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C-Terminal Sequencing of Peptides and Proteins by Successive Degradation with Heptafluorobutyric Anhydride Vapor

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A chemical C-terminal sequencing method of proteins, involving cyanogen bromide cleavage of proteins, selective isolation of the C-terminal peptide and the C-terminal successive degradation with heptafluorobutyric anhydride vapor is presented. FAB/MS analysis of the C-terminal truncated peptide mixture clearly indicated the C-terminal sequence of a protein.

We have reported a carboxypeptidase mimetic degradation by the action of perfluoroacyl anhydride vapor on a solid peptide possibly through the intermediary formation of oxazolones at the C-termini.[1] The resulted degradation mixture was analyzed by fast atom bombardment mass spectrometry (FAB/MS) which permits the C-terminal sequence assignment by the differences of molecular masses of the truncated fragments.

It is difficult to apply this method to protein C-terminal sequencing because usual proteins are too big to analyze by FAB/MS directly. Electrospray ionization (ESI)/MS, has been successfully applied to direct measurement of protein molecular weights although an analysis of a sample containing a mixture of similar molecular masses is not always easy.

We tried to fragment a protein and selectively isolate the C-terminal peptide. After various trials, we selected the classical cyanogen bromide cleavage specific for methionyl peptide bond.[2] The cleaved peptide fragments were selectively fractionated by covalent bond formation with the *N*-(2-aminoethyl)-3-aminopropyl glass (APG, LKB Biochrom Ltd.) into non-C-terminal fragments and the C-terminal peptide.[3,4] The C-terminal peptide was successively degraded with heptafluorobutyric anhydride (HFBAA)[5] and

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analyzed by FAB/MS (JMS-HX110, JEOL).

Both 5 nmoles of bovine adrenal medulla dodecapeptide (Peptide Institute Inc., Minoh) and sheep myoglobin (Sigma Chem. Co., USA) were dissolved in 100 μl of 70% formic acid separately and reacted with equal weight of cyanogen bromide at room temperature for 16 h. The mixtures were vacuum evaporated and added with 30 μl of anhydrous trifluoroacetic acid (TFA). Each mixture was kept for 30 min at room temperature and again evaporated to ensure the formation of homoserine lactone at the cleaved C-termini.

The products were dissolved in 10 μl of water and then added with 30 μl of *N,N'*-dimethylformamide (DMF). The solution was mixed with 50 mg of APG, pre-equilibrated with a 2% triethylamine DMF solution and incubated for 2 h at 45°C. The C-terminal peptide was left unbound but the all other peptide fragments, having the C-terminal lactone, were covalently bound to APG. The APG was washed with each two 100 μl portions of DMF and 0.1 M pyridine collidine buffer (pH 8.2). The unbound and washed fractions were collected and evaporated in a small test tube (6 x 40 mm). The following processes were carried out in a glove box continuously flushed with dry nitrogen gas to avoid moisture as completely as possible. The tube was then placed in a reaction vessel (19 x 100 mm, Pierce USA). Hundred μl of a 15% (v/v) HFBAA acetonitrile solution available from Nacalai Tesque (Kyoto) was added to the vessel, evacuated (10^{-2} Torr) and sealed under cooling with liquid nitrogen. The reaction was carried out at -20°C for 1 h. The reaction product was evaporated and treated with vapor of 10% aqueous pyridine at 100°C for 30 min in order to hydrolyze the *O*-acyl derivatives and the oxazolone. The final product was dissolved in 50% acetic acid and mixed with an equal volume of the matrix consisting of glycerol, thioglycerol, 3-nitrobenzyl alcohol and TFA (1:2:1:0.5 v/v) and analyzed by FAB/MS.

The bovine adrenal medulla dodecapeptide was cleaved and the C-terminal heptapeptide (6-12) was isolated while the N-terminal pentapeptide was removed by the APG treatment. Figure 1(a) shows the FAB/MS spectrum of the successive degradation products by HFBAA vapor, where all the acylated C-terminal truncated peptide ions $[M+H]^+$ are clearly shown. Acyl peptides 6-12, 6-10 and 6-8 accompany -18 ions by dehydration even after water treatment, and 6-8 and 6-7 accompany -46 ions probably due to decarboxylation. The -18 ions may cause misjudgment of amino acids between Phe and Glu, Asp and Pro and Met and Leu or Ile.

Sheep myoglobin was subjected to the C-terminal sequencing including the isolation of the C-terminal peptide (143-153) and HFBAA degradation. Figure 1(b) is a FAB/MS spectrum of the degraded fragments; $[M+H]^+$ 1342.8 is the acylated and di-dehydrated peptide 143-153. The dehydration may be due

to that of the glutamine-152 residue into glutamic-γ-nitrile. The second dehydration residue may occur at glutamic acid-145 which possibly forms *N*-substituted [2-aminoacyl glutarimide].[6)]

The FAB/MS spectra demonstrated that the present combined techniques are practically applicable to protein C-terminal sequencing when methionine residue(s) is located in the C-terminal region. The HFBAA vapor degraded the protein but the C-terminal truncated protein fragments were not

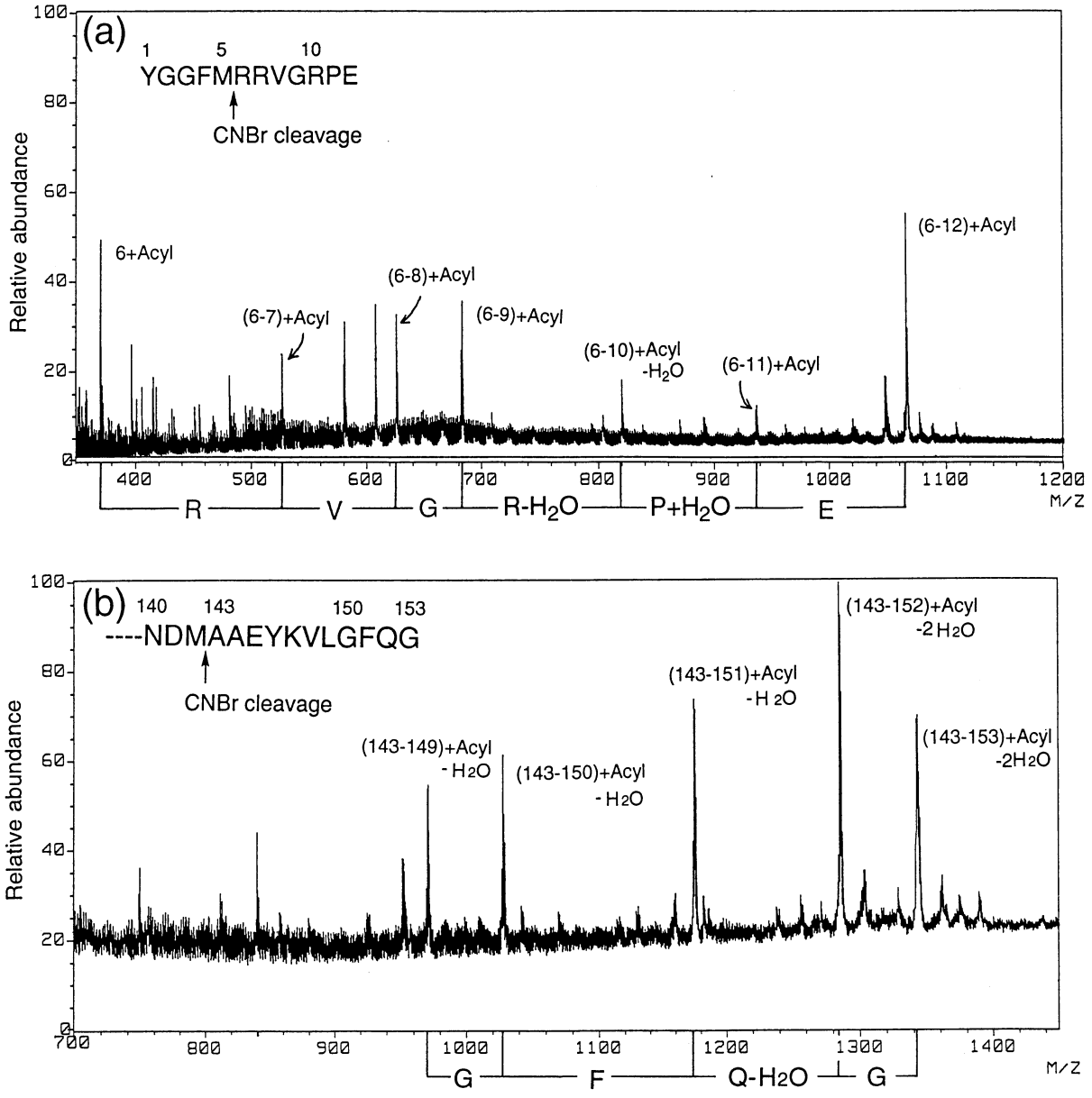

Fig.1. FAB/MS spectrum of successively degraded C-terminal peptides. (a)C-terminal peptides of bovine adrenal medulla dodecapeptide. (b)C-terminal peptides of sheep myoglobin.

analyzable by FAB/MS in too big mass-size nor by ESI/MS in accuracy. In the ESI/MS multicharged ions overcome the size problem but a more accurate measurement is require to detect the small differences in mass numbers due to variation of amino acid residues in truncated fragments.

In the previous work[7], the mixture of truncated protein fragments by the treatment with HFBAA was cleaved by CNBr and the product mixture was directly analyzed by FAB/MS. That method was successfully applied for a few proteins. However, in many cases, as FAB/MS spectrum becomes too complicate to analyze and mutual suppression of ionization in FAB/MS[8] were often observed, the present technique including isolation of the C-terminal peptide is therefore proposed.

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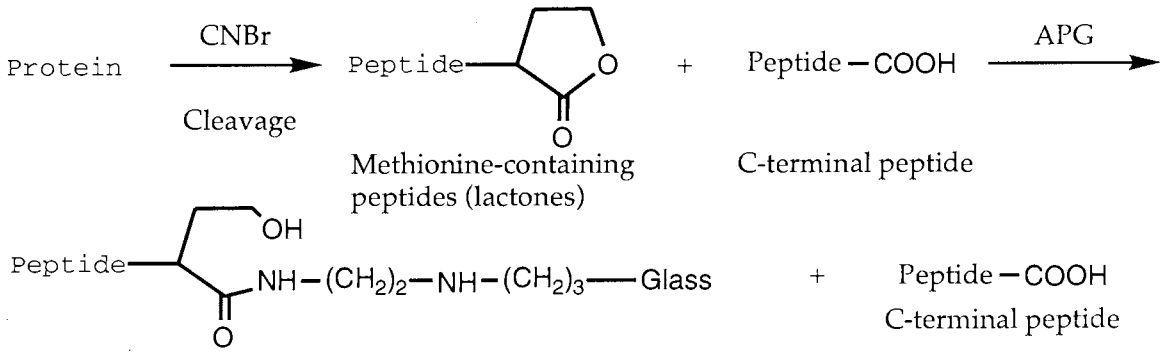

5) We avoid to use pentafluoropropionic anhydride[1] because the increment of mass by the acylation (146) is similar to residual weight of Phe (147).
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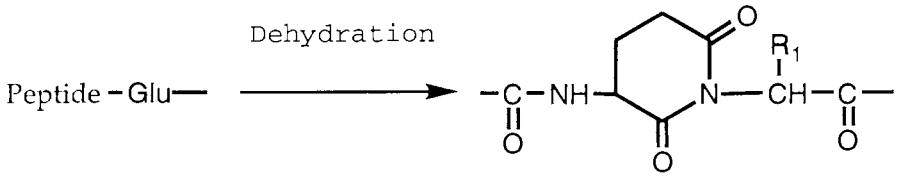

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