Total Structures of Colistin Minor Components

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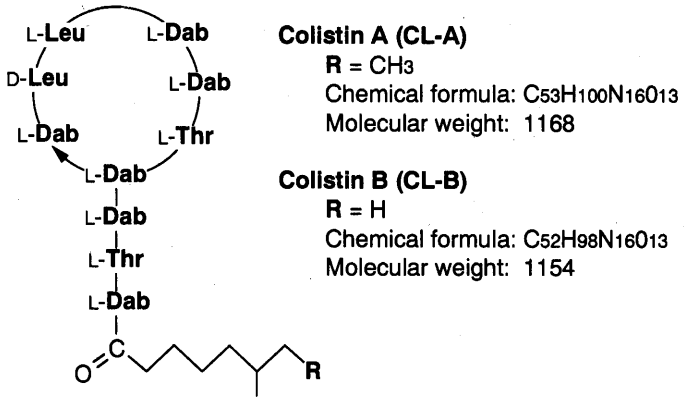
Structural characterization of the colistin (CL) components were carried out using Frit-fast atom bombardment liquid chromatography/mass spectrometry (Frit-FAB LC/MS), tandem mass spectrometry (MS/MS) and the amino acid analysis proposed by MARFEY, and the total structures of 4 minor components including the absolute configuration of the constituent amino acids were proposed. The structures of the minor components were the same as those of the main component colistin A or B except that L-leucine is replaced by L-valine or L-isoleucine.

Colistin (CL) is a peptide antibiotic produced by *Bacillus polimyxa* var. *colistinus* that exhibits antibacterial activity against Gram-negative organisms[1]. Colistins A (CL-A) and B (CL-B) are the main components of CL and their structures have been elucidated by SUZUKI *et al.*[2,3] as acylated linear-ring peptides as shown in Fig. 1. The two main components of CL have a common peptide moiety including L-2,4-diaminobutyric acid (Dab) which is a characteristic constituent amino acid of polymyxin group antibiotics, and they differ from each other only in the fatty acid moiety attached to the *N*-terminal amino acid. CL also includes some minor components. Although the amino acid composition and *N*-terminal fatty acid of the minor components have been reported[4~6], the total structures including the absolute configuration of the constituent amino acids have not yet been elucidated.

In previous studies[7~9], we characterized the structures of the bacitracin (BC) minor components using Frit-fast atom bombardment (FAB) liquid chromatography/mass spectrometry (LC/MS), tandem mass spectrometry (MS/MS) and the amino acid analysis proposed by MARFEY (MARFEY's method)[10], and the total structures of 13 minor components were successfully elucidated[9]. Since the structures of the BC components are similar to those of CL, it is considered that the total structures

Fig. 1. Structures of colistins A and B.

Dab; 2,4-diaminobutyric acid.



Colistin A (CL-A)
R = CH_3
Chemical formula: $C_{53}H_{100}N_{16}O_{13}$
Molecular weight: 1168

Colistin B (CL-B)
R = H
Chemical formula: $C_{52}H_{98}N_{16}O_{13}$
Molecular weight: 1154

of these minor components can be elucidated, if these characterization techniques are applied to CL. On the other hand, we have previously established the separation conditions for the CL components using HPLC and isolation conditions for CLs-A and -B using preparative high-speed countercurrent chromatography (HSCCC)[11]. These conditions are expected to be helpful to smoothly characterize the minor components of CL in this study.

This paper describes the structural characterization of the CL minor components using the techniques of Frit-FAB LC/MS, MS/MS and MARFEY's method.

Experimental

Materials

Acetone, acetonitrile, *n*-butanol, glycerol, hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), trifluoroacetic acid (TFA) were all analytical grade reagents. Colistin sulfate (19600 unit/mg) and 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA)[10] were purchased from Kyowa Hakko Kogyo (Tokyo, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Standard of amino acids were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Frit-FAB LC/MS

The HPLC separation of CL components was performed on a Chromatorex Phenyl column (Fuji Silysia, Ltd., Kasugai, Japan) (5 μm, 250 × 4.0 mm i.d.) using a LC-100P HPLC pump (Yokogawa Analytical Systems, Tokyo, Japan). An acetonitrile - 0.01 M TFA aqueous solution (1 : 3) was used as the mobile phase at the flow rate of 0.5 ml/minute.

The mass spectrometer and the data system used were a JMS-AX505W (JEOL, Tokyo, Japan) and a JMA-DA5000 (JEOL), respectively. The temperature of the ion source was kept at 60°C and a neutral xenon beam was used as the primary beam for the ionization of the sample by FAB. The acceleration voltages of the primary and secondary beams were adjusted to 5 and 3 kV, respectively. The LC/MS data were obtained by scanning from m/z 100 to m/z 1200 at the cycle time of 5.5 sec. The total ion current chromatogaph (TIC) range was set to m/z 300 ~ 1200.

The HPLC and the mass spectrometer were interfaced by a laboratory made flow splitter, connection tubing (fused silica 100 cm × 0.06 mm i.d.) and Frit-FAB probe (JEOL). The effluent from the HPLC was split at the ratio of 4 : 500 and a smaller portion of the effluent was introduced into the mass spectrometer through the connection tubing at a flow rate of 4 μl/minute.

Isolation of Minor Components

According to our previous report[11], 20 mg of commercial CL complex was separated by preparative HSCCC. After removing the corresponding portion of CLs-A and -B (tube No. 86 ~ 107 and 121 ~ 170), the fractionated effluent from the HSCCC was combined and evaporated to dryness to yield the mixture of enriched minor components. The HSCCC separation was replicated and the resulting mixture was separated by preparative HPLC to yield the isolated minor components. The separation was performed on a Chromatorex Phenyl HPLC column (5 μm, 250 × 20.0 mm i.d.) using an acetonitrile - 0.01 M TFA aqueous solution (1 : 3) as the mobile phase at a flow rate of 10.0 ml/minute. The sample of enriched minor components was dissolved in 1 ml of water and a 200 μl aliquot of the solution was injected into the HPLC. The eluent was monitored at the wavelength of 210 nm, and the eluent of the minor components (retention time 8.3 ~ 8.7 minutes for component No. 1, 9.6 ~ 10.3 minutes for No. 2, 11.4 ~ 12.4 minutes for No. 4 and 13.0 ~ 14.2 for No. 5) were collected and lyophilized after removal of the acetonitrile by a rotary evaporator. The yield of each minor component was 0.5 ~ 1.0 mg.

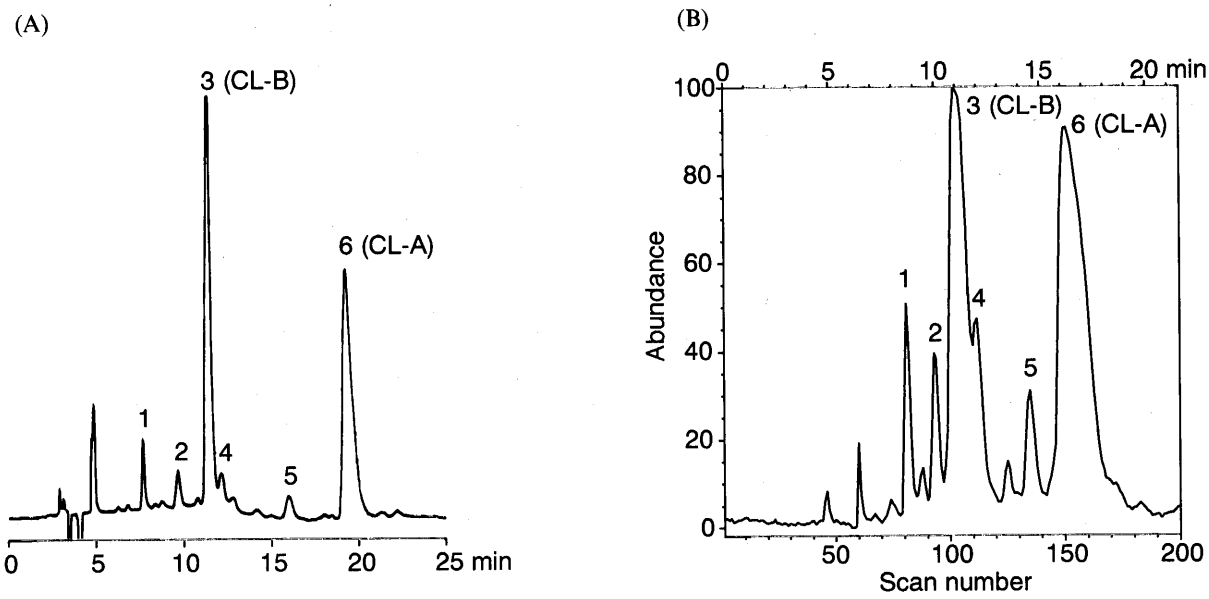
MS/MS Analysis

MS/MS was carried out using a JMS-HX/HX 110A tandem mass spectrometer (JEOL). The sample was ionized under FAB conditions and the resulting protonated molecule $[M+H]^+$ was selected as the precursor ion at the first mass spectrometer. The precursor ion was decomposed at the collision cell under collision induced decomposition (CID) conditions and the resulting product ions were separated and detected at the second mass spectrometer. The product ion spectrum was obtained by processing the mass data using a JMA-DA7000 data system (JEOL). The fast atom beam was operated at 6 kV using a xenon gas and the first mass spectrometer was operated at a 10 kV accelerating potential. The floating cell was used at a 5 kV voltage. A mixture of glycerol and 1 N HCl (1 : 1) was used as the matrix for the ionization of samples under FAB conditions, and helium was used as the collision gas.

Determination of Absolute Configuration of Constituent Amino Acid

In a sealed vial, 0.2 mg of sample was hydrolyzed at 110°C for 24 hours with 100 μl of distilled 6 N HCl

Fig. 2. Separations of commercial colistin complex.



(A) HPLC profile. Column, Chromatorex Ph (5 μm, 250 × 4.6 mm i.d.); mobile phase, acetonitrile - 0.01 M TFA aqueous solution (24 : 76); flow rate, 1.0 ml/minute; detection; 210 nm; sample size, 5 μg. (B) Total ion current chromatogram of Frit-FAB LC/MS. Column, Chromatorex Ph (5 μm, 250 × 4.0 mm i.d.); mobile phase, acetonitrile - 0.01 M TFA aqueous solution. (1 : 3) containing 1% glycerel; flow rate, 0.5 ml/minute; primary beam, Xe0; acceleration voltage, 5 kV (primary) and 3 kV (secondary); scan range, m/z 100 ~ 1200; scan cycle, 5.5 sec; ion source, Frit-FAB, ion source temperature, 60°C; splitter, flow type, laboratory made; split ratio, 4 : 500; TIC range, m/z 300 ~ 1200; sample size, 50 μg.

aqueous solution. The resulting amino acid solution was evaporated to dryness and the residue was dissolved in 50 μl of water. The amino acid solution was transferred into a 2-ml reaction vial, and 20 μl of 1 M $NaHCO_3$ aqueous solution and 1% FDAA acetone solution were added to the vial. The vial was capped and incubated at 37°C for 60 minutes in a water bath. After the addition of 20 μl of 1 N HCl aqueous solution into the vial to stop the reaction, the reaction mixture was evaporated to dryness. The residue, derivatized amino acids, was dissolved in 1 ml of methanol and then a 2 μl aliquot of the solution was injected into the HPLC system. The separation was performed on a Inertsil C8 HPLC column (5 μm, 150 × 4.6 mm i.d.) (GL-Science, Tokyo, Japan) using acetonitrile 0.01 M TFA aqueous solution as the mobile phase in the gradient elution mode (acetonitrile, 20% - 40%, 30 minutes followed by a hold at 40% for 10 minutes). The flow rate of the mobile phase was 1.0 ml/minute and the monitoring wavelength was set at 340 nm.

Results and Discussion

Frit-FAB LC/MS Analysis of Commercial CL

In a previous study[11], we have established HPLC conditions for CL components using a phenyl-type HPLC column and an acetonitrile-TFA aqueous solution as the mobile phase. Because these conditions can separate 4 minor components in a commercial CL complex as shown in Fig. 2 (A) without any non-volatile solute in the mobile phase, we adapted the conditions to Frit-FAB LC/MS. As a matrix for the FAB ionization, glycerol was added to the mobile phase at the concentration of 1%. Fig. 2 (B) shows the total ion current chromatogram (TIC) of a commercial CL complex. Although the resolution between peaks No. 3 and 4 was insufficient on the TIC, satisfactory mass spectra containing protonated molecules ($[M+H]^+$ and $[M+2H]^{2+}$) and some fragment ions were obtained from peaks No. 1, 2, 3, 4, 5 and 6. The molecular weights of the 4 minor components determined from their protonated molecules are listed in Table 1. The mass spectra of CL-B (peak No. 3) and the minor component of peak No. 1 are shown in Figs. 3 (A) and (B), respectively.

As shown in Fig. 3, the mass spectrum patterns were

Fig. 3. Background subtracted FAB mass spectra of CL components by Frit-FAB LC/MS and their diagnostic fragmentation pattern.

(A) CL-B and (B) peak No. 1.

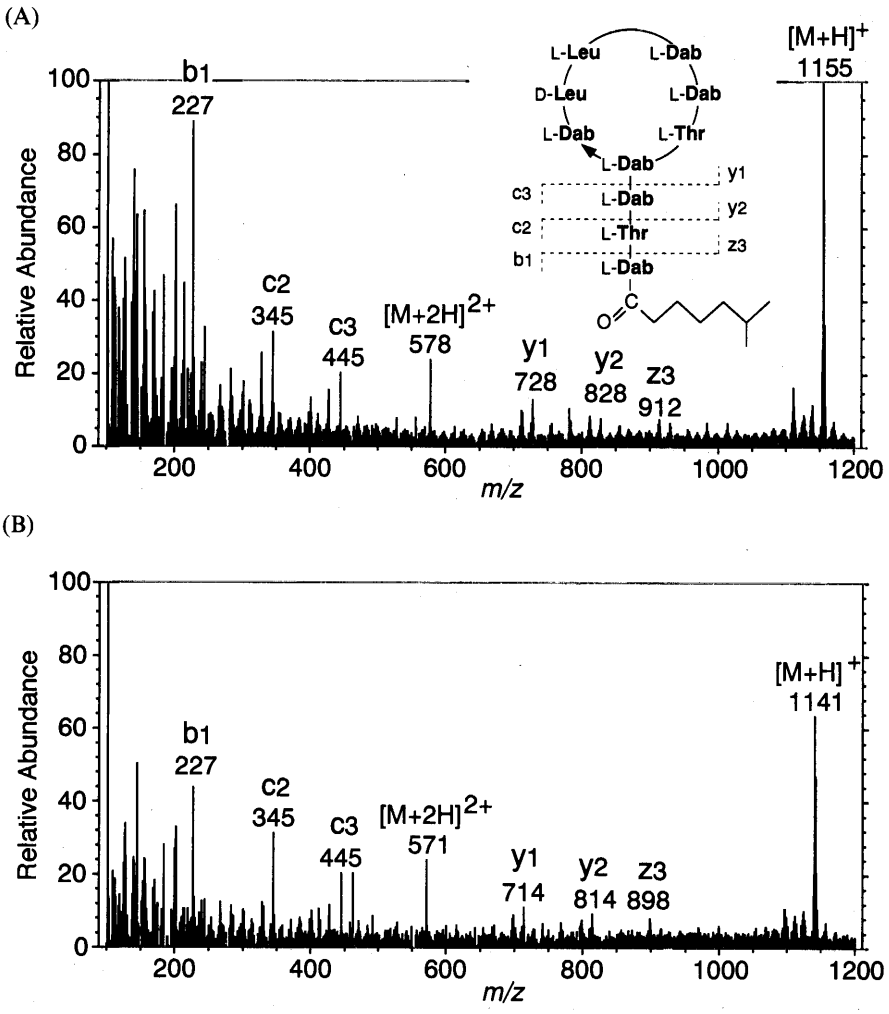


Table 1. Molecular weights and fragment ions of CL components observed in Frit-FAB LC/MS and product ion spectra.

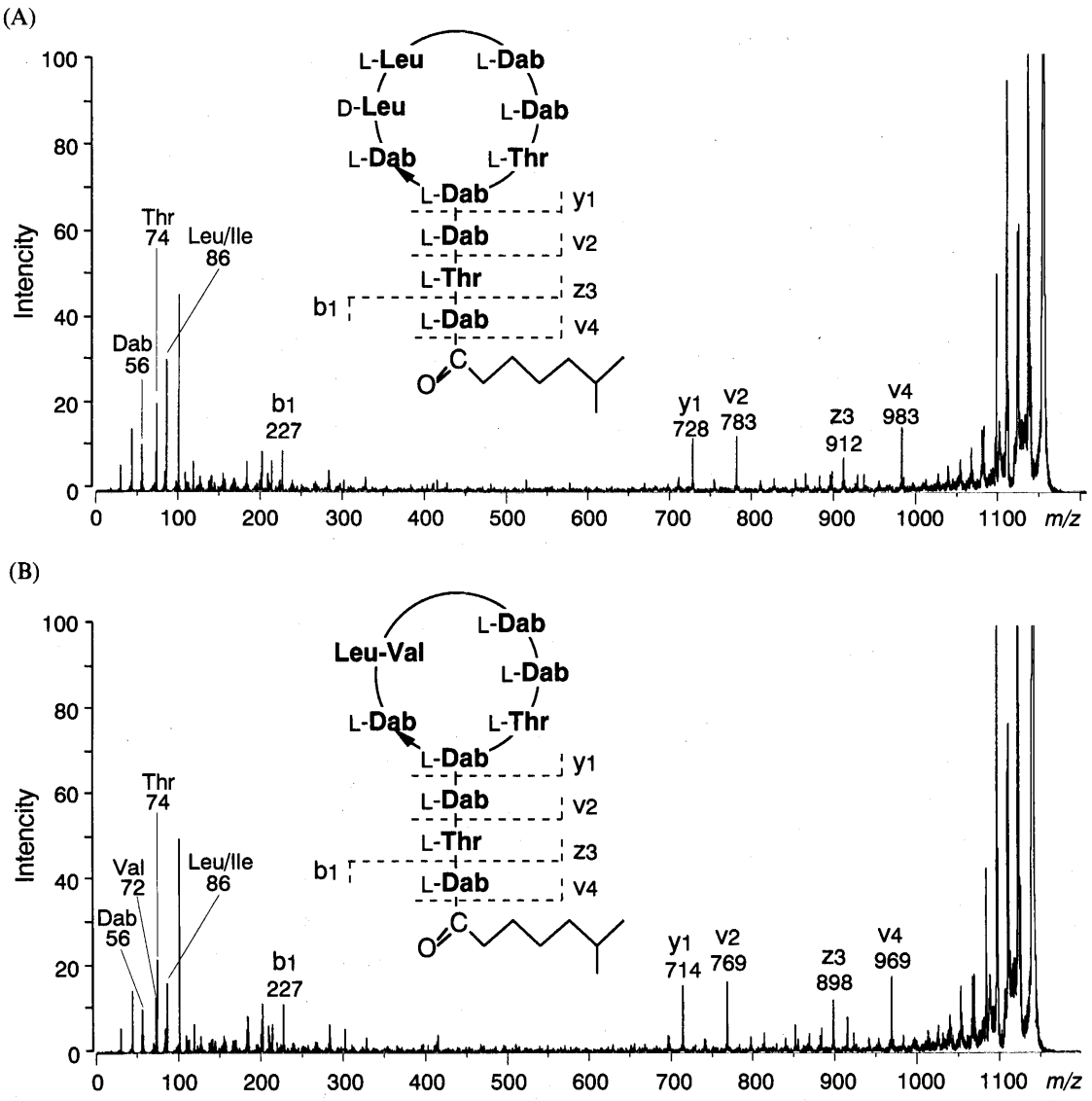
Peak No.	M.W.	*N*-Terminal ions (*m/z*)			*C*-Terminal ions (*m/z*)					Immonium ions (*m/z*)			
		b1	**c2**	**c3**	**y1**	**y2**	v2	**z3**	v4	Dab	Val	Thr	Leu/Ile
1	1140	227	345	445	714	814	769	898	969	56	72	74	86
2	1154	227	345	445	728	828	783	912	983	56	–	74	86
3 (CL-B)	1154	227	345	445	728	828	783	912	983	56	–	74	86
4	1154	241	359	459	714	814	769	898	969	56	72	74	86
5	1168	241	359	459	728	828	783	912	983	56	–	74	86
6 (CL-A)	1168	241	359	459	728	828	783	912	983	56	–	74	86

Bold typed ions, observed in Frit-FAB LC/MS spectra; ions with under line, observed in product ion spectra.

very similar among the 6 components of CL. From the similarity in the mass spectra and the molecular weights of each component, it is considered that the main and minor components of CL are homologous compounds and they differ only from one another by one or two constituent amino acids and/or *N*-terminal fatty acids. Further, we investigated the diagnostic fragment ions in each mass spectrum to obtain information concerning the amino acid sequence and *N*-terminal fatty acid. The fragment ions of the CL components were assigned according to the nomenclature proposed by BIEMANN[12] and the resulting sequence ions are listed in Table 1. Although the CL components did not yield the distinct diagnostic ion of the ring peptide moieties under the Frit-FAB LC/MS conditions, they were cleaved at the side chain peptide moieties to yield strong *N*-terminal (b1, c2 and c3) and weak *C*-terminal (y1, y2 and z3) series ions as shown in Fig. 3. Table 1 shows that the components of peaks No. 1, 2 and 3 (CL-B), and of peaks No. 4, 5 and 6 (CL-A) yield the same *N*-terminal series ions among each group, and the ions of the former group are 14 mass units lower than those of the latter group. On the other hand, the components of peaks No. 1 and 4 yield the same *C*-terminal series ions which are

Fig. 4. Product ion spectra of $[M+H]^+$ from isolated CL components and their fragmentation patterns.

(A) CL-B and (B) peak No. 1.



Mass spectrometer, JMS-HX/HX 110A; data system, JMA-DA7000; Ion source, FAB; primary beam, Xe^0; acceleration voltage, 6 kV (primary) and 10 kV (secondary); floating cell voltage, 5 kV; matrix, glycerol - 1 N HCl aq. solution (1 : 1); collision gas, helium.

Table 2. Absolute configuration of constituent amino acid in CL components.

Peak No.	Dab	Thr	Val	Ile	Leu
1	L	L	L	–	D
2	L	L	–	L	D
3 (CL-B)	L	L	–	–	D,L
4	L	L	L	–	D
5	L	L	–	L	D
6 (CL-A)	L	L	–	–	D,L

Fig. 5. General structures of CL components.

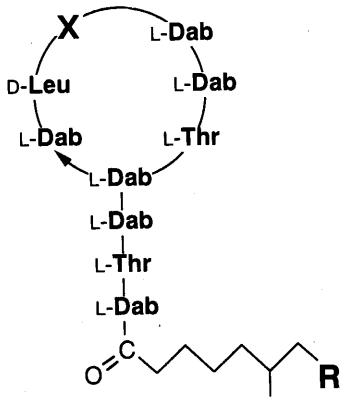


Table 3. Proposed structures of CL components.

Peak No.	M.W.	X[a]	R[b]
1	1140	L-Val	H
2	1154	L-Ile	H
3 (CL-B)	1154	L-Leu	H
4	1154	L-Val	CH_3
5	1168	L-Ile	CH_3
6 (CL-A)	1168	L-Leu	CH_3

a, b; See Fig. 5

14 mass units lower than those of the other 4 components. These *N*- and *C*-terminal series ions and the molecular weights in Table 1 suggest that the structures of peaks No. 1 and 4 are the same as those of CLs-B and -A, respectively, but one amino acid in their ring peptide moieties are replaced by that less one methylene unit. However, no structural difference was found in the above LC/MS data between peak No. 2 and CL-B, and between peak No. 5 and CL-A.

MS/MS Analysis of Isolated Components

To examine the fragmentation of the minor components in detail, an MS/MS analysis of the isolated components was carried out. Figs. 4 (A) and (B) show the product ion spectra of CL-B and the minor component of peak No. 1 under CID conditions and the assignment of the product ions. Although the fragmentation pattern was somewhat different from that by LC/MS, some sequence ions, especially the *C*-terminal ions, were observed more distinctly in the spectra. In addition, immonium ions that originated from the constituent amino acids were clearly detected in the low *m/z* range of the spectra. The assignable prominent ions in the spectra are listed in Table 1 together with those by LC/MS. The *N*- and *C*-terminal ions supported the structures suggested by the above LC/MS experiment, but no additional information concerning the structures of the components was provided by these ions. However, a characteristic immonium ion of valine is found at *m/z* 72 in the spectra of peaks No. 1 and 4 as shown in Fig. 4 (B) and this ion indicates the presence of valine in the ring peptide moieties of peaks No. 1 and 4 instead of one leucine of CLs-A and -B. ELVERDAM *et al.*[6] reported that valine, norvaline and isoleucine were found in each of 3 minor components of CL and these minor components contained one leucine. This report suggests the possibility that one isoleucine exists in the ring peptide moieties of peaks No. 2 and 5 instead of leucine. CLs-A and -B include L- and D-leucines in their ring peptide moieties. However, it was difficult to determine which leucine is replaced by another amino acid in the minor components, because there are no sequence ions of the ring peptide moieties in the product ion spectra.

Amino Acid Analysis of Isolated Components

The absolute configuration of the constituent amino acids in each isolated component was determined to structurally characterize the minor components by MARFEY's method, because it is simple, rapid and requires only a few hundred micrograms of sample for the analysis. After hydrolysis of the isolated components, the resulting mixture of the constituent amino acids was derivatized with FDAA and was analyzed by HPLC. As shown in Table 2, L-Dab, L-threonine and D-leucine were found in all components, and L-valine or L-isoleucine was only found in each minor component instead of L-leucine in CLs-A and -B. These results suggest that the components of CL have the general structure shown in Fig. 5 and the components are different from each other in one constituent amino acid labeled "X" and/or *N*-terminal fatty acid labeled "R". The variations in "X" and "R" are summarized in Table 3. It is considered that the proposed structures are reasonable in consideration of the elution order of the components under the reversed phase HPLC conditions. The proposed structures of peaks No. 2 and 5 are identical to those of circulins B and A, respectively, which are the main components of a peptide antibiotic circulin and their structures have been proposed by SUZUKI *et al.*[13,14].

Conclusion

The total structures of 4 minor components of CL including the absolute configuration of the constituent amino acids were elucidated based on the LC/MS analysis results of a commercial CL complex, and MS/MS and amino acid analysis results of the isolated CL components. The structures of the minor components were the same as that of the main component CL-A or -B except that L-leucine is replaced by L-valine or L-isoleucine. The techniques used in this study were useful for the structural characterization of peptides, especially in the case that a sample contains D-amino acid and/or that the content of the target component in a sample mixture is low.

Acknowledgments

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