

Enzymatic Pathway for the Bacterial Degradation of the Cyanobacterial Cyclic Peptide Toxin Microcystin LR

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An isolated bacterium, identified as a new *Sphingomonas* species, was demonstrated to contain a novel enzymatic pathway which acted on microcystin LR, the most common cyanobacterial cyclic peptide toxin. Degradation of microcystin LR was mediated by at least three intracellular hydrolytic enzymes. The use of classic protease inhibitors allowed (i) the classification of these enzymes into general protease families and (ii) the *in vitro* accumulation of otherwise transient microcystin LR degradation products. The initial site of hydrolytic cleavage of the parent cyclic peptide by an enzyme that we designate microcystinase is at the 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda)-Arg peptide bond. Two intermediates of microcystin LR enzymatic degradation have been identified; one is linearized (acyclo-) microcystin LR, NH₂-Adda-Glu(iso)-methyldehydroalanine-Ala-Leu-β-methylaspartate-Arg-OH, and the other is the tetrapeptide NH₂-Adda-Glu(iso)-methyldehydroalanine-Ala-OH. The intermediate degradation products were less active than the parent cyclic peptide; the observed 50% inhibitory concentrations for crude chicken brain protein phosphatase were 0.6 nM for microcystin LR, 95 nM for linear LR, and 12 nM for the tetrapeptide. These linear peptides were nontoxic to mice at doses up to 250 μg/kg. Ring opening of the potent hepatotoxin microcystin LR by bacterial microcystinase effectively renders the compound nontoxic by dramatically reducing the interaction with the target protein phosphatase.

Microcystins are a group of cyclic heptapeptide hepatotoxins produced by cyanobacteria belonging to the genera *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria* (5). Structural variants of microcystin, most of which are highly toxic, contain different amino acids at two nonconserved positions in the cyclic heptapeptide and demethylated or acetylated amino acid residues. Almost 50 variants of microcystin have been identified, and several others have yet to be fully characterized (24, 28). The most common of these, microcystin LR, has the structure cyclo-D-alanine-L-leucine-erythro-β-methyl-D-isoaspartic acid-L-arginine-Adda-D-isoglutamic acid-N-methyldehydroalanine (Mdha), where Adda is the novel amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (3, 5, 15).

The molecular basis of the toxicity of the microcystins is inhibition of hepatocyte protein phosphatases 1 and 2A (17, 29). This causes cyokeratin hyperphosphorylation which leads to microfilament disassembly, cell rupture, and hemorrhaging of the liver *in vivo* in mammals (8). Of significant concern is the possibility that chronic exposure to low concentrations of microcystins in drinking water supplies may promote tumor growth in the human liver (4, 7, 21, 27).

Investigations into the persistence and degradation of these cyanobacterial toxins are becoming increasingly important as the significance and frequency of cyanobacterial blooms in domestic water supplies increase and the potential long-term health effects become clearer. In a recent study, we demonstrated that microcystin LR persisted in surface waters for 2 weeks before endemic bacterial populations degraded the tox-

ins, and small amounts were still detectable even after more than 1 month (13). Further work resulted in the isolation of a single bacterial strain that utilized microcystin LR as a sole source of carbon and nitrogen for growth (11). Tentatively identified previously as a *Pseudomonas* sp., this bacterium has now been correctly classified as a new *Sphingomonas* species (unpublished data). Cell extracts (CE) of this strain contained an enzyme activity, referred to as microcystinase activity, that degraded microcystin LR and RR *in vitro*, but not the closely related pentapeptide nodularin. Enzyme activity was expressed constitutively when the bacterium was grown on medium containing peptone and yeast extract or glucose and yeast extract.

It has been demonstrated previously that microcystin can be degraded by complex natural populations of microorganisms from diverse ecosystems, such as sewage effluent (16), lake sediment, and water (13, 23). However, nothing is known about the dynamics of this degradation process in terms of the pathways followed or enzymes involved. In this paper we describe the partial characterization of a mixture of hydrolytic enzymes from the *Sphingomonas* strain mentioned above and identify intermediate products which support the proposition that there is a metabolic pathway for the metabolism of microcystin LR.

MATERIALS AND METHODS

Strain growth and maintenance. Because of the difficulty and cost of purifying large quantities of microcystin, the *Sphingomonas* strain was routinely maintained on peptone-yeast extract medium as a way to conserve microcystin LR. Microcystinase activity was expressed constitutively on this medium (11). The strain has been deposited in the Australian Collection of Microorganisms as strain ACM 3962.

CE preparation. Cultures of the bacterial strain were inoculated into peptone-yeast extract medium and incubated overnight at 27°C. The cultures were harvested in the late logarithmic (growth) phase and centrifuged to obtain a pellet, the supernatant was decanted, and an equal volume of 0.05 M potassium phosphate buffer (pH 6.90) was added. The cells were resuspended and repelleted,

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and the supernatant was discarded. This washing protocol was repeated three times. The resuspended cells were sonicated at 4°C with a Braun Labsonic L sonicator (output, 150 W). The cell debris was pelleted by centrifugation at 18,000 rpm for 20 min in a Beckman model J21 centrifuge equipped with a model JA 20 rotor at 4°C, and the CE was decanted and used for the enzyme activity assays.

Enzyme activity assays. Enzyme assays were performed by using 0.25 ml of CE and microcystin LR at final concentrations ranging from 25 to 75 μM . Microcystin LR was purified from an Australian bloom of *Microcystis aeruginosa* as described previously by Jones et al. (11). Phosphate buffer (see above) was used to make the assay volume up to 1.5 ml. All assay mixtures were prepared in 1.5-ml Eppendorf tubes (on ice), vortexed, and incubated at 30°C in a water bath. Samples (200 μl) were removed periodically, centrifuged at 15,000 rpm (Beckman Microfuge E centrifuge) for 5 min at 4°C, and then analyzed by high-performance liquid chromatography (HPLC), using standard conditions as described below.

Enzyme inhibition. Enzyme inhibitors were prepared as follows (22). Leupeptin (Sigma) purified from *Streptomyces roseus* was prepared freshly as a 10 mM stock solution in Milli-Q water and was used at an assay concentration of 100 μM . 1,10-Phenanthroline (Sigma) was prepared as a 200 mM stock solution in methanol and was used at assay concentrations of 1 and 10 mM. L-trans-Epoxy-succinyl-leucylamide-(4-guanidino)-butane (E-64) (Sigma) was prepared as a 10 mM stock solution in Milli-Q water and was used at an assay concentration of 100 μM . Pepstatin (Sigma) was prepared as a 10 mM stock solution in dimethyl sulfoxide and was used at an assay concentration of 100 μM . Phenylmethanesulfonyl fluoride (PMSF) (Sigma) was prepared as a 200 mM stock solution in dimethyl sulfoxide and was used at an assay concentration of 1 mM. Benzamidine hydrochloride (Aldrich) was prepared as a 100 mM stock solution in Milli-Q water and was used at an assay concentration of 1 mM. Trypsin inhibitor (from soybean; Sigma) was prepared as a 15-mg/ml stock solution, in Milli-Q water and was used at an assay concentration of 1 mg/ml. EDTA was prepared as a 100 mM stock solution in water and was used at assay concentrations of 1 and 10 mM.

In most cases the CE (0.25 ml), buffer, and required inhibitor were preincubated at 30°C for 2 h; the only exception was the preparation containing PMSF, which was preincubated for 30 min. Then microcystin LR was added, and the preparations were vortexed. Samples (200 μl) were periodically removed, and 100 μl of each sample was injected onto the HPLC system by using standard conditions.

Microcystin analysis. Microcystin LR and its degradation products were quantified by reverse-phase HPLC (Waters) by using a Spherisorb ODS-2 C_{18} column (5 μm ; 250 by 4.6 mm; Alltech). The mobile phase consisted of 20% acetonitrile in 8 mM aqueous ammonium acetate. The acetonitrile content was raised to 35% with a 20-min linear gradient. Further details of the HPLC system used have been described previously by Jones et al. (11).

Preparation and purification of degradation products A and B. PMSF was added to 10 ml of standard CE (after sonication 16 times [5 s each]) to give a final concentration of 1 mM. After equilibration of the extract at 30°C, microcystin LR was added to a final concentration of 140 μM . The solution was frozen after the assay established that microcystin LR had disappeared and that degradation product A was present in approximately stoichiometric amounts. A similar method was used for the preparation of degradation product B, except that EDTA (1 mM) was used as an inhibitor.

The degradation products were purified by preparative HPLC by using a Waters $\mu\text{Bondapak C}_{18}$ column (150 by 19 mm). The optimum conditions used for the system for baseline separation of peaks were an isocratic run of 28% acetonitrile-ammonium acetate at a flow rate of 4.5 ml/min and detection at 240 nm.

Assay vials containing either degradation product A or degradation product B were thawed and filtered (pore size, 0.2 μm) before injection onto the column. Samples (400 μl) of each extract were manually injected onto the preparative HPLC system described above, and the fraction corresponding to each degradation product was individually collected. The pooled fractions for product A or B were separately desalted by using a glass column (inside diameter, 25 mm) packed with 1 g of Waters Preparative C_{18} medium (125 Å [12.5 nm]; mesh size, 55 to 105 μm). The column was prewashed with 1 column volume of methanol and 3 column volumes of Milli-Q water. The fractions were diluted with Milli-Q water to a volume of 100 ml, passed through the preparative C_{18} column, and washed once with 10 ml of Milli-Q water and once with 10 ml of 30% methanol. The degradation products were eluted four times with 5 ml of 100% methanol and pooled. Samples were analyzed by HPLC to confirm the presence and purity of the degradation product. Concentration of the samples was performed by rotary evaporation at 40°C.

Amino acid analysis. The parent microcystin and degradation products A and B were separately hydrolyzed in 6 M HCl at 105°C under a vacuum for 24 h prior to amino acid analysis. The released amino acids were pre-column derivatized with phenylisothiocyanate to produce the phenylthiocarbamyl amino acids. The derivatives were applied to a Beckman Ultrasphere PTH 5- $\mu\text{m C}_{18}$ column (250 by 4.6 mm), which was run at 45°C. Solvent A consisted of 0.14 M sodium acetate (pH 6.45) containing 0.5 ml of triethylamine per liter and 0.2 mg of EDTA per liter. Solvent B consisted of 60% acetonitrile and 40% water. The derivatives were eluted over an 18-min period with a 0 to 46% solvent B linear gradient. The flow rate was maintained at 1 ml/min.

Ion spray mass spectrometry (MS). A triple-quadrupole mass spectrometer (model API III; Sciex, Thornhill, Ontario, Canada) was used in this investigation. A pneumatically assisted electrospray (ion spray) interface was used for sample introduction into the atmospheric pressure ionization source of the mass spectrometer. The interface sprayer was operated at a positive potential of 5.6 kV. Positive ions generated by the ion evaporation process entered the analyzer of the mass spectrometer through an interface plate and subsequently through a 100- μm hole in the sampling orifice. To aid desolvation of highly charged droplets and to prevent particulate matter from entering the analyzer region, a curtain gas of ultrapure nitrogen (1 to 2 liters/min) was applied between the interface plate and the sampling orifice. The analyzer region of the mass spectrometer was kept at 2×10^{-5} torr (ca. 267×10^{-5} Pa) by using a helium cryopump.

Full-scan single MS experiments were performed by scanning quadrupole 1 from mass/charge ratio (m/z) 100 to m/z 1,200. For tandem (MS-MS) experiments, collision-induced dissociation (CID) of the parent precursor ion was affected by bombardment with ultrapure argon. Bombardment was carried out in quadrupole 2 with a collision cell gas thickness of 300×10^{12} atoms per cm and the collision energy (Q-2 rod offset voltage) typically set at -20 V. The resulting CID (product ion) spectra were obtained by scanning quadrupole 3 from m/z 100 to m/z 1,200.

Liquid chromatography-MS was performed by introducing the liquid chromatography eluent directly into the mass spectrometer ion source through a fused silica capillary (50 cm by 75 μm [inside diameter]). Degradation products A and B were analyzed by a combination of single MS, MS-MS, and liquid chromatography-MS. Data were entered into an Apple Macintosh IIx computer and were processed by using the software package MacSpec (Sciex).

Protein phosphatase inhibition assay. Inhibition of crude chicken brain protein phosphatase was determined by using the radioassay ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$) method, as described previously (13).

Mouse bioassay. Intraperitoneal injection of purified microcystin LR and degradation products into adult white mice was performed by Mandy Choice, University of New England, who used previously described methods (12).

RESULTS

Enzyme and inhibitor studies. In our earlier experiments (11), microcystin LR degradation assays were carried out with *Shingomonas* CE in phosphate buffer only. In those assays, loss of microcystin LR was observed in HPLC chromatograms, and no new peaks corresponding to the formation of intermediate breakdown products of microcystin LR were observed. The rate of microcystin loss was shown to be directly proportional to the volume of added CE, which is consistent with an enzymatically mediated process.

Evidence for the involvement of more than one enzyme in the degradative pathway was first obtained from experiments intended to assess the role of periplasmic enzymes in the degradative process. These experiments required the addition of EDTA to enhance release of periplasmic proteins. Although little activity was found in the periplasmic fraction, control assays (performed with complete CE and EDTA) revealed the transient appearance of one intermediate product and the stable accumulation of a second. These intermediates can be seen clearly as separate peaks in time sequence HPLC chromatograms and were designated degradation products A and B (Fig. 1).

To confirm and characterize the formation of degradative intermediates, assays were carried out with a range of common protease inhibitors, which were incubated with CE prior to addition of microcystin LR. These enzyme inhibitors also provide a crude method which can be used to classify the proteases assumed to be present in the CE into general families (22). Addition of the inhibitors benzamidine (1 mM), leupeptin (100 μM), E-64 (100 μM), pepstatin (100 μM), and soybean trypsin inhibitor (1 mg/ml) had no effect on microcystin LR degradation. Inhibitory effects were observed with 1,10-phenanthroline (10 mM), which caused >90% inhibition of microcystin LR degradation. 1,10-Phenanthroline at a lower concentration (1 mM) and EDTA at concentrations of 10 and 1 mM did not significantly inhibit the primary degradation reaction, but these compounds completely inhibited a subsequent degradation re-

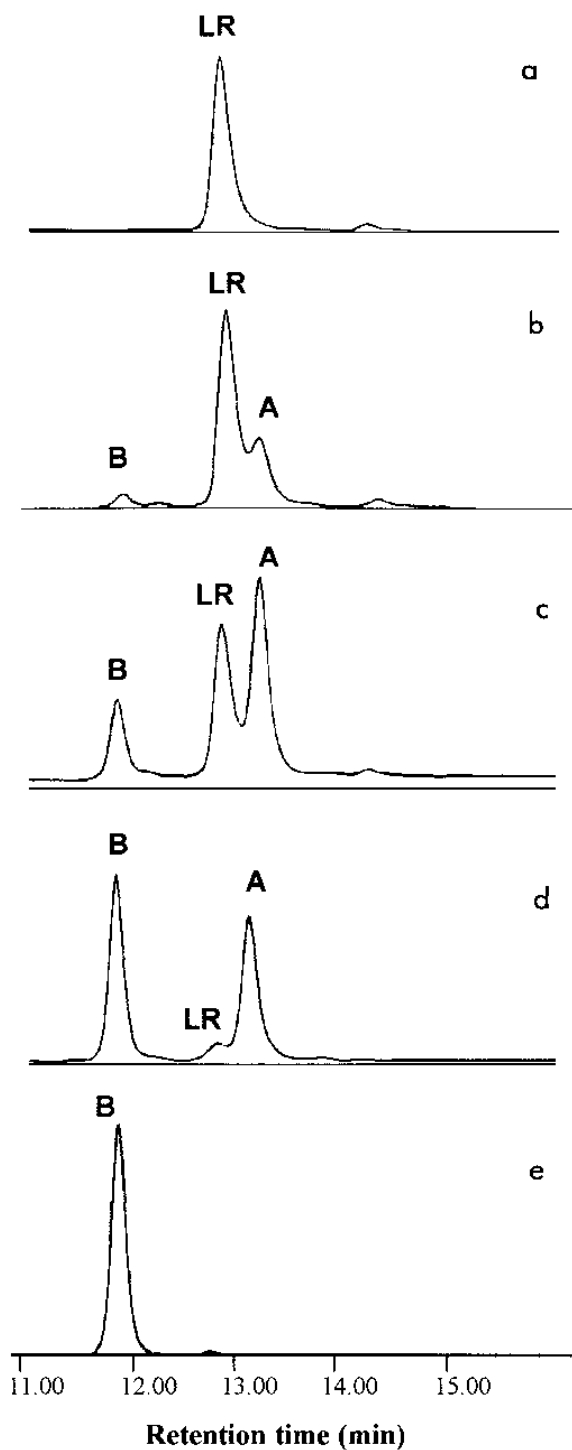


FIG. 1. HPLC chromatograms at time zero (a), 1 h (b), 3 h (c), 8 h (d), and 20 h (e), illustrating degradation of microcystin LR incubated with CE in the presence of EDTA (1 mM). Transient formation of degradation product A occurs before accumulation of degradation product B. As shown previously by Jones et al. (11), the rate of breakdown of microcystin LR was proportional to the CE concentration. In this experiment, an approximately 1/10 dilution of CE was used, which resulted in slower formation of degradation products.

action(s), resulting in transient accumulation of product A and apparent stoichiometric accumulation of product B (assuming that the molar absorptivity of the degradation products is similar to that of the parent microcystin LR) (Fig. 1). Addition of

PMSF (1 mM), an inhibitor of all serine proteases and some cysteine proteases (22, 26), resulted in the accumulation of only degradation product A.

Identification of intermediate degradation products. Degradation products A and B had UV spectra that were very similar, although not identical, to the UV spectrum of the parent microcystin LR. The λ_{\max} of degradation product A was 234 nm (compared with a λ_{\max} of 238 nm for microcystin LR), and product A had a slightly higher relative A_{200} (Fig. 2A). Similarly, degradation product B had a λ_{\max} of 234 nm, but a slightly lower A_{200} than microcystin LR (Fig. 2B). The 4,6-conjugated diene of the Adda amino acid side chain on microcystin contributes to the characteristic spectrum and is an important determinant of the retention and elution characteristics of microcystin LR in reverse-phase HPLC analysis (11). Removal of Adda or destruction of its diene conjugated double bond would produce a compound which is UV transparent at 210 to 240 nm. We concluded, therefore, that following enzymatic modification of microcystin, the Adda side chain remained intact.

Total amino acid determination and ion spray MS (single and triple quadrupole) were used to identify the degradation products. Milligram amounts of degradation products A and B were enzymatically prepared by using appropriate inhibitors and were purified by preparative reverse-phase HPLC, as described previously.

The quantitative results of the amino acid analysis are presented in Table 1. Alanine was used for normalization, and the results are presented as apparent molar ratios relative to alanine. The amino acids glutamate, alanine, arginine, and leucine were identified in total amino acid digests of pure microcystin LR, as expected. A peak identified as β -methylaspartate (Masp) by comparison with the results of Harada et al. (10) was also detected. The Adda residue was not observed in the standard amino acid analysis, while the Mdha residue was

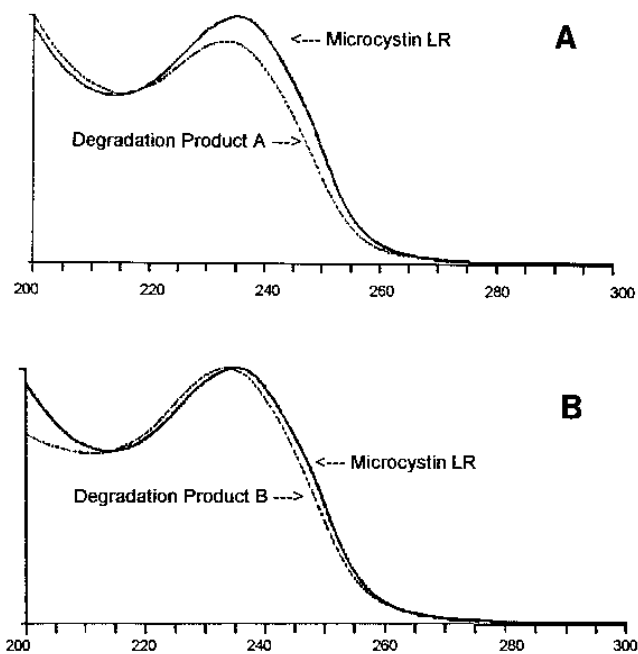


FIG. 2. UV absorption spectra between 200 and 300 nm for microcystin LR and degradation product A (A) and for microcystin LR and degradation product B (B). Spectra were obtained at 5-nm intervals, and the y axis is normalized absorbance.

TABLE 1. Apparent amino acid ratios for microcystin LR and degradation products A and B

Compound	Molar ratio ^a				
	Glutamate	Alanine	Arginine	Leucine	Masp
Microcystin LR	1.20	1.00	0.76	1.07	0.51
Product A	1.20	1.00	0.85	1.11	0.52
Product B	0.69	1.00	— ^b	—	—

^a The level of alanine was defined as 1.00. Unspecified amino acids are present at ratios of <0.05.

^b —, not detected.

converted to methylamine and was only a minor peak, which is consistent with the results of previous studies (10, 19). Degradation product A contained the five major amino acids found in microcystin LR, whereas only glutamate and alanine were identified in degradation product B.

The ion spray mass spectral analysis of microcystin LR revealed a major ion at m/z 995.4, corresponding to the $[M+H]^+$ protonated molecular ion (10, 19). Whereas little fragmentation was observed with the parent (cyclo-) microcystin LR, degradation products A and B showed significant fragmentation, suggesting that they were hydrolyzed linear (acyclo) products of microcystin LR.

Degradation product A exhibited a protonated molecular ion at m/z 1,013.5 and a base peak at m/z 862.0. This, in conjunction with the results of the amino acid analysis, indicated that product A was hydrolyzed (i.e., linearized) microcystin LR ($[M+H_2O+H]^+$). A peak at m/z 507 was consistent with the $[M+H_2O+2H]^{2+}$ structure, while a peak at m/z 1,127.5 was consistent with the trifluoroacetic acid (TFA) adduct $[M+H_2O+H+TFA]^+$. The peak at m/z 862.0 (M-151) corresponds to the loss of the terminal phenylethylmethoxy group from Adda (M-135), a common loss fragment of microcystins (19), plus the amino NH_2 group from Adda due to a radical fragmentation rearrangement. The presence of this ion is thus indicative of the linear microcystin product containing N-terminal Adda.

Degradation product B exhibited a protonated molecular ion at m/z 615 with a minor peak at m/z 598. An $[M+H]^+$ ion at m/z 615 was consistent with the loss of Arg-Masp-Leu from hydrolyzed (acyclo-) microcystin LR ($[M+H_2O+H]^+$, 1,013) and was also consistent with the amino acid analysis data. The ion at m/z 598 corresponds to the loss of ammonia from degradation product B.

To confirm the proposed structures of degradation products A and B, an MS-MS fragmentation analysis was performed with precursor ions m/z 1,013 and m/z 615, respectively. A series of fragment ions arising from CID of the precursor ion was recorded for each compound and for microcystin LR (precursor ion at m/z 995) (Fig. 3 and Tables 2 through 4). The product ions detected for degradation product A were those measured previously for linear (acyclo-) microcystin LR by Choi et al. (6). Ions at m/z 879, 862, 756, and 726 correspond to various fragment losses from Adda, as shown in Table 3. The presence of carboxy-terminal arginine was demonstrated by ions m/z 571 (Mdha-Ala-Leu-Masp-Arg-OH + 2H) and m/z 488 (Ala-Leu-Masp-Arg-OH + 2H) compared with ions which were similar but lacked -OH from microcystin LR MS-MS data (m/z 553 and 470) (Fig. 3A).

Product B exhibited ions at m/z 509, 425, and 297 (Fig. 3C), which corresponded to the sequential loss of amino acids (and the Adda amino group) from the proposed parent compound Adda-Glu-Mdha-Ala-OH. The presence of alanine at the car-

boxy terminus was demonstrated by the fragment ion at m/z 173, which corresponded to Mdha-Ala-OH + 2H. This ion is directly comparable to the ion at m/z 155 (Mdha-Ala) in the product ion CID spectrum of microcystin LR (Fig. 3A). A fragment ion at m/z 173, identified similarly as Mdha-Ala-OH + 2H, was also reported by Choi et al. (6); this ion was derived from the related linear heptapeptide Leu-Masp-Arg-Adda-Glu-Mdha-Ala-OH.

Mass spectral data, together with amino acid analysis data, indicate that degradation product A was linear (acyclo-) microcystin LR with an N-terminal Adda moiety [i.e., NH_2 -Adda-Glu(iso)-Mdha-Ala-Leu-Masp-Arg-OH] and that degradation product B was the tetrapeptide NH_2 -Adda-Glu(iso)-Mdha-Ala-OH.

Toxicity of degradation products. Mouse bioassay analyses showed that both degradation products were nontoxic at doses up to 250 μg kg of mouse⁻¹. This compares with a 50% lethal dose of 50 μg kg⁻¹ for (cyclo-) microcystin LR (14). A lack of larger amounts of purified products prevented mouse dosing at higher concentrations.

The molecular activity of the two degradation products was tested by performing an in vitro protein phosphatase activity assay. Both linear peptide degradation products exhibited significantly less activity toward chicken brain protein phosphatase than the parent (cyclo-) microcystin LR (Fig. 4). The observed 50% inhibitory concentrations for linear (acyclo-) microcystin LR (95 nM) and the linear tetrapeptide (12 nM) were 160 and 20 times lower, respectively, than the 50% inhibitory concentration of microcystin LR (0.6 nM).

DISCUSSION

Previously, we reported that the bacterium isolated from irrigation drainage water with the ability to degrade the cyanobacterial toxin microcystin LR appeared to be a member of the genus *Pseudomonas*. Subsequent chemotaxonomic work has confirmed that the organism is a new species in the genus *Sphingomonas* (unpublished data). Although this *Sphingomonas* sp. was originally isolated by classical enrichment culture techniques, with microcystin LR as the sole carbon and nitrogen source, microcystinase activity was subsequently expressed constitutively at high levels in cells grown in peptone-yeast extract medium in the absence of microcystin (11). This at first seemed a little surprising. However, we now believe that the microcystin is recognized by the *Sphingomonas* sp. as an oligopeptide similar to the oligopeptides found in peptone, which is a complex mixture of linear and, probably, cyclic oligopeptides. From the viewpoint of microcystin LR degradation, the enzymes are present fortuitously and are required for normal cellular growth and metabolism when the bacterium is grown on peptone medium.

The appearance of intermediate degradation products A and B during assays performed in the presence of EDTA suggested that a suite of enzymes was present in the CE and that these enzymes acted sequentially to degrade microcystin LR. From the inhibitor studies, we postulate that at least three enzymes are involved in the breakdown of microcystin LR. These are enzyme 1, which catalyzes the conversion of microcystin to product A; enzyme 2, which catalyzes the conversion of product A to product B; and enzyme 3, which catalyzes the breakdown of product B.

The combination of mass spectral data, MS-MS data, and amino acid analysis data enabled us to identify both isolated degradation products. The MS molecular ions at m/z 1,013 for product A and at m/z 615 for product B supported the amino acid analysis data (Table 1) in defining the compounds as

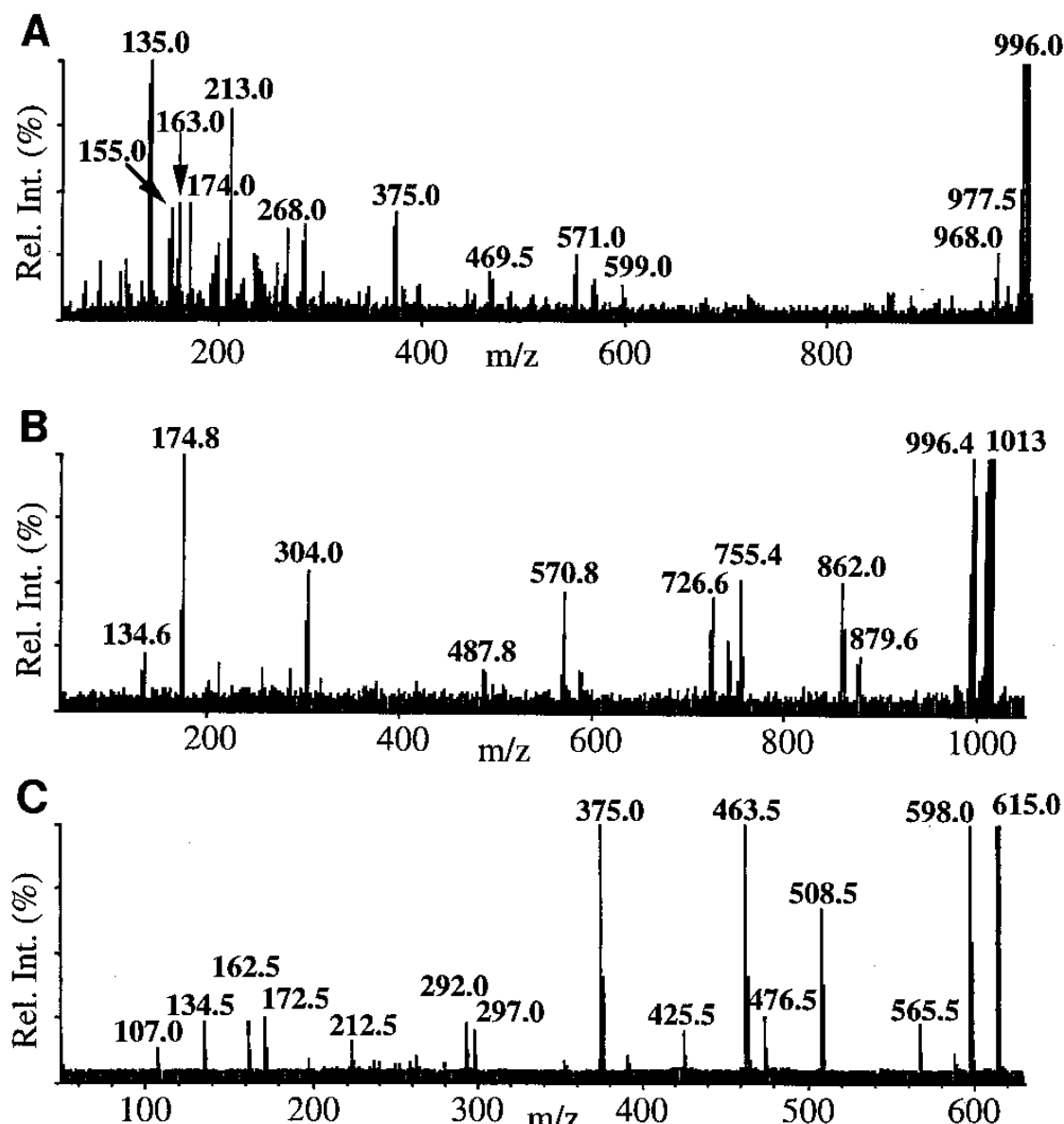


FIG. 3. CID MS-MS spectra of microcystin LR (A), degradation product A (B), and degradation product B (C). Rel. Int., relative intensity.

linearized (acyclo-) microcystin LR (N-terminal Adda) and the tetrapeptide $\text{NH}_2\text{-Adda-Glu(iso)-Mdha-Ala-OH}$, respectively. The fragment ions in the MS-MS spectra (Fig. 3) for both compounds could be assigned to predicted or previously reported fragment ions for microcystin LR hydrolysis products (6) and confirmed these identifications.

The mass spectra of degradation products A and B also revealed a major fragment ion at $M^+ - 151$ rather than at $M - 135$, which corresponds to the loss of $\text{PhCH}_2\text{CHCOCH}_3$ from Adda in (cyclo-) microcystins (19). We suggest that this ion corresponds to a loss of $\text{PhCH}_2\text{CHCOCH}_3$ from Adda plus the Adda N-terminal amino group ($M - 16$) of the hydrolyzed microcystin because of electron delocalization from the 4,6-conjugated diene. A series of fragment ions corresponding to the loss of the Adda amino group can be observed in the MS-MS data for both degradation product A and degradation product B (Fig. 3). Along with other MS-MS data this provides

TABLE 2. MS-MS parent ion and daughter ions for microcystin LR

m/z	Identity
995	M + H (cyclo Adda-Glu-Mdha-Ala-Leu-Masp-Arg)
599	Arg-Adda-Glu + H
553	Mdha-Ala-Leu-Masp-Arg + H
470	Ala-Leu-Masp-Arg + H
398	Mdha-Ala-Leu-Masp + H
375	$\text{C}_{11}\text{H}_{14}\text{O-Glu-Mdha} + \text{H}$
292	$\text{C}_{11}\text{H}_{14}\text{O-Glu} + \text{H}$
268	Mdha-Ala-Leu + H
213	Glu-Mdha + H
163	$\text{C}_{11}\text{H}_{14}\text{O} + \text{H}$
155	Mdha-Ala + H
135	$\text{PhCH}_2\text{CHOME}$

TABLE 3. MS-MS parent ion and daughter ions for degradation product A

<i>m/z</i>	Identity
1,013.....	M + H (Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH + H)
996.....	M + H - NH ₃
879.....	M + H - PhCH ₂ CHOMe
862.....	M + H - NH ₂ - PhCH ₂ CHOMe
756.....	CH ₃ CHCO-Glu-Mdha-Ala-Leu-Masp-Arg-OH + 2H
726.....	CO-Glu-Mdha-Ala-Leu-Masp-Arg-OH - 2H
571.....	Mdha-Ala-Leu-Masp-Arg-OH + 2H
488.....	Ala-Leu-Masp-Arg-OH + 2H
304.....	Masp-Arg-OH + 2H
286.....	Masp-Arg + H
213.....	Glu-Mdha + H
175.....	Arg-OH + 2H
135.....	PhCH ₂ CHOMe

evidence which identifies the carboxy-terminal arginine and alanine in degradation products A and B, respectively, evidence that Adda is N terminal and, therefore, evidence that microcystin hydrolysis occurs at the Adda-Arginine peptide bond.

Further support for this proposition can be obtained by comparing our results with the results of Choi et al. (6) for several linear peptides isolated from *M. aeruginosa*. We defined degradation product A as acyclo-microcystin LR on the basis of hydrolysis of a single peptide bond in the parent cyclic peptide. The tetrapeptide product B is derived by further hydrolysis of product A. Therefore, product A corresponds to one of two possible linear heptapeptides on the basis of hydrolysis of microcystin LR at either the Adda-Arg or Ala-Leu peptide bond. Choi et al. (6) purified both possible linear heptapeptides, and each of their sequences was established by fast atom bombardment MS-CID MS, which resulted in different fragmentation patterns. The fragmentation pattern of product A illustrated in Fig. 3B determined by ion spray MS-CID MS contains several prominent ions identical to those in the fast atom bombardment MS-CID MS spectrum of Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH (6). This includes peaks at *m/e* 571 (Mdha-Ala-Leu-Masp-Arg-OH), 488 (Ala-Leu-Masp-Arg-OH), 304 (Masp-Arg-OH), 175 (Arg-OH), and 135 (PhCH₂CHOCH₃), as well as peaks at *m/e* 726 and 756 corresponding to C-C fragmentation in the N-terminal Adda. The arginine peak (*m/e* 175) of product A is very prominent, which is consistent with a C-terminal position, while there is no peak for a C-terminal Mdha-Ala-OH fragment (*m/e* 173). In con-

TABLE 4. MS-MS parent ion and daughter ions for degradation product B

<i>m/z</i>	Identity
615.....	M + H (Adda-Glu-Mdha-Ala-OH + H)
598.....	M + H - NH ₃
566.....	Adda (- NH ₂ - MeOH)-Glu-Mdha-Ala-OH + H
509.....	Adda (- NH ₂)-Glu-Mdha + H
477.....	Adda (- NH ₂ - MeOH)-Glu-Mdha + H
464.....	M + H - PhCH ₂ CHOMe - NH ₂
426.....	Adda (- NH ₂)-Glu + H
375.....	C ₁₁ H ₁₄ O-Glu-Mdha + H
297.....	Adda (- NH ₂) + H
292.....	C ₁₁ H ₁₄ O-Glu + H
213.....	Glu-Mdha + H
173.....	Mdha-Ala-OH + 2H
163.....	C ₁₁ H ₁₄ O + H
135.....	PhCH ₂ CHOMe

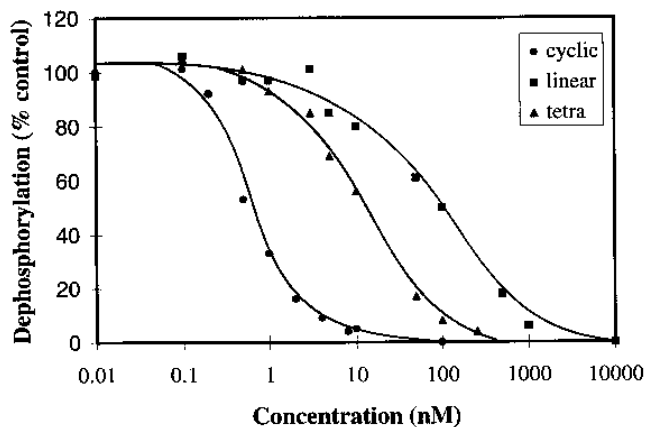


FIG. 4. Protein phosphatase inhibition assay for microcystin LR (cyclic) and its degradation products, linear microcystin LR (linear) and the tetrapeptide NH₂-Adda-Glu(iso)-Mdha-Ala-OH (tetra).

trast, the electrospray interface MS-CID MS spectrum of product A (Fig. 3B) has no peaks in common with the fast atom bombardment MS-CID MS spectrum of Leu-Masp-Arg-Adda-Glu-Mdha-Ala determined by Choi et al. (6). These comparisons establish without a doubt that the acyclo heptapeptide product A has an N-terminal Adda residue and that the initial hydrolytic cleavage of microcystin LR by microcystinase takes place at the Adda-Arg peptide bond.

Addition of the inhibitors benzamidine, leupeptin, E-64, pepstatin, and soybean trypsin inhibitor had no effect on microcystin LR degradation. Benzamidine and soybean trypsin inhibitor are both reversible inhibitors of the trypsin-like enzymes of the serine family. Leupeptin also inhibits trypsin-like serine proteases and most cysteine proteases. Leupeptin and E-64 are inhibitors of proteases of the cysteine family, while pepstatin is a specific inhibitor of aspartic proteases, including cathepsin D, pepsin, and renin (20, 26). Since these inhibitors had no effect on degradation, these protease families were not considered active in the degradation of microcystin LR. EDTA and 1,10-phenanthroline target metalloproteases and metal-activated proteases by chelating or sequestering the active site metal atom, commonly zinc (26). Thus, it appeared that both enzyme 1 and enzyme 3 are metalloproteases or metal-activated proteases and that enzyme 3 is more susceptible to inhibition by EDTA. Enzyme 2, which was inhibited by PMSF, was assumed not to be a cysteine protease because of a lack of inhibition by leupeptin and E-64, but rather was assumed to be a serine protease. Serine proteases may be divided into two classes, mammalian serine protease family I (including chymotrypsin and trypsin) and bacterial serine protease family II, which includes subtilisin (20). Since the inhibitors benzamidine, leupeptin, and soybean trypsin inhibitor, which are specific for trypsin-like enzymes belonging to serine protease family I, had no effect, enzyme 2 was suspected to be a member of bacterial serine protease family II. These families differ from each other in their three-dimensional structure despite having a common active site geometry and enzymatic mechanism (20).

On the basis of the product identifications and the results of the inhibitor studies, a pathway for microcystin LR degradation can be proposed (Fig. 5). In this pathway, the three enzyme activities identified act sequentially to degrade microcystin LR. In this paper we refer to enzyme 1, which is responsible for the ring opening of microcystin LR, as microcystinase.

The environmental significance of microcystinase is highlighted by the 160-fold reduction in the activity of the parent

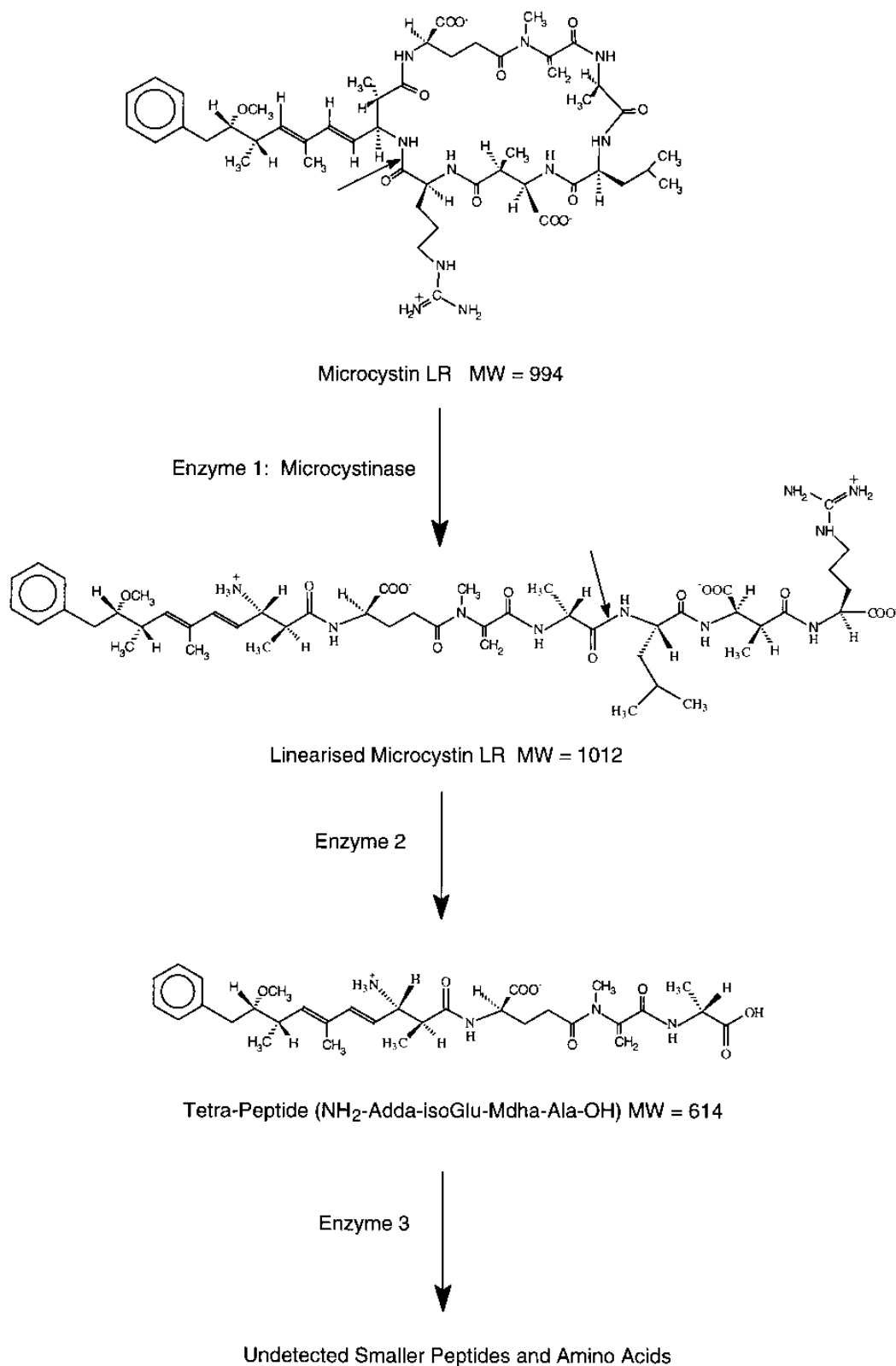


FIG. 5. Proposed degradation pathway of microcystin LR, showing the formation of identified intermediate products. The small arrows indicate sites of peptide hydrolysis. MW, molecular weight.

(cyclic) microcystin LR when it is converted to the linear (acyclo) structure. This is presumably due to an alteration in the interaction between the molecule and protein phosphatase. It is interesting that the tetrapeptide product is more toxic than the linear microcystin and is only about 20 times less toxic than the cyclic form (Fig. 4). It is known that the conjugated diene on Adda, with 4E,6E stereochemistry, and the intact glutamate residue are essential features for maintenance of protein phosphatase interaction and toxicity (1). Alterations to other portions of the molecule have little effect on toxicity, except in the case of the doubly arginine-substituted molecule microcystin RR, which is about 5 to 10 times less toxic than most other microcystins (1). Rudolph-Bohner et al. (25) deduced three possible molecular structures for microcystin LY. These authors demonstrated that a consistent feature of the predicted structures was a compact planar array of the peptide backbone Adda-Glu-Mdha. Both linear microcystin LR degradation products have this backbone. The crystal structure of the protein phosphatase 1 (PP-1)-microcystin LR complex was published recently (9), and microcystin interactions with PP-1 were found in three distinct regions. The Adda residue of microcystin LR fills a hydrophobic groove at the PP-1 active site, leucine interacts with the C-terminal groove, and water bridges provide indirect coordination with two metal ions at the active site by the carboxylate group and carbonyl oxygen of microcystin LR. A covalent linkage formed between Mdha and an adjacent cysteine residue on PP-1, which contributed to the stability of the toxin-enzyme complex, was judged to be not essential for inhibition on the basis of prior blocking of the thiol group on the enzyme (30) and blockage of the reactive Mdha group in microcystin (18). The conformation of microcystin when it is bound to the active site of PP-1 (9) is very similar to its conformation in solution (2). This probably increases the affinity of microcystin for PP-1 by reducing the entropy lost upon binding. The tetrapeptide can preserve the enzyme-inhibitor interactions involving the Adda side chain, glutamate, and Mdha residues. The three extra residues in the linear peptide presumably provide little extra binding energy but have a significant entropic cost. Hence, the tetrapeptide has a stronger affinity for PP-1 than the acyclo-microcystin has.

Because of the increasing concern regarding cyanobacterial blooms in reticulated water supplies, the persistence of toxins in such supplies is of particular importance. A recent study described the persistence of microcystins in natural waters for more than 1 month before the endemic bacterial population degraded the toxins to safe levels (13). Degradation of microcystins by complex natural populations of microorganisms obtained from diverse ecosystems, such as sewage effluent (16), lake sediment, and water (11, 23), has been demonstrated previously. We have partially described a pathway by which an isolated *Sphingomonas* sp. strain utilizes at least three enzymes to metabolize the cyanobacterial toxin microcystin LR. Whether there are other enzymes in this pathway or indeed if there are other pathways for microcystin degradation is uncertain at this stage. For example, using HPLC alone, we would not be able to detect any pathway which involved oxidation of the 4,6-diene or hydrolytic cleavage of Adda from the peptide. However, the apparent stoichiometric formation of linear microcystin LR and tetrapeptide suggests that this is the only microcystin breakdown pathway in this *Sphingomonas* strain. The first enzyme in the pathway, microcystinase, appears to be the most important not only because it opens the otherwise highly stable cyclic peptide, but also because, as a consequence of this, it results in a 160-fold reduction in the activity of the molecule. We also have characterized the molecular genetics of the degradative enzymes, and microcystinase appears to be

a novel metalloprotease (unpublished data). It is hoped that the bacterium or specific enzymes can be used in a bioremediation process to remove toxins from important water supplies.

REFERENCES

1. An, J., and W. W. Carmichael. 1994. Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. *Toxicol* **32**:1495-1507.
2. Bagu, J. R., F. D. Sonnichsen, D. Williams, R. J. Anderson, B. D. Sykes, and C. F. B. Holmes. 1995. Comparison of the solution structures of microcystin-LR and motuporin. *Nature (London) Struct. Biol.* **2**:114-116.
3. Carmichael, W. W. 1992. Cyanobacteria secondary metabolites—the cyanotoxins. *J. Appl. Bacteriol.* **72**:445-459.
4. Carmichael, W. W. 1994. The toxins of cyanobacteria. *Sci. Am.* **270**:64-72.
5. Carmichael, W. W., C. L. A. Jones, N. A. Mahmood, and W. C. Theiss. 1985. Algal toxins and water based diseases. *Crit. Rev. Environ. Control.* **15**:275-313.
6. Choi, B. W., M. Namikoshi, F. Sun, K. L. Rinehart, W. W. Carmichael, A. M. Kaup, W. R. Evans, and V. R. Beasley. 1993. Isolation of linear peptides related to the hepatotoxins nodularin and microcystins. *Tetrahedron Lett.* **34**:7881-7884.
7. Falconer, I. R. 1991. Tumour promotion and liver injury caused by oral consumption of cyanobacteria. *Environ. Toxicol. Water Qual.* **6**:177-184.
8. Falconer, I. R., and S. K. Yeung. 1992. Cytoskeletal changes in hepatocytes induced by *Microcystis* toxins and their relation to hyperphosphorylation of cell proteins. *Chem. Biol. Interact.* **81**:181-196.
9. Goldberg, J., H. Huang, Y. Kwon, P. Greengard, A. C. Nairn, and J. Kuriyan. 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature (London)* **376**:745-753.
10. Harada, K., K. Matsuura, M. Suzuki, M. F. Watanabe, S. Oishi, A. M. Dahlem, V. R. Beasley, and W. W. Carmichael. 1990. Isolation and characterisation of the minor components associated with microcystin LR and RR in the cyanobacterium (blue-green algae). *Toxicol* **28**:55-64.
11. Jones, G. J., D. G. Bourne, R. L. Blakeley, and H. Doelle. 1994. Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Nat. Toxins* **2**:228-235.
12. Jones, G. J., I. F. Falconer, and R. M. Wilkins. 1995. Persistence of the cyclic peptide toxins in dried cyanobacterial crusts from Lake Mokoan, Australia. *Environ. Toxicol. Water Qual.* **10**:19-24.
13. Jones, G. J., and P. T. Orr. 1994. Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Res.* **28**:871-876.
14. Krishnamurthy, T., W. W. Carmichael, and E. W. Sarver. 1986. Toxic peptides from freshwater cyanobacteria (blue-green algae). I. Isolation and purification of peptides from *Microcystis aeruginosa* and *Anabaena flos-aquae*. *Toxicol* **24**:865-873.
15. Krishnamurthy, T., L. Szafraniec, D. F. Hunt, J. Shabanowitz, J. R. Yates, C. R. Hauer, W. W. Carmichael, O. Skulberg, G. A. Codd, and S. Missler. 1989. Structural characterization of toxic cyclic peptides from blue-green algae by tandem mass spectrometry. *Proc. Natl. Acad. Sci. USA* **86**:770-774.
16. Lam, A. K. Y., P. M. Fedorak, and E. E. Prepas. 1995. Biotransformation of the cyanobacterial hepatotoxin microcystin LR, as determined by HPLC and protein phosphatase bioassay. *Environ. Sci. Technol.* **29**:242-246.
17. MacKintosh, C., K. A. Beattie, S. Klumpp, P. Cohen, and G. A. Codd. 1990. Cyanobacterial microcystin LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **264**:187-192.
18. Moorhead, G., R. W. MacKintosh, N. Morrice, T. Gallagher, and C. MacKintosh. 1994. Purification of type 1 protein (serine/threonine) phosphatases by microcystin-Sepharose affinity chromatography. *FEBS Lett.* **356**:46-50.
19. Namikoshi, M., K. L. Rinehart, and R. Sakai. 1992. Identification of 12 hepatotoxins from Homer Lake bloom of cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. *J. Org. Chem.* **57**:866-872.
20. Neurath, H. 1982. The diversity of proteolytic enzymes, p. 1-12. In R. J. Beynon and J. S. Bond (ed.), *Proteolytic enzymes: a practical approach*. IRL Press, Oxford.
21. Nishiwaki-Matsushima, R., T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W. W. Carmichael, and H. Fujike. 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J. Cancer Res. Clin. Oncol.* **118**:420-424.
22. North, M. J. 1982. Comparative biochemistry of the proteinases of eucaryotic microorganisms. *Microbiol. Rev.* **46**:308-340.
23. Rapala, J., K. Lahti, K. Sivonen, and S. I. Niemela. 1994. Biodegradability and adsorption on lake sediments of cyanobacterial hepatotoxins and anatoxin-a. *Letts. Appl. Microbiol.* **19**:423-428.
24. Rinehart, K. L., M. Namikoshio, and B. W. Choi. 1994. Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). *J. Appl. Phycol.* **6**:159-176.

25. **Rudolf-Bohner, S., D. F. Mierke, and L. Moroder.** 1994. Molecular structure of the cyanobacterial tumour-promoting microcystins. *FEBS Lett.* **349**:319–323.
26. **Salvesen, G., and H. Nagase.** 1982. Inhibition of proteolytic enzymes, p. 83–102. *In* R. J. Beynon and J. S. Bond (ed.), *Proteolytic enzymes: a practical approach*. IRL Press, Oxford.
27. **Shun-Zhang, Y.** 1989. Drinking water and primary liver cancer, p. 30. *In* Z. G. Tang, M. C. Wu, and S. S. Xia (ed.), *Primary liver cancer*. Springer-Verlag, Berlin.
28. **Sivonen, K., M. Namikoshi, W. R. Evans, W. W. Carmichael, F. Sun, L. Rouhiainen, R. Luukkainen, and K. L. Rinehart.** 1992. Isolation and characterization of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. *Appl. Environ. Microbiol.* **58**:2495–2500.
29. **Yoshizawa, S., R. Matsushima, M. F. Watanabe, K. Harada, A. Ichihara, W. W. Carmichael, and H. Fujiki.** 1990. Inhibition of protein phosphatases by microcystin and nodularin associated with hepatotoxicity. *J. Cancer Res. Clin. Oncol.* **116**:609–614.
30. **Zhang, Z., S. Zhao, S. Deans-Zirattu, G. Bai, and E. Y. C. Lee.** 1993. Mutagenesis of the catalytic subunit of rabbit muscle protein phosphatase-1. *Mol. Cell. Biochem.* **128**:113–119.