Determination of Amino Acid Sequence of Peptides by the Combination of Mass Spectrometry and Edman Degradation. Alternate Use of *p*-Chloro- and *p*-Bromophenylisothiocyanate Followed by Modification of Shortened Peptide

Asao MURAI and Yoko TAKEUCHI

Central Research Laboratories, Ajinomoto Co. Inc., Suzuki-cho, Kawasaki-ku, Kawasaki 210

(Received April 30, 1975)

It was shown that the alternate use of *p*-chloro- and *p*-bromophenylisothiocyanate for Edman degradation makes it possible to determine the amino acid sequence of peptides more unambigously by mass spectrometry, *i.e.*, by the doublet molecular ions of phenylthiohydantoin derivatives of amino acids with the intensity ratio inherent to the natural abundance of each halogen atom. The amino acid sequence of the shortened peptide could also be surely determined by the deuterium labeled mixed acylation by shifting the "sequence determining peaks" to higher mass region where no interfering peaks for the interpretation of the mass spectra would appear as well as labeling them as doublets with equal intensities by the distance of 2 mass units. Two examples, a dodecapeptide H-(Pro-Pro-Gly)$_4$-OH, and an octapeptide angiotensin II, were presented.

In recent years, the combination of Edman degradation[1] with mass spectrometry is well recognized as a useful method for the determination of amino acid sequence of peptides.[2] The advantages of the method might be considered as follows; the phenylthiohydantoins produced from the sequential degradation of peptide with phenylisothiocyanate are the most desirable derivatives for the unambigous identification of amino acids by mass spectrometry, and after suitable modification the residual shortened peptide can readily be determined the amino acid sequence in one step by mass spectrometry.[3,4] Thiohydantoin derivatives of amino acids can also be identified by gas chromatography-mass spectrometry.[5,6] An automatic sequenator using Edman degradation with reaction and inlet device directly connected to mass spectrometer has already been reported.[7]

Tschesche, Wachter[8] and Weygand, Obenmeier[9] reported that the N-terminal amino acids could be more clearly identified by converting to their *p*-bromophenylthiohydantoin [PTH(Br)] derivatives by mass spectrometry because the doublet molecular and fragment ions appear on their mass spectra with approximately equal intensities by the distance of two mass units due to the isotopic bromine atoms, ^{79}Br and ^{81}Br.

It seems that the alternate use of *p*-bromo- and *p*-chlorophenylisothiocyanate [PIT(Br) and PIT(Cl), respectively] for the sequential Edman degradation followed by mass spectrometric identification of the products [amino acid-PTH(Br) or PTH(Cl)] allows more unambigous sequence determination by checking the intensity ratio of their molecular and fragment ion pairs inherent to the natural abundances of each halogen atom. This is especially advantageous for the cases where the extraction of PTH derivative is incomplete or the application to the peptides in which the same amino acid neighbours with each other.

The authors also wish to report the method for the determination of amino acid sequence of peptides shortened by the consecutive Edman degradations; mixed acylation of peptide with normal and deuterium labeled long chain acyl groups to discriminate the "sequence determining peaks" (due to the cleavages of CO–N bonds with positive charges on each carbonyl group) by marking with doublet as well as shifting them to higher mass region where no undesirable contaminant peaks would appear.

Results and Discussion

Alternate Edman Degradation with PIT(Cl) and PIT(Br). A dodecapeptide, H-(Pro-Pro-Gly)$_4$-OH, was applied to the alternate sequential Edman degradations with PIT(Cl) and PIT(Br) followed by acylation and permethylation of the shortened peptide. Series of mass spectra of the PTH(Cl or Br) derivatives obtained from first through sixth Edman degradation are illustrated in Fig. 1. The mass spectrum of the derivative of the N-terminal amino acid indicates the clear molecular ion pair at *m/e* 266 and 268 with an intensity ratio of 3 : 1 and this readily leads to identification the N-terminal amino acid as proline. The spectrum of the second Edman degradation products clearly indicates the second amino acid as proline, too, by the molecular ion pair at *m/e* 310/312 with the equal intensity ratio of proline-PTH(Br). The mass spectrum of the third Edman degradation products using PIT(Cl) exibits three doublet peaks of molecular ions. One pair of the doublet at *m/e* 226/228 with the intensity ratio of 3 : 1 must be the molecular ion of the third amino acid-PTH(Cl), glycine. Another doublets at *m/e* 266/268 (3 : 1) and *m/e* 310/312 (1 : 1) should be attributed to the first and second amino acid, proline and proline, by the incomplete extraction of the PTH derivatives in the first and second stage of the Edman degradations. A similar result can be deduced from the spectrum of the fourth Edman degradation products using PIT(Br), in which the new pair of doublet at *m/e* 310/312 (1 : 1) is indicative of the fourth amino acid, proline. The same procedures of identification of amino acid-PTH derivatives by the alternate Edman degradations resulted the determination of the sequence from first to sixth amino acid as Pro–Pro–Gly–Pro–Pro–Gly. The residual shortened peptide was then acetylated, esterified and permethylated by the method

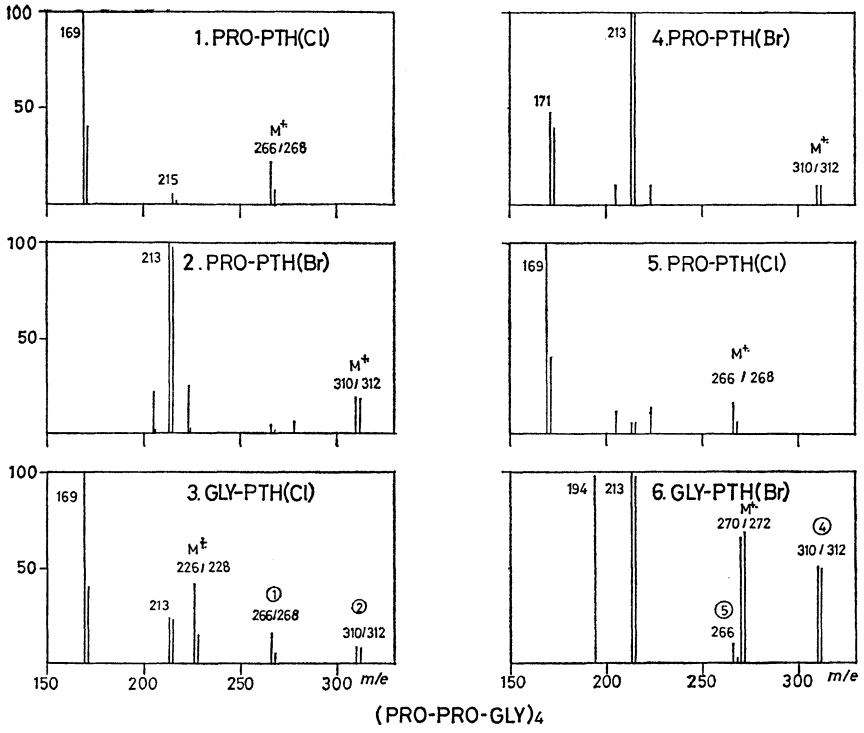
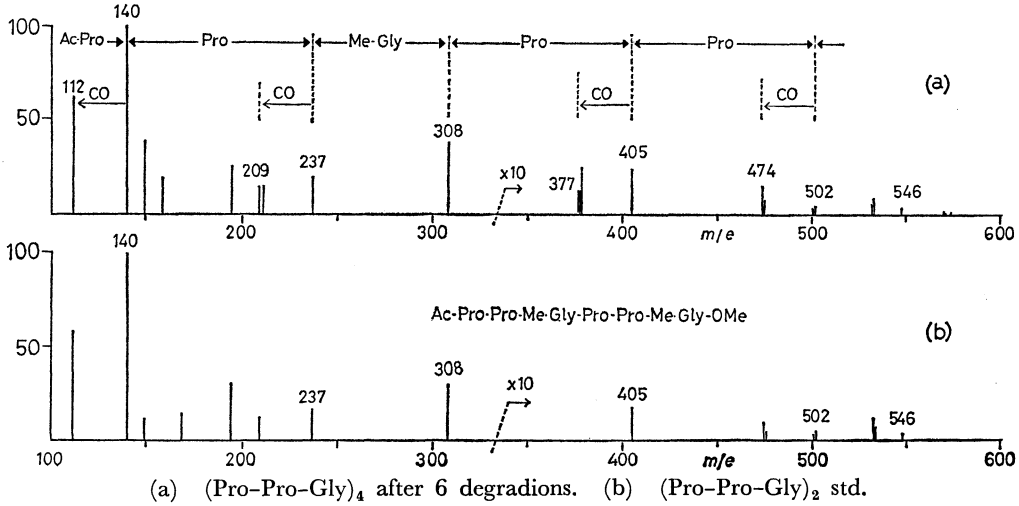


Fig. 1. Series of mass spectra of amino acid-PTH (Cl or Br) derivatives from 1st to 6th Edman degradations of (Pro-Pro-Gly)$_4$. The peaks under encircled numerals represent the molecular ions of the PTH derivatives from corresponding order of degradation.



(a) (Pro-Pro-Gly)$_4$ after 6 degradions. (b) (Pro-Pro-Gly)$_2$ std.

Fig. 2. Mass spectra of the N-acetylated and permethylated peptides.
Upper: residual peptide after 6 degradations of (Pro-Pro-Gly)$_4$, lower: standard peptide (Pro-Pro-Gly)$_2$.

of Vilkas and Lederer[10] and determined the amino acid sequence by its mass spectrum as Pro–Pro–Gly–Pro-Pro–Gly. The mass spectra of the modified shortened peptide after six degradations and the standard peptide, H–(Pro–Pro–Gly)$_2$–OH, are shown in Fig. 2. The complete amino acid sequence of this dodecapeptide was then ascertained by combining with the results from alternate Edman degradation as H–(Pro–Pro–Gly)$_4$–OH

Mixed Long-chain Acylation with Unsaturation or Deuterium Labeling. Consecutive Edman degradation procedures often accumulate undesirable by-products or other artifacts by which the mass spectra are contaminated at the mass region of lower "sequence determining peaks" ($\sim m/e$ 300) of an acetylated peptide derivatives. It seems that the interpretation of mass spectra for the sequence determination must be much simplified by introducing the mixture of saturated and unsaturated acyl groups to the peptides not only because the molecular and fragment peaks would be marked by doublets by the distance of two mass units but because they would be shifted to the higher mass region where no peaks from contamination would appear. Mixed acylation

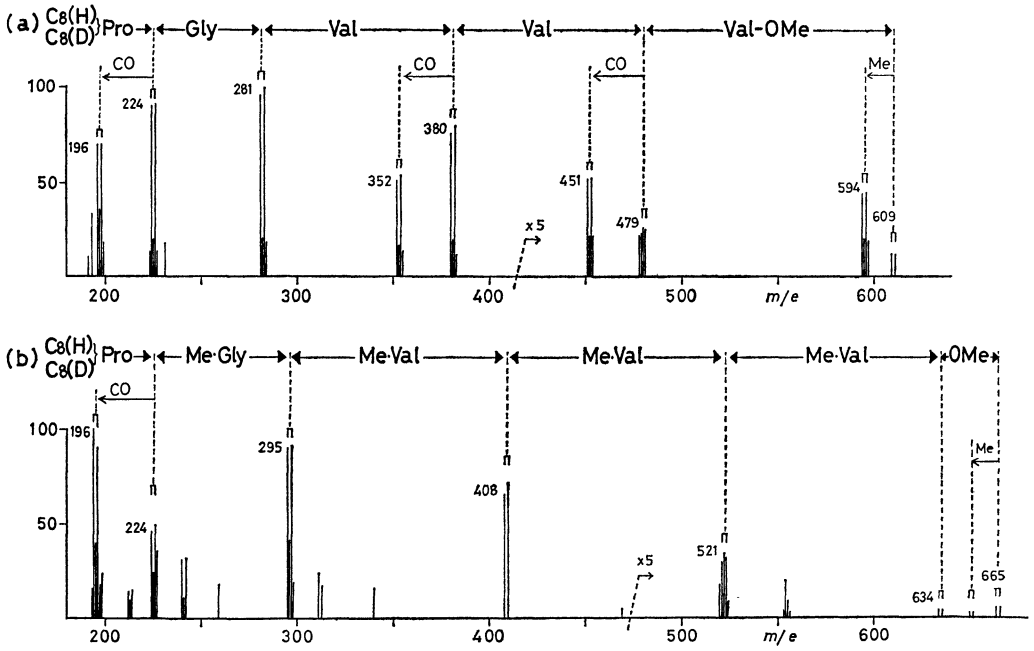


Fig. 3. Mass spectra of mixed acylated Pro-Gly-Val-Val-Val methyl ester with(b) and without(a) permethylation. $C_8(H) = C_7H_{15}CO$, $C_8(D) = C_7H_{13}D_2CO$

of peptides with C_2H_5COCl/C_2H_3COCl, $C_7H_{15}COCl/C_7H_{13}COCl$, and $C_{17}H_{35}COCl/C_{17}H_{33}COCl$ were investigated. A C_8-mixed acylation was sufficient for the "sequence determining peak" shifting. The intensity ratio of the doublets, however, differed significantly among the peaks of the molecular and fragment ions within a spectrum and moreover, the ratio varied not only with vaporization temperature but with peptide to peptide. Similar results were obtained from C_3- or C_{18}-mixed acylation.

Marking the molecular and sequence peaks by doublets was successful by the acylation of peptides with equivalent mixture of *n*-octanoyl chloride and *n*-octanoyl-3,3-d_2 chloride. Figure 3 shows the mass spectra of deuterium labeled-acylated Pro–Gly–Val–Val–Val methyl ester with (b) and without (a) permethylation. The lowest "sequence determining peaks" appear at *m/e* 224/226 (acylprolyl ion). The fragment ions appearing in higher mass region than that of the acylprolyl ion were enough simple and clear for the interpretation of sequence determination.

Permethylation of long-chain acylated peptide esters usually lowers the relative intensities of the molecular ions and does not seem to give an additional advantage on the spectra for the interpretation of sequence determination. The vaporization temperature, however, is lowered by 30—40° by permethylation.

Combination of Alternate Edman Degradation and Deuterium Labeled Long-chain Acylation of the Residual Peptide. A typical example for the sequence determination of peptides by sequential Edman degradation followed by esterification and deuterium labeled long-chain acylation of the shortened peptides is shown in Fig. 4. An octapeptide, angiotensin II, was easily proved its amino acid sequence after six alternate Edman degradations followed by deuterium labeled acylation.

There can be the case where the shortened peptide, even in the case of tripeptide such as Val–Val–Val, is extracted together with the PTH derivative by ethyl acetate by the usual separation procedures[5,6] in Edman degradation. As in these cases, the mixture of the extracted PTH derivative and the residual peptide was directly applied to esterification and *N*-acylation, then the mass spectra of the mixture were recorded at the suitable vaporization temperatures at which the PTH and peptide derivatives were separately evaporated. Alternatively, PHT derivatives can be well and selectively extracted from the residual peptides with toluene–ethyl acetate (1 : 1) instead of ethyl acetate.

Experimental

Peptides. H–(Pro–Pro–Gly)$_2$–OH, H–(Pro–Pro–Gly)$_4$–OH, and angiotensin II were purchased from Protein Research Foundation.

n-*Octanoic-3,3-d_2 Acid.* A similar method of synthesis of *n*-heptanoic-3,3-d_2 acid by Katoh *et al.*[13] was used with the starting material of *n*-hexanoic acid in place of *n*-valeric acid.

Edman Degradation. Sequential degradation of peptides with PIT (Cl or Br) was done by the method of Tschesche and Wachter.[8] PIT(Cl) and PIT(Br) were synthesized by the method described elsewhere.[11]

Peptide Modification. The shortened peptide (1—5 mg) was esterified with HCl-saturated methanol (10 ml) at room temperature for 2 hr and the excess reagents were evaporated off. To the residue, 2 ml of dried pyridine containing acid chloride 1.2—2 molar equivalent to the free amino groups was added and the solution was allowed to stand for 2 hr at room temperature. After the solvent was evaporated, the residue was dissolved in chloroform and was washed with

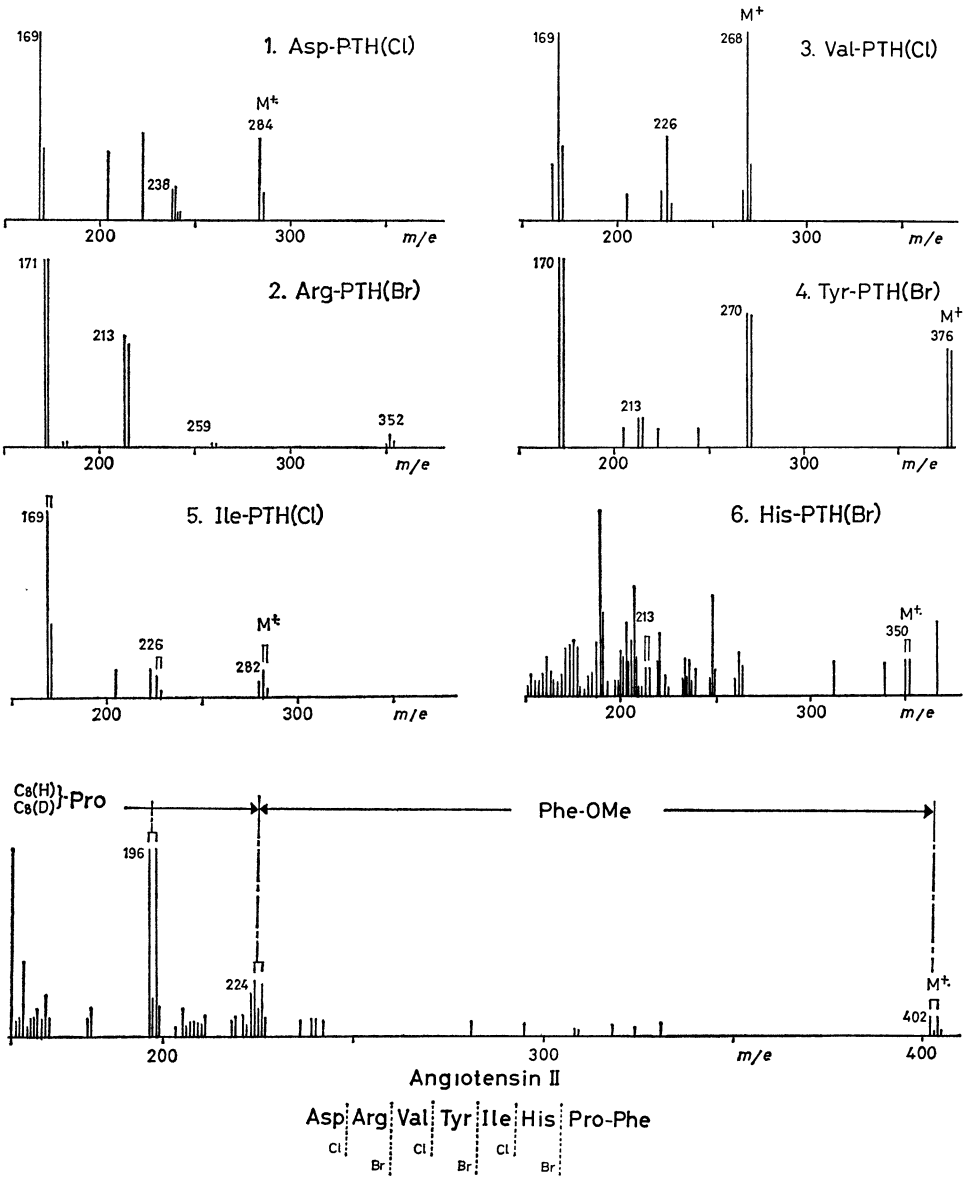


Fig. 4. Mass spectra of PTH(Cl or Br) derivatives from 1st to 6th Edman degradations of angiotensin II and residual peptide after esterification and deuterium labeled mixed acylation.

aqueous $NaHCO_3$ solution. The chloroform layer was then dried over anhydrous Na_2SO_4, and the solvent was evaporated. The oily residue was directly introduced into the ion source of mass spectrometer. Permethylation, if necessary, was carried out according to the method of Thomas[12] or Vilkas.[10]

Mass Spectrometry. A Hitachi RMU-6E Mass Spectrometer with 70 eV ionizing energy, 80 μA of emission current, and ion source temperature of 220° was used.

References

1) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).
2) N. A. Aldanova, E. I. Vinogradova, S. A. Kazaryan, B. V. Rozynov, and M. M. Shemyakin, *Biochem. USSR.*, **35**, 742 (1970).
3) M. M. Shemyakin, *Pure Appl. Chem.*, **17**, 313 (1968).
4) E. Lederer, *ibid.*, **17**, 489 (1968).
5) H. Hagenmaier, W. Ebbighausen, G. Nicholson, and W. Votsch, *Z. Naturforsch.*, **25b**, 681 (1970).
6) R. Burgus, M. Butcher, M. Amos, N. Ling, M. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale, and R. Guillemin, *Proc. Nat. Acad. Sci. U.S.A.*, **69**, 278 (1972).
7) R. E. Lovins, J. Craig, T. Fairwell, and C. McKinney, *Anal. Biochem.*, **47**, 539 (1972).
8) H. Tschesche and E. Wachter, *Eur. J. Biochem.*, **16**, 187 (1970).
9) F. Weygand and R. Obermeier, *ibid.*, **20**, 72 (1971).
10) E. Vilkas and E. Lederer, *Tetrahedron*, **26**, 3089 (1968).
11) G. J. M. Van der Kerk, C. W. Pluygers, and G. de Vries, "Organic Syntheses," Vol. 45 (1965), p. 19.
12) D. W. Thomas, B. C. Das, S. D. Gero, and E. Lederer, *Biochem. Biophys. Res. Commun.*, **32**, 519 (1968).
13) M. Katoh, D. A. Jaeger, and C. Djerassi, *J. Amer. Chem. Soc.*, **94**, 3107 (1972).