STRUCTURAL CHARACTERIZATION OF BACITRACIN COMPONENTS BY FRIT-FAST ATOM BOMBARDMENT (FAB) LIQUID CHROMATOGRAPHY/ MASS SPECTROMETRY (LC/MS)

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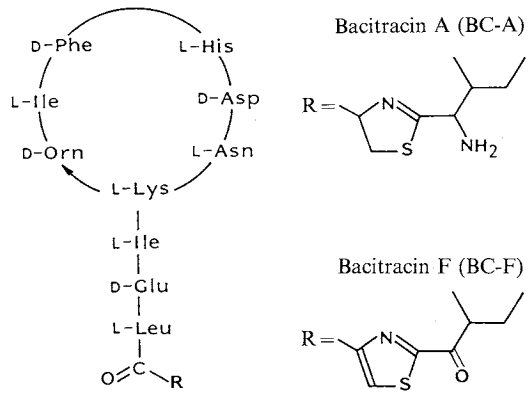
The structural characterization of minor components of bacitracin (BC) complex was carried out using a technique of liquid chromatography/mass spectrometry (LC/MS). Satisfactory total ion current chromatogram of BC complex and excellent mass spectra of many components were given by Frit-fast atom bombardment (FAB) LC/MS analytical system, and the structures of 13 minor components could be proposed. The 13 minor components were classified into two groups, bacitracin A (BC-A) related components and bacitracin F (BC-F) related components depending on their common *N*-terminal moieties. The structures of BC-A related components and BC-F related components were the same as those of BC-A and BC-F, respectively, except that one to three of isoleucine and leucine residues are replaced by valines. The BC-F related components were degradation products of BC-A related components through the same degradation process as that of BC-A.

Bacitracin (BC) is a complex of cyclic peptide antibiotics produced by *Bacillus subtilis* and *Bacillus licheniformis*, and exhibits an inhibitory activity against Gram-positive bacteria[1]. Commercial BC complex consists of many similar peptides which have been classified into bacitracin A (BC-A), BC-B, -C, -D, -E, -F and -G on the basis of separation by countercurrent distribution[2~6]. BC-A is a main component of BC complex and BC-F is a degradation product of BC-A. BC-A is different from BC-F in the *N*-terminal moiety composed of cysteine and isoleucine. The structures of these two components had been proposed as shown in Fig. 1[7,8], and the proposed structures had been confirmed by synthesis[9~17]. However, the structures of other minor components have never been published except the partial structure of BC-B[1]. Concerning the biological properties, it was reported only that BC-A, -B and -C have antimicrobial activity and BC-C and -F show nephrotoxicity[18,19].

In our previous reports, we separated commercial BC complex by TLC and HPLC, and demonstrated that more than twenty components are contained in the complex[20,21]. In another report, we isolated BC-A and -F from commercial BC complex by preparative high-speed countercurrent chromatography (HSCCC) and characterized the isolated components by liquid secondary ion mass spectrometry (LS/MS) and tandem mass spectrometry

Fig. 1. Structures of bacitracins A and F.



(MS/MS) under fast atom bombardment (FAB) conditions[22]. The structural information from the resulting liquid secondary ion mass and tandem mass spectra supported the proposed structures of BC-A and -F, suggesting the applicability of FAB-MS, LS/MS and MS/MS to the structural characterization of other minor components. While these techniques are not usable without the isolation of minor components from the sample complex, a combination of liquid chromatography and mass spectrometry enables us to obtain the mass spectrum of each individual component even if the sample is in a complex mixture. We have applied a Frit-FAB liquid chromatography/mass spectrometry (LC/MS) to the analysis of commercial BC complex and obtained a satisfactory total ion current chromatogram (TIC) and background subtracted FAB mass spectra of BC-A and -F in our previous study[23]. Because the mass spectra of BC-A and -F contained some structurally informative fragment ions, the LC/MS system would be available for the characterization of minor components without the isolation process. This paper describes the techniques for analysis of BC components using Frit-FAB LC/MS and the structural characterization of the minor components by the LC/MS.

Experimental

Materials

Acetonitrile, chloroform, ethanol (95%), glycerol, methanol and trifluoroacetic acid (TFA) were of analytical reagent grade. Bacitracin was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.).

Frit-FAB LC/MS Conditions

The separation of BC components was performed on Chromatorex Phenyl (Fuji-Davison Ltd., Kasugai, Japan) (5 μm, 250 × 4.0 mm i.d.) using a LC-100P HPLC pump (Yokogawa Electric, Tokyo, Japan). Acetonitrile-water containing 0.01 M TFA and 1% glycerol was used as a mobile phase under linear gradient elution mode (acetonitrile, 26~44%, 70 minutes) at the flow rate of 0.5 ml/minute. A LC-100U UV-detector (Yokogawa Electric) was used for measurement of HPLC profiles at the wavelength of 234 nm.

The mass spectrometer and the data system used were a JMS-AX505W (Jeol, Tokyo, Japan) and a JMA-DA5000 (Jeol), respectively. The temperature of ion source was kept at 60°C and neutral xenon beam was used as a primary beam for the ionization of sample by FAB. The acceleration voltages of the primary and secondary beams were adjusted to 3 and 5 kV, respectively. The LC/MS data were obtained by scanning from m/z 100 to m/z 1,500 at the cycle time of 6.5 seconds. The TIC range was set to m/z 300~1,500.

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 HPLC was split at the ratio of 4:500 and the smaller portion of the effluent was introduced into the mass spectrometer through the connection tubing at a flow rate of 4 μl/minute[23].

Preparation of Enriched Sample

According to our previous report[24,25], 5 g of commercial BC complex was separated by a cross-axis countercurrent chromatography (X-axis CCC) and the following fractions were obtained; Fr. 1 (300 mg), Fr. 2 (56 mg), Fr. 3 (110 mg), Fr. 4 (135 mg), Fr. 5 (2.146 g), Fr. 6 (1.320 g), Fr. 7 (191 mg) and Fr. 8 (144 mg). The sample for the LC/MS analysis of BC-A related components was prepared by mixing the Frs. 5, 6 and 7 at the ratio of 1:2:3, and that of BC-F related components was prepared by mixing the Frs. 1, 2 and 3 at the ratio of 1:4:6.

Results and Discussion

Separation of BC Complex

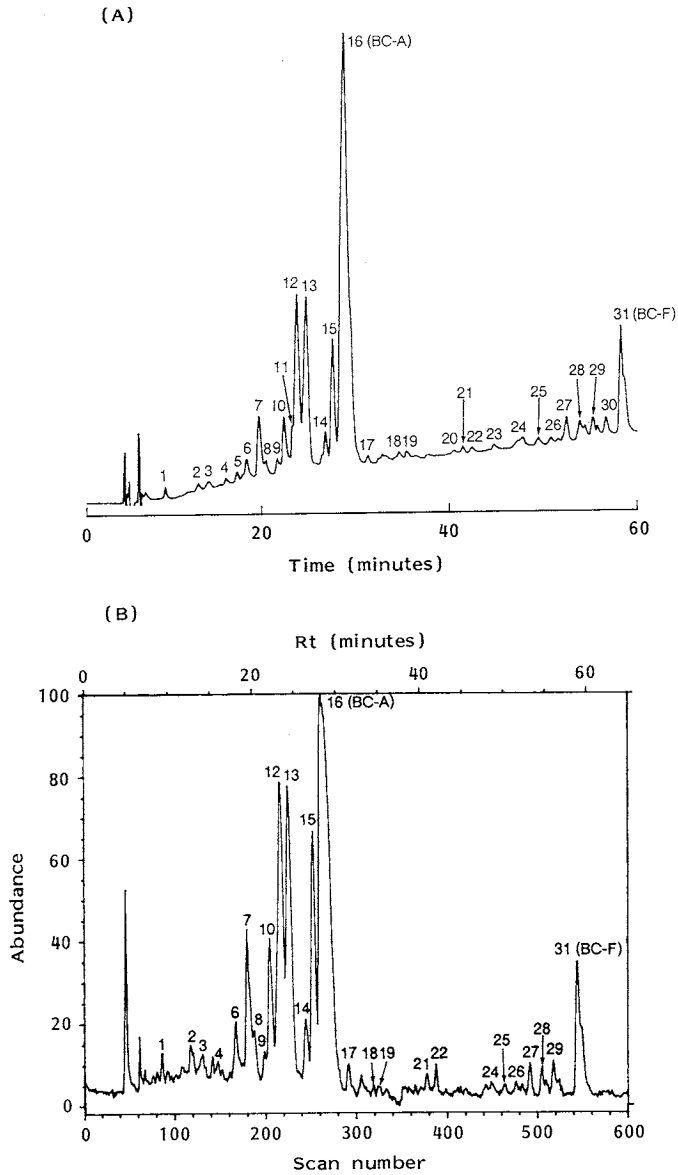
TUJI and ROBERTSON reported a HPLC condition using a gradient elution mode[26]. We also established

an optimum HPLC condition for the separation of BC components using isocratic elution mode[21]. However, these conditions were not suitable for the Frit-FAB LC/MS system because of involatile salts in the mobile phases. In order to operate Frit-FAB LC/MS smoothly, a suitable matrix is required to add

Fig. 2. Separations of BC complex.

(A) HPLC profile. Column, Chromatorex Phenyl (5 μm, 250 × 4.0 mm i.d.); mobile phase, acetonitrile-water, containing 0.01 M TFA and 1% glycerol; gradient rate, acetonitrile 26~44% (70 minutes), linear; flow rate, 0.5 ml/minute; detection wavelength, 234 nm; sample, commercial BC complex 50 μg.

(B) Total ion current chromatogram. Primary beam, Xe^0; primary acceleration, 5 kV; secondary acceleration, 3 kV; scan range, m/z 100~1,500; scan cycle, 6.5 seconds; ion source, Frit-FAB, ion source temperature, 60°C; splitter, flow type, laboratory made; split ratio, 4:500; TIC range, m/z 300~1,500; sample, commercial BC complex 100 μg.



into a mobile phase and no involatile salt is used in the mobile phase. So a mobile phase containing acetonitrile and 0.01 M TFA aqueous solution was used under the gradient elution mode in consideration of the polarities among the components. As a matrix for the ionization of sample under the FAB conditions, glycerol was used at the concentration of 1% of the mobile phase, because it gave the satisfactory FAB mass spectra of BC-A and -F in our previous study[22,23]. The HPLC separation of the components was scarcely affected by the addition of glycerol in the mobile phase. As a result of the optimization of elution conditions with C_{18}, C_8 and phenyl type HPLC columns, a satisfactory separation was obtained using a phenyl type HPLC column and a linear gradient elution of acetonitrile from 26% to 44% for 70 minutes at a flow rate of 0.5 ml/minute. The HPLC profile of BC components under the optimized conditions is shown in Fig. 2(A). Commercial BC complex (50 μg) was separated into more than 30 peaks with good resolution under the conditions. Successively, the HPLC system was connected with the mass spectrometer using Frit-FAB LC/MS interface, 100 μg of commercial BC complex was applied to LC/MS analysis and the resulting TIC is shown in Fig. 2(B). The components of BC were successfully separated on the TIC with almost the same resolution as that of the HPLC profile, except that the peak height of BC-A is relatively lower compared with those of other minor components, suggesting the saturation of ionization caused by overloading sample to the ion source.

Molecular Weights of BC Components

Fig. 3 shows the background subtracted FAB mass spectrum at the peak top of BC-A on the TIC and the fragmentation patterns of BC-A that was confirmed by MS/MS in our previous study[22]. The background subtraction is one of the advantageous techniques of LC/MS over the standard MS, so that good-quality mass spectrum could be obtained without peaks of matrix. Not only the protonated molecule but also doubly protonated molecule and some sequence ions were observed in the mass spectrum. In order to elucidate the molecular weights (MW) of other minor components, the mass spectra at each peak

Fig. 3. Background subtracted FAB mass spectrum of BC-A by Frit LC/MS and its diagnostic fragmentation pattern.

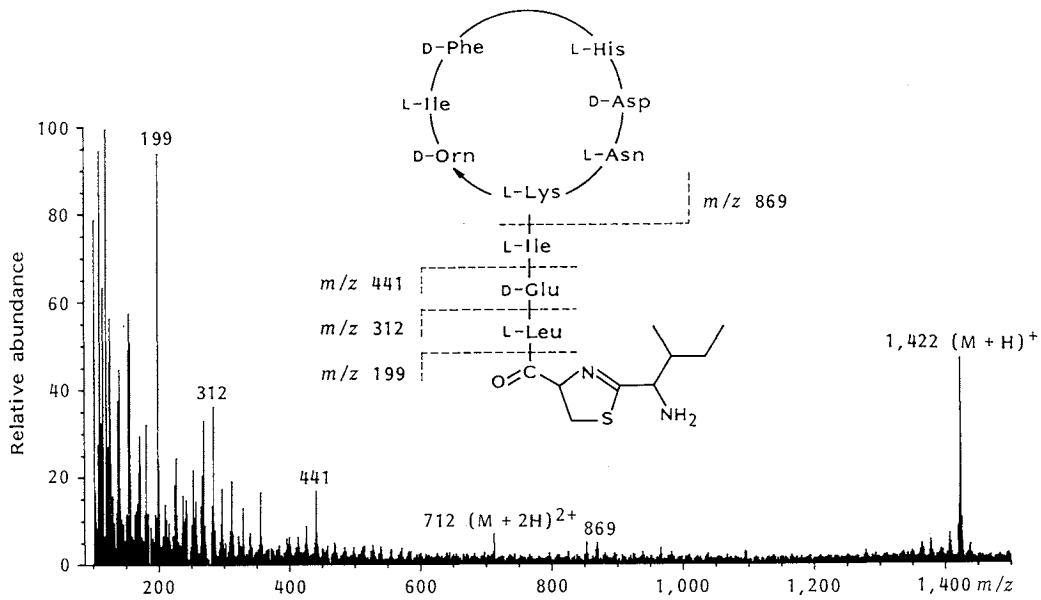


Fig. 4. Background subtracted FAB mass spectrum of peak No. 7 by Frit LC/MS.

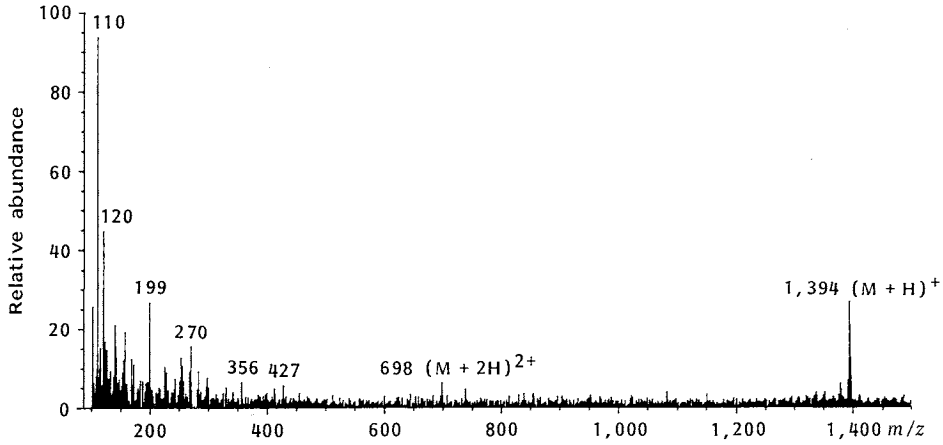
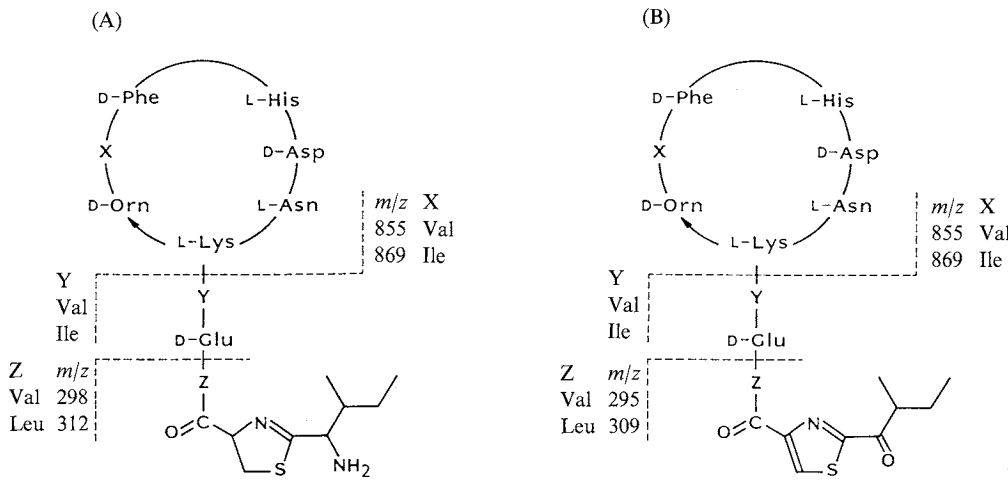


Fig. 5. General structures of BC components and their fragmentation patterns.

(A) BC-A related components. (B) BC-F related components.



top were examined in detail. A typical mass spectrum of a minor component (peak No. 7) is shown in Fig. 4. The singly and doubly protonated molecules were found at *m/z* 1,394 and 698, respectively, indicating that the MW is 1,393. Similarly, the MW of 13 minor components could be determined as listed in Table 1.

Table 1. Molecular weights of BC components.

Peak No.	MW	Peak No.	MW
6	1,379	24	
7		25	1,390
10	1,393	26	
11		27	
12		28	1,404
13	1,407	29	
15		31 (BC-F)	1,418
16 (BC-A)	1,421		

NEWTON and ABRAHAM reported that valine is contained in BC-B, -D and -E as a constituent amino acid[2]. Another paper described the structure of BC-B as very similar to that of BC-A, except that one of isoleucines is replaced by valine[1]. Table 1 shows that there are differences in the number of methylene unit among the components of No. 6~16,

suggesting that the differences are due to those of constituent amino acids, leucine/isoleucine and valine, on an assumption that not only one isoleucine but every isoleucine and leucine residues are replaceable by valines. Accordingly, the structures of these components are the same as that of BC-A except the following differences: Peaks No. 12, 13 and 15 contain one valine instead of leucine or isoleucine residues, peaks No. 7, 10 and 11 contain two valines, and peak No. 6 contains three valines instead of any isoleucine and leucine residues.

It is known that BC-A decomposes to give BC-F by way of an oxidative deamination process at the *N*-terminal moiety and that the decomposition is accelerated in aqueous solution[1]. CRAIG and KONIGSBERG suggested that BC-B is converted into a BC-F type component more rapidly than BC-A[6]. Since these minor components have the same *N*-terminal moieties as that of BC-A, it was considered that they are converted into their degradation products through the same process as that of BC-A, and that such degradation products like BC-F are contained in commercial BC complex. The MW of such degradation products are estimated to be three mass units lower than those of the original components and to be one to three methylene units lower than that of BC-F. Although the 6 components with MW of 1,390 (peaks No. 24, 25 and 26) and of 1,404 (No. 27, 28 and 29) are corresponding to the degradation products of 6 components of MW 1,393 and 1,407, respectively, a component corresponding to the degradation product of the component with MW of 1,379 (peak No. 6) could not be found out in the commercial BC complex, probably because of low content.

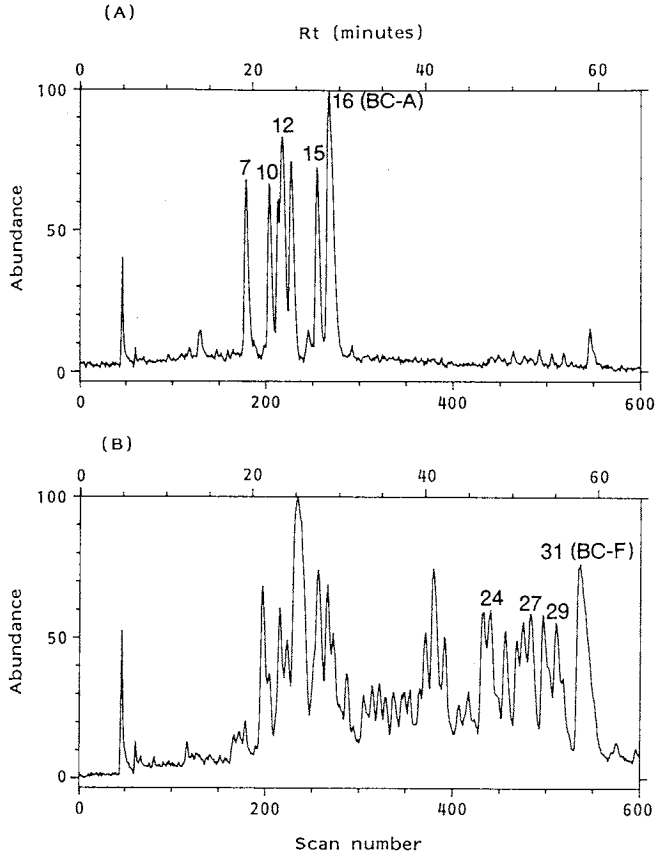
To confirm the relationship between the original components and their degradation products mentioned above, the HPLC profiles of BC complex were compared before and after standing in aqueous solution (1,000 ppm) at room temperature for three months. The peak heights of a group (peaks No. 24, 25, 26, 27, 28, 29 and BC-F) were gradually increased with decreasing the peak heights of another group (peaks No. 6, 7, 10, 11, 12, 13, 15 and BC-A). Since the members of each group have the common *N*-terminal moieties and exhibit the similar retention behavior on HPLC, each group is called "BC-A related components" and "BC-F related components" depending on their *N*-terminal moieties. The members of BC-A related components and BC-F related components have the common structures as shown in Fig. 5(A) and (B), respectively, and they are different from one another in the constituent amino acids labeled "X", "Y" and "Z". The amino acids labeled "X" and "Y" are either isoleucine or valine and that of "Z" is either leucine or valine. It was evident from the MW that the three amino acids of the component of peak No. 6 are all valines. However, additional information of amino acid sequences was required for the structural characterization of other components.

Determination of Amino Acid Sequence

Since the structures of these minor components are very similar to those of BC-A and -F, it was expected that they are cleaved at the peptide bonds to yield some diagnostic sequence ions as shown in Fig. 5 under FAB-MS conditions[22]. Although the Frit-FAB mass spectra of the components of peaks No. 7, 10, 11, 12, 13, 15, 24, 25, 26, 27, 28 and 29 were investigated to obtain the information of amino acids sequences, no satisfactory results were obtained, because most of the sequence ions were too weak to be distinguished from the background noise in the mass spectra. In our previous report, 5 g of commercial BC complex were separated by a preparative X-axis CCC[24,25]. Although BC-A was successfully isolated into one fraction, other fractions were still mixtures composed of some minor components. According to the report[24,25], commercial BC complex was separated into 8 fractions (Fr. 1 to 8) and the content of

Fig. 6. TIC of the enriched samples containing (A) BC-A related components and (B) BC-F related components.

Conditions, see Fig. 2.

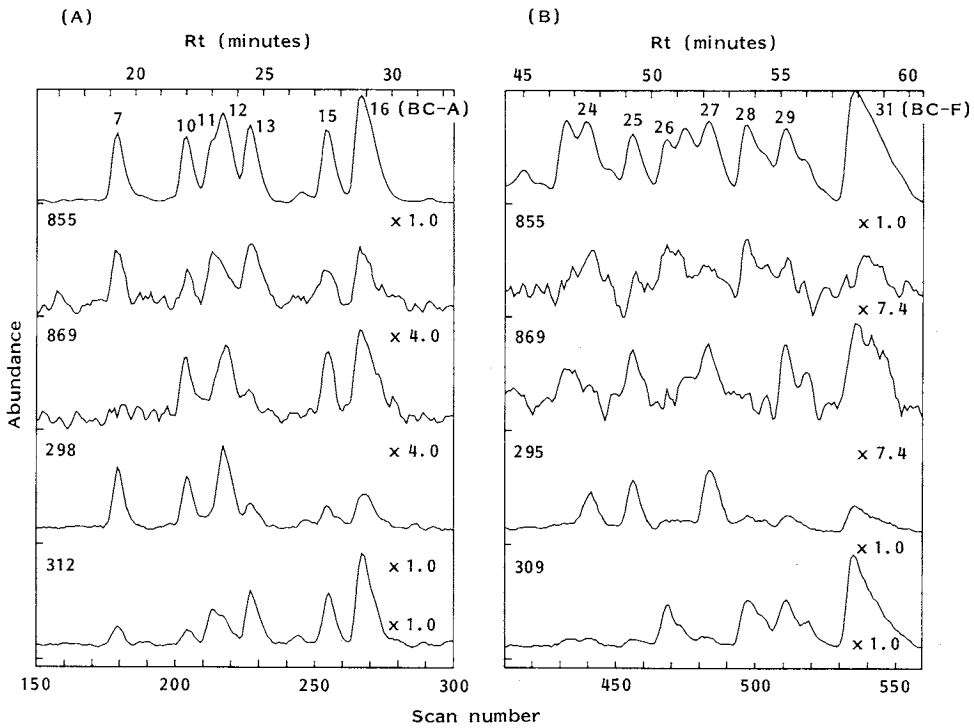


each fraction was investigated by HPLC. As a result, the members of BC-A related components were mainly contained in Frs. 5, 6 and 7, and those of BC-F related components were in Frs. 1, 2 and 3. These fractions may be used to prepare the sample mixture in which the desired minor components are conveniently enriched.

Fig. 6(A) shows the TIC of BC-A related components (200 μg) that was prepared by mixing properly the above fractions containing mainly the BC-A related components. The satisfactory mass spectra containing molecular ion species and useful sequence ions were obtained with good intensities at the peak top of each component, indicating that the desired components had been satisfactorily enriched in the sample mixture. The fragment ions at *m/z* 298, 312, 855 and 869 were set as the selected ions for mass chromatography on the basis of the fragmentations as shown in Fig. 5(A). Fig. 7(A) shows the enlarged TIC and mass chromatograms. The peaks at *m/z* 869 and 312 were higher than those of *m/z* 855 and 298 at the retention time of BC-A, indicating strongly that the constituent amino acids labeled "X" and "Z" of BC-A are isoleucine and leucine, respectively. Because the correct amino acid sequence of BC-A was indicated by this method, the amino acids labeled "X" and "Z" of other minor components could be distinguished by comparing the peak heights at *m/z* 869 and 312 with those of *m/z* 855 and 298, respectively,

Fig. 7. Enlarged TIC and mass chromatograms of the enriched samples at selected ions, *m/z* 869, 855, 312, 309, 298 and 295.

(A) BC-A related components. (B) BC-F related components. Conditions, see Fig. 2.



at the retention time of each component, and the third amino acids labeled "Y" can be consequently deduced. For example, in the case of component of peak No. 7 (MW 1,393), both amino acids labeled "X" and "Z" were determined to be valines, because the peaks of *m/z* 855 and 298 are higher than those of *m/z* 869 and 312, respectively. Therefore, the last amino acid labeled "Y" was determined to be isoleucine. The three amino acids of other components were determined by the same manner.

Table 2. Proposed structures of BC components.

Peak No.	MW	General structure	X	Y	Z
6	1,379		Val	Val	Val
7	1,393		Val	Ile	Val
10	1,393		Ile	Val	Val
11	1,393	Fig. 5 (A)	Val	Val	Leu
12	1,407		Ile	Ile	Val
13	1,407		Val	Ile	Leu
15	1,407		Ile	Val	Leu
16 (BC-A)	1,421		Ile	Ile	Leu
24	1,390		Val	Ile	Val
25	1,390		Ile	Val	Val
26	1,390		Val	Val	Leu
27	1,404	Fig. 5 (B)	Ile	Ile	Val
28	1,404		Val	Ile	Leu
29	1,404		Ile	Val	Leu
31 (BC-F)	1,418		Ile	Ile	Leu

The BC-F related components (400 μg) enriched fraction was similarly prepared and applied to LC/MS analysis. Although an efficient enrichment could not be achieved in this case as shown in Fig. 6(B), some informative sequence ions were observed in each mass spectrum of BC-F related components. The fragment ions at *m/z* 295, 309, 855 and 869 were selected on the basis of fragmentation of Fig. 5(B) to distinguish the amino acid labeled "X" and "Z". Fig. 7(B) shows the resulting mass chromatograms of the selected ions and the constituent amino acids labeled "X", "Y" and "Z" were determined by the same manner as described above. From the LC/MS

analysis the structures of the 12 minor components (peaks No. 7, 10, 11, 12, 13, 15, 24, 25, 26, 27, 28 and 29) can be proposed, whose proposed structures and MW are summarized with those of BC-A and -F in Table 2.

Conclusion

The Frit-FAB LC/MS system was successfully used for the structural characterization of minor components in commercial BC complex, and the structures of 13 minor components were proposed. The Frit-FAB LC/MS system was quite effective to separate and characterize the BC components in a short time. The fractions of BC complex separated by the preparative X-axis CCC in our previous study were very useful to prepare the sample mixture in which desired minor components are effectively enriched, and the satisfactory LC/MS data were given by using the enriched samples. Most of the minor components were very similar to BC-A and -F in structure and they were classified into two groups, BC-A related components and BC-F related components depending on their *N*-terminal moieties. The structures of BC-A related components were the same as that of BC-A, except that one to three of isoleucine and leucine residues are replaced by valines. The BC-F related components were degradation products of BC-A related components through the same degradation process as that of BC-A.

The structures of BC minor components proposed previously by NEWTON *et al.* and CRAIG *et al.* were ambiguous due to insufficient separation of the components by the countercurrent distribution[2,22]. Although the structures of the minor components were proposed by means of Frit-FAB LC/MS without isolation process in the present study, an additional study including analysis of the detailed fragmentation under FAB-MS conditions and determination of absolute configuration of the individual constituent amino acids is required to confirm the total structures. We are carrying out the isolation of these minor components using preparative HPLC, characterization of the isolated components by MS/MS and determination of absolute configurations of the constituent amino acids. These results will be reported elsewhere.

Acknowledgments

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