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Application of Mass Spectrometry to Sequence Analysis of Pyroglutamyl Peptides from Snake Venoms: Contribution to the Confirmation of the Amino Acid Sequence of Bradykinin-potentiating Peptides B, C and E isolated from the Venom of *Agkistrodon halys blomhoffii*[1,2]

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The amino acid sequences of the three bradykinin-potentiating peptides from the venom of *Agkistrodon halys blomhoffii* (potentiators B, C, and E), which had been previously presented by Kato and Suzuki using conventional stepwise techniques, were smoothly confirmed by means of mass spectrometry coupled with enzymatic cleavage techniques. In the course of the sequencing, it was suggested that the preparation of potentiator B previously purified is contaminated by a small amount of the peptides with analogous amino acid sequences.

The results showed the usefulness of mass spectrometric method in the sequence determination of naturally occurring peptides having N-terminal pyroglutamic acid and abundant proline residues. Mass spectrometric method was also effective for the detection of a small amount of contaminating peptide in the sample.

Bradykinin–potentiating peptides from the venoms of *Agkistrodon halys blomhoffii*[4] and *Bothrops jararaca*[5] have recieved much attention owing to their properties to potentiate specifically the actions of bradykinin and to inhibit angiotensin-converting enzyme. All of these peptides have pyroglutamic acid at N-terminus and proline at C-terminus, and their proline content is extremely high except one (BPP5a)[5a] of the peptides isolated from the venom of *Bothrops jararaca*.

Although the sequence analysis of the bradykinin-potentiating peptides was considerably difficult according to the conventional degradation methods, Kato and Suzuki[4] deduced the primary structures of three peptides, potentiators B, C and E, obtained from the venom of *Agkistrodon halys blomhoffii*;

potentiator B: Pyr–Gly–Leu–Pro–Pro–Arg–Pro–Lys–Ile–Pro–Pro
potentiator C: Pyr–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro
potentiator E: Pyr–Lys–Trp–Asp–Pro–Pro–Pro–Val–Ser–Pro–Pro

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1) Part of this paper was presented at the 9th Symposium of Peptide Chemistry at Shizuoka College of Pharmacy, Shizuoka (Japan), 25th, November, 1971.

2) The abbreviated designations of amino acids, peptides and their derivatives mentioned in the paper are those from *Biochemistry*, **5**, 2585 (1966); **6**, 362 (1967). Amino acid symbols except Gly denote the L configuration. Pyr=pyrrolidone carboxylic acid, Dec(OH)=3-hydroxydecanoic acid.

3) Location: *a*) *Takara-machi, Kanazawa*; *b*) *Yamada-Kami, Suita, Osaka.*

4) *a*) H. Kato and T. Suzuki, *Experientia*, **25**, 694 (1969); *idem*, *Proc. Japan Acad.*, **46**, 176 (1970); *idem*, *Biochemistry*, **10**, 972 (1971); *b*) H. Kato and T. Suzuki, *Experientia*, **26**, 1205 (1970); *idem*, *Ciência e Cultura*, **23**, 527 (1972).

5) *a*) S.H. Ferreira, D.C. Bartelt, and J.J. Greene, *Biochemistry*, **9**, 2583 (1970); *b*) M.A. Ondetti, N.J. Williams, E.F. Sabo, J. Pluščec, E.R. Weaver, and O. Kocy, *Biochemistry*, **10**, 4033 (1971).

The peptides of these structures were synthesized by Kimura and Sakakibara[6] and identified with the corresponding natural peptides by measuring electrophoretic mobility, *Rf* value on paper chromatogram and biological activity.

However, the high proline content made difficult to identify these peptides with the synthetic ones. We have already experienced that a synthetic peptide Pyr–Gly–Leu–Pro–Pro–Gly–Pro–Ile–Pro–Pro, which is missing proline residue at position 7 of potentiator C, showed similar behaviors to potentiator C on paper electrophoresis and chromatography (unpublished data). Furthermore, Sakakibara, *et al.*[7] reported that some synthetic peptides analogous to bradykinin had bradykinin-potentiating activity. Therefore, it is more profitable to use mass spectrometric method for obtaining further confirmation of the structures of the bradykinin-potentiating peptides. Mass spectrometry has advantages in distinguishing acidic amino acids from their amides and in applicability to the peptides whose amino terminal are blocked. Particularly, bradykinin-potentiating peptides have pyrolgutamic acid and prolyl-proline or prolyl-prolyl-proline sequence in their molecules and mass spectrometry would provide a direct approach allowing assignment of the complete sequences of the peptides.

The present paper describes the results of sequence analyses of three bradykinin-potentiating peptides, potentiators B, C, and E by direct mass spectrometry in conjunction with enzymatic degradation methods.

Experimental

Materials——The bradykinin-potentiating peptides (potentiators B, C, and E) were isolated from the lyophylized venom of *Agkistrodon halys blomhoffii* as reported previously.[4a] The peptide fragments of potentiator B (B–C–1, B–C–3, and B–S–4) and potentiator E (E–T–1, E–T–1a, and E–T–1b) were obtained from the digest of each potentiator with collagenase, *Streptomyces griseus* protease or trypsin, according to the methods of Kato and Suzuki.[4] The homogeneity of the samples used was checked by high-voltage paper electrophoresis at pH 3.5 (pyridine: acetic acid: water, 1:10: 289, v/v) and thin–layer chromatography (1-butanol: acetic acid: water, 3: 1: 1, v/v).

Methods——Modification of arginine-containing peptide to the corresponding ornithine peptide was carried out with a minor modification of the published procedure[8]: The peptide (1—2 mg) was dissolved in a mixture (2 ml) of hydrazine hydrate and water (1: 1, v/v), and kept for 1 hr at 95°. The reaction mixture was evaporated *in vacuo* with the repeated addition of water to remove a trace of hydrazine. Then the residue was dried *in vacuo* for 10 hr at 40°.

Acetylation was performed in acetic anhydride–acetic acid (1: 1, v/v) at room temperature by the method of Agarwal, *et al.*[9]

3-Hydroxydecanoyl group has been found to be useful as an N-terminal tag for mass spectrometric analysis of long chain peptides.[10] To a solution of sample (1 mg) in water (0.7 ml) $NaHCO_3$ (4.5 μmoles) and N-3-hydroxydecanoyloxysuccinimide[11] (50 μmoles) in dioxane (0.7 ml) were added. The mixture was stirred for 24 hr at room temperature, diluted with water (1 ml), acidified with 1N HCl and extracted with AcOEt (10 ml). The organic solvent layer was washed with water, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was rinsed twice with hot *n*-hexane.

N-Acylpeptide methyl ester was prepared by treating N-acylpeptide with an excess of a solution of CH_2N_2 in ether (2 ml) or with 0.1N methanolic HCl at 20° for 12 hr.[9]

Permethylation was carried out by the method of Hakomori, as adapted by Thomas.[12]

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[6] T. Kimura and S. Sakakibara, Abstracts of 23rd Annual Meeting of the Japan Chemical Society, Tokyo, Apr. 1970, III, p. 1851; *idem*, Abstracts of 8th Symposium on Peptide Chemistry, Osaka, Nov. 1970, p. 151.

[7] S. Sakakibara, M. Naruse and T. Kimura, Abstracts of 8th Symposium on Peptide Chemistry, Osaka, Nov. 1970, p. 160.

[8] M.M. Shemyakin, Yu. A. Ovchinnikov, I.I. Vinogradova, M. Yu. Feigina, A.A. Kiryushkin, N.A. Aldanova, Yu. B. Alakhov, M.M. Lipkin, and B.V. Rosinov, *Experientia*, 23, 428 (1968).

[9] K.L. Agarwal, G.W. Kenner, and R.C. Sheppard, *J. Am. Chem. Soc.*, **91**, 3096 (1969).

[10] T. Uyehara, S. Nagai, K. Okada, and M. Hiramoto, Abstracts of 92nd Annual Meeting of the Japan Pharmaceutical Society, Osaka, Apr. 1972, III, p. 48.

[11] M. Hiramoto, K. Okada, S. Nagai, and H. Kawamoto, *Chem. Pharm. Bull.* (Tokyo), **19**, 1303 (1971).

[12] D.W. Thomas, *Biochem. Biophys. Res. Commun.*, 33, 483 (1968).

Mass spectra were determined using Nipon Denshi model JMS-O1SG mass spectrometer with the direct inlet system at probe temperatures ranging from 130° to 280°.

Result

1. Potentiator B

Potentiator B was acetylated and permethylated. The spectrum of the product yielded the sequence Pyr–Gly–Leu–Pro, but no structural information was obtained beyond the fourth residue from the amino end.

In order to overcome an unfavorable effect of the guanidine group of arginine residue,[13] potentiator B was hydrazinolyzed to the corresponding ornithine peptide, which was then acetylated and permethylated. The resulting derivative (I) underwent the normal fragmentation of amino acid type to give sequence determining peaks at *m/e* 98, 126, 197, 324, 421, 518, 702, 799, and 997 as shown in Fig. 1, which permitted to identify the sequence of the first eight amino acid residues of the peptide as Pyr–Gly–Leu–Pro–Pro–Arg–Pro–Lys. The presence of N-terminal pyroglutamic acid was clearly proved by the intense *m/e* 98 (N-methyl-pyrrolidone ion) and 126 (N-methyl-pyrrolidone carbonyl ion) peaks.[14]

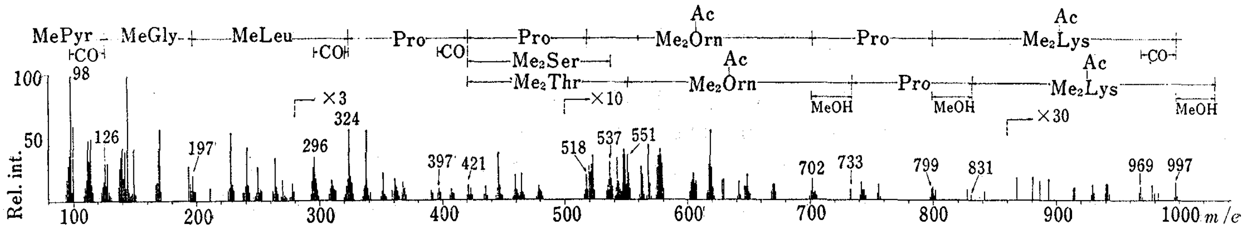

Fig. 1. Mass Spectrum of the Product (I) obtained after Consecutive Treatments of Potentiator B with N_2H_4, Ac_2O, and CH_3I

The digestion of potentiator B with collagenase resulted in the formation of two peptides, B-C-1 (ninhydrin reaction negative, Sakaguchi reaction positive) and B-C-3 (ninhydrin reaction positive, Sakaguchi reaction negative), which could be separated by electrophoresis. The former was successively treated with hydrazine, acetic anhydride and diazomethane to convert into the corresponding acetylated ornithine peptide methyl ester (II) and the latter was treated with N-3-hydroxydecanoyloxysuccinimide followed by esterification with diazo-

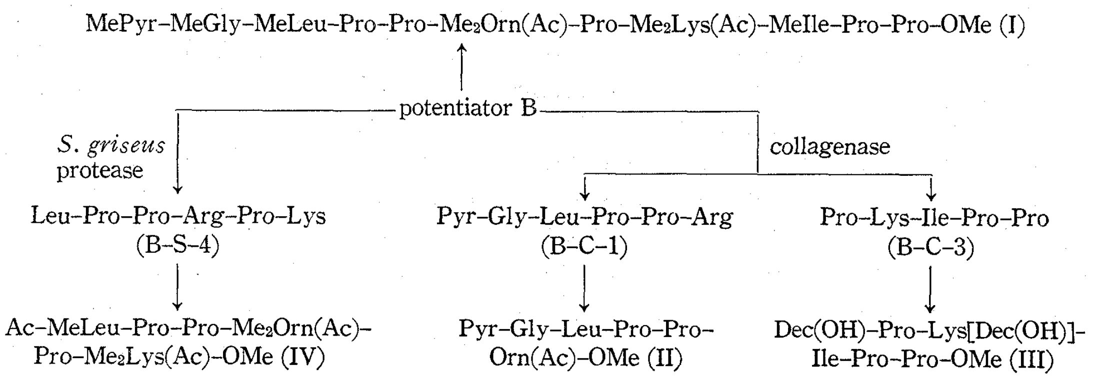

Chart 1. Derivatives of Potentiator B and Its Proteolytic Fragments Employed in Mass Spectrometric Analysis

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[13] D.W. Thomas, D.C. Das, S.D. Géro, and E. Lederer, *Biochem. Biophys. Res. Commun.*, **32**, 519 (1968); M.M. Shemyakin, Yu. A. Ovchinnikov, and A.A. Kiryushkin, "Mass Spectrometry–Techniques and Applications," ed. by G.W.A. Milne, Wiley-Interscience, New York, 1971, p. 289.

[14] *a*) G.H. de Haas, F. Franěk, B. Keil, D.W. Thomas, and E. Lederer, *FEBS Letters*, **4**, 25 (1969); *b*) W.R. Gray and U.E. del Valle, *Biochemistry*, **9**, 2134 (1970).

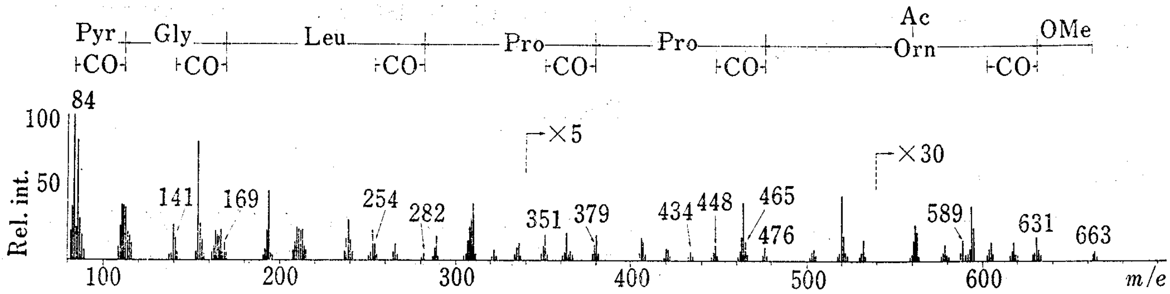

Fig. 2. Mass Spectrum of the Product (II) obtained after Consecutive Treatments of B-C-1 with N_2H_4, Ac_2O, and CH_2N_2

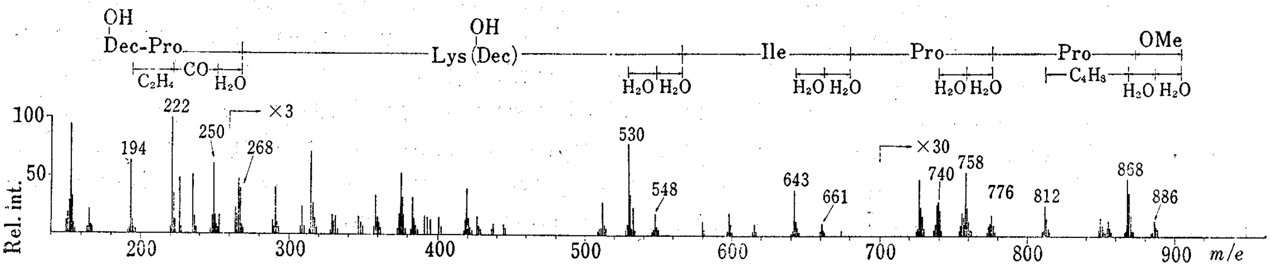

Fig. 3. Mass Spectrum of 3-Hydroxydecanoylated B-C-3 Methyl Ester (III)

methane to form bis(3-hydroxydecanoyl) pentapeptide methyl ester (III) (Chart 1). The mass spectra of the derivatives, II and III, (Fig. 2 and 3) showed a series of sequence determining peaks as well as the expected molecular ion peak at *m/e* 663[15] (in II) or (M^+-H_2O) peak at *m/e* 886 (in III). The results led to the expected structures of B-C-1 and B-C-3 to be Pyr–Gly–Leu–Pro–Pro–Arg and Pro–Lys–Ile–Pro–Pro which correspond to the N- and C-terminal sequences of the parent molecule, respectively. From these results and the amino acid composition[4a)] the complete sequence of potentiator B could be mass-spectrometrically identified as Pyr–Gly–Leu–Pro–Pro–Arg–Pro–Lys–Ile–Pro–Pro, compatible with that previously assigned by conventional methods.

When potentiator B was hydrolysed with a protease from *Streptomyces griseus*, 4 peptide fragments were isolated by high voltage paper electrophoresis of the hydrolysate.[4a)] One of them, B-S-4, was consecutively hydrazinolyzed, acetylated and permethylated to convert into the permethylated N-triacetyl ornithine peptide (IV), which was then subjected to mass spectrometry. As shown in Fig. 4, the spectrum contained the sequence peaks for the expected hexapeptide (Leu–Pro–Pro–Arg–Pro–Lys) at *m/e* 170, 267, 363, 548, 645, and 874 (M^+). In addition, two further serial peaks also appeared at *m/e* 382, 565, 663, 861 and 892, and 396, 548, 645, 677 and 906. The sequences of Leu–Pro–Ser–Arg–Pro–Lys and Leu–Pro–Thr–Arg–Pro–Lys, in which a proline residue at position 3 of B-S-4 is replaced by serine and threonine, respectively, seemed to account for these peaks. This might reveal the contamination of some minor components in potentiator B preparation[16] which have not been separated. Peaks attributable to the above minor components appeared also in the spectra of the other peptide fragments from potentiator B; for example, the spectrum of II (Fig. 2) showed the peak at *m/e* 465 ($C_{21}H_{31}O_7N_5$, Calcd., 465.222; Found, 465.221), which corresponds to the fragment ion [Pyr–Gly–Leu–Pro–Ser]$^+$ [17] accompanied by transfer of a

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15) The peak is accompanied by *m/e* 589 peak which is due to the lactam resulting from a ring closure of the C-terminal ornithine [D.W. Thomas, B.C. Das, S.D. Géro, and E. Lederer, *Biochem. Biophys. Res. Commun.*, 32, 519 (1969)].

16) Not more than 0.1 residue of serine and 0.2 residue of threonine were found in the hydrolysate with 6N HCl.

17) Peaks attributable to successive losses of H_2O and CO from this fragment appeared at *m/e* 448 and *m/e* 420, respectively. The peak at *m/e* 434 probably corresponds to a fragment ion [Pyr–Gly–Leu–Pro–Thr–H_2O–CO]$^+$ from the minor component containing threonine.

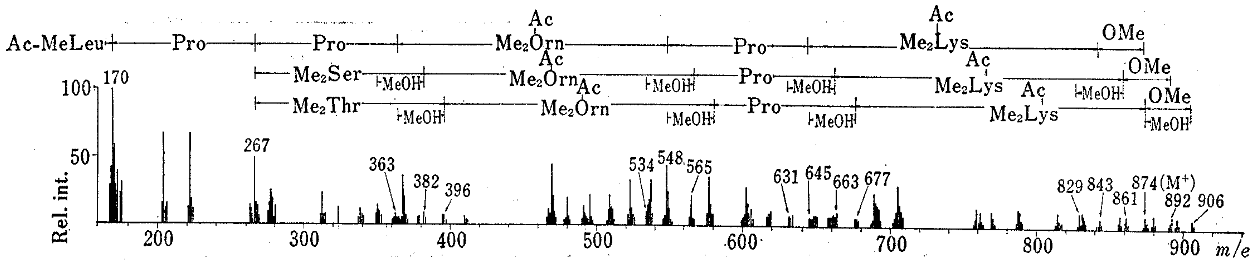

Fig. 4. Mass Spectrum of the Product (IV) obtained after Consecutive Treatments of B-S-4 with N_2H_4, Ac_2O, and CH_3I

hydrogen, and the spectrum of I (Fig. 1) showed a group of peaks at *m/e* 537 (or 518), 551, 733, and 831 which can be ascribed to the fragment ions from the above minor components,[18] as depicted in the figure. New unknown oligopeptides have recently been separated by Dowex 50×2 column chromatography of a preparation of potentiator B, the fact of which might reflect the above minor components. Efforts for the isolation and identification of them are now in progress.

2. Potentiator C

Potentiator C, which has no trifunctional amino acid, was permethylated directly. After extraction and washing, the sample solution was dried and the product was introduced directly into the mass spectrometer without further purification. It was sufficiently volatile to give a good spectrum, as shown in Fig. 5. It contained a set of intense peaks attributable to cleavage of peptide bonds, at *m/e* 197, 324, 421, 518, 589, 686, 783, and 910, which permitted to identify the order of nine residues from the N-terminus of the parent peptide as Pyr–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile. The most intense peak at *m/e* 98 was due to N-terminal pyroglutamic acid. Fragments with the remaining two proline residues which were indicated by the amino acid composition[4a] were not detected, however, the residues must be situated at the C-terminal position of the molecule. Thus the total structure of the derivative of potentiator C could be readily confirmed by means of mass spectrometry as follows: MePyr–MeGly–MeLeu–Pro–Pro–MeGly–Pro–Pro–MeIle–Pro–Pro–OMe.

Therefore, the structure of potentiator C, Pyr–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro, was confirmed mass-spectrometrically.

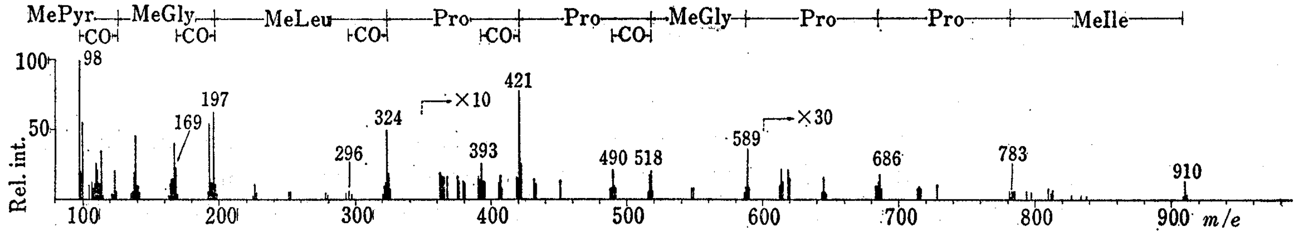

Fig. 5. Mass Spectrum of Permethylated Potentiator C

3. Potentiator E

Potentiator E seemed to be particularly unsuitable for sequencing by mass spectrometric method, since the amino acid analysis of the peptide[4b] showed that it contains trifunctional amino acid residues. These trifunctional groups cause generally some difficulties in volatility and thermal instability of the peptide. Nevertheless, the spectrum (Fig. 6) of the acetylated potentiator E dimethyl ester (V) yielded the sequence Pyr–Lys–Trp–Asp–Pro without any trouble; sequence peaks for the first five amino acids appeared at *m/e* 84, 281, 469, 570, and 694. The expected N-terminal pyroglutamic acid was obviously indicated

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[18] Peak due to the serine containing component was only partially observable (*i.e.* at *m/e* 537); this seems probably to be ascribed to the low volatility of the component.

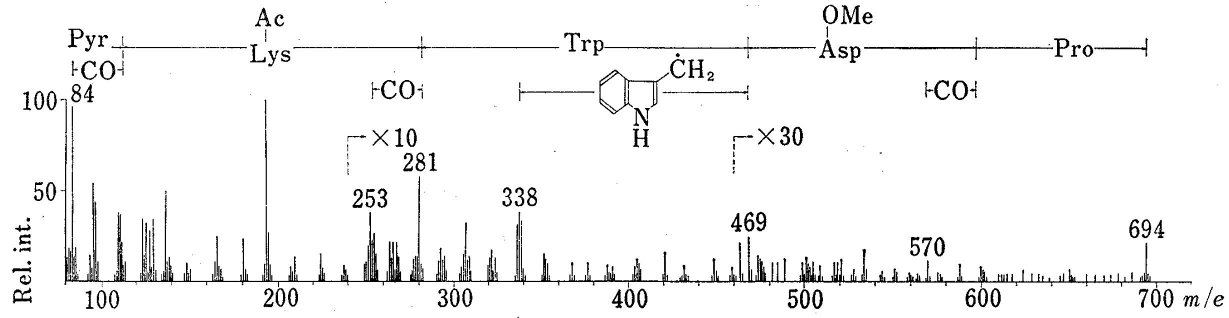


Fig. 6. Mass Spectrum of Acetylated Potentiator E Dimethyl Ester (V)

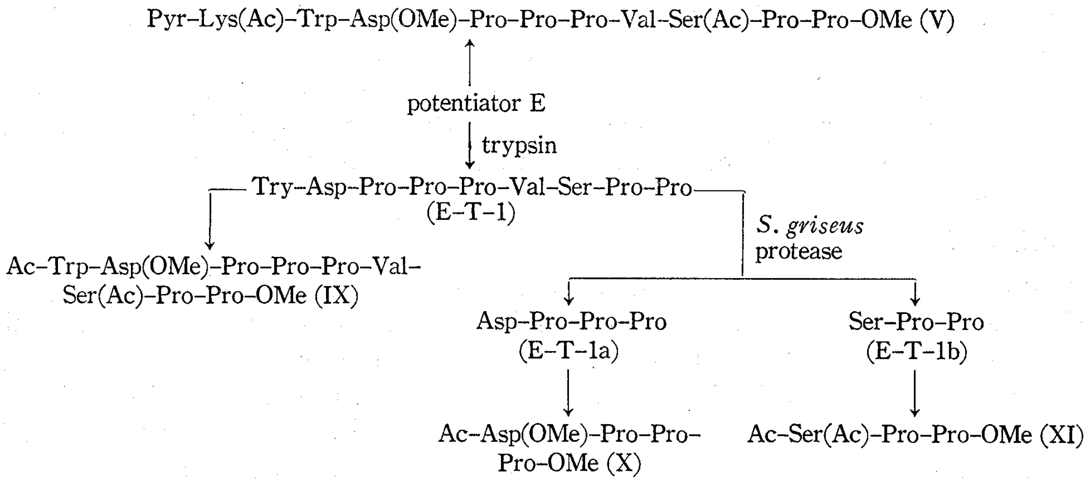
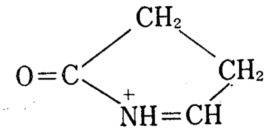
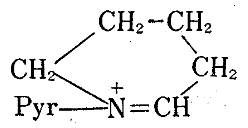


Chart 2. Derivatives of Potentiator E and its Proteolytic Fragments Employed in Mass Spectrometric Analysis

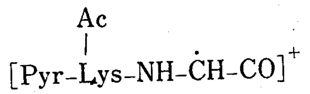
by an intense peak at m/e 84 due to the pyrrolidone fragment (VI).[14] Abundant ions at m/e 194 and 338 correspond to the ion (VII) arised by cyclization from the lysine residue of the [Pyr–Lys(Ac)]$^+$ fragment and the ion (VIII) formed by the loss of the tryptophan side chain from [Pyr–Lys(Ac)–Trp]$^+$, respectively.[19]



VI



VII



$$[\text{Pyr-}\overset{\text{Ac}}{\overset{|}{\text{Lys}}}\text{-NH-}\dot{\text{C}}\text{H-CO}]^+$$

VIII

To obtain further informations for the sequence of potentiator E, a nonapeptide (E-T-1) isolated from the tryptic digest of potentiator E was subjected to acetylation and followed by esterification with diazomethane. As shown in Fig. 7, the resulting acetylated E-T-1 dimethyl ester (IX) was cleaved to give all of the sequence ions for six amino acid residues from the N-terminus at m/e 201, 330, 421, 519, 551, and 617, except one of the ions at m/e 455 or 427. The peaks beyond the third amino acids have partly or completely suffered the loss of the side chain of the tryptophan residue.[13]

From this analysis, the sequence of the first six amino acids of E-T-1 was confirmed as Trp–Asp–Pro–Pro–Pro–Val. The digestion of E-T-1 with the *Streptomyces griseus* protease yielded two components, E-T-1a and E-T-1b, which could be separated by paper chromatography (n-BuOH: pyridine: AcOH: H_2O=15: 10: 3: 12, v/v). Each of the components was esterified and acetylated. The mass spectra of the respective products (X) and (XI)

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[19] M.M. Shemyakin, Yu. A. Ovchinnikov, A.A. Kiryushkin, E.I. Vinogradova, M.I. Miroshinikov, Yu. B. Alakhov, V.M. Lipkin, Yu. B. Shvetsuv, N.S. Selfson, B.V. Rosinov, V.N. Bachrarev, and V.M. Burikov, *Nature*, **211**, 361 (1966).

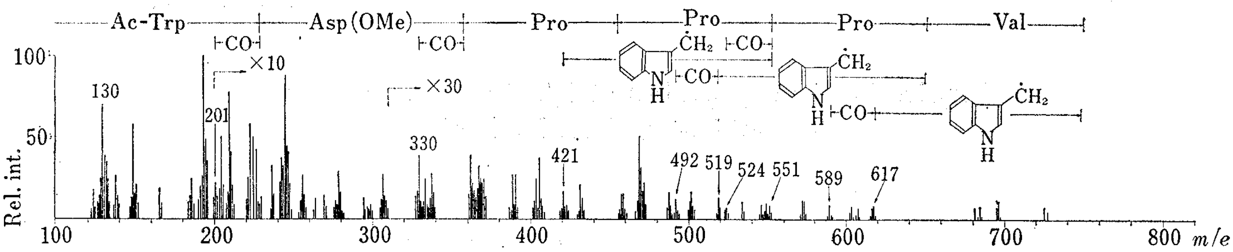

Fig. 7. Mass Spectrum of Acetylated E-T-1 Dimethyl Ester (IX)

were given in Fig. 8. Sequence peaks were obvious for X at *m/e* 172, 269, 366, 463, and 494 (M^+) and for XI at *m/e* 172, 269, 366, and 397 (M^+). Besides these, the spectrum of X also exhibited the characteristic loss of acetic acid from all fragments containing O-acetylated serine residue.[20] Thus the structures of E-T-1a and E-T-1b were established to be as follows: E-T-1a: Asp–Pro–Pro–Pro; E-T-1b: Ser–Pro–Pro.

Referring to the result of the amino acid composition, the sequence of E-T-1 was concluded as Trp–Asp–Pro–Pro–Pro–Val–Ser–Pro–Pro.

From the results obtained with the intact molecule and E-T-1, the structure of potentiator E could be determined as Pyr–Lys–Trp–Asp–Pro–Pro–Pro–Val–Ser–Pro–Pro, which was in full accord with that which has been established by conventional methods.

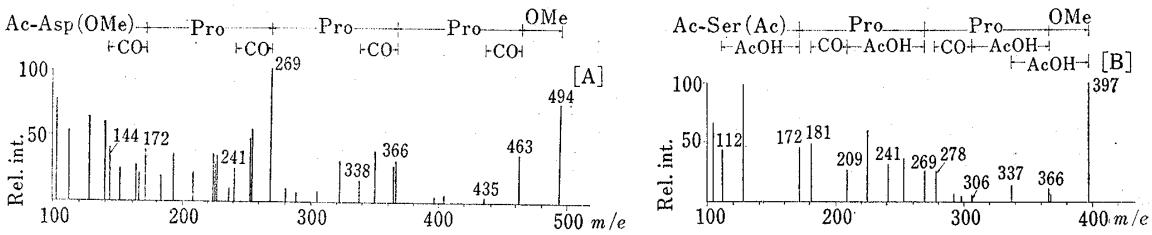

Fig. 8. Mass Spectra of [A] Acetylated E-T-1a Methyl Ester (X) and [B] Acetylated E-T-1b Methyl Ester (XI)

Discussion

The results obtained in this paper has presented the unambiguous evidence supporting the original assignments of the structures of potentiators B, C, and E, and an illustration of much usefulness of mass spectrometry in sequence analysis of oligopeptides with analogous structures. By mass spectrometry total amino acid sequences of the bradykinin-potentiating peptides examined were smoothly confirmed without the recourse to conventional stepwise techniques. As in the cases of potentiators B and C, it was possible to obtain the sequence information of the eight or nine amino acid residues from the N-terminus from one mass spectrum, which allowed assigning a complete or most part of sequence of these potentiators.

Moreover, the ability of mass spectrometry to distinguish the components of a mixture without their separation presents another advantage of this method, leading to the finding of the possible minor components. This was experienced in the sequence analysis of potentiator B. This experience may be analogous to the mass spectrometric findings of peptide contaminants in the N-terminal peptide from a λ chain of a pig immunoglobulin[14a,21] and in the tetrapeptide from the hydrolysate of silk fibroin of *Bombyx mori*.[22]

Acknowledgement Thanks are due to Miss T. Tsuji of our faculty for the technical assistance in mass spectrometric analyses.

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[20] E. Lederer, *Pure Appl. Chem.*, **17**, 489 (1968).

[21] F. Franěk, B. Keil, O.W. Thomas, and E. Lederer, *FEBS Letters*, **2**, 309 (1969).

[22] A.J. Geddes, G.N. Graham, and H.R. Morris, *Biochem. J.*, **114**, 695 (1969).