

Co-production of Microcystins and Aeruginopeptins by Natural Cyanobacterial Bloom

Ken-ichi Harada,¹ Tsuyoshi Mayumi,¹ Takayuki Shimada,¹ Kiyonaga Fujii,¹ Fumio Kondo,² Ho-dong Park,³ Mariyo F. Watanabe⁴

¹Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468-8503, Japan

²Aichi Prefectural Institute of Public Health, Kita, Nagoya 462-8576, Japan

³Department of Environmental Science, Faculty of Science, Shinshu University, Matsumoto 390-0802, Japan

⁴Department of Environmental System, Faculty of Geo-environmental Sciences, Rissho University, 1700 Magechi, Kumagaya, Saitama 360-0194, Japan

Received 22 August 2000; revised 20 December 2000; accepted 28 March 2001

ABSTRACT: The relationship between *Microcystis* composition and the production of microcystins and nontoxic peptides in bloom cells, which was regularly collected in Lake Suwa, Japan, in the summer season from 1991 to 1994, was investigated. In order to determine the structures of the nontoxic peptides, we collected large amounts of bloom materials from the same lake on July 23, 1991, and isolated three nontoxic peptides. They were named as aeruginopeptins 917S-A, -B, and -C, and their structures were mainly determined by a mass spectrometry/mass spectrometry (MS/MS) technique as 19-membered cyclic depsipeptides possessing the Ahp (3-amino-6-hydroxy-2-piperidone) moiety. An analysis of the microcystins and aeruginopeptins in the collected bloom cells and their *Microcystis* composition suggested that the *M. aeruginosa* large cell size produces both microcystins and aeruginopeptins, and the production of both compounds is genetically closely related. © 2001 by John Wiley & Sons, Inc. *Environ Toxicol* 16: 298–305, 2001

Keywords: *Microcystis*; microcystins; aeruginopeptins; Lake Suwa; MS/MS

INTRODUCTION

Toxic blooms of cyanobacteria occur in eutrophicated lakes, ponds, and rivers all over the world. Cyanobacteria have produced acute toxins such as hepatotoxic peptides, microcystins, and nodularins, and neurotoxic alkaloids, anatoxin-a, anatoxin-a(s), and saxitoxins (Sivonen and Jones, 1999). Another hepatotoxin, cylin-

drospormopsin, was also isolated from several cyanobacteria such as *Cylindrospermopsis* and *Umezakia*. *Microcystis*, *Oscillatoria* (*Planktothrix*), *Anabaena*, *Aphanizomenon*, and *Nodularia* produce these toxins and have been responsible for the death of wild and domestic animals by drinking water contaminated with blooms (Kuiper-Goodman et al., 1999). The principal species under investigation is *Microcystis aeruginosa*, which has been frequently cited in animal poisoning incidents and produces potently hepatotoxic microcystins. The toxins consist of seven amino acids, and approximately 60

Correspondence to: Ken-ichi Harada; e-mail: kiharada@meijo-u.ac.jp.

variants have been isolated so far (Sivonen and Jones, 1999; Harada, 1996). They inhibit protein phosphatases 1 and 2A in a manner similar to okadaic acid and have a tumor-promoting activity on the rat liver (Kuiper-Goodman et al., 1999). In 1996 a toxic accident causing the death of 50 patients occurred in Brazil due to microcystins in the water used for haemodialysis (Jochimsen et al., 1998; Pouria et al., 1998).

Microcystins are known to be produced by four genera of cyanobacteria, *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), and *Nostoc* (Sivonen and Jones, 1999). Recently, it was found that these genera also produce other types of nontoxic peptides in addition to microcystins. They show characteristic biological activities, which are structurally classified into several groups: 19-membered cyclic depsipeptides possessing a 3-amino-6-hydroxy-2-piperidone (Ahp) moiety, 19-membered cyclic peptides possessing an ureido linkage, tricyclic depsipeptides, and linear peptides (Namikoshi and Rinehart, 1996). In order to elucidate the biosynthetic relationship between hepatotoxic peptides, microcystins and nodularin, and other nontoxic peptides, we have carried out the isolation and structural determination of nontoxic peptides produced together with hepatotoxic peptides by the toxic and nontoxic cyanobacterial genera, *Microcystis* (Harada et al., 1993), *Anabaena* (Fujii et al., 1996; Harada et al., 1995), *Oscillatoria* (*Planktothrix*) (Fujii et al., 2000a), *Nostoc* (Fujii et al., 1999), and *Nodularia* (Fujii et al., 1997a; 1997b). From these experiments the following findings were obtained: (1) nontoxic peptides are always produced by toxic cyanobacteria that co-produce hepatotoxic peptides; (2) no such peptides were detected from neurotoxic cyanobacteria that produce anatoxin-a; and (3) other types of compounds than peptides are isolated from nontoxic cyanobacteria. In these experiments, laboratory strains have been always used.

We also studied the relationship between the cyanobacterial composition and the concentration of microcystin in the warm season for 4 years (1991–1994) in a eutrophic lake, Lake Suwa, Japan (Park et al., 1998). In this lake *Microcystis* has been usually predominant in the warm season, and the species composition of *Microcystis* rapidly changed during this experiment period. The concentration of microcystin was closely related to the species composition of *Microcystis*. That is, when *Microcystis aeruginosa* was predominant, the concentration of microcystin was relatively high, whereas its concentration was diminished when other species such as *M. viridis* and *M. wesenbergii* were dominant. During this experiment, we also found that a few compounds other than microcystins were present in these bloom samples, whose structures were similar to

those of aeruginopeptins from two laboratory strains of toxic *M. aeruginosa* (Harada et al., 1993). In this report, we describe the isolation and structural determination of nontoxic peptides from bloom samples and the relationship between the production of microcystins and isolated nontoxic peptides, and the species composition of *Microcystis*.

MATERIALS AND METHODS

Cell Materials

Microcystis cells for the determination of the microcystin and aeruginopeptin content and cell composition were collected from the center of Lake Suwa at 10-day intervals from June to October in the years 1991–1994, using a plankton net (40- μ m mesh size). The cells were lyophilized before use. *Microcystis* cells for isolation of the aeruginopeptins were collected in Lake Suwa on July 23, 1991, and they were also lyophilized before use.

Toxicity and Enzyme Inhibitory Tests

The toxicity of aeruginopeptins 917S was tested according to Kondo et al.'s method (1992). Serine protease inhibitory activity assay was carried out according to Shin et al.'s method (1996).

Isolation of Aeruginopeptins 917S-A, -B, and -C

Lyophilized cells (100 g) were extracted three times with 2 L of 5% acetic acid (aq.) for 30 min while stirring. The combined extracts (6 L) were centrifuged for 5 min at 15,000g and then the supernatant was applied to a preconditioned octadecylsilylanized (ODS) silica gel column (120 \times 40 mm ID, Chromatorex ODS, Fuji Silsia Chemical, Tokyo, Japan). The column was rinsed with water (1 L) and 20% methanol–water (2 L) and then eluted with methanol (2 L) to give a fraction containing the microcystins and aeruginopeptins. The desired fraction was separated to give 917S-A (25.2 mg), -B (11.7 mg), and -C (6.5 mg) by the combination of the following chromatographies: silica gel; chloroform:methanol:water (65:25:5 and 65:15:5, lower phase), ethyl acetate:isopropanol:water (8:1:2, upper layer) as the mobile phase and TOYOPEARL HW-40F (900 \times 11 mm ID); methanol as the mobile phase. The three compounds were obtained as a colorless amorphous powder and their principal physicochemical properties are shown in Table I.

Structural Determination of Aeruginopeptins 917S-A, -B, and -C

The molecular weights of the three aeruginopeptins were determined by fast atom bombardment mass spectrometry (FABMS) using the following conditions: instrument, JEOL (Tokyo, Japan) JMS-HX110; primary ion, xenon; accelerating voltage, 10 kV (primary), 5 kV (secondary); matrix, glycerol. The ^1H - and ^{13}C -NMR spectra were measured using the following conditions: instrument, JEOL JMN-GX400, JEOL JMN-GSX400, and Bruker AMX-500; solvent, CD_3OD (for ^1H -NMR), $^{12}\text{CD}_3\text{OD}$ (for ^{13}C -NMR); reference, tetramethylsilane. The sequence of the constituent amino acids were determined using the product ion spectra obtained by tandem mass spectrometry under the following conditions: instrument, JEOL JMS-HX110/HX110; primary ion, xenon; accelerating voltage, 10 kV (primary), 5 kV (secondary); matrix, glycerol; collision gas, helium; floating cell, 3.03 kV. The specific rotation was recorded at 27°C at the sodium D line. The absolute configuration of constituent amino acids was determined using Marfey's method (Marfey, 1985). One hundred micrograms of aeruginopeptin was hydrolyzed at 110°C for 24 hr with 50 μL of 6 *M* hydrochloric acid. The solution was evaporated to dryness and then dissolved in 50 μL of water. To this amino acid solution was added 50 μL of 1 *M* sodium bicarbonate and then 100 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in acetone. This solution was vortexed and incubated at 35°C for 1 hr and then was quenched by the addition of 50 μL of 1 *M* hydrochloric acid. After dilution with acetonitrile (800 μL), 1 μL of the FDAA derivative was analyzed using the following high-performance liquid chromatography (HPLC) conditions: pump, TOSOH CCPS pumps (Tokyo, Japan); detector, TOSOH UV-8020; column, Cosmosil

5Ph (150 \times 4.6 mm ID, Nacalai Tesque, Kyoto, Japan); column oven, 40°C; mobile phase, (A) 0.1 *M* ammonium acetate (aq., pH 3) and (B) acetonitrile under linear gradient conditions; (B) 15 \rightarrow 55%; flow rate, 1.0 mL/min; detection, UV (340 nm).

Determination of Concentrations of Microcystins and Aeruginopeptins in Collected Cyanobacteria

Lyophilized cells (50–100 mg) were extracted three times with 10 mL of 5% acetic acid (aq.) for 30 min with stirring. The combined extracts were centrifuged for 5 min at 1700*g* and the supernatant was applied to a preconditioned ODS silica gel cartridge (0.5 g, Bakerbond SPE 7020-03, Phillipsberg, NJ). The cartridge was rinsed with water (10 mL) and 20% methanol–water (15 mL) and then eluted with methanol (15 mL) to give a fraction containing microcystins and aeruginopeptins. The desired fraction was subjected to HPLC under the following conditions: for microcystins; pump, Shimadzu LC-9A (Kyoto, Japan); detector, Shimadzu SPD-6A, Cosmosil 5C18 column (150 \times 4.6 mm ID, Nacalai Tesque, Kyoto, Japan); mobile phase, methanol: 0.05 *M* phosphate buffer (pH 3.0) = 58:42; flow rate was 1.0 mL/min; detection, ultraviolet (UV) (238 nm) and for aeruginopeptins; pump, JASCO 880PU (Tokyo, Japan); detector, JASCO 875UV; column, Cosmosil 5Ph (150 \times 4.6 mm ID, Nacalai Tesque); mobile phase, 0.1 *M* ammonium acetate : acetonitrile = 68:32; flow rate, 1.0 mL/min; detection, UV (230 nm). The quantification was carried out using standard solutions (20, 60, and 100 ng/5 mL in methanol) and (100, 300, and 500 ng/5 mL in methanol) of microcystins and aeruginopeptins, respectively.

TABLE I. Physicochemical properties and constituent amino acids of aeruginopeptins 917S-A, -B, and -C

Peptide	Molecular Formula (High Resolution MS Data) Molecular Weight	$[\alpha]_D$	UV (MeOH) λ_{max} (nm) (ϵ)	Constituent Amino Acid ^a
A	$\text{C}_{54}\text{H}_{72}\text{N}_8\text{O}_{15}$ (<i>m/z</i> 1055.5060, $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$, $\Delta - 3.0$ mmu) 1072	-20.5° (c 0.164, MeOH)	278 (4586) 225 (22869) ^b	L-Thr, L-Glu, L-Ile, L-Tyr, L-Leu
B	$\text{C}_{54}\text{H}_{76}\text{N}_8\text{O}_{15}$ (<i>m/z</i> 1041.5370, $[\text{M} + \text{H} - 2\text{H}_2\text{O}]^+$, $\Delta - 7.0$ mmu) 1076	-33.2° (c 0.127, MeOH)	277 (3997) 224 (21904) ^b	L-Thr, L-Glu, L-Ile, L-Leu
C	$\text{C}_{51}\text{H}_{74}\text{N}_8\text{O}_{14}$ (<i>m/z</i> 1005.5060, $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$, $\Delta + 1.0$ mmu) 1022	-40.3° (c 0.118, MeOH)	276 (2516) 224 (16431) ^b	L-Thr, L-Glu, L-Ile, L-Leu

^aThese were confirmed by Marfey method.

^bShoulder.

RESULTS

In a preliminary experiment, small amounts of lyophilized cells collected from Lake Suwa, Japan, on July 23, 1991, were subjected to the usual analysis procedure including extraction with 5% acetic acid, clean-up using an ODS silica gel cartridge and analysis by thin layer chromatography (TLC) and HPLC (Harada et al., 1988a; 1988b). Figure 1 shows a typical HPLC chromatogram of the desired fraction from a collected sample, suggesting that three unknown compounds were present together with microcystins-LR and -RR in this bloom material. In order to isolate these unknown compounds, lyophilized cells (100 g) were treated according to the procedure shown in Materials and Methods based on the results mentioned above. Finally, each isolated compound was purified using TOYOPEARL HW-40F to give A (25.2 mg), B (11.7 mg), and C (6.5 mg).

They showed no toxicity to mice up to 1 mg/kg and a very weak inhibitory activity to chymotrypsin. The physicochemical properties of the isolated compounds are summarized in Table I. They were designated as aeruginopeptins 917S-A, -B, and -C because their structures are very similar to those of the four aeruginopeptins as described earlier (Harada et al., 1993), and they were isolated from the cyanobacteria in Lake Suwa on July, 1991 (917S). Aeruginopeptin 917S-A is a peptide compound with a molecular weight of 1072,

and its composition was established by high-resolution positive ion fast atom bombardment mass spectrometry (FABMS). The constituent amino acids detected from the acid hydrolyzate were Thr, Glu, Ile, Tyr, and Leu, and they had all the L-configuration based on the analysis results using Marfey's method (Fujii et al., 1997c; Marfey, 1985). The $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra of aeruginopeptin 917S-A also supported that the amino acid sequence and overall structure of this compound was similar to that of aeruginopeptin 95-A (Harada et al., 1993). The sequence of the constituent amino acids was firmly determined by the mass spectrometry/mass spectrometry (MS/MS) method. Figure 2 shows the product ion spectrum of m/z 1055 ($M + H - \text{H}_2\text{O}$) $^+$ from aeruginopeptin 917S-A under MS/MS conditions. The observed product ions are assigned based on the fragmentation pattern of aeruginopeptin 95-A (Fig. 3 and Table II) (Harada et al., 1993, Fujii et al., 2000b). These results indicated that aeruginopeptin 917S-A has the structure shown in Figure 4, which is a 19-membered cyclic depsipeptide possessing Ile, MeTyr, Leu, Ahp, Tyr, Thr, Gln, and Hpla (4-hydroxyphenyl-lactic acid). The absolute configurations of Ahp and Hpla were determined elsewhere (Harada et al., 1996, 2000), as shown in Figure 4.

Aeruginopeptins 917S-B and -C are very similar peptides to aeruginopeptin 917S-A, as shown in Table I. The molecular weight of aeruginopeptin 917S-B is 1076, which is four mass units higher than that of aeruginopeptin 917S-A, and Tyr is lost when compared to the constituent amino acids of aeruginopeptin 917S-A. Although the product ion spectrum was not effectively measured, the observed diagnostic ions are summarized in Table II. This mass spectrometric behavior is quite similar to that of aeruginopeptin 95-B and 228-B that both have tetrahydrotyrosine (ThTyr) as a constituent amino acid (Harada et al., 1993). This result indicated that Tyr in aeruginopeptin 917S-A is replaced by ThTyr in aeruginopeptin 917S-B (Fig. 4). It was found from the amino acid analysis that aeruginopeptin 917S-C has 2 mol of Leu. The diagnostic ions from the MS/MS experiment are shown in Table II. The m/z values of the product ions, *b*, *c*, *o*, and *p* are 50 mass units lower than the corresponding ions from aeruginopeptin 917S-A, indicating that Tyr in aeruginopeptin 917S-A has replaced by Leu in aeruginopeptin 917S-C (Fig. 4) (Fujii et al., 2000b).

In order to investigate the relationship between the production of microcystins and aeruginopeptins 917S, and cell composition, cyanobacterial cells were collected from the center of Lake Suwa at 10-day intervals from June to October in the years 1991–1994. The intra- and extracellular microcystin and aeruginopeptin concentrations of bloom materials in 1991 are shown in Figure 5(a). The highest concentrations of microcystins

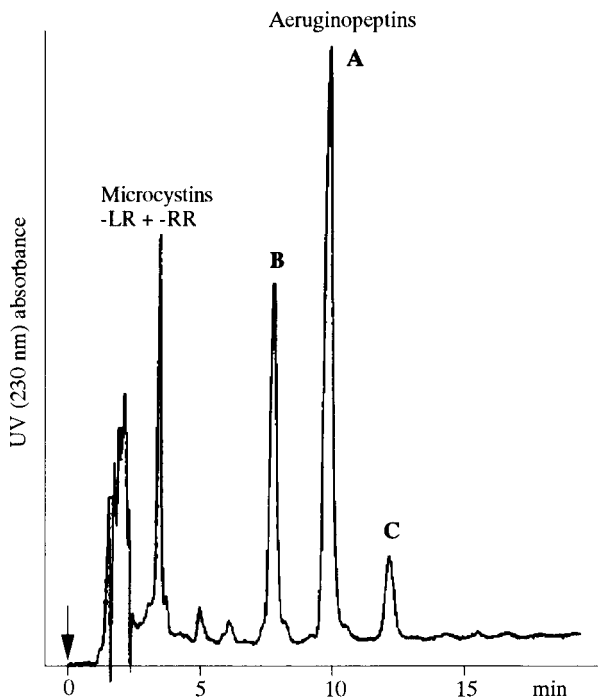


Fig. 1. High-performance liquid chromatogram of the desired fraction from a sample collected on July 23, 1991.

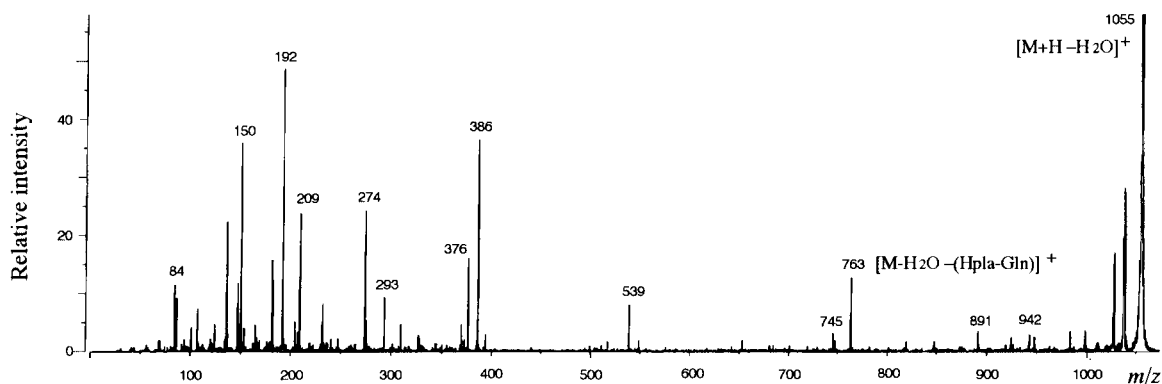


Fig. 2. Product ion spectrum of m/z 1055 ($M + H - H_2O$)⁺ from aeruginopeptin 917S-A under MS/MS conditions.

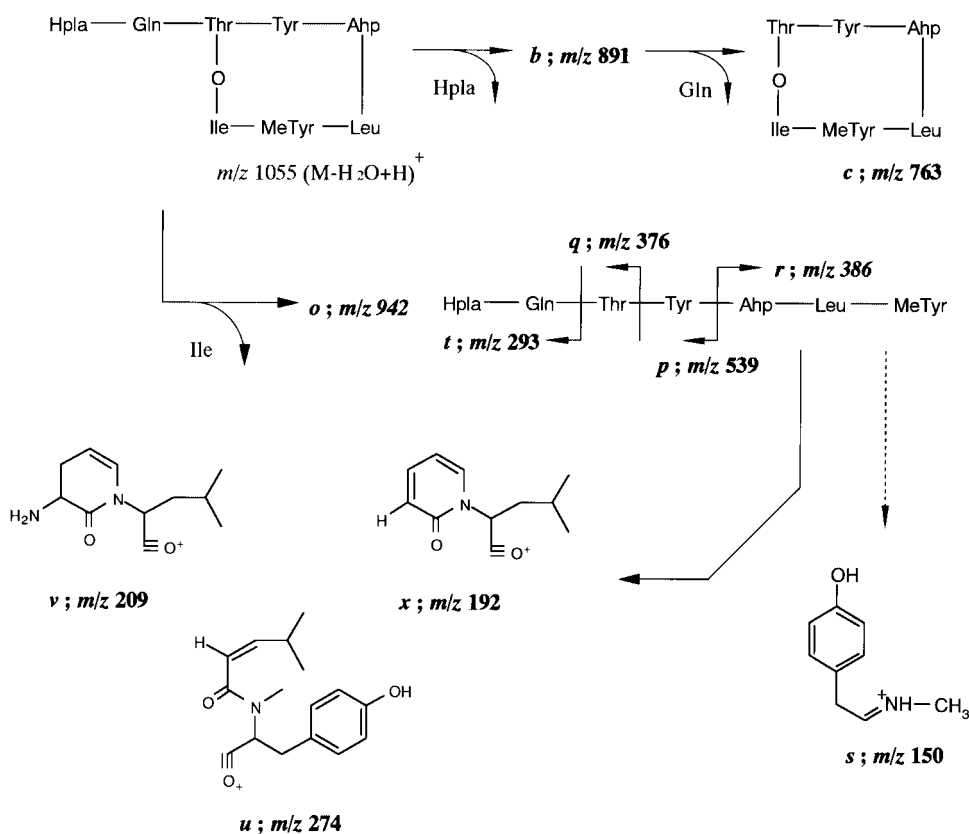


Fig. 3. Diagnostic ions for the sequencing observed in the product ion spectrum of aeruginopeptin 917S-A.

TABLE II. Diagnostic ions observed in the product ion spectra of aeruginopeptins 917S-A, -B, and -C

	($M - H_2O + H$) ⁺	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>o</i>	<i>p</i>	<i>q</i>	<i>r</i>	<i>s</i>	<i>t</i>	<i>u</i>	<i>v</i>	<i>x</i>
917S-A	1055	—	891	763	—	942	539	376	386	150	293	274	209	192
917S-B	($M - 2H_2O + H$) ⁺ 1041	—	—	749(-14)	666	—	—	376	386	150	293	274	209	192
917S-C	($M - H_2O + H$) ⁺ 1005	—	841(-50)	713(-50)	—	892(-50)	489(-50)	376	386	150	293	274	209	192

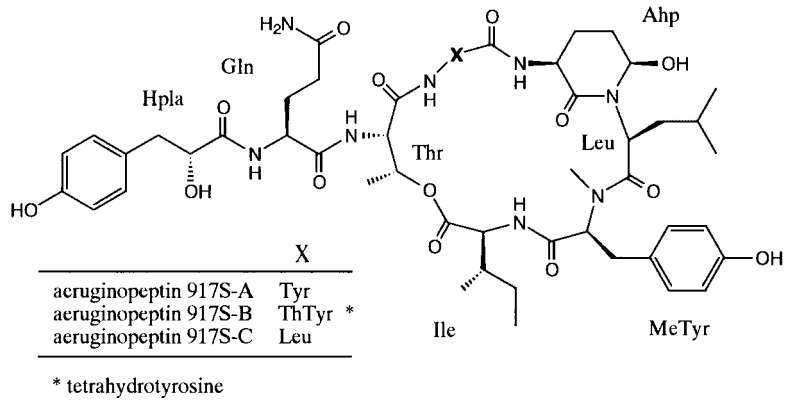


Fig. 4. Structures of aeruginopeptins 917S-A, -B, and -C.

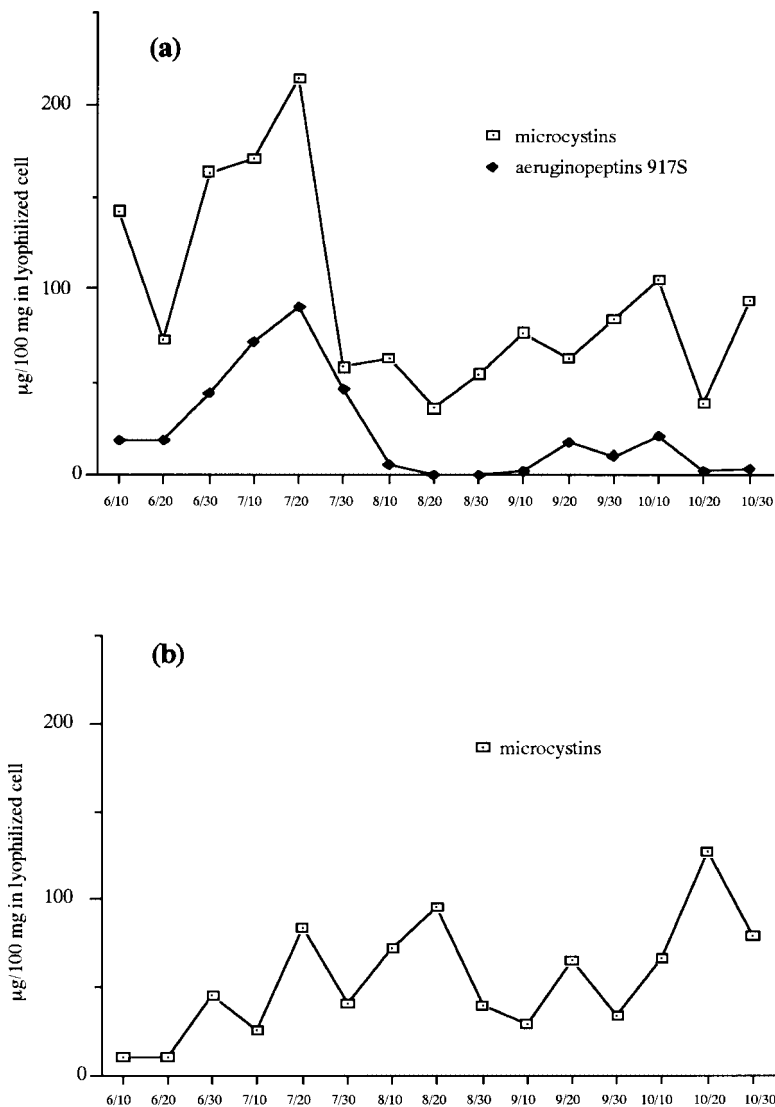


Fig. 5. Intra- and extracellular microcystin and aeruginopeptin concentrations of bloom materials in (a) 1991 and (b) 1992.

and aeruginopeptins were observed on July 20. Thereafter, the concentration of microcystins and aeruginopeptins continued to range from 50 to 100 and below 50 $\mu\text{g}/100$ mg cells, respectively. Figure 5(b) shows the intra- and extracellular microcystin concentrations of bloom materials in 1992 and the concentration ranged around 50 $\mu\text{g}/100$ mg cells except in early June and late October. However, no aeruginopeptins were detected in 1992 [Fig. 5(b)]. In 1993, the concentrations of microcystins were much lower and aeruginopeptins were not detected. The concentrations of aeruginopeptins were not determined in 1994.

DISCUSSION

In the present experiment we isolated aeruginopeptins 917S-A, -B, and -C from bloom materials collected from Lake Suwa on July 23, 1991. We had purified previously four aeruginopeptins from two toxic laboratory strains, *M. aeruginosa* M228 and TAC 95 (Harada et al., 1993), and their structures were quite similar to those of aeruginopeptins 917S-A, -B, and -C (Fig. 4). Aeruginopeptin 917S-A is identical to microcystilide A from *M. aeruginosa* NO-15-1840, which showed a weak toxicity and cytotoxicity (Tsukamoto et al., 1993). Although almost all cyclic depsipeptides containing the Ahp moiety inhibit serine proteases such as trypsin, chymotrypsin, elastase, plasmin, and thrombin (Namikoshi and Rinehart, 1996), aeruginopeptins 917S showed weak activity against chymotrypsin as did the four aeruginopeptins isolated earlier.

It was found in previous experiments the relationship between the *Microcystis* composition and the production of microcystins that is briefly summarized below (Park et al., 1998). While *M. aeruginosa* large cell size groups produce higher amounts of microcystins, the small size group, which was later separated into two species, *M. ichthyoblade* and *M. novacekii*, showed toxin production variations. All strains of *M. viridis* contained lower amounts of microcystins, and *M. wesenbergii* was found to be nontoxic. Indeed, the period of production of the large amounts of the microcystins coincides with that of the appearance of *M. aeruginosa* (large) in 1991 [Fig. 5(a)]. In 1992, *M. viridis* was predominant, and the concentration of microcystins ranged around 50 $\mu\text{g}/100$ mg cells [Fig. 5(b)] that corresponded to the August to October period in 1991.

In addition, we found an interesting phenomenon that the production of aeruginopeptins is superimposable with that of microcystins apart from the production amount in 1991 [Fig. 5(a)], whereas no aeruginopeptins were found in the bloom samples collected in 1992 [Fig. 5(b)]. These results strongly suggested that aeruginopeptins are simultaneously produced together

with microcystins by *M. aeruginosa* (large), and the production of toxic and nontoxic peptides is closely related to the species of *Microcystis*. In order to confirm this finding, we analyzed four *M. viridis* strains and two *M. wesenbergii* strains, but no aeruginopeptin-type compound was detected in these strains.

Recently, many groups have isolated bioactive but nontoxic peptides from cyanobacteria, and Nanikoshi and Rinehart (1996) classified these peptides into four groups. To elucidate the biosynthetic relationship between the hepatotoxic peptides, microcystins and nodularin, and other nontoxic peptides such as aeruginopeptins, we have also isolated the nontoxic peptides produced from the toxic or nontoxic cyanobacterial strains, *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc*, and *Nodularia* and have determined their structures (Fujii et al., 1996, 1997a, 1997b, 1999, 2000a; Harada et al., 1993, 1995). While these peptides, except for microviridin I, are produced by toxic cyanobacteria that co-produce hepatotoxic peptides, no such peptides were detected from the nontoxic and neurotoxic cyanobacteria producing anatoxin-a. Because microviridins were also produced by the toxic strains (Okino et al., 1995), the production of nontoxic peptides may be related to that of the hepatotoxic peptides.

Microcystins are synthesized nonribosomally via peptide synthetases (Döhren et al., 1997; Marahiel et al., 1997). Dittmann et al. (1997) cloned the microcystin-synthetase genes, *mcyA* and *mcyB* from the *M. aeruginosa* strain, PCC7806, which produces nontoxic peptides, cyanopeptolins A and B (Martin et al., 1993) in addition to desmethylmicrocystin-LR and microcystin-LR. Cyanopeptolins are a 19-membered cyclic depsipeptide possessing the Ahp moiety and are similar to those of aeruginopeptins apart from their N-terminus. They also prepared a mutant after the disruption of these genes and confirmed that this mutant strain does not produce microcystins but still produces cyanopeptolins (Dittmann et al., 1997). This result revealed that the peptide synthetase for microcystins and cyanopeptolins are independently present in this toxic cyanobacteria. Nishizawa et al. (1999) also identified three genes (*mcyA*, *mcyB*, *mcyC*) encoding microcystin synthetases from toxic *M. aeruginosa* K-139, which produced desmethylmicrocystin-LR and microcystin-LR (Harada et al., 1991). Recently, it was found that this strain also produces an aeruginopeptin-type compound that will be reported elsewhere. They also reported that the microcystin nonproducing strains of *Microcystis* are divided into two groups: one possesses no *mcy* genes and the other possesses the *mcy* genes (Nishizawa et al., 2000). Very recently, a procedure using two polymerase chain reaction (PCR) methods was proposed for the rapid and sensitive detection of the

toxigenic strains of *Microcystis*, and many cyanobacterial samples were examined using this method (Neilan et al., 1999).

Based on our results obtained to date and the genetic studies mentioned above, the following conclusions were suggested: *M. aeruginosa* can be divided into toxic and nontoxic groups. The former has always synthetase genes for both microcystins and nontoxic peptides, and the latter is further classified into two groups, one of which has the genes but no expression while the other does not possess the genes. *M. viridis* produces lower amounts of microcystins but does not produce nontoxic peptides, suggesting the lack of synthetase genes. The function of microcystin in cyanobacterial cells is still unclear. The problem why nontoxic peptides such as aeruginopeptins and cyanopeptolins are produced together with microcystins by toxic cyanobacteria should be also considered.

We thank Dr. Kazuo Hirayama (Central Research Laboratory, Ajinomoto Co., Japan) for measurement of MS/MS spectra and Professor Shirai (School of Agriculture, Ibaraki University, Japan) for helpful discussion.

REFERENCES

- Dittmann, E.; Neilan, B. A.; Erhard, M.; Döhren, H.; Börner, T. *Mol Microbiol* 1997, 26, 779–787.
- Döhren, H.; Keller, U.; Vater, J.; Zoehner, R. *Chem Rev* 1997, 97, 2675–2705.
- Fujii, K.; Harada, K.-I.; Suzuki, M.; Kondo, F.; Ikai, Y.; Oka, H.; Carmichael, W. W.; Sivonen, K. Harmful and Toxic Algal Bloom; Intergovernmental Oceanographic Commission of UNESCO: Sendai, Japan, 1996; pp 559–562.
- Fujii, K.; Sivonen, K.; Adachi, K.; Noguchi, Y.; Sano, H.; Hirayama, K.; Suzuki, M.; Harada, K.-I. *Tetrahedron Lett* 1997a, 38, 5525–5528.
- Fujii, K.; Sivonen, K.; Adachi, K.; Noguchi, Y.; Shimizu, Y.; Sano, H.; Hirayama, K.; Suzuki, M.; Harada, K.-I. *Tetrahedron Lett* 1997b, 38, 5529–5532.
- Fujii, K.; Ikai, Y.; Oka, H.; Suzuki, M.; Harada, K.-I. *Anal Chem* 1997c, 69, 5146–5151.
- Fujii, K.; Sivonen, K.; Kashiwagi, T.; Hirayama, K.; Harada, K.-I. *J Org Chem* 1999, 64, 5777–5782.
- Fujii, K.; Sivonen, K.; Naganawa, E.; Harada, K.-I. *Tetrahedron* 2000a, 56, 725–733.
- Fujii, K.; Maymi, T.; Noguchi, Y.; Kashiwagi, T.; Akashi, S.; Sivonen, K.; Hirayama, K.; Harada, K.-I. *J Mass Spectrom Soc Jpn* 2000b, 48, 56–64.
- Harada, K.-I. *Toxic Microcystis*; CRC Press: Boca Raton, FL, 1996; 103–148.
- Harada, K.-I.; Suzuki, M.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W.; Rinehart, Jr., K. L. *Toxicon* 1988a, 26, 433–439.
- Harada, K.-I.; Matsuura, K.; Suzuki, M.; Oka, H.; Watanabe, M. F.; Oishi, S.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W. *J Chromatogr* 1988b, 448, 275–283.
- Harada, K.-I.; Ogawa, K.; Matsuura, K.; Nagai, H.; Murata, H.; Itezono, M.; Nakayama, N.; Shirai, M.; Nakano, M. *Toxicon* 1991, 29, 479–489.
- Harada, K.-I.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Watanabe, M. F. *Tetrahedron Lett* 1993, 34, 6091–6094.
- Harada, K.-I.; Fujii, K.; Shimada, T.; Suzuki, M.; Sano, M.; Adachi, K.; Carmichael, W. W. *Tetrahedron Lett* 1995, 36, 1511–1514.
- Harada, K.-I.; Fujii, K.; Hayashi, K.; Suzuki, M.; Ikai, Y.; Oka, H. *Tetrahedron Lett* 1996, 37, 3001–3004.
- Harada, K.-I.; Shimizu, Y.; Kawakami, A.; Norimoto, M.; Fujii, K. *Anal Chem* 2000, 72, 4142–4147.
- Jochimsen, E. M.; Carmichael, W. W.; An, J.; Cardo, D. M.; Cookson, S. T.; Holmes, C. E. M.; Antunes, B. C.; Filho, D. A.; Lyra, T. M.; Barreto, V. S. T.; Azevedo, S. M. F. O.; Jarvis, W. R. *N Engl J Med* 1998, 338, 873–878.
- Kondo, F.; Ikai, Y.; Oka, H.; Okumura, M.; Ishikawa, N.; Harada, K.-I.; Matsuura, K.; Murata, H.; Suzuki, M. *Chem Res Toxicol* 1992, 5, 591–596.
- Kuiper-Goodman, T.; Falconer, I.; Fitzgerald, J. *Toxic Cyanobacteria in Water*; E & FN Spon: London, 1999; pp 113–153.
- Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. *Chem Rev* 1997, 97, 2651–2673.
- Marfey, P. *Carlsberg Res Commu* 1985, 49, 591–596.
- Martin, C.; Oberer, L.; Ino, T.; König, W. A.; Busch, M.; Wecksser, J. *J Antibiotics* 1993, 46, 1550–1556.
- Namikoshi, M.; Rinehart, K. L. *J Indust Microbiol* 1996, 17, 373–384.
- Neilan, B. A.; Dittmann, E.; Rouhiainen, L.; Bass, R. A.; Schaub, V.; Sivonen, K.; Börner, T. *J Bacteriol* 1999, 181, 4089–4097.
- Nishizawa, T.; Asayama, M.; Fujii, K.; Harada, K.-I.; Shirai, M. *J Biochem* 1999, 126, 520–529.
- Nishizawa, T.; Ueda, A.; Asayama, M.; Fujii, K.; Harada, K.-I.; Ochi, K.; Shirai, M. *J Biochem* 2000, 127, 779–789.
- Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron* 1995, 51, 10679–10686.
- Park, H.-D.; Iwami, C.; Watanabe, M. F.; Harada, K.-I.; Okino, T.; Hayashi, H. *Environ Toxicol Water Qual* 1998, 13, 61–72.
- Pouria, S.; de Andrade, A.; Cavalcanti, R. L.; Barreto, V. T.; Ward, C. J.; Preiser, W.; Poon, G. K.; Neild, G. H.; Codd, G. A. *Lancet* 1998, 352, 21–26.
- Shin, H.; Murakami, M.; Matsuda, H.; Yamaguchi, K. *Tetrahedron* 1996, 52, 8159–8168.
- Sivonen, K.; Jones, G. *Toxic Cyanobacteria in Water*; E & FN Spon: London, 1999; pp 41–111.
- Tsukamoto, S.; Painuly, P.; Young, K. A.; Yang, X.; Shimizu, Y.; Cornell, L. *J Am Chem Soc* 1993, 115, 11046–11047.