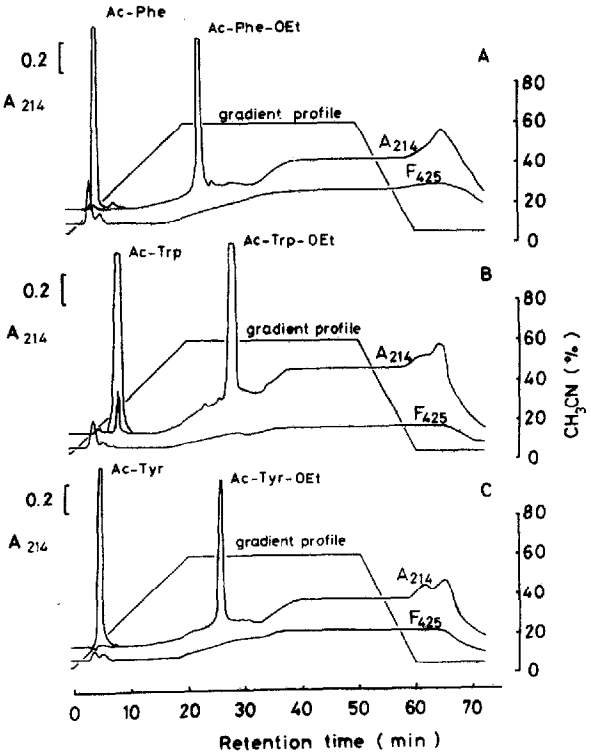


Fig. 2. HPLC of $NH_2$-blocked amino acid esters. The $NH_2$-blocked amino acids and their esters used are shown. Experimental conditions as in Fig. 1.

*Identification of an $NH_2$-terminal myristyl-peptide from the catalytic subunit of cAMP-dependent protein kinase type II*

The catalytic subunit of cAMP-dependent protein kinase type II partially purified from bovine heart was digested with pepsin[2], and the digest was subjected directly to HPLC. As Fig. 5 shows that the digest was separated into two peaks, a large peak occurring at the mobile phase hold-up time position and a small peak appearing at a retention time of 19 min. The small peak, *o*-phthalaldehyde negative, was pooled and lyophilized. The amino acid composition of the acid hydrolysate of this peak was in dose agreement with that (Gly 1.0, Asp 1.0 and Ala 1.0) of the $NH_2$-terminal blocked peptide separated from the peptic digest of the catalytic subunit of cAMP-dependent protein kinase type II[2]. The fatty acid in the acid hydrolysate was identified as myristic acid by HPLC after derivatization with *p*-bromophenacyl bromide. These results coincided with the chemical compositions of the $NH_2$-terminal portion (myristyl-Gly-Asn-Ala) of the catalytic subunit of cAMP-dependent protein kinase type II[1,2].

The described method may be suitable for the identification and sequence determination of long chain fatty acyl-peptides constituting $NH_2$-terminal portions of proteins.

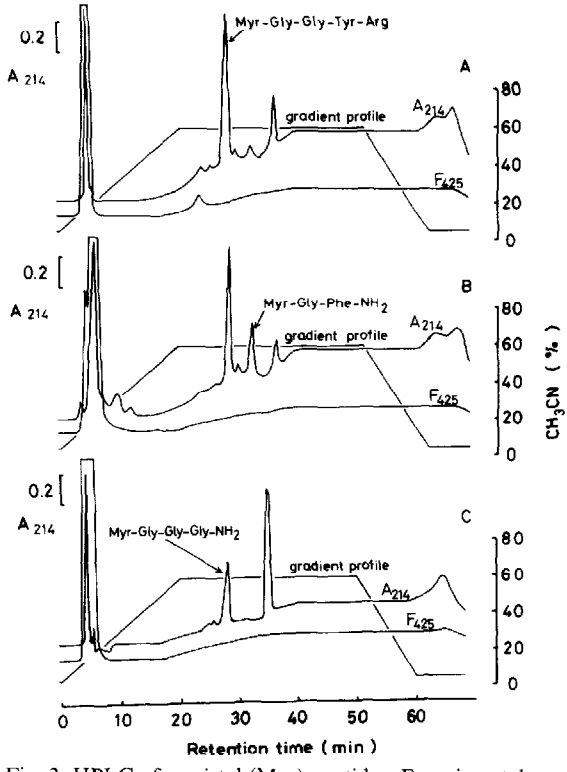


Fig. 3. HPLC of myristyl (Myr) peptides. Experimental conditions in Fig. 1.

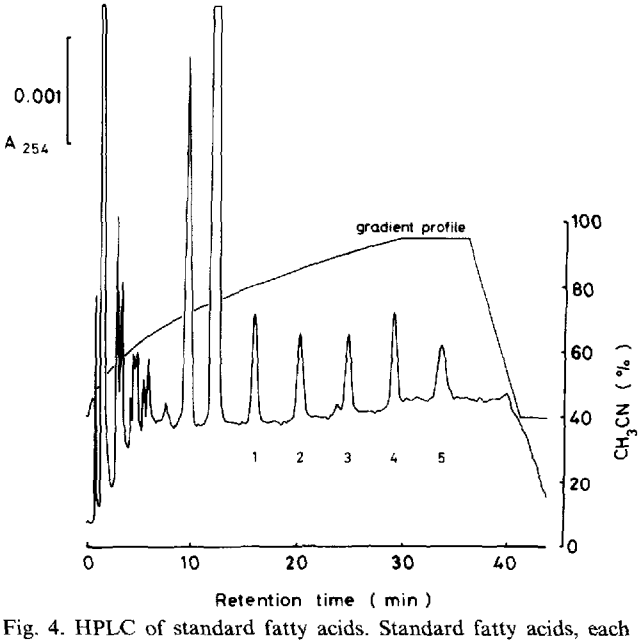


Fig. 4. HPLC of standard fatty acids. Standard fatty acids, each 100 pmol, were derivatized with *p*-bromophenacyl bromide[13]. Column: Radial-Pak $C_{18}$ cartridge (particle size 10 $\mu$m, 100 × 8 mm I.D.). Eluents: A, 40% acetonitrile; B, 95% acetonitrile. Gradient: 0–100% B in 30 min in a convex curve (number 5), and then 100% B for 5 min. Detector: UV (254 nm) at 0.001 a.u.f.s. Flow-rate: 3.0 ml/min.

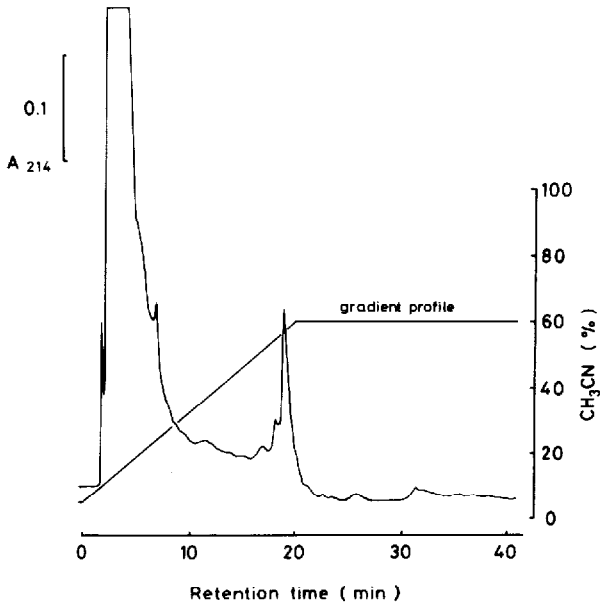


Fig. 5. HPLC of a peptic digest of the catalytic subunit of the cAMP-dependent protein kinase type II from bovine heart prepared as described[2]. Experimental conditions as in Fig. 1.

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