Acetylation of peptides with deuterium labelled acetic anhydride for the fast atom bombardment mass spectrometry.

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The specific and simplified acetylation method for the α-amino group of a peptide with deuterium labelled anhydride mixture was studied, using some model peptides. The method devised for the N-terminus analysis of a peptide by fast atom bomberdment (FAB) mass spectrometry. The N-terminal α-amino groups of peptides, Tyr-Gly-Gly-Phe-Leu and Ala-Lys-Asn-Phe-Phe, could be acetylated specifically at the range between pH 4.0 and pH 7.0. Below pH 3.0, no acetylation of the peptides were observed. The ionic species of the buffer solutions were also effective on the yields of acetylation of the peptides. The acetylation products were analyzed by HPLC and further by FAB mass spectrometry. With combination of these acetylation technique, it was possible to distinguish between the N-terminal fragment ions and the C-terminal fragment ions on FAB mass spectra.
Keywords : Acetylation, Amino acid sequencing, Deuterium label, FAB mass spectrometry.

1. Introduction

Various attempts for the amino acid sequencing by the mass spectrometric method has been tested[1-5]. Shimonishi et al.[1] reported on the use of FD mass spectrometry in conjunction with Edman degradation. Tsugita et al.[2] reported on the method with partial acid hydrolysis of a peptide. These approaches use the mass spectrometry of the $[M+H]^+$ of the original peptide and its consecutive chemical degradation products. Another approaches use the mass spectra of fragment ions induced by the collision disociation mass spectrometry[3,4]. However the one of the drawbacks of these approaches would be the insufficient accuracy at the assignment of complicated signals of fragment ions from large peptides. To clear this drawback, Takao et al.[5] reported ^{18}O labelling of carboxyl terminal of a peptide with triptic digests in $H_2{}^{18}O$. So we studied on the simplified conditions wich could applicable for the amino acid sequencing by FAB mass spectrometry based on the double labelling method of duterium. In this paper, the specificity of the acetyla tion of the terminal α-amino groups of peptides with a mixture of acetic anhydride and acetic anhydride-d_6 (1:1) was analyzed by HPLC and FAB mass spectrometry. The acetylated fragment ions showed doublet signals with 3 mass units difference, due to the labeling by ^2H at their acetyl residue. On the other hands, the C-terminal fragment ions showed singlet signals. This approach gave the possible future the peptide sequencing method.

2.Materials and Methods

2.1. Materials

Acetic anhydride (special grade) was purchased from Wako Pure Chemical Industries, LTD. Acetic anhydride-d_6 (99+

atom % D) was purchased from Aldrich Chemical Company Inc. Mixed acetic anhydride reagent (H-D reagent) was prepared by 0.5ml of each and stored in desicator at room temperature untill use. Peptides (Tyr-Gly-Gly-Phe-Leu and Ala-Lys-Asn-Phe-Phe) were purchased from Seikagaku Kogyo CO., LTD. Ammonium acetate (special grade), sodium dihydrogenphosphate (special grade), trifluoroacetic acid (TFA, analytical grade) and acetonitrile (HPLC grade) were purchased from Kanto Chemical CO., INC.

2.2. Apparaturs and Methods

Acetylation of the Peptides - The standard acetylation conditons were described as following ; Peptides (0.5 mg) were dissolved in 50μl of 0.3M ammonium acetate solution (pH4.0) and 1μl of acetic anhydride or H-D reagent were mixed at room temperature[5),6)]. After 5 min, the solution was frozen in liquid nitrogen immeadiaterly and lyophilized. Other conditions were discrived in the each sections.

HPLC Conditions - HPLC system was a Waters 600E system which was composed of a 600E system controller, a 610 pump, a WISP 700S automatic sample injector and a 990J photodiode array detector. The column was a μ - bondasphere 5μ C_{18} -100 A (3.9 mm i.d. x 150 mm). Tyr-Gly-Gly-Phe-Leu ([Leu^5] -enkephalin) and its derivatives were eluted with 30 % CH^3CN contained 0.1 % TFA at a flow rate of 1.0 ml/min. Ala-Lys-Asn-Phe-Phe and its derivatives were eluted with 25% CH^3CN contained 0.1 % TFA at a flow rate of 1.0 ml/min. The detection was at the wave length 230 nm.

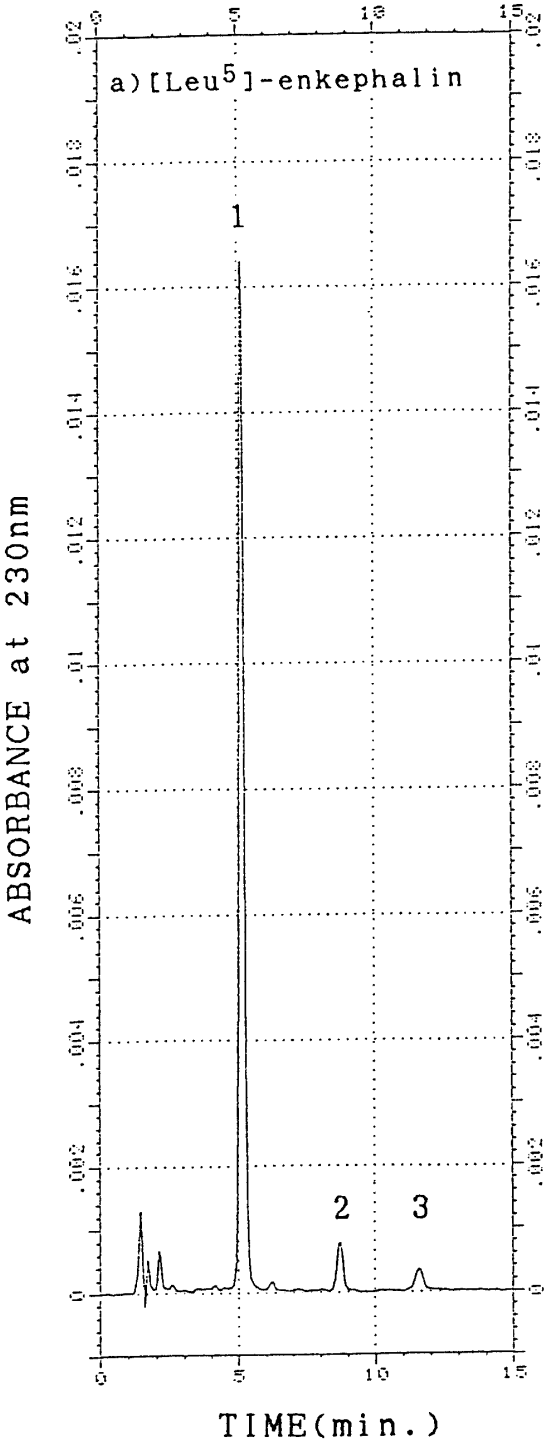
Measurement of FAB Mass Spectrum - FAB mass spectra were measured by a JEOL JMS-HX110 double-focussing mass spectrometer. Xenon was used for the source of the fast atom beam (6 KeV). The mass spectrometer was operated at 10 KeV accelating voltage. Sample preparation was performed as following ; Samples were dissolved in 10μl of 0.1% TFA solution. Then, 1μl of sample solution was mixed with 1μl of glycerol on target stage.

3. Results and Discussion

3.1. Chromatograms of Reaction Products of Peptides with Acetic Anhydride

Tyr-Gly-Gly-Phe-Leu and Ala-Lys-Asn-Phe-Phe were used for the model peptides, because these peptides respectively have two kinds of acetylation sites. Tyr-Gly-Gly-Phe-Leu has N-terminal α-amino group and phenolic hydroxyl group of tyrosine, and Ala-Lys-Asn-Phe-Phe has N-terminal α-amino group and ε-amino group of lysine for the sites of acetylation. Tyr-Gly-Gly-Phe-Leu and Ala-Lys-Asn-Phe-Phe (each 0.5mg) were dissolved in 50μl of 0.3M sodium phosphate buffer (pH 4.0), respectively. One μl of acetic anhydride was then added to the peptide solutions. After 40min, the solutions were frozen in liquid nitrogen and lyophilized. Then, acetylation products were dissolved in 50μl of eluent and analyzed by HPLC.

Chromatogram of Tyr-Gly-Gly-Phe-Leu reacted with acetic anhydride at pH 4.0, was shown in fig. 1. All acetylation products were eluted within 15 min. In this elution conditions, 3 peaks (5.2min, 8.8min and 11.7min) were detected. The molecular mass ions of peaks (5.2min, 8.8min and 11.7min) were gave 598, 598 and 640, respectively (shown in Fig.2). It suggested that peak of 5.2min and 8.8min were monoacetylated Tyr-Gly-Gly-Phe-Leu and peak of 11.7min was diacetylated Tyr-Gly-Gly-Phe-Leu. Furthermore, O-acetylated



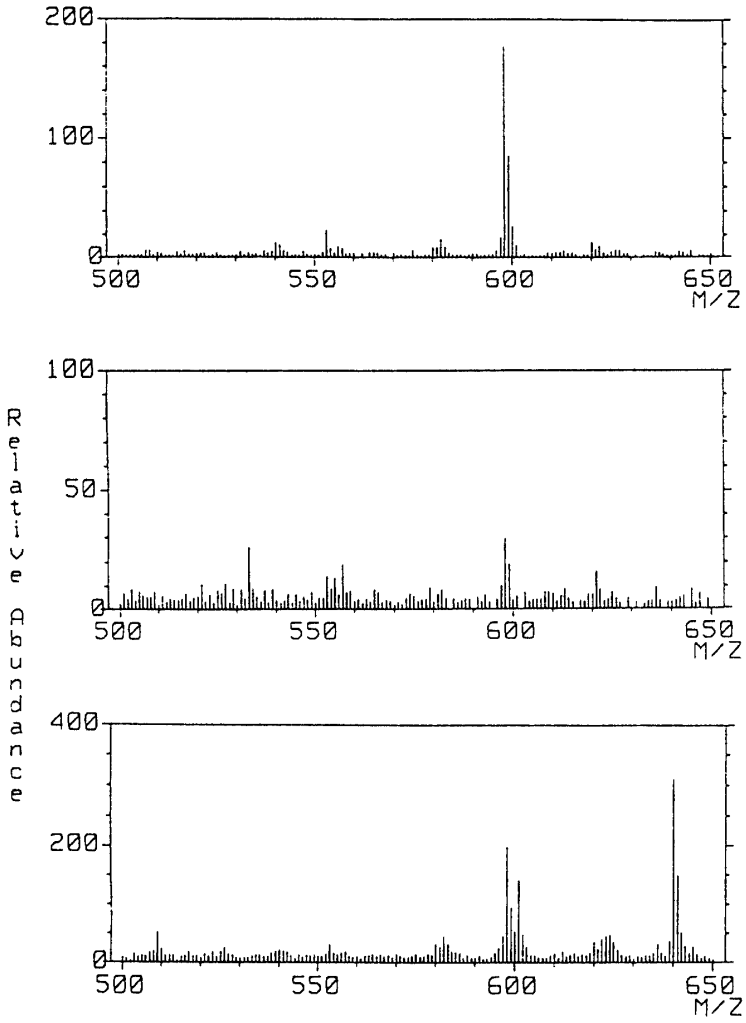


Fig.1. Chromatogram of the acetylated [Leu^5] -enkephalin.

[Leu^5] -enkephalin was reacted with acetic anhydride in 0.3M sodium phosphate buffer (pH 4.0) for 40min. The chromatography was performed at following conditions ; column ; μ-bondasphere 5μ C_{18}-100A (3.9 mm i.d. x 150 mm). flow rate ; 1.0 ml/min(isocratic). eluent ; 30% CH_3CN contained 0.1% TFA.

Each peak was N-acetyl [Leu^5] -enkephalin (1), O -acetyl [Leu^5] -enkephalin (2), N,O-diacetyl [Leu^5] -enkephalin (3), respectry.

Fig.2. Mass spectra of each peak of the acetylated [Leu^5] -enkephalin.

The FAB mass spectra of each peak of the acetylated [Leu^5] -enkephalin (shown in fig.1.) was measured.

(1)peak 1 of the acetylated [Leu^5] -enkephalin

(2)peak 2 of the acetylated [Leu^5] -enkephalin

(3)peak 3 of the acetylated [Leu^5] -enkephalin

tyrosine in acetylated Tyr-Gly-Gly-Phe-Leu was deacetylated by hydroxylamine at pH 7.5[7),8)], then, peak of 5.2min was increased, while peak of 11.7min was decreased (not data shown). Therefore, 5.2min, 8.8min and 11.7min were assigned as N-acetyl Tyr -Gly-Gly-Phe-Leu, O-acetyl Tyr-Gly-Gly -Phe-Leu and N,O-diacetyl Tyr-Gly-Gly -Phe-Leu, respectively. The Tyr-Gly-Gly -Phe-Leu was eluted at 3.4min.

Figure 3 shows chromatogram of acetylated products of Ala-Lys-Asn-Phe-Phe at pH 4.0. The molecular mass ions of peaks (3.0min, 4.3min and 5.9min) were

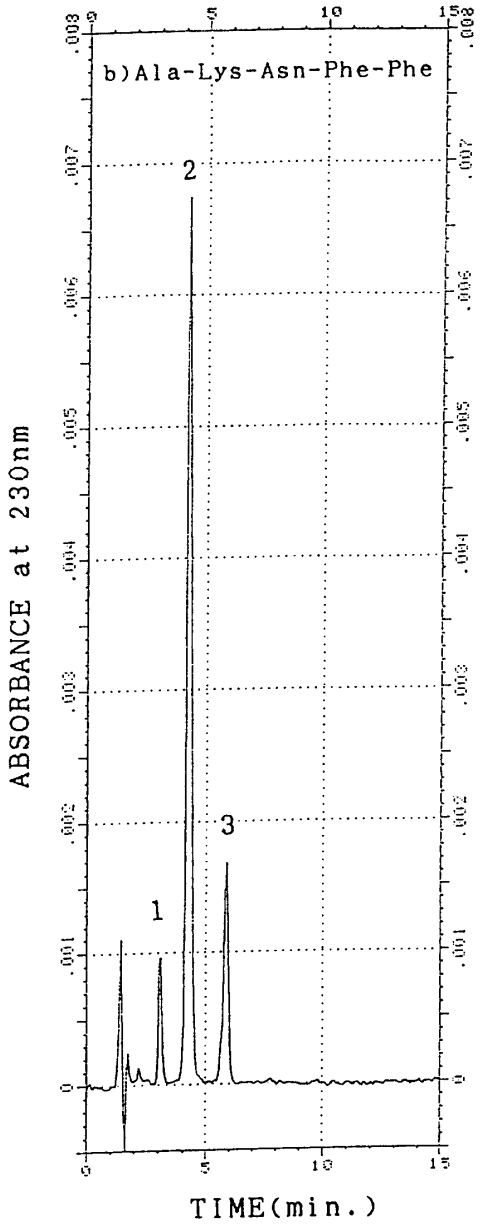


Fig.3. Chromatogram of the acetylated Ala-Lys-Asn-Phe -Phe.

Ala-Lys-Asn-Phe-Phe was reacted with acetic anhydride in 0.3M sodium phosphate buffer (pH 4.0) for 40min. The chromatography was performed at following conditions; column; μ-bondasphere 5μ C_{18}-100A (3.9 mm i.d. x 150 mm). flow rate; 1.0 ml/min (isocratic). eluent; 25% CH_3CN contained 0.1% TFA.

Each peak was Ala-Lys-Asn-Phe-Phe (1), α-N-acetyl Ala-Lys-Asn-Phe-Phe (2) and α,ε-N,N-diacetyl Ala -Lys-Asn-Phe-Phe (3), respectively.

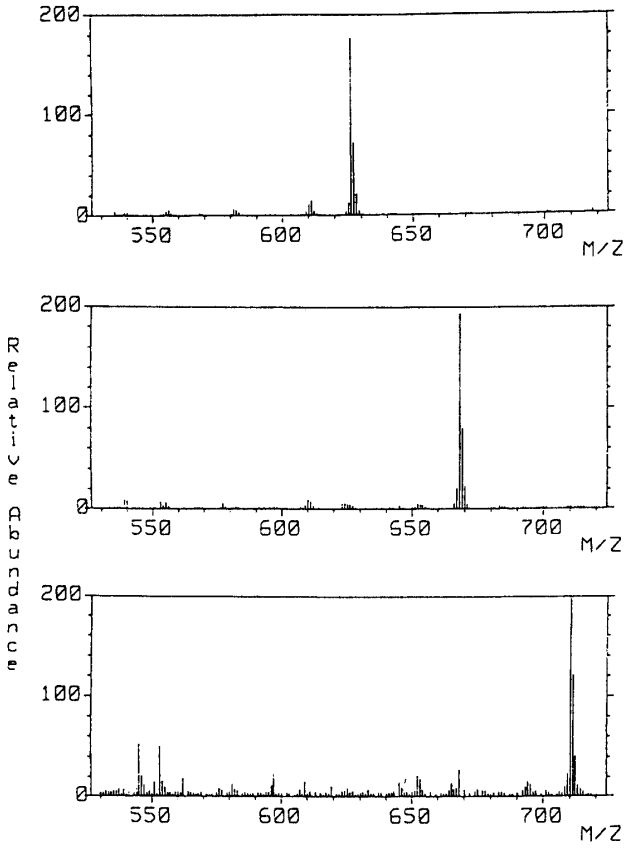


Fig.4. Mass spectra of each peak of the acetylated Ala -Lys-Asn-Phe-Phe.

The FAB mass spectra of each peak of the acetylated Ala-Lys-Asn-Phe-Phe (shown in fig.3.) was measured.
(1)peak 1 of the acetylated Ala-Lys-Asn-Phe-Phe
(2)peak 2 of the acetylated Ala-Lys-Asn-Phe-Phe
(3)peak 3 of the acetylated Ala-Lys-Asn-Phe-Phe

gave 626, 668 and 710 (shown in Fig.4), respectively. Then, monoacetyl Ala-Lys-Asn-Phe-Phe and α,ε-N,N-diacetyl Ala-Lys-Asn-Phe-Phe were eluted at 4.3min and 5.9min, respectively. The monoacetyl Ala-Lys-Asn-Phe-Phe had fragment ions (m/z=539, 555), which derived from α-N acetyl Ala-Lys-Asn-Phe-Phe. Therefore, the peak of 3.0min was assigned to α-N acetyl Ala-Lys-Asn-Phe-Phe. However, ε-N acetyl Ala-Lys-Asn-Phe-Phe was not detected.

3.2 Effect of pH on the Acetylation Products of Peptides

Figure 5 shows yield of acetylation products of peptides, Tyr-Gly-Gly-Phe-Leu and Ala-Lys-Asn-Phe-Phe, at various pH value. Below pH 3.0, the peptides were not acetylated. Above pH 4.0, the terminal α-amino groups of peptids were acetylated efficiently. On the other hands, the ε-amino group and the phenolic hydroxyl group were acetylated higher pH value than the terminal α-amino group. The maximum yield of N-acetyl Tyr-Gly-Gly-Phe-Leu and α-N-acetyl Ala-Lys-Asn-Phe-Phe were 92.0% and 74.5%, respectively. Therefore, the optimal pH value to obtain N-acetyl peptide was pH 4.0.

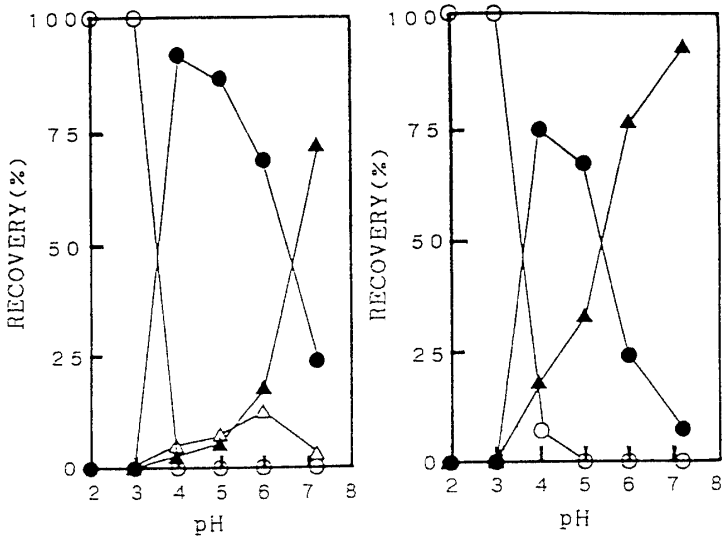


Fig.5. Effect of pH value on the acetylation of the peptides.
(a) [Leu^5] -enkephalin
○ ; [Leu^5] -enkephalin,
● ; N-acetyl [Leu^5] -enkephalin,
△ ; O-acetyl [Leu^5] -enkephalin,
▲ ; N,O-diacetyl [Leu^5] -enkephalin
(b) Ala-Lys-Asn-Phe-Phe
○ ; Ala-Lys-Asn-Phe-Phe
● ; α-N-acetyl Ala-Lys-Asn-Phe-Phe
▲ ; α,ε-N,N-diacetyl Ala-Lys-Asn-Phe-Phe
The acetylation reaction of the peptides with acetic anhydride was performed in 0.3M sodium phosphate buffer for 40 min.

3.3 Time Course of Acetylation

The time course of acetylation of the terminal amino groups of the peptides, reacted at pH 4.0, was shown in fig.6. One mg of Tyr-Gly-Gly-Phe-Leu and Ala-Lys-Asn-Phe-Phe were dissolved in 100μl of 0.3M sodium phosphate buffer (pH 4.0), respectively. Acetic anhydride (2μl) was then added to peptide solutions. At intervals of 1, 3, 5, 7, 10, 20, 30 and 40min, portions (10μl) of the reaction mixtures were removed in duplicate and lyophilized immediately. Acetic anhydride reacted rapidly with the terminal amino groups of pe-

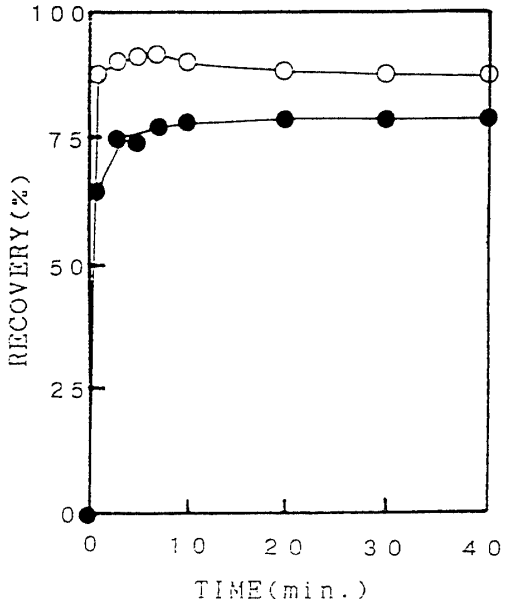


Fig.6. Rate of the acetylation of the terminal amino groups of the peptides.
○ ; N-acetyl [Leu^5] -enkephalin
● ; α-N-acetyl Ala-Lys-Asn-Phe-Phe
The acetylation reaction of the peptides with acetic anhydride was performed in 0.3M sodium phosphate buffer (pH 4.0).

ptides. The acetylation reaction virtually completed within 5min.

TABLE 1 Effect of Diferent buffer Ions on Acetylation of α-Amino Group of Peptides.

Buffer*1	yield of α-N acetyl peptides(%) [Leu⁵]-enkephaline	Ala-Lys-Asp-Phe-Phe
sodium phosphate	92.0	75.3
ammonium acetate	93.9	82.2
ammonium citrate	80.8	55.7
ammonium oxarate	85.3	71.9

*1 buffer: 0.3M, pH4.0

3.4 Effect of Buffer Ionic Species on the Acetylation

The effect of buffer ions on the acetylation of the peptides at pH 4.0 were examined (shown in table 1). The acetylation conditions were same as that of effect of pH value, except for reaction buffer. Four buffers, 0.3M sodium phosphate, 0.3M ammonium citrate, 0.3M ammonium oxalate and 0.3M ammonium acetate were used as reaction buffer. All reaction buffers were adjusted at pH 4.0. The presence of 0.3M ammonium acetate (pH 4.0) increased the yields of the peptides acetylated the terminal amino groups (Tyr-Gly-Gly-Phe-Leu 93%, Ala-Lys-Asn-Phe-Phe 82%), when compared with the results observed in 0.3M sodium phosphate, ammonium citrate and ammonium oxalate at pH 4.0. It has another advantage, to use 0.3M ammonium acetate buffer (pH 4.0) as reaction buffer. In the case of using sodium phosphate buffer as the reaction buffer, the presence of sodium ions complicates the FAB mass spectrum. But, there are no sodium ions in ammonium acetate buffer, therefor, the FAB mass spectrum is clearly. The acetylation reaction in ammonium acetate buffer (pH 4.0) also completed within 5min (not data shown).

The peptides were subjected to react with acetic anhydride at various peptide concentrations. The acetylation reaction was performed in 0.3M ammonium acetate adjusted at pH 4.0. The peptide concentration was changed from 10ng to 10μg per μl against at a constant concentration of acetic anhydride (0.2μmol per μl), but the peptide concentration was not affected the yield of the terminal amino group acetylated peptides (fig.7). Hence, to obtained maximal yields of the terminal amino group acetylated peptides, it is need to control the reaction pH value.

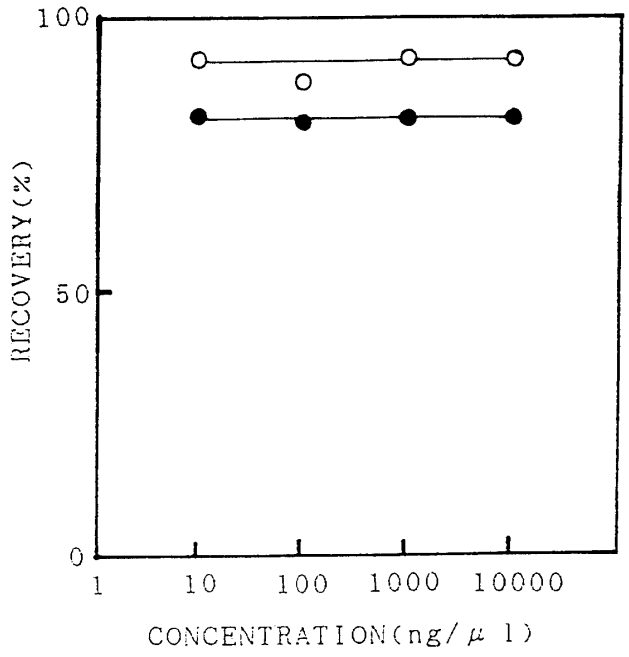


Fig.7 Effect of the peptide concentration on the acetylation of the terminal amino groups of the peptides.
○ : N-acetyl [Leu⁵] -enkephalin.
● : αN-acetyl Ala-Lys-Asn-Phe-Phe.
The acetylation reaction of the peptides with acetic anhydride was performed in 0.3M ammonium acetate buffer (pH 4.0).

3.5 Analysis of Acetylated Peptide by FAB Mass Spectrometry

The acetylation of the terminal amino

group of the peptide was applied to the amino acid sequencing by fast atom bombardment mass spectrometry. The acetylation of the ter minal amino group of Ala -Lys-Asn-Phe-Phe was performed with acetic anhydride and a mixture of acetic anhydride and acetic anhydride-d_6 (1:1), in ammonium acetate buffer (pH 4.0) at room temperature. After 5min, the solutions were immediately frozen in liquid nitrogen and lyophilized. The lyophilized samples were dissolved in 10μl of 25% CH_3CN contained 0.1% TFA and analyzed on reverse phase chromatography, respectively. Figure 8 shows chromatograms of Ala-Lys -Asn-Phe-Phe reacted with acetic anhydride (a) and with a mixture of acetic anhydride and acetic anhydride-d_6 (1:1) (b). The reverse phase chromatography could not separated α-N acetylated and α-N-d_3 acetylated Ala-Lys-Asn-Phe-Phe. Presence of acetic anhydride-d_6 was not affected the yield of each acetylation products.

The FAB mass spectra of the α-N acetylated Ala-Lys-Asn-Phe-Phe separated by HPLC were measured by FAB mass spectrometry. Figure 9 shows FAB mass spectra of α-N acetylated Ala-Lys-Asn-Phe -Phe treated with acetic anhydride (a) and

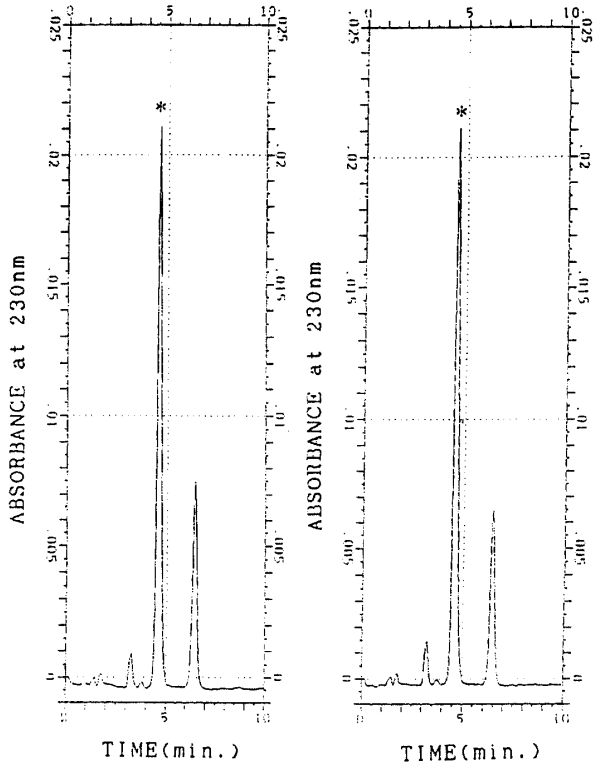


Fig.8 Chromatograms of acetylated Ala-Lys-Asn-Phe -Phe reacted with acetic anhydride and a mixture of acetic anhydride and acetic anhydride-d_6 (1:1).

The acetylation of Ala-Lys-Asn-Phe-Phe was performed with acetic anhydride (a) and a mixture of acetic anhydride and acetic anhydride-d_6 (1:1) (b). The chromatography conditions were the same as fig.1. The elent was 25% CH_3CN contained 0.1% TFA. The peak, marked *, was fractionated and its FAB mass spectrum was measured, respectively (shown in fig.6).

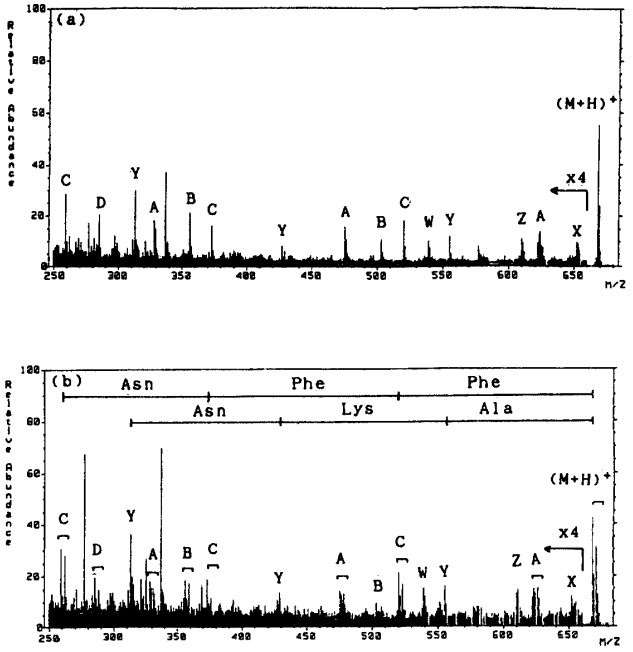


Fig.9. FAB mass spectra of α-N-acetylated Ala-Lys -Asn-Phe-Phe reacted with acetic anhydride and a mixture of acetic anhydride and acetic anhydride-d_6 (1:1).

α-N-acetyl Ala-Lys-Asn-Phe-Phe reacted with acetic anhydride and a mixture of acetic anhydride and acetic anhydride-d_6 (1 : 1) were separated by HPLC and fractionated (shown in fig.5). Each fraction was lyophilized, then, dissolved in 10 μl of 0.1% TFA and its FAB mass spectrum of α-N-acetyl Ala-Lys-Asn-Phe-Phe was measured, respectively.

(a) : acetylated with acetic anhydride.

(b) : acetylated with a mixture of acetic anhydride and acetic anhydride-d_6 (1:1).

with a mixture of acetic anhydride and acetic anhydride-d_6 (1:1) (b). The molecular mass ion and sequence ions of α-N acetylated Ala-Lys-Asn-Phe-Phe treated with acetic anhydride showed singlet signals (fig. 9-a). On the other hands, the molecular mass ion and sequence ions delived from N-terminus of α-N acetylated Ala-Lys-Asn-Phe-Phe treated with a mixture of acetic anhydride and acetic anhydride-d_6 (1:1) showed a doublet signals (fig. 9-b) due to partial incorporation of ^2H into the α-N acetyl residue. The sequence ions derived from the C-terminus of α-N acetylated Ala-Lys-Asn-Phe -Phe treated with a mixture of acetic anhydride and acetic anhydride-d_6 (1:1) gave singlet signals, because they doese not include the N-terminal acetyl residue. Thus, under these conditions the N-terminus and the C -terminus sequence ions were distinguished as the doublet signals and the singlet signals, clearly. Therefore, the acetylation of an α-amino group of the peptide with a mixture of acetic anhydride and acetic anhydride-d_6 (1:1) is useful to assign each fragment ions on a mass spectrum.

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