Fungal Metabolites. XVI.[1] Structures of New Peptaibols, Trichokindins I—VII, from the Fungus *Trichoderma harzianum*

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New peptaibols, trichokindins I—VII, have been isolated from the fungus *Trichoderma harzianum*. Their structures were characterized by spectrometric methods. Trichokindins, which are 18-residue peptides containing one to three isovaline residues, were found to induce Ca^{2+}-dependent catecholamine secretion from bovine adrenal medullary chromaffin cells.

Keywords *Trichoderma harzianum*; peptaibol; catecholamine; α-aminoisobutyric acid; isovaline; amino alcohol

Trichoderma species are soil fungi present throughout the world and are potentially useful as biocontrol agents because of their mycoparasitic activity. So far a number of peptides named peptaibols, such as alamethicins,[2] suzukacillins[3] and trichosporins,[4] have been isolated from this species, as well as hypelcins[5] from the stroma of the perfect stage, *Hypocrea*. Peptalbols are characterized by an acylated N-terminus, the presence of an amino alcohol at the C-terminus and a high content of α-aminoisobutyric acid (Aib). Peptaibols are well-known membrane modifiers, inducing voltage-gated ion channel formation,[6] membrane perturbation[7] and hemolysis,[8] and thus are considered to exhibit their biological activities, *i.e.*, uncoupling of oxidative phosphorylation,[9] by modifying membrane structures. Recently, it was reported by Garcia *et al.* and us that alamethicin and trichosporins induce release of catecholamines from adrenal chromaffin cells.[10] Physiologically, catecholamine secretion is caused by calcium ion influx and therefore the effects of these peptides on catecholamine release are presumably due to formation of artificial calcium ion channels. To explore secretory mechanisms, it would be useful to have available a series of peptaibols which are differ in length and conformation.

We have recently isolated from *Trichoderma harzianum* two new peptaibol groups named trichokindins (TK) and trichorozins (TZ), which consist of 18 and 11 residues, respectively. In this paper, we describe the isolation of these peptaibols, together with the structure determination of the isovaline (Iva)–rich TKs. The catacholamin-releasing activity of a main component, TK-VII, is discussed.

Experimental

General Procedure All melting points were measured on a Yanagimoto micro melting point apparatus without correction. All NMR experiments were carried out by using Bruker AC-300 and AM-600 spectrometers at room temperature. Samples were dissolved in 0.5 ml of CD_3OH containing tetramethylsilane (TMS) as an internal standard. Circular dichroism (CD) spectra were recorded on a JASCO J-720 spectropolarimeter. High-performance liquid chromatography (HPLC) was performed on Shimadzu LC-6A and LC-8A systems using YMC packed octadecyl silica (ODS) column (YMC Co., Ltd.), AM-313 (6 mm i.d. × 250 mm) for analytical HPLC and SH-345 (20 mm i.d. × 250 mm) for semi-preparative HPLC. Positive-ion fast atom bombardment mass spectrometry (FAB-MS) and positive-ion fast atom bombardment mass spectrometry/mass spectrometry (FAB-MS/MS) were carried out on a Finnigan MAT 70 triple-stage quadrupole mass spectrometer. Glycerol–thioglycerol (1 : 1) was used as a matrix. Samples were bombarded with 8 kV xenon atoms and then product ions were collided with argon atoms whose energy was 20—40 eV at a pressure of 0.8—1.2 mTorr.

Cultivation of *Trichoderma harzianum* *T. harzianum* isolated from soil collected at Nara (Japan) was grown in Petri dishes containing a 10-ml aliquot of medium prepared by dissolving 39 g of potato dextrose agar in 1000 ml of distilled water. The dishes were allowed to stand at 28°C for two weeks.

Isolation of TZs and TKs Conidia were collected from 3000 Petri dishes by washing with 3000 ml of MeOH, and allowed to stand at room temperature for several hours. After filtration and evaporation of the filtrate, the residue was partitioned between 1000 ml of EtOAc and 500 ml of H_2O. The organic phase was dried over Na_2SO_4 and evaporated to give 1.7 g of a brown residue. The residue was chromatographed on Sephadex LH-20 (Pharmacia, 20 mm i.d. × 1200 mm) and eluted with

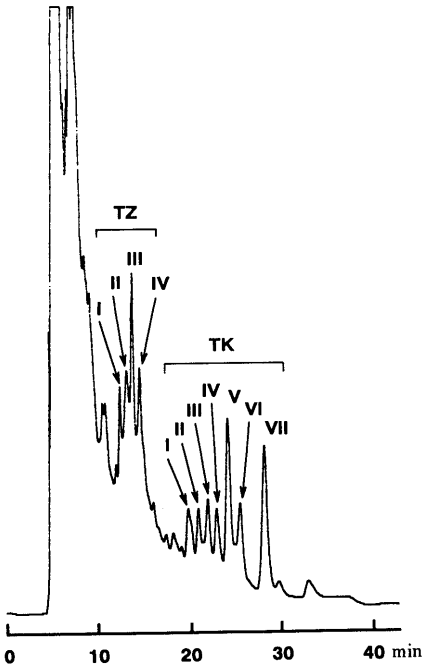

Fig. 1. HPLC Chromatogram of a Peptide mixture after Chrmatography on Sephadex LH-20 (TK: Trichokindin, TZ: Trichorozin)

HPLC conditions: mobile phase, MeOH–H_2O (85 : 15); flow rate, 1 ml/min; UV detection, 220 nm; column, YMC packed ODS AM-313 (6 mm i.d. × 250 mm); column temperature, 40 °C.

MeOH (0.1 ml/min). The peptide-containing fractions were collected, treated with activated charcoal and evaporated to afford 370 mg of a crude peptide mixture. The mixture was purified by semi-preparative HPLC to yield 6.8—23.8 mg of TZs and 7.0—20 mg of TKs (Fig. 1). The HPLC conditions were as follows: mobile phase, MeOH–H_2O (85 : 15); flow rate, 5 ml/min; UV detection, 220 nm; column temperature, 40 °C.

Determination of Amino Acid Compositions and Absolute Configurations of Amino Acids and Amino Alcohols For amino acid analyses, samples (0.5—1 mg) were hydrolyzed in 6 N HCl at 110 °C for 20—24 h. Each hydrolysate was analyzed on an automatic amino acid analyzer.

The absolute configurations of amino acids and leucinol (Lol) or isoleucinol (Iol) of each sample were established in the following manner. The acid hydrolysate was refluxed in absolute MeOH–thionyl chloride (10 : 1, 2 ml) for 3 h. After removal of the solvent and reagent, the residue was treated with a solution of 3, 5-dinitrobenzoyl chloride (*ca.* 1 mg) and triethylamine (one drop) in EtOAc (2 ml) and the mixture was stirred for 24 h. The resulting *N*-3,5-dinitrobenzoate methyl esters of amino acids and *N*-3,5-dinitrobenzoate of Lol or Iol were analyzed by HPLC with a column having an optically active stationary phase [conditions: mobile phase, *n*-hexane–1,2-dichloromethane–ethanol (94 : 5 : 1 for Pro, 91 : 7.5 : 1.5 for Leu, Ile and Iva, 88 : 10 : 2, for Glu and Ala, 92 : 4 : 4 for Lol, Iol and Ser); flow rate, 1 ml/min; UV detection, 254 nm; column, Sumipax OA-4100 (4 mm i.d. × 250 mm, Sumika Chemical Analysis Service Ltd.); column temperature, 35 °C]. Retention times were compared with those of the derivatives of standard amino acids and Lol or Iol.

Catecholamine Secretion Measurement of catecholamine-releasing activity was carried out in the same manner as described previously.[10b]

Results and Discussion

Characterization of TKs TKs, obtained as single peaks on HPLC chromatograms, exhibited typical IR absorptions for peptides (3300, 1660 and 1530 cm^{-1}). However, these compounds were negative to ninhydrin reagent and were not esterified with diazomethane. These facts indicate that TKs do not have free amino and carboxy groups in the molecules, and their N- and C-terminal amino acids are protected. In fact, the one- and two-dimensional ^1H-NMR spectra of the main component, TK-VII, showed the existence of an acetyl group and Iol. In addition, nine quaternary carbon signals arising from six Aib and three Iva residues were observed in its distortionless enhancement by polarization transfer (DEPT) spectra. Therefore, TKs were considered to belong to the class of peptaibols. Furthermore, the amino acid proportions of TKs were the same except for Aib and Iva residues, which cannot be determined by a routine automatic amino acid analyzer. This result suggested that the amino acid sequences of TKs are very similar, as is usually observed for peptaibols.

By analyzing the derivatives of acid hydrolysates with HPLC, the absolute configurations of normal amino acids and amino alcohols were determined as the *S*-form, whereas Iva was the *R*-form. In the course of this procedure, it was found that TK-II apparently contained both Iol and Lol in the molecule. This result suggested that TK-II is composed of at least two components. In addition, NMR and FAB-MS analyses indicated that TK-I, III and V are also mixtures of two components which differ only in the locations of Aib and Iva residues in the sequences (*vide infra*). Further purification was tried under various HPLC conditions, but without success. Thus, structure elucidation of TK-I, II, III and V was done with mixtures as was done in the case of minor components of trichosporin-Bs.[4c] The numbers of Aib and Iva residues in TK-IV, VI and and VII were determined from the DEPT spectra, whereas those of the other peptides were estimated from the mass spectra. The characteristics of these compounds are summarized in Table I.

Structures of TK-IV, VI and VII The FAB mass spectrum (Fig. 2a) of the main component, TK-VII, has a mass region where no fragment ions are observed above *m/z* 1200 and exhibits preferred cleavage of an Aib–Pro bond as depicted in Fig. 2b. Some sequence-specific ions from the N-terminal oligopeptide (*m/z* 1151) were observed, though the sequence was, in part, ambiguous. On the other hand, diagnostic ions for the C-terminal oligopeptide (*m/z* 626) were not recognized clearly above *m/z* 300. So, collision-induced decomposition (CID)[11] was used to obtain the complete sequence. For the C-terminal peptide, the *m/z* 626 ion was selected and collided to afford the *m/z* 211 ions, which is generated through successive losses of Iol, Gln and 2 Aib and was identified as Pro–Leu (Fig. 3). In addition, the CID spectrum of the *m/z* 211 ion gave the *m/z* 70 ion, which is produced by the loss of CO (28 amu) from the proline acylium ion (*m/z* 98, not shown).[4b] Therefore, the C-terminal amino acid sequence was determined as Pro–Leu–Aib–Aib–Gln–Iol. Similarly, two fragment ions, *m/z* 1151 and 470, were subjected to CID to afford the complete N-terminal sequence. The *m/z*

TABLE I. Properties of TKs I—VII

	TK-Ia, b	TK-IIa	TK-IIb	TK-IIIa, b	TK-IV	TK-Va, b	TK-VI	TK-VII
Molecular formula	$C_{81}H_{142}N_{20}O_{22}$	$C_{81}H_{142}N_{20}O_{22}$	$C_{82}H_{144}N_{20}O_{22}$	$C_{82}H_{144}N_{20}O_{22}$	$C_{82}H_{144}N_{20}O_{22}$	$C_{82}H_{144}N_{20}O_{22}$	$C_{83}H_{146}N_{20}O_{22}$	$C_{83}H_{146}N_{20}O_{22}$
MH^+ (observed)	1749	1749	1763	1763	1763	1763	1777	1777
mp (°C)	226—228	220—223		235—239	235—238	225—228	238—242	223—246
Molecular ellipticity [θ] (deg·cm²·mol⁻¹)								
207 nm	—	—		—	−2140000	—	−2224000	−2175000
221 nm	—	—		—	−1678000	—	−1704000	−1814000
Amino acid ratios								
Ser	1	1	1	1	1	1	1	1
Glu	2	2	2	2	2	2	2	2
Ala	2	2	2	2	2	2	2	2
Leu	2	2	2	2	2	2	2	2
Pro	1	1	1	1	1	1	1	1
Aib	8	8	7	7	7	7	6	6
Iva	1	1	2	2	2	2	3	3
Lol			1	1			1	
Iol	1	1			1	1		1

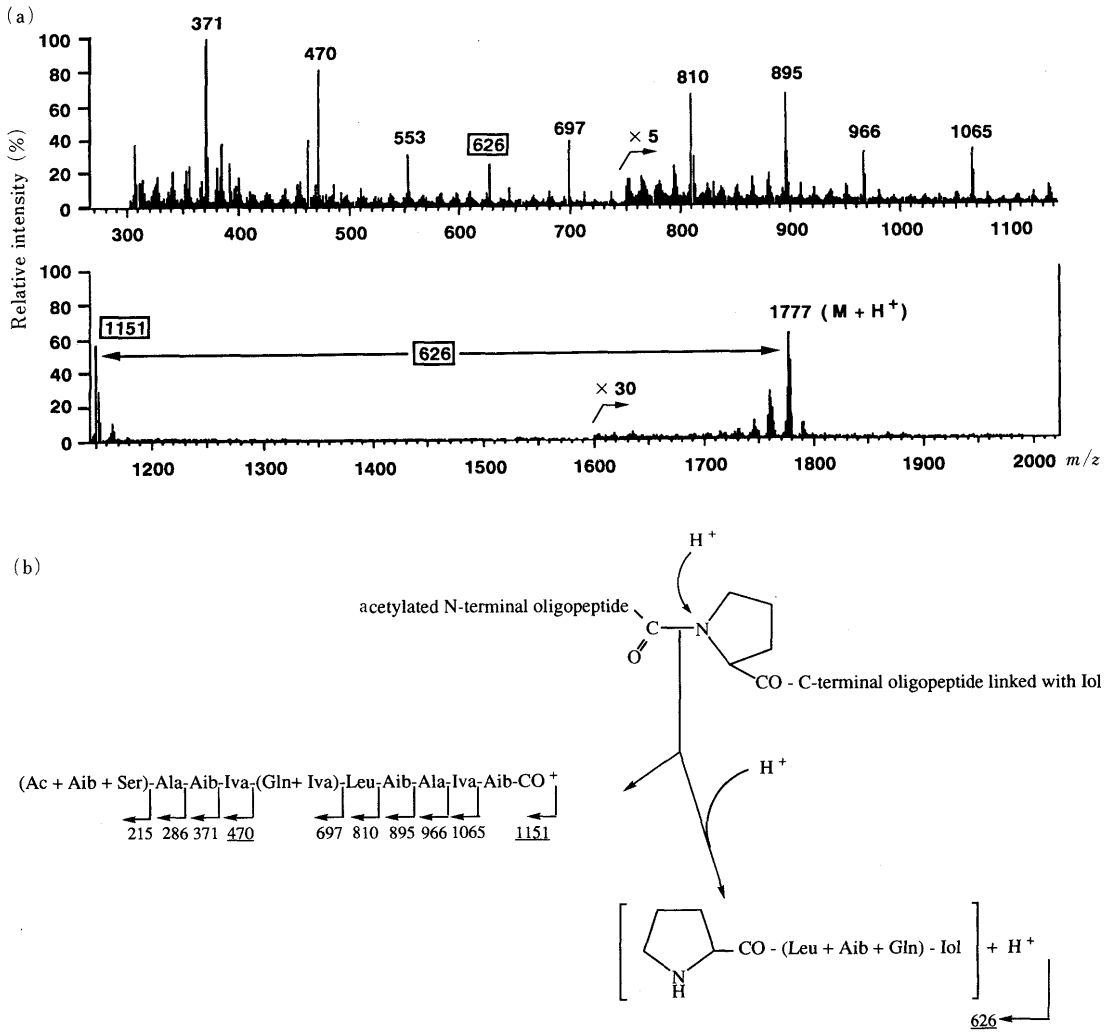

Fig. 2. Positive-Ion FAB Mass Spectrum of TK-VII (a) and Production of the C-Terminal Oligopeptide Ion (b)

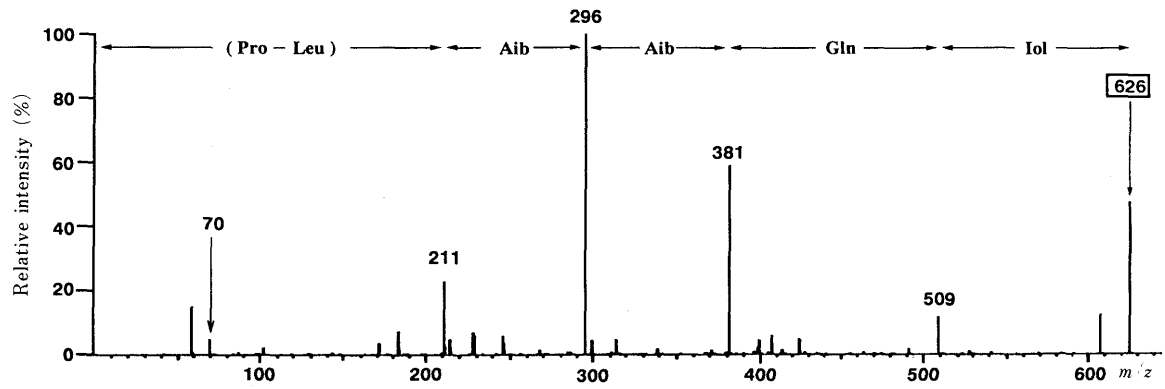

Fig. 3. Product Ions from the *m/z* 626 Ion by CID

The *m/z* 70 ion, which was of minor abundance, was clearly confirmed by CID of the *m/z* 211 ion.

1151 ion did not give product ions below *m/z* 371, but afforded the *m/z* 598 ion, which allows us to assign Gln and Iva to positions 6 and 7, respectively (not shown). On the other hand, the *m/z* 470 ion completed the N-terminal pentapeptide: Ac-Aib–Ser–Ala–Aib–Iva (Fig. 4). By connecting the N- and C-terminal oligopeptides, the whole primary structure was obtained as follows; Ac-Aib–Ser–Ala–Aib–Iva–Gln–Iva–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Iol. The characteristic feature of TK-VII as a peptaibol is the presence of more than two D-Iva residues in the molecule. The structures of TK-IV and VI were also determined as shown in Table II.

Structures of the Remaining Peptides The structures of TK-I—III and V were determined by detailed CID analyses, which indicated that each of them consists of two components. Figure 5a shows the FAB mass spec-

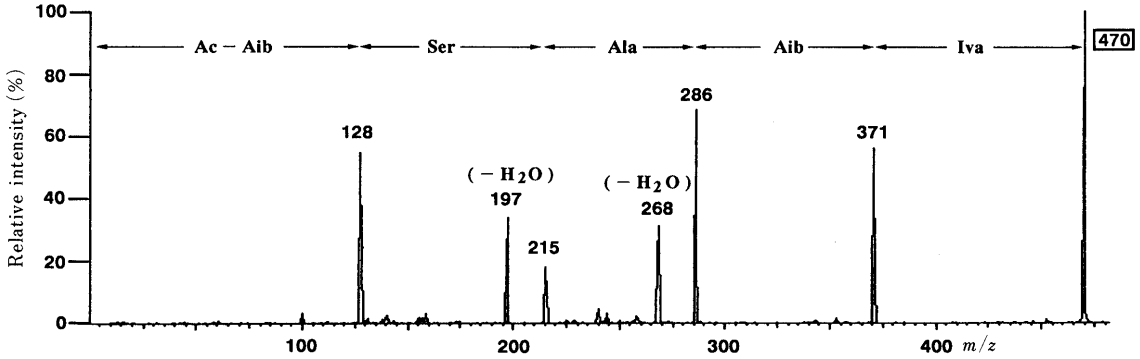

Fig. 4. Product Ions from the m/z 470 Ion by CID

TABLE II. Primary Structures of TKs I—VII

	Positions[a] 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
TK-Ia	Ac–Aib–Ser–Ala–Aib–Aib–Gln–Iva–Leu–Aib–Ala–Aib–Aib–Pro–Leu–Aib–Aib–Gln–Iol
TK-Ib	Ac–Aib–Ser–Ala–Aib–Iva–Gln–Aib–Leu–Aib–Ala–Aib–Aib–Pro–Leu–Aib–Aib–Gln–Iol
TK-IIa	Ac–Aib–Ser–Ala–Aib–Aib–Gln–Aib–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Iol
TK-IIb	Ac–Aib–Ser–Ala–Aib–Iva–Gln–Iva–Leu–Aib–Ala–Aib–Aib–Pro–Leu–Aib–Aib–Gln–Lol
TK-IIIa	Ac–Aib–Ser–Ala–Aib–Aib–Gln–Iva–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Lol
TK-IIIb	Ac–Aib–Ser–Ala–Aib–Iva–Gln–Aib–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Lol
TK-IV	Ac–Aib–Ser–Ala–Aib–Iva–Gln–Iva–Leu–Aib–Ala–Aib–Aib–Pro–Leu–Aib–Aib–Gln–Iol
TK-Va	Ac–Aib–Ser–Ala–Aib–Aib–Gln–Iva–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Iol
TK-Vb	Ac–Aib–Ser–Ala–Aib–Iva–Gln–Aib–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Iol
TK-VI	Ac–Aib–Ser–Ala–Aib–Iva–Gln–Iva–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Lol
TK-VII	Ac–Aib–Ser–Ala–Aib–Iva–Gln–Iva–Leu–Aib–Ala–Iva–Aib–Pro–Leu–Aib–Aib–Gln–Iol

a) The arrows denote the positions at which the heterogeneity of amino acids is observed.

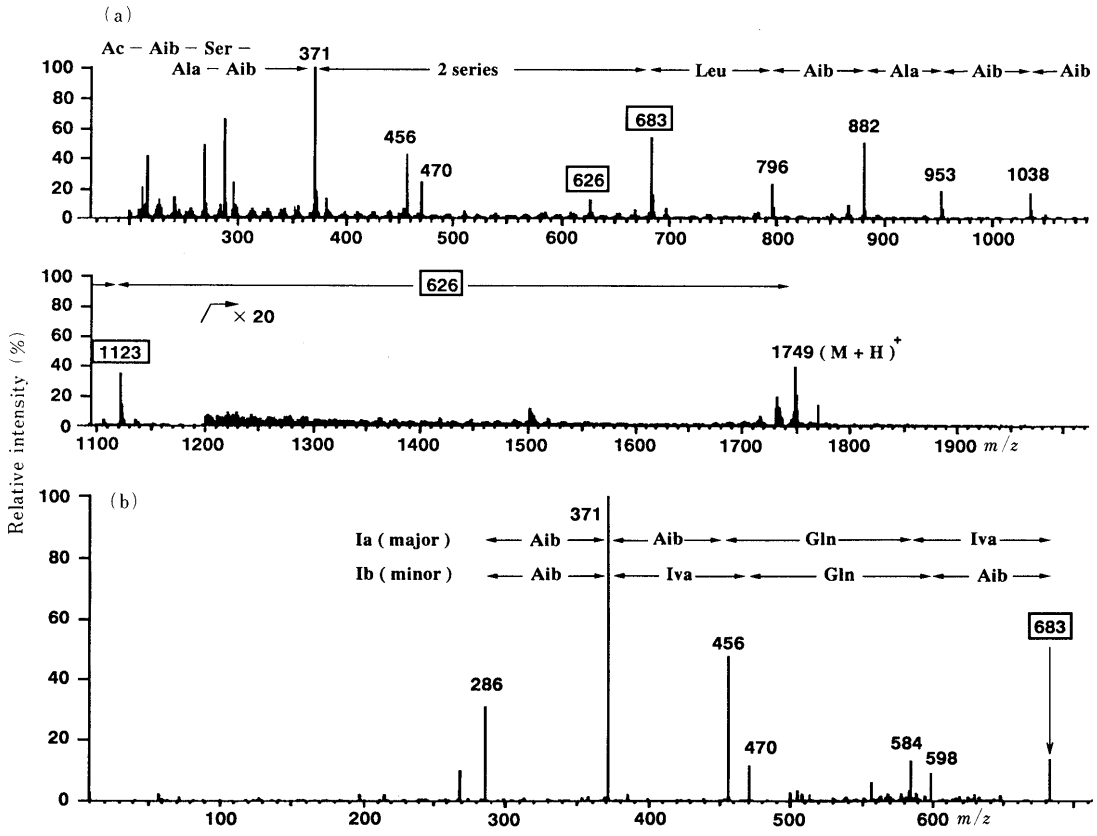

Fig. 5. Positive-Ion FAB Mass Spectrum of TK-I (a) and Product Ions from the m/z 683 Ion by CID

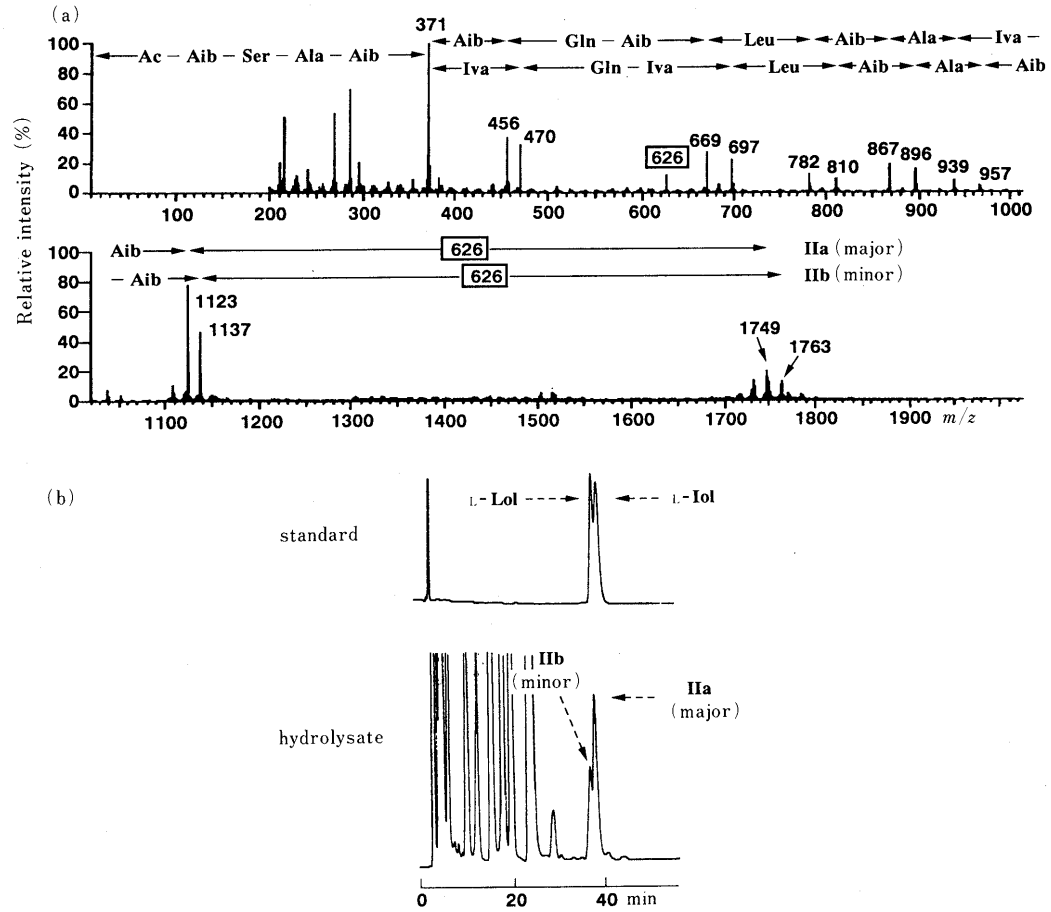

Fig. 6. Positive-Ion FAB Mass Spectrum of TK-II (a) and Separation of L-Lol and Iol Derivatives on HPLC

trum of TK-I; the presence of two ion series was deduced between *m/z* 683 and 371 based on the observation of two fragment ions at *m/z* 456 and 470. In fact, the CID spectrum of the N-terminal oligopeptide fragment ion, *m/z* 683, gave two acylium ions, *m/z* 598 and 584 which indicated the existence of two sequences, Gln–Iva and Gln–Aib (Fig. 5b). For convenience, the peptide which showed a greater ion abundance in the CID spectrum was designated as Ia and the other as Ib. The C-terminal oligopeptide sequence was characterized by CID of the *m/z* 626 ion, which afforded the same fragment ions as those of the peptides mentioned above. The structures of the constituent peptides of TK-III and V were also determined similarly (Table II).

Figure 6a shows the FAB mass spectrum of TK-II. Unlike TK-I, III and V, TK-II was found to be a mixture composed of two peptides whose molecular weights are different (*m/z* 1763 and 1749). Apparently the difference results from the N-terminal oligopeptide moieties (*m/z* 1137 and 1123), since the only C-terminal oligopeptide ion observed was *m/z* 626, as was in the case of TK-I, III and V. The CID method unambiguously gave the N-terminal sequences of IIa (major) and IIb (minor) as shown in Fig. 6a. On the other hand, CID of the *m/z* 626 ion afforded the same fragment ions as those of the other peptide, indicating that the C-terminal sequences of IIa and IIb differ from each other only in the amino alcohols. Figure 6b shows an HPLC chromatogram of the acid hydrolysate derivatives of TK-II, suggesting that Iol is linked with the major component (TK-IIa) based on a comparison of peak intensities of Iol and Lol.

As described above, these Iva–rich peptaibols, TKs, all consist of 18 amino acids, including one amino alcohol.

Catecholamine Secretion Trichosporin-Bs, which are 20-residue peptides and take a fully helical structure, release catecholamines from bovine adrenal chromaffin cells in a Ca^{2+}-dependent manner. In view of the pore-forming mechanism of alamethicin in lipid bilayers, trichosporin-Bs presumably form ion channels in the chromaffin cells by aggregation of several helical molecules. We examined TK-VII, since it was homogeneous and the most hydrophobic of TKs; hydrophobicity is considered to be important for interaction between membranes and peptaibols.[9d] When bovine adrenal chromaffin cells were incubated with 10 μM of TK-VII in the presence and absence of Ca^{2+}, 27% and 5% of the total catecholamines in the cells were secreted into the medium, respectively. The basal secretions were 3.4% and 2.7% in the presence and absence of Ca^{2+}, respectively. These results indicate that TK-VII also releases catecholamines from the cells in a Ca^{2+}-dependent manner without fusion of membranes, at least up to 10 μM. Therefore, it is suggested that TK-VII forms ion channels spanning the cell membranes, even though TK-VII is a shorter molecule than trichosporin-Bs.

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