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# Bacterial Degradation of Microcystins and Nodularin

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Microcystins and nodularins produced by cyanobacteria are potent hepatotoxins and tumor promoters. They are, respectively, cyclic heptapeptides and cyclic pentapeptides containing a characteristic $\beta$-amino acid residue, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E* ),6(*E*)-dienoic acid (Adda). Strain B-9 isolated from Lake Tsukui, Japan, degrades microcystin-LR, which is the most toxic among the microcystins, to nontoxic Adda as an end product. In the present study, we characterized the bacterial degradation process of the cyclic peptide hepatotoxins by liquid chromatography/ion trap tandem mass spectrometry. The use of protease inhibitors with a B-9 cell extract indicated that the degradation process of microcystin-LR consists of sequential enzymatic hydrolyses of Arg-Adda, Ala-Leu, and then Adda-Glu peptide bonds into two known nontoxic intermediate degradation products and then Adda, respectively. Subsequently, additional microcystins and nodularin were compared with microcystin-LR on substrate specificity. The cyclic peptides containing the Arg-Adda peptide bond were almost completely degraded to Adda as well as microcystin-LR, whereas microcystin-LF containing the Phe-Adda peptide bond instead of Arg-Adda peptide bond and 6(*Z*)-Adda-microcystin-LR and -RR which are geometrical isomers of the Adda residue were barely degraded. These results indicated that the degrading enzymes selectively hydrolyzed the Arg-Adda peptide bond as the initial ring opening of the cyclic peptide hepatotoxins, microcystins and nodularin.

## Introduction

In the natural environment, many wild and domestic animals have died from ingesting water contaminated with the hepatotoxins, microcystins (MCs) (*1*−*3*). MCs[1] are produced by freshwater cyanobacteria belonging to the genera *Microcystis*, *Anabaena*, *Nostoc*, *Planktothrix*, and *Anabaenopsis* in addition to *Radiocystis* and terrestrial cyanobacterium *Hapalosiphon*. Their potent hepatotoxicity and tumor-promoting activity are considered to be caused by inhibitions of protein serine/threonine phosphatases 1 and 2A (*4, 5*). In 1996, MCs caused the death of over 50 patients at a hemodialysis clinic in Caruaru, Brazil (*6, 7*). However, no remedy has been reported so far. The structures of cyclic heptapeptides consist of cyclo-D-Ala-$R_1$-D-MeAsp(iso)-$R_2$-Adda-D-Glu(iso)-Mdha (Figure 1). Adda is a characteristic $\beta$-amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid, Mdha is *N*-methyldehydroalanine, and D-MeAsp is *erythro*-$\beta$-methyl-D-Asp. $R_1$ and $R_2$ are variable L-amino acids, and microcystin-LR (MCLR), which is one of the most common and most toxic among the MCs, contains L-Leu (L) and L-Arg (R), respectively.

In research on MC detoxication in the aquatic ecosystem, biodegradation has received a lot of attention (*8, 9*). An MC-degrading bacterium, *Sphingomonas* sp. strain MJ-PV, has been isolated from Australian natural water (*10*). Degradative activity was measured in a cell extract, and the use of several protease inhibitors indicated that the enzymatic degradation pathway of MCLR consisted of hydrolyses of the Arg-Adda and then Ala-Leu peptide bonds, and a linearized MCLR (H-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-OH) and then a tetrapeptide (H-Adda-Glu-Mdha-Ala-OH) were formed as the intermediate degradation products, respectively (*11*). After characterization of a gene cluster encoding the MC-degrading enzymes, their hydrolases and a following peptidase were termed MlrA, MlrB, and MlrC, respectively (*12*). The strain MJ-PV has been deposited in the Australian Collection of Microorganisms as strain ACM-3962.

Recently, more MC-degrading bacteria have been isolated, especially from Japanese lakes (*13*−*15*). Interestingly, all of them appeared to be identified as *Sphingomonas* sp., however, not all strains of the *Sphingomonas* genus are able to degrade MCs. We isolated an MC-degrading bacterium strain B-9 in 1997 from Lake

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[1] Abbreviations: MC, microcystin; NOD, nodularin; MCLR, microcystin-LR; 3-DMMCLR, 3-desmethylmicrocystin-LR; 6(*Z*)-MCLR, 6(*Z*)-Adda-microcystin-LR; MCLR-Cys, MCLR cysteine conjugate; DHMCLR, dihydromicrocystin-LR; MCRR, microcystin-RR; 6(*Z*)-MCRR, 6(*Z*)-Adda-microcystin-RR; MCLF, microcystin-LF; Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid; Mdha, *N*-methyldehydroalanine; Mdhb, *N*-methyldehydrobutyrine; LC/$MS^n$, liquid chromatography/tandem mass spectrometry; ITMS, ion trap mass spectrometer; ESI, electrospray ionization; QqQMS, triple quadrupole mass spectrometer; RIC, reconstructed ion chromatograms; PMSF, phenylmethylsulfonyl fluoride.

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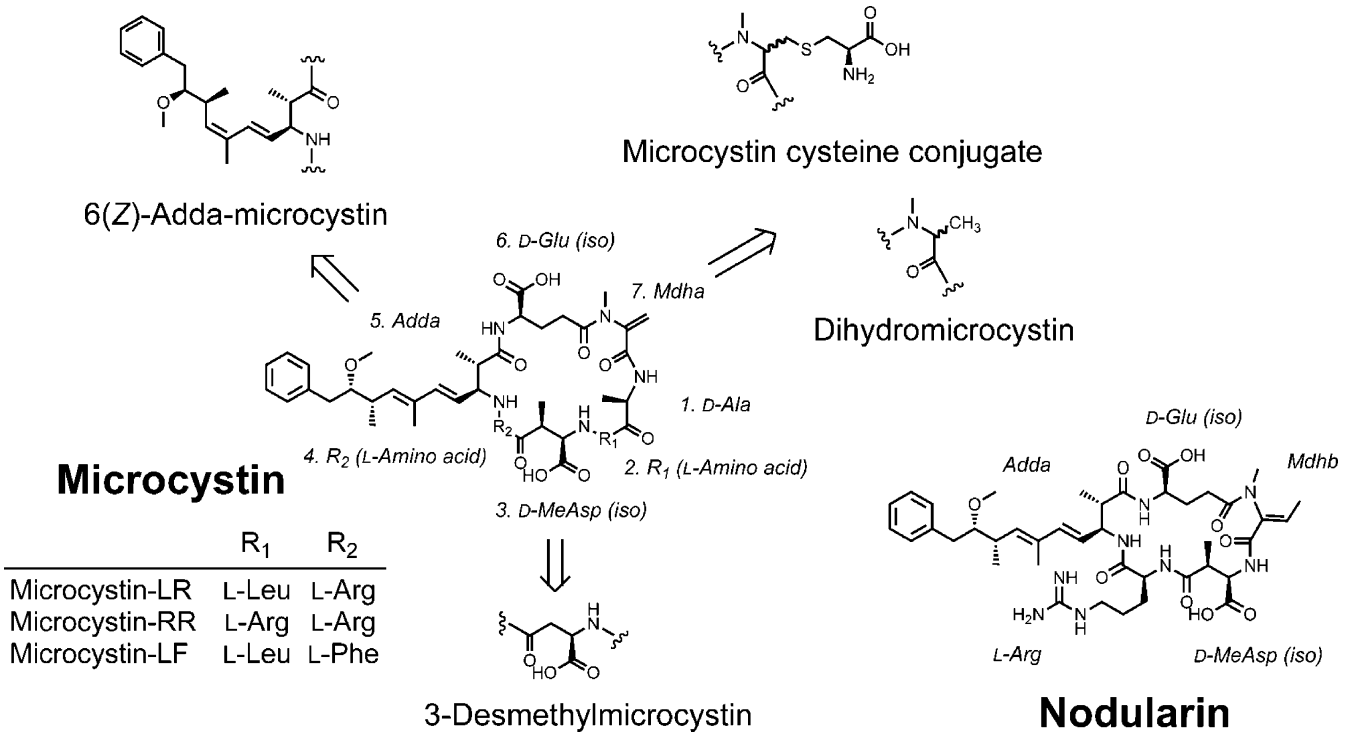


**Figure 1.** Structure of cyclic peptide hepatotoxins, microcystins (MCs) and nodularin (NOD), used in this bacterial degradation study. In addition to NOD, eight MC variants, microcystin-LR (MCLR), 6(*Z*)-Adda-microcystin-LR (6(*Z*)-MCLR), 3-desmethylmicrocystin-LR (3-DMMCLR), dihydromicrocystin-LR (DHMCLR), MCLR cysteine conjugate (MCLR−Cys), microcystin-RR (MCRR), 6(*Z*)-Adda-microcystin-RR (6(*Z*)-MCRR), and microcystin-LF (MCLF), were used. Adda is a characteristic $\beta$-amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid, Mdha is *N*-methyldehydroalanine, Mdhb is *N*-methyldehydrobutyrine, and D-MeAsp is *erythro*-$\beta$-methyl-D-Asp.

Tsukui, Japan. The degradative activity was in the cell extract, which was the case for the Australian strain ACM-3962, and the B-9 cell extract degraded MCLR to three products. An end product, nontoxic Adda, has been isolated by sequential chromatography and identified using various spectral data including NMR and MS spectral data (*16*). Although the Adda residue is essential for the acute toxicity and the protein phosphatase inhibitory activity of MCs (*17, 18*), the isolated Adda showed neither. Two intermediate degradation products were not fully confirmed.

In addition to MCs, nodularin (NOD) produced by a brackish cyanobacterium *Nodularia* sp. is a hepatotoxic pentapeptide containing the Adda residue (*19*). We expect that the degradative activity of the strain B-9 can be utilized for the biological treatment of the harmful MCs and NOD in natural water. In addition, the degrading enzymes have the potential to be a medical countermeasure of MCs. In the present study, therefore, we have characterized the enzymatic MC-degrading system in the B-9 cell extract. The degradation process of MCLR to Adda was confirmed using protease inhibitors as demonstrated in the previous study of the strain ACM-3962 (*11*); after that, the substrate specificities of the MC-degrading enzymes were investigated by liquid chromatography/tandem mass spectrometry (LC/MS$^n$).

## Materials and Methods

**Caution**: *MCs and NOD are hazardous because of their potent hepatotoxicity and tumor-promoting activity and should be handled carefully*.

**Toxins, Adda, and Chemicals.** MCLR was isolated from lyophilized cyanobacterial cells collected from Laguna de Bay, Philippines (*3, 20*). In the same way, 3-desmethylmicrocystin-LR (3-DMMCLR), 6(*Z*)-Adda-microcystin-LR (6(*Z*)-MCLR), microcystin-RR (MCRR), and 6(*Z*)-Adda-microcystin-RR (6(*Z*)-MCRR) were isolated from cyanobacterial cells collected from Lake Suwa, Japan. Dihydromicrocystin-LR (DHMCLR) was prepared by a modification of the method described previously (*21*). Mdha residue of MCLR was reduced with sodium borohydride in water instead of 2-propanol. MCLR cysteine conjugate (MCLR−Cys) was prepared via Michael-type addition to Mdha residue of MCLR (*22*). Microcystin-LF (MCLF) was kindly provided by Dr. Jussi Meriluoto. NOD was purchased from WAKO Pure Chemicals (Osaka, Japan). These structures are shown in Figure 1. Adda was isolated previously as the end product of the MCLR degradation by the B-9 cell extract (*16*). The purity of all cyclic peptides was more than 95% on HPLC analysis, but that of 6(*Z*)-MCLR was poor (43%) because of possible isomerization during a long storage at 4 °C.

Dithiothreitol, DMSO, phenylmethanesulfonyl fluoride (PMSF), EDTA, HPLC grade methanol, and formic acid for column chromatography were purchased from Nacalai tesque (Kyoto, Japan). Water was purified from distillate using a Barnstead E-pure 3 (Dubuque, IA).

**Preparation of Cell Extract of MC-Degrading Bacterium Strain B-9.** Culture of the strain B-9 was inoculated into 250 mL of a standard medium composed of peptone, yeast extract, and glucose, and incubated at 27 °C on an orbital shaker at 200 rpm for 3 days. After centrifugation for 30 min at 3000*g*, the cells were harvested as a pellet and were resuspended in 50 mL of 50 mM Tris-HCl buffer (pH 7.6) containing 0.5 mM dithiothreitol, followed by extraction using French pressure cell press (Thermo IEC, Rochester, NY) under a working pressure of 10 000 psi. Protein concentration of the resulting extract was 2.98 ±0.27 mg/mL (±S. E., $n = 8$) determined using a protein assay reagent (Bradford, bovine serum albumin, Bio-Rad, laboratories, Hercules, CA).

**Preparation of Intermediate Degradation Products of MCLR.** MCLR was dissolved in water at 1.0 mg/mL. Fifty microliters of this solution was added to 500 $\mu$L of the cell

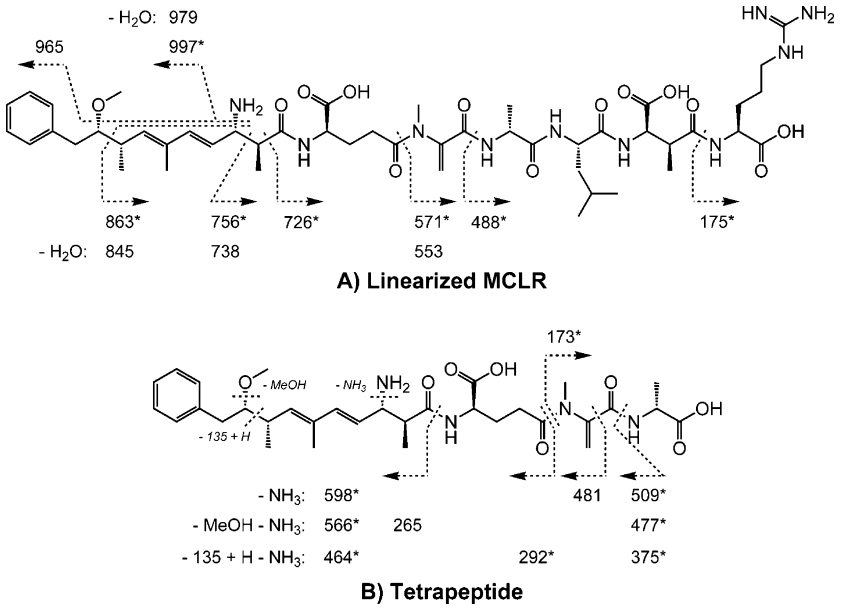


**Figure 2.** Proposed fragmentation patterns for the intermediate degradation products of MCLR. The MCLR degradation process by the B-9 cell extract was partially inhibited by the protease inhibitors and accumulated intermediate products were analyzed using ESI−ITMS. (A) LC/MS$^n$ analysis ($n$ = 1−4) of the linearized MCLR and (B) LC/MS$^n$ analysis ($n$ = 1−3) of the tetrapeptide were carried out under 30−40% and 25−35% of collision energies, respectively. The fragment ions indicated by asterisks (*) have been observed using ESI-QqQMS, previously (*11*).

extract which was preincubated for 30 min with protease inhibitors, PMSF or EDTA, at a resulting concentration of 10 mM. These inhibitors have been used previously in a similar study of the Australian strain (*11*). After incubation at 27 °C for 24 h, each 50 μL of these mixtures was added to 50 μL of methanol containing 0.2% formic acid to quench the degradation process and stored at −80 °C. Before MS analysis, supernatants after centrifugation for 10 min at 15 000*g* were filtrated with an Ultrafree-MC membrane filter unit (hydrophilic PTFE, 0.20 μm, Millipore, Bedford, MA).

**Degradation of MCs and NOD.** MCs and NOD, except for MCLF, were dissolved in water at 1.0 mg/mL, respectively. Fifty microliters of the mixtures were added to 500 μL of the cell extract and incubated at 27 °C. In MCLF (and MCLR as the positive control), 25 μL of the DMSO solution at 2.0 mg/mL and 25 μL of water were added to 500 μL of the cell extract and incubated. After 0, 24, and 48 h, 50 μL of these mixtures was added to 50 μL of methanol containing 0.2% formic acid and stored at −80 °C. As stated above, the centrifugation and the filtration were performed before MS analysis.

**Ion Trap Tandem Mass Spectrometry.** MCs, NOD, and their degradation products were analyzed using an LC/ion trap mass spectrometer (ITMS). LC separation was performed with an Agilent 1100 HPLC system (Agilent technologies, Palo Alto, CA). Five microliters of the filtrated sample was loaded onto a TSK-gel Super-ODS column (2 μm, 2.0 × 100 mm, TOSOH, Tokyo, Japan) at 40 °C. The mobile phase was water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). The flow rate was 200 μL/min. The entire eluate was directed into a mass spectrometer where it was diverted to waste for 5 min after injection to avoid introduction of salts to the ion source.

MS analysis was accomplished on a Finnigan LCQ Deca XP plus ITMS (Thermo electron, San Jose, CA) equipped with an electrospray ionization (ESI) interface. ESI conditions in the positive ion mode were as follows: capillary temperature, 300 °C; sheath gas flow rate, 50 arb; aux/sweep gas flow rate, 5 arb; ESI source voltage, 5.0 kV; capillary voltage, 42 V; tube lens offset, 20 V. In the collision-induced dissociation (CID) mode, excitation of the ions was accomplished through collisions with helium. Different collision energies were used. An isolation width for the precursor ion was always set at 3.0 amu.

The LC/ITMS was operated in one of the following methods: (1) LC/MS$^n$ analysis ($n$ = 1−4) for the intermediate degradation products of MCLR in the inhibition studies; (2) MS$^n$ analysis ($n$ = 1−5) after direct infusion for the isolated Adda (*16*); and (3) LC/MS analysis for MCs, NOD, and their degradation products. In the methods of 1 and 2, various scan ranges were used according to the molecular weights of the analytes. Using the method of 3, the scan range was between *m/z* 100 and 1500. LC gradient elution conditions were initially 50% B to 100% B at 5 min and then 100% B from 5 to 10 min in the method of 1 and initially 40% B to 90% B at 30 min in the method of 3.

## Results

**MS$^n$ Analysis of Degradation Products of MCLR by the B-9 Cell Extract.** The degradation process of MCLR by the B-9 cell extract was investigated using LC/ITMS. LC/MS analyses showed that the preincubations of the B-9 cell extract with the protease inhibitors PMSF and EDTA accumulated respective intermediate degradation products of MCLR ($[M + H]^+$ at *m/z* 1014 and 615, data not shown). The fragment ions obtained from subsequent LC/MS$^n$ analyses were similar to those of the linearized MCLR (H-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-OH) and the tetrapeptide (H-Adda-Glu-Mdha-Ala-OH) reported as the intermediate degradation products resulting from the Australian strain ACM-3962 (*11*) (Figure 2). Even though different mass spectrometers, ESI−ITMS providing MS$^n$ spectra and ESI-triple quadrupole mass spectrometer (QqQMS) providing MS$^2$ spectra, were used, the results indicated that the intermediate degradation products of MCLR by the strains ACM-3962 and B-9 were identical.

ESI-MS$^n$ analysis after direct infusion for Adda showed several accompanying ions in MS$^1$ spectrum (Figure 3B). Subsequent MS$^n$ ($n$ = 2−5) analyses supported that one of them, at *m/z* 663, was a dimeric ion $[2M + H]^+$, and three of them, at *m/z* 265, 283, and 315, were fragment ions (Figure 3C−E). The fragment ion at *m/z* 315 corresponds to the loss of an ammonia $[M + H - NH_3]^+$, and the others at *m/z* 283 and 265 correspond to the further loss of a methanol $[M + H - NH_3 - MeOH]^+$ and a

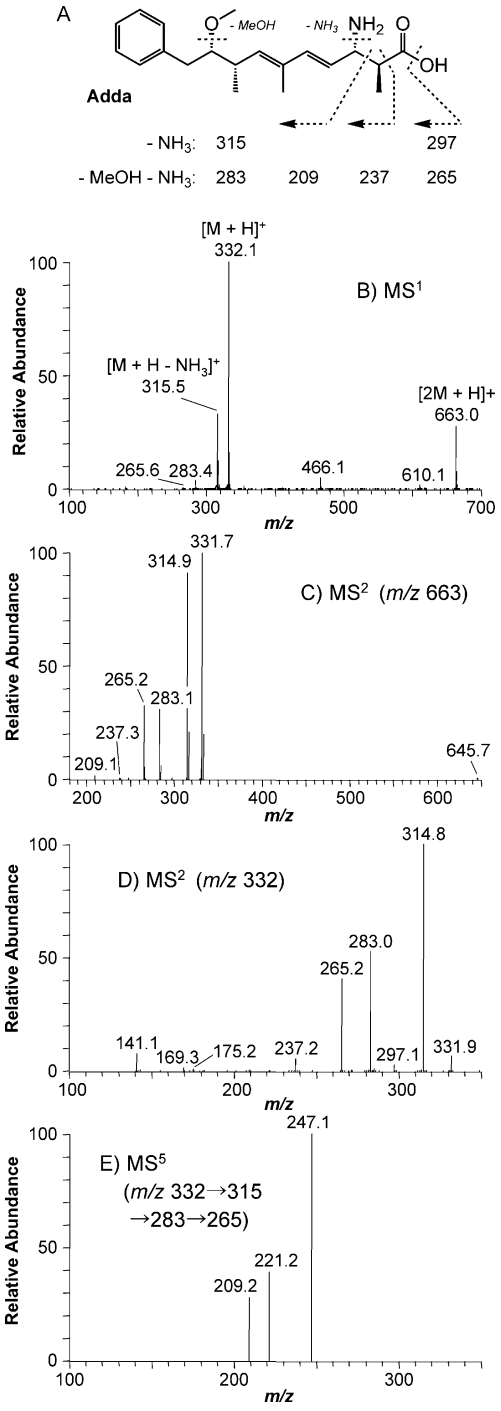


**Figure 3.** $MS^n$ analysis after direct infusion for Adda. Adda was isolated and identified previously as an end product of the MCLR degradation by the B-9 cell extract (*16*). ESI-$MS^n$ ($n$ = 1−5) spectra of Adda (B−E) and proposed fragmentation pattern (A) are shown. Mass-to-charge ratio ($m/z$) of the precursor ions is indicated in the spectra, respectively. Collision energies were between 20 and 35%. This $MS^n$ analysis indicated that the ESI of Adda produced the fragment ions at $m/z$ 265, 283, and 315 and the dimeric ion at $m/z$ 663 in $MS^1$ spectrum (A).

methanol and a water $[M + H - NH_3 - MeOH - H_2O]^+$, respectively (Figure 3A).

Consequently, the study using the protease inhibitors showed that the degradation process of MCLR consisted of sequential hydrolyses of the Arg-Adda, Ala-Leu, and then Adda-Glu peptide bonds, and the linearized MCLR, the tetrapeptide, and then Adda were formed, respectively (Figure 4).

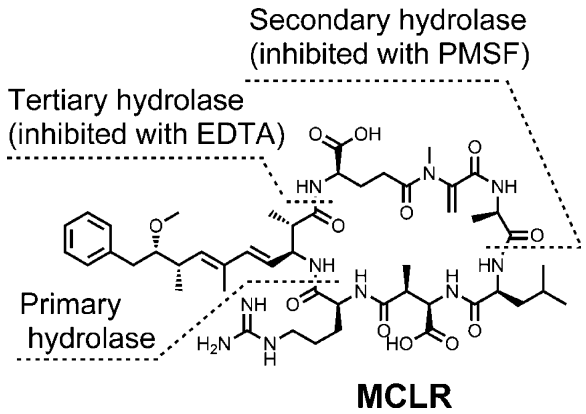


**Figure 4.** Proposed sequential hydrolyses of MCLR by the B-9 cell extract. The proposed degradation process of MCLR consisted of sequential hydrolyses of the Arg-Adda, Ala-Leu, and then Adda-Glu peptide bonds, and the linearized MCLR, the tetrapeptide, and then Adda were formed, respectively. PMSF inhibited the secondary hydrolase and the following tertiary hydrolase was also not active, and therefore the linearized MCLR was accumulated. EDTA inhibited the tertiary hydrolase, and therefore the tetrapeptide was accumulated.

**Comparison of Degradation Behavior of MCs and NOD by the B-9 Cell Extract.** LC/MS analyses of eight of the MC variants, NOD, and their expected degradation products corresponding to the linearized MCLR, the tetrapeptide, and Adda were performed at 0, 24, and 48 h after the beginning of the degradation (the structures of MCs and NOD are shown in Figure 1). Reconstructed ion chromatograms (RIC) of MCRR containing the Arg residue instead of the Leu residue of MCLR and the expected degradation products are shown in Figure 5A. As well as MCLR, MCRR was smoothly degraded within 24 h, and the tetrapeptide and Adda were produced. The expected linearized MCRR was not detected within 48 h. In contrast, the geometrical isomer 6(*Z*)-MCRR was barely degraded within 48 h (Figure 5B). However, the expected degradation products, especially 6(*Z*)-Adda, were observed. On ESI-MS spectra, Adda was accompanied by several ions containing the above-mentioned fragment ions (at $m/z$ 265, 283, and 315) and the dimeric ion (at $m/z$ 663), whereas the expected 6(*Z*)-Adda was accompanied by no fragment ions and a slight dimeric ion (Figure 6). However, their ESI-$MS^2$ spectra showed similar fragmentation patterns (data not shown), supporting the idea that one of the degradation products of 6(*Z*)-MCRR was 6(*Z*)-Adda, as expected, and that the amino group and the methoxy group of 6(*Z*)-Adda formed a rigid cyclic structure by their intramolecular hydrogen bond.

MCLF containing the Phe residue instead of the Arg residue of MCLR was also barely degraded, and the slight expected degradation products were observed (Figure 7A). MCLF has no basic group unlike the other MCs and therefore the $H_2O$ adduct ($[M + H + H_2O]^+$ $m/z$ 1004.5) and the sodium adduct ($[M + Na]^+$ $m/z$ 1008.5) ions are observed more intensely than the protonated molecule ($[M + H]^+$ $m/z$ 986.5) on ESI-MS spectra (data not shown). Although RIC at $m/z$ 1004.5 provided an intense peak at the same retention time ($t_R$) as the peak of MCLF, the ion at $m/z$ 1004.5 should not correspond to the hydrolyzed MCLF but rather to the $H_2O$ adduct. The cyclic pentapeptide NOD was smoothly degraded; however, only Adda of the expected degradation products was slightly observed just 24 h after (Figure 7B). 3-DMMCLR

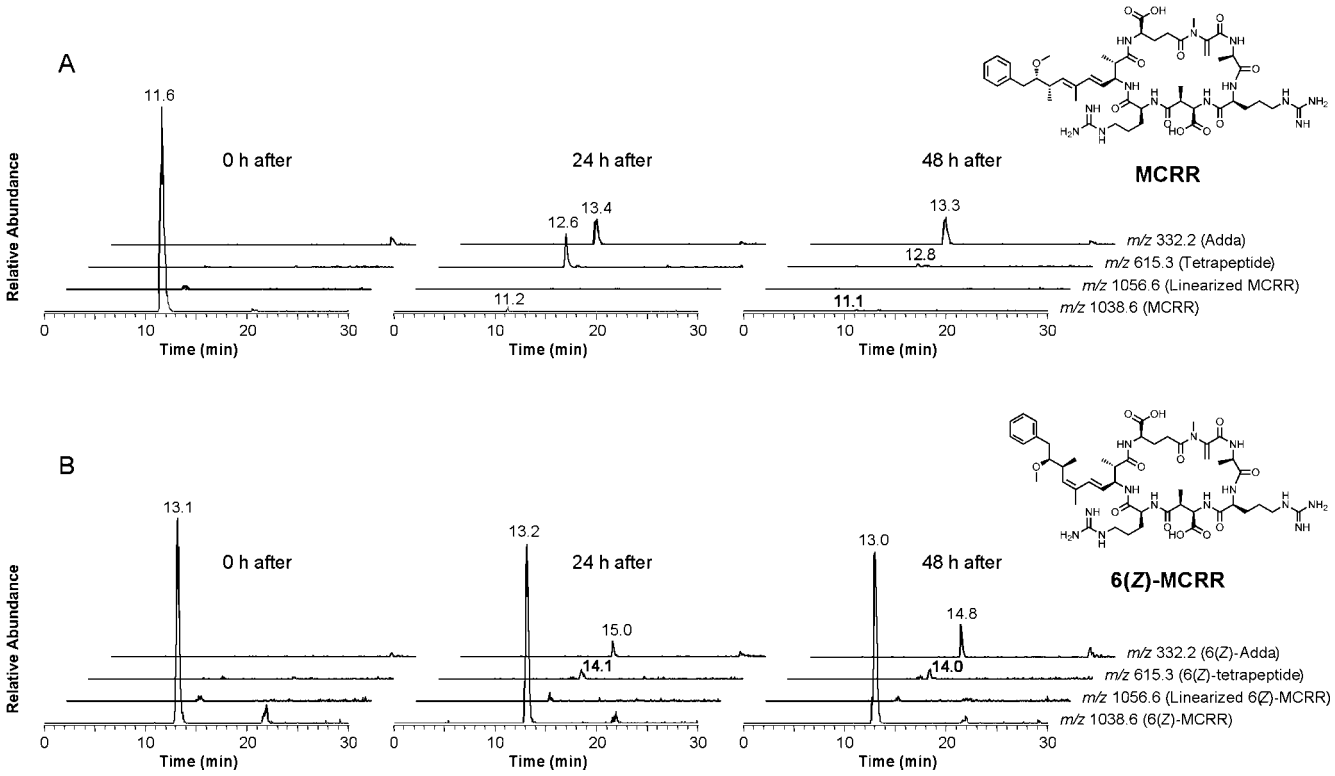


**Figure 5.** LC/MS analysis of the degradation processes of MCRR and 6(*Z*)-MCRR by the B-9 cell extract. RIC of MCRR and 6(*Z*)-MCRR and their expected degradation products (theoretical *m/z* ± 1.0 Da) 0, 24, and 48 h after the beginning of the degradations are shown. (A) MCRR was almost completely degraded within 24 h, whereas (B) the geometrical isomer 6(*Z*)-MCRR was barely degraded within 48 h.

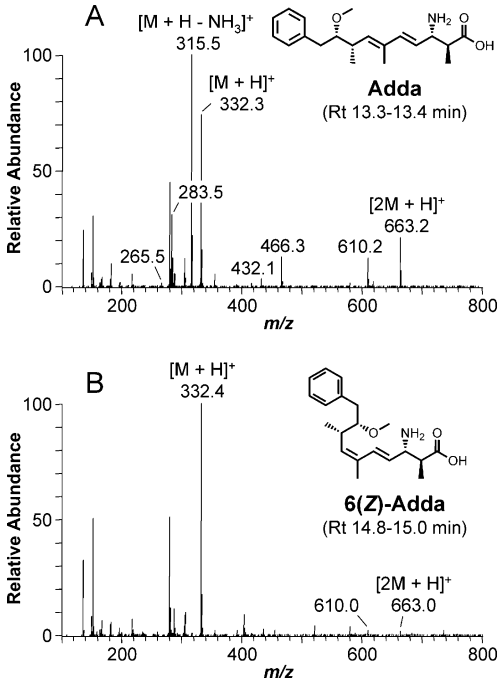


**Figure 6.** Comparison of ESI-MS spectra between Adda and the geometrical isomer 6(*Z*)-Adda by LC/MS analysis. Adda and 6(*Z*)-Adda shown in this figure were produced from MCRR and 6(*Z*)-MCRR, respectively. The ions indicated by *m/z* were observed at the same $t_R$. (A) The ESI of Adda produced the fragment ions corresponding to the loss of an ammonia and the further loss of a methanol at *m/z* 265, 283, and 315 and the dimeric ion at *m/z* 663. (B) However the ESI of 6(*Z*)-Adda produced no fragment ions and the slight dimeric ion. Refer to Figure 3.

and two of the Mdha-modified MCLR, DHMCLR and MCLR−Cys, were smoothly degraded within 48 and 24 h, respectively (Figure 8, chromatographic data not shown). Despite the poor purity (43%), 6(*Z*)-MCLR was used in this study and was barely degraded.

Retention times of the expected degradation products of MCs and NOD are summarized in Table 1. As expected, Adda ($t_R$ 13.2−13.6 min) was produced from all of the cyclic peptides containing the Adda residue, and the tetrapeptide ($t_R$ 12.5−12.9 min) from all of the cyclic peptides containing the corresponding sequence, Adda-Glu-Mdha-Ala. The expected Mdha-modified tetrapeptides, the dihydrotetrapeptide ($t_R$ 13.2−13.5 min) and the tetrapeptide cysteine conjugate ($t_R$ 9.7 min), were later and earlier eluted than the tetrapeptide, respectively. Their $t_R$ was not contradictory to that of their precursor cyclic peptides, the later eluted DHMCLR ($t_R$ 16.3 min) and the earlier eluted MCLR−Cys epimers ($t_R$ 13.7 and 15.2 min) than MCLR ($t_R$ 15.7−15.8 min). Similarly, it was consistent that the geometrical isomers 6(*Z*)-MCLR ($t_R$ 16.2−16.3 min) and 6(*Z*)-MCRR ($t_R$ 13.0−13.2 min), and their expected degradation products, the 6(*Z*)-Adda-tetrapeptide ($t_R$ 13.6−14.1 min) and the 6(*Z*)-Adda ($t_R$ 14.6−15.0 min), were later eluted than their corresponding peptides, MCLR, MCRR ($t_R$ 11.1−11.6 min), the tetrapeptide, and Adda, respectively. The cyclic pentapeptide NOD contains no corresponding sequence to the tetrapeptide. All of the expected linearized MCs and NOD were barely observed or not observed at all. Consequently, the other expected degradation products of the MCs and NOD were observed by LC/MS analysis.

## Discussion

In our previous study, it was found that the cell extract of the strain B-9 degraded MCLR to three products, and the end product of them was the characteristic β-amino

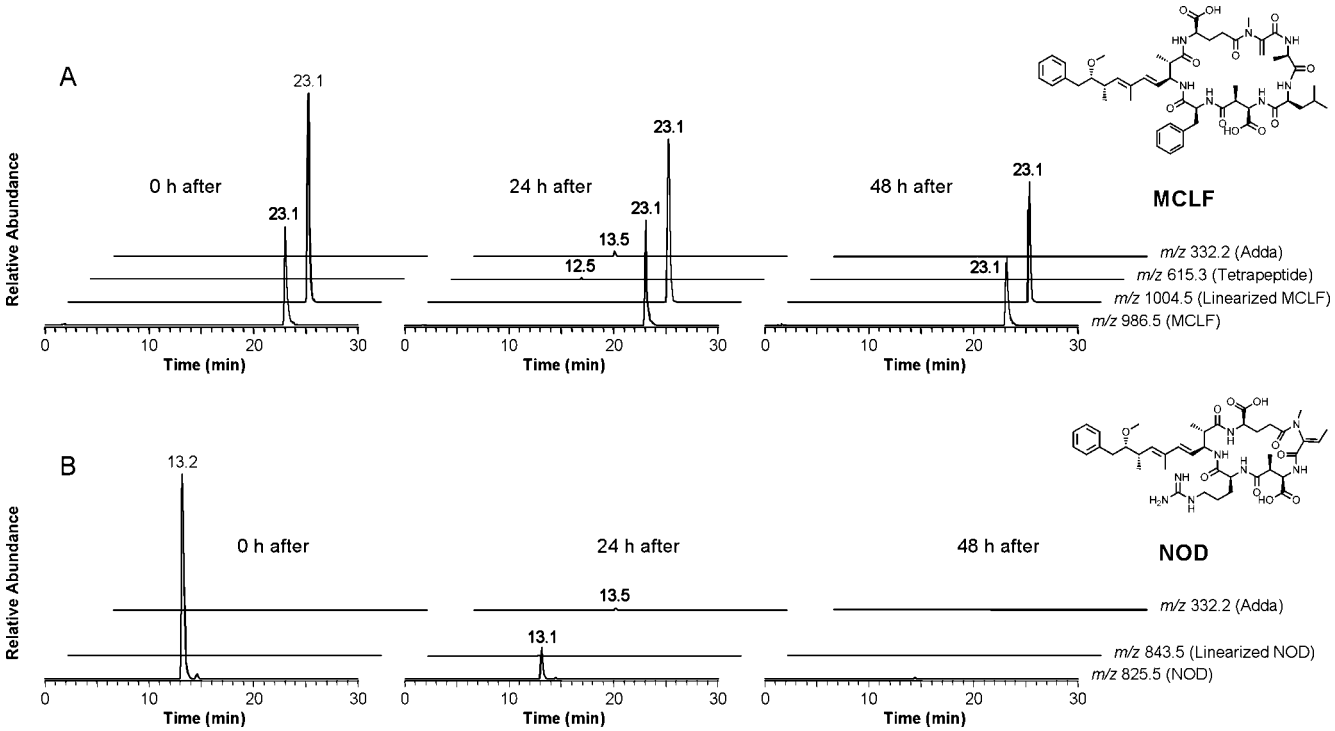


**Figure 7.** LC/MS analysis of the degradation processes of MCLF and NOD by the B-9 cell extract. RIC of MCLF and NOD and their expected degradation products (theoretical $m/z \pm 1.0$ Da) 0, 24, and 48 h after the beginning of the degradations are shown. (A) MCLF containing the Phe-Adda peptide bond was barely degraded within 48 h, whereas (B) the cyclic pentapeptide NOD containing the Arg-Adda peptide bond was remarkably degraded within 24 h.

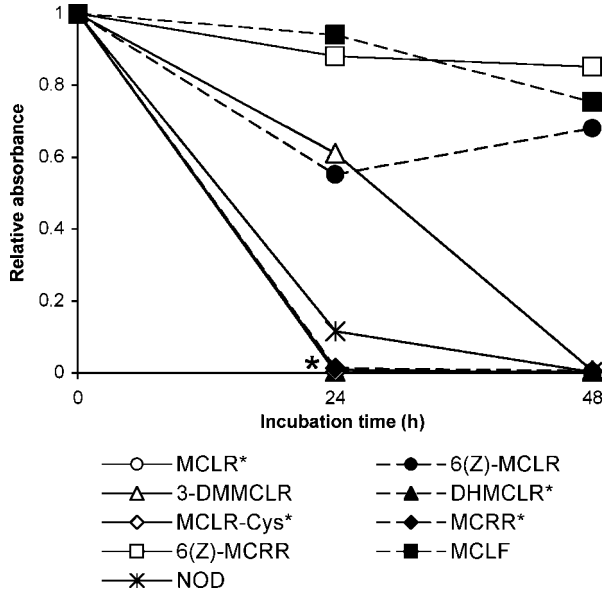


**Figure 8.** Degradation of MCs and NOD by the B-9 cell extract. Quantitative data were obtained from the peak areas on the RIC (refer to Figures 5 and 7). * MCLR, DHMCLR, MCLR–Cys, and MCRR were almost completely degraded within 24 h. 6(*Z*)-MCLR, 6(*Z*)-MCRR, and MCLF were barely degraded within 48 h.

**Table 1. Retention Time ($t_R$) of MCs, NOD, and Their Expected Degradation Products on LC/MS Analysis**

| | $t_R$ (min) | | | |
|---|---|---|---|---|
| microcystin and nodularin | precursor cyclic peptide | linearized peptide | tetrapeptide | Adda |
| MCLR | 15.7–15.8 | N. D.[a] | 12.5–12.9 | 13.5–13.6 |
| 6(*Z*)-MCLR | 16.2–16.3 | 13.9 | 13.6–13.8 | 14.6–14.9 |
| 3-DMMCLR | 16.2–16.4 | 13.7–13.8 | 12.6 | 13.2–13.3 |
| DHMCLR | 16.3 | 14.8 | 13.2–13.5 | 13.4–13.5 |
| MCLR–Cys | 13.7, 15.2 | 13.1, 13.8 | 9.7 | 13.4–13.5 |
| MCRR | 11.1–11.6 | N. D. | 12.6-12.8 | 13.3–13.4 |
| 6(*Z*)-MCRR | 13.0–13.2 | N. D. | 14.0–14.1 | 14.8–15.0 |
| MCLF | 23.1 | N. D. | 12.5 | 13.5 |
| NOD | 13.1–13.2 | N. D. | | 13.5 |

[a] N. D.: not detected.

acid Adda (*16*). Two intermediate products, the linearized MCLR (H-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-OH) and the tetrapeptide (H-Adda-Glu-Mdha-Ala-OH), which had been reported as the intermediate degradation products by *Sphingomonas* sp. strain ACM-3962, were determined by LC/MS analysis (*11, 12*). The MC-degrading enzymes of the strain ACM-3962, MlrA, MlrB, and MlrC, hydrolyzed the Arg-Adda, Ala-Leu, and an unknown peptide bond of MCLR, respectively. MlrB inhibition with the serine protease/cysteine protease inhibitor PMSF and MlrC inhibition with the metalloprotease/metal-activated protease inhibitor EDTA accumulated the intermediate degradation products, the linearized MCLR and the tetrapeptide, which were identified by $MS^2$ analysis, respectively. In the strain B-9, however, the presumed intermediate products were not confirmed, and the initially hydrolyzed peptide bond of the cyclic heptapeptide MCLR was unknown. In the present study, therefore, partial inhibition study of the B-9 cell extract and $MS^n$ analysis were performed as well as the study on the ACM-3962 cell extract. Consequently, the intermediate degradation products of MCLR by the strain B-9 were identical to those of the strain ACM-3962, the linearized MCLR and the tetrapeptide. It was suggested that the degradation process of MCLR by the strain B-9 consisted of the sequential enzymatic hydrolyses of the Arg-Adda, Ala-Leu, and then Adda-Glu peptide bonds (Figure 4). Three of the degradation products have been reported as nontoxic (*11, 16*); therefore, the primary hydrolysis is

essential for the detoxication. The MCLR degradation study by the B-9 culture also showed two of the nontoxic degradation products, the tetrapeptide and Adda (data not shown), indicating that the strain B-9 actually detoxified MCLR by the sequential hydrolyses in the cell.

Subsequently, the substrate specificity of the MC-degrading enzymes in the B-9 cell extract was investigated using the additional seven MCs and NOD, in comparison with MCLR. Three of them, 6(*Z*)-MCLR, 6(*Z*)-MCRR, and MCLF, were barely degraded within 48 h (Figures 1 and 8). The five degradable MCs and NOD contain the Arg-Adda peptide bond, whereas the barely degradable three MCs contain the Arg-6(*Z*)-Adda or the Phe-Adda peptide bond instead. Although the substitution of the Arg residue for the Leu residue (MCRR), the modifications of the Mdha residue (DHMCLR, MCLR−Cys, and NOD), and the loss of the Ala-Leu residues (NOD) did not interfere with the degradations of the cyclic peptides, the desmethylation of the MeAsp residue followed by the Arg residue (3-DMMCLR) interfered in some degree. It was suggested that the primary hydrolase recognized the MeAsp-Arg-Adda peptide sequence and selectively hydrolyzed the Arg-Adda peptide bond.

Because the expected degradation products of MCs and NOD corresponding to those of MCLR were observed by LC/MS analysis (Table 1), the MC-degrading enzymes appeared to hydrolyze all of the used cyclic peptides in the same degradation process. The linearized MCs were barely observed or not observed at all, so the rate of secondary hydrolysis was possibly greater than that of primary and tertiary hydrolyses. In addition, the smooth degradation of MCRR to the tetrapeptide indicated that the secondary hydrolase was able to hydrolyze the Ala-Arg peptide bond in addition to the Ala-Leu peptide bond (Figure 5A). Similarly, the accumulation of 6(*Z*)-Adda after the degradations of 6(*Z*)-MCLR and 6(*Z*)-MCRR indicated that the tertiary hydrolase was able to hydrolyze the 6(*Z*)-Adda-Glu peptide bond in addition to the Adda-Glu peptide bond (Figure 5B, chromatographic data of 6(*Z*)-MCLR not shown).

The Australian strain ACM-3962 had been the only other strain of which the degradation products and the MC-degrading enzymes were characterized (*11, 12*). The cell extract of this strain degraded the cyclic heptapeptide MCLR and MCRR, but not the cyclic pentapeptide NOD, which was degraded by the B-9 cell extract as stated above. While the degradation processes of MCLR by the strains ACM-3962 and B-9 were similar, their degradation enzymes were not identical. In Japanese lakes, some MC-degrading bacteria have been isolated. The strain Y2 identified as *Sphingomonas* sp. with very low probability (0.17%) was isolated from Lake Suwa, Japan (*13*). Culture of this strain degraded MCLR, MCRR, and microcystin-YR (MCYR). The analysis of the population dynamics of the strain Y2 in *Microcystis* mucilage suggested that the strain Y2 responded to changes in the concentration of MCs and degraded the MCs immediately after the releases from *Microcystis* cells (*23*). Culture of *Sphingomonas* sp. strain MD-1 isolated from Lake Kasumigaura, Japan, degraded MCLR, MCRR, and MCYR, but not NOD (*14*). Both of the Japanese strains possessed *mlrA* homologues (*24*). The primary hydrolase of the strain B-9 was similar to MlrA at the hydrolysis of the Arg-Adda peptide bond but was not similar in the substrate specificity, particularly to NOD. Culture of *Sphingomonas* sp. strain 7CY isolated from Lake Suwa, Japan, degraded hydrophobic microcystin-LY (MCLY), microcystin-LW (MCLW), and MCLF in addition to MCLR and MCRR (*15*). Therefore, the MC degradation of this culture did not require the Arg-Adda peptide bond as in the B-9 cell extract. Interestingly, the culture and cell extract of this strain did not degrade nodularin-Har (NOD-Har) containing the homoarginine residue instead of the Arg residue of NOD; however, incubation with MCRR allowed the strain to degrade NOD-Har. The strain might require an enzyme induced during the degradation of MCRR to utilize NOD-Har as nutrition. Similarly, cultivation with MCs probably induces another MC-degrading enzyme of the strain B-9.

## Conclusions

In the present study, we have characterized the enzymatic MC-degrading system of the strain B-9 by LC/MS$^n$. Consequently, the proposed degradation process of MCLR consisted of the sequential hydrolyses of the Arg-Adda, Ala-Leu, and then Adda-Glu peptide bonds, and three of the nontoxic degradation products were formed. These hydrolyses were carried out by unique enzymes that degraded NOD in addition to MCs. The primary hydrolase selectively hydrolyzed the Arg-Adda peptide bond, but not the Arg-6(Z)-Adda or the Phe-Adda peptide bond. To use this strain for biological treatment of MCs and NOD in natural water, these data of the degradative characteristics should be required. Furthermore, the active substances of the bacteria possess the possibility to be applied as the remedy for the MC intoxication by future biotechnologies.

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