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Release and Biodegradation of Microcystins in Blue-Green Algae, *Microcystis* PCC7820*

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Abstract

Microcystis cells synthesize hepatotoxins and frequently become dominant in Japanese fresh water blooms. The toxins termed microcystins are cyclic heptapeptides and expose to risks with death for animals and humans. The decomposition of microcystins which are stable in ordinary condition, in blue-green algae, *Microcystis* PCC 7820, was investigated under aseptic condition. The release of microcystin-LR, -LY, -LW, and -LF was observed after cell lysis and persisted in culture medium where the microcystin-LR concentration reached 5.5 mg/*l*. These results indicated that the release of these toxins would not be due to physiological secretion, suggesting that biodegradation of microcystins should occur in natural condition. Microcystins were degraded at eighth day after adding water sample from lake Suwa, but not after adding boiled water sample from Lake Suwa, indicating that microorganism(s) indigenous to Lake Suwa would involve in the degradation process. The decomposition of microcystins was more notable in the presence of bed sediment and mud collected from local sources, in which microcystin-LR, -LY, -LW, and -LF were decomposed completely in nine days. These results suggest that microorganisms in natural surroundings should be potent agents for the removal of microcystins from drinking water for animals and humans.

Introduction

Heavy water-bloom forming blue-green algae exhibit buoyancy owing to the presence of gas vesicles inside the cells. They are distributed world-wide in eutrophic fresh waterbodies for drinking purposes (FALCONER et al., 1983; CODD et al., 1995). The outbreak of *Microcystis* has been also reported in Japan (WATANABE et al., 1988; 1989; 1991; PARK et al., 1993a; 1998). Among blue-green algae, *Microcystis* particularly become dominant in Japanese fresh water blooms (PARK et al., 1993b) and most *Microcystis* species produce

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toxins as secondary metabolites (CARMICHAEL et al., 1992) although their physiological significance is not clear. FRANCIS (1878) firstly introduced the episode in which cyanobacterial toxins caused deaths of wild and domestic animals, following the ingestion of toxic blue-green algal scums from blooms.

Microcystis species have been exposing humans to a health hazard. For example, the cyanobacterial toxins are thought to be an agent for high incidence of primary liver cancer in China (UENO et al., 1996), and are the most probable cause of death among individuals receiving dialysis treatment in Brazil (JOCHIMSEN et al., 1998). Most *Microcystis* species produce toxins termed microcystins (CARMICHAEL et al., 1988a) which are cyclic heptapeptides (CARMICHAEL et al., 1988b). The chemical structure is composed of five common amino acids and two variable L-amino acids (BOTES et al., 1984). Over sixty components of slightly modified structures have been isolated (KAYA, 1998; OHTAKE et al., 1998; KRISHNAMURTHY et al., 1989; HARADA et al., 1990). LD_{50} values (i.p. mouse) were reported to range from 50 to 600μg/kg (SIVONEN, 1996; WATANABE et al., 1988; CARMICHAEL, 1992). Microcystins inhibit protein phosphatases 1 and 2A in a similar manner to okadaic acid (MACKINTOSH et al., 1990; YOSHIZAWA et al., 1990; ERIKSSON et al., 1990) and potent tumor promoters (NISHIWAKI-MATSUSHIMA et al., 1992; FALCONER and BUCKLEY, 1989).

Water blooms commonly decreases along with a drop in water temperature in early autumn. Microcystins stay inside the cells, but they are released into natural waters after cell lysis (BERG et al., 1987; WATANABE et al., 1992; PARK et al., 1998). Microcystins released from cells have slightly persisted in lake water (WATANABE et al., 1992), and are chemically stable in aqueous solution. Microcystin-LR is not degraded by copper sulfate treatment (KENEFICK et al., 1993) and by exposure with sunlight alone (TSUJI et al., 1994). There have never been reported that released microcystins in waterbodies persisted for a long period, suggesting that these toxins might be diluted by water flowed in, and/or decomposed by microbiological actions. *Microcystis* cells produce hydrophilic and hydrophobic toxins. Degradation of hydrophilic microcystin-RR, -YR, and -LR in surface waters has been demonstrated in laboratory condition (KENEFICK et al., 1993; WATANABE et al., 1992; BERG et al., 1987), but that of hydrophobic ones has never been investigated. The decomposition of hydrophobic as well as hydrophilic microcystins released from *Microcystis* cells are discussed in the present study.

Material and Methods

Organism

A toxic strain *Microcystis* PCC 7820 was obtained from Pasteur Culture Collections,

Paris, France. The strain was originally isolated from Loch Balgavies, Scotland (RIPPKA and HERDMAN, 1992).

The alga was grown in a 19 liter glass bottle containing 18 liters of BG11 medium (omitting Na_2CO_3 and $Na_2SiO_3 \cdot 9H_2O$, replacing EDTA (disodium magnesium) with EDTA (disodium) and Citric acid with Citric acid$\cdot H_2O$) (ALLEN, 1968) under continuous illumination at 117μE/m^2/s on the surface of the bottle at about 25°C with aerating sterile air (1.4-2.5 liters/min).

Extraction of Toxins

A portion of cell suspension (100 or 500 ml) was taken from the bottle once every 5 or 7 days and filtrated through a glass fiber filter (GF/C, ϕ90mm, Whatman, England). Cells on the filter were dried up in auto dry desiccator (Sanplatec Corp., Tokyo) and the filtrate was retained. The dried cells were suspended in 70 % (v/v) methanol-0.1 % (v/v) trifluoroacetic acid (TFA) and stirred for 1h at room temperature. The extracted sample and the filtrate were centrifuged at 6,100$\times g$ at 4 °C for 10 min. Both supernatants were separately applied to Sep-Pak Vac C18 cartridges (500mg, 3cc, Waters, Massachusetts, U.S.A.) which had been previously conditioned with 10 ml of HPLC-grade methanol and 20 ml of water. The cartridges were then washed with 10 ml of 10, 20 and 30 % (v/v) aqueous methanol in series, followed by elution with 40 ml of methanol. The eluates were evaporated by a rotary evaporator (R-144 and B-480, Buchi-Shibata, Tokyo), suspended in 1 ml of methanol, and centrifuged (Chibitan, XX42CF06T, Tomy, Tokyo). The supernatants (10 μl) were applied to high performance liquid chromatograph (HPLC).

High Performance Liquid Chromatography (HPLC) Analysis

Microcystins were identified on analytical HPLC with a photodiode array detector (Waters). The equipment consists of a model 600 multisolvent delivery system, a model 486 tunable absorbance detector, a model 996 photodiode array detector, a column heater module, Deck computer system, and a symmetry C18 column (4.6 × 250 mm, Waters).

The eluent was a combination of Milli-Q water (Millipore) and acetonitrile, both containing 0.05 % (v/v) TFA, and a linear gradient condition was shown in Fig. 1 according to the method of LAWTON et al. (1994). The flow rate was 1 ml/min at 30°C for analysis and an absorbance at 238 nm was monitored. The procedure of the HPLC system was followed by the method previously described by LAWTON et al. (1994).

The HPLC data were analyzed by Milennium 2010-J computer software version 2.10 (Waters).

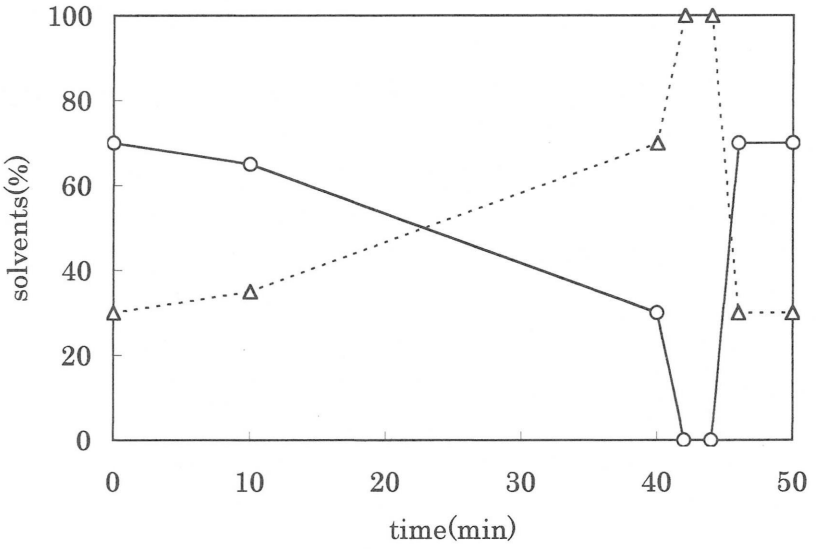

Fig. 1. A linear gradient of solvents on HPLC.
solvent (○): H_2O-0.05% (v/v) TFA
solvent (△): acetonitrile-0.05% (v/v) TFA

Biodegradation of Toxins

To study biodegradation of microcystins, surface water and soil samples were collected from local sources including Lake Suwa, Suwa city, Nagano prefecture, Japan, Funakoshi Oh-ike and rice field, Shimizu city, Shizuoka prefecture, Japan. Bed sediment and mud samples suspended in distilled water, and water samples were passed through filter papers (Advantec, Tokyo).

Known amounts of microcystins were added to an Erlenmeyer flask containing 100 m*l* of raw lake sample water, filtrated sample water, boiled sample water or distilled water. The flasks were kept in the dark at about 25 °C. An aliquot (1 m*l*) of samples was withdrawn from flasks at intervals and centrifuged. The supernatants were analyzed on HPLC.

Results

The distribution of intra- and extra-cellular microcystins was investigated in order to evaluate the quantity of toxins released from *Microcystis* cells.

Figure 2(a) shows the growth of *Microcystis* PCC7820 cells under continuous illumination for 120 days. The cells grew linearly for 20 days, and then, growth rate declined in the ensuing 55 days (Fig. 2(a)). A rapid decrease in A_{720} was observed after 75 days, resulted from cell lysis (Figure 2(a)). Figure 2(b) shows the changes in concentrations of hydro-

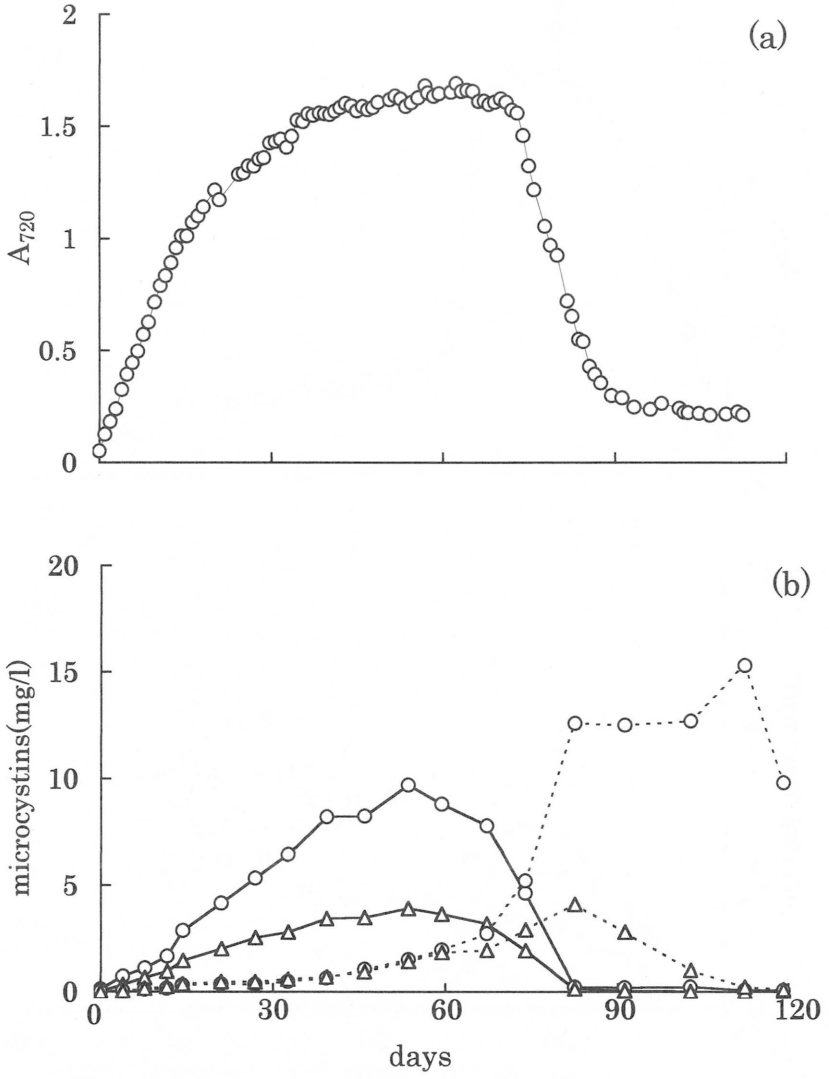

Fig. 2. Growth of *Microcystis* PCC7820 cells under continuous illumination with microcystin concentration (-LR(○), -(LY + LW + LF) (△)) in the cells (solid line) and in the medium (dotted line).
(a) growth curve
(b) distribution of microcystins

philic microcystin-LR and hydrophobic microcystin-LY, -LW, and -LF inside the cells and in the medium during growth. The intracellular microcystin concentrations increased along with A_{720}, and then dropped when the cells started to lyse (Fig. 2(a) and Fig. 2(b)). On the other hand, drastic release of microcystin-LR to the medium coincided with the decrease in intracellular microcystin-LR (Fig. 2(b)). The aspect of change in concentrations of

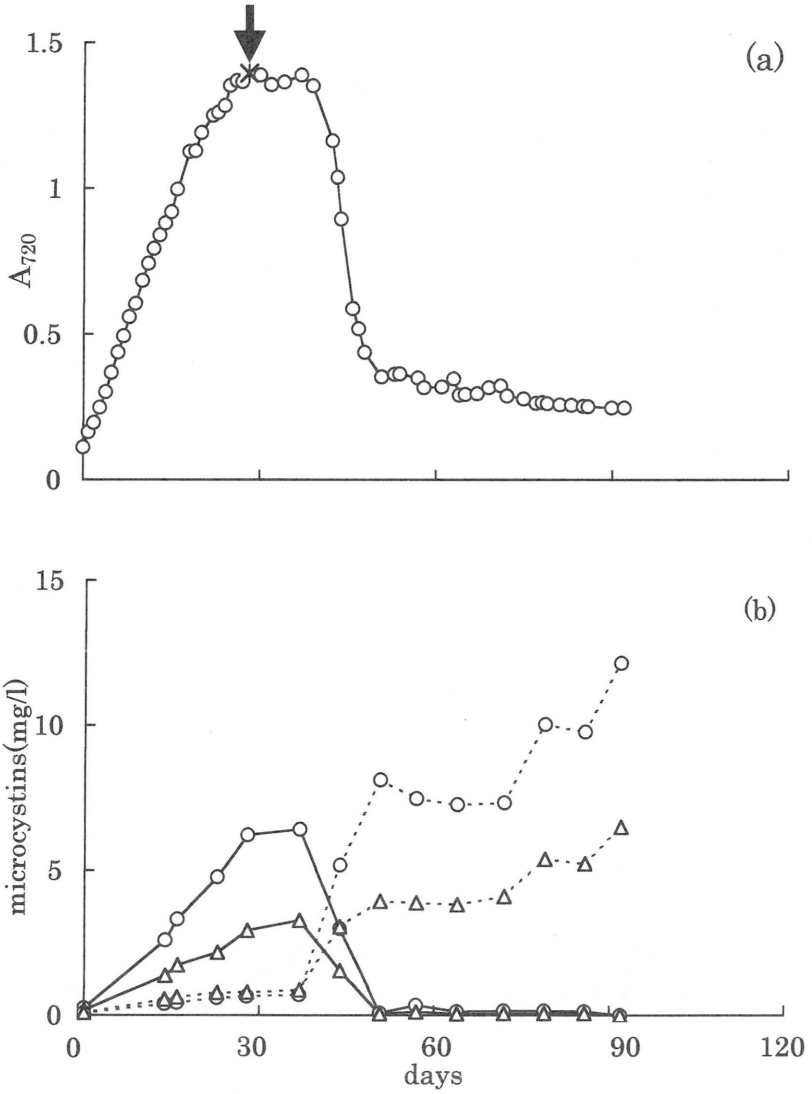

Fig. 3. Growth of *Microcystis* PCC7820 cells with microcystin concentration (-LR(○), -(LY + LW + LF) (△)) in the cells (solid line) and in the medium (dotted line). Lights were turned off at the 28th day(*).
(a) growth curve
(b) distribution of microcystins

hydrophobic microcystin-LY, -LW, and -LF were similar to that of microcystin-LR (Fig. 2(b)).

Figure 3(a) shows the growth of *Microcystis* cells. Illumination was turned off at the 28th day (A_{720}=1.394). Linear growth was observed in the first 28 days (Fig. 3(a)). The value of A_{720} was kept constant for the following 10 days after turning lights off, and

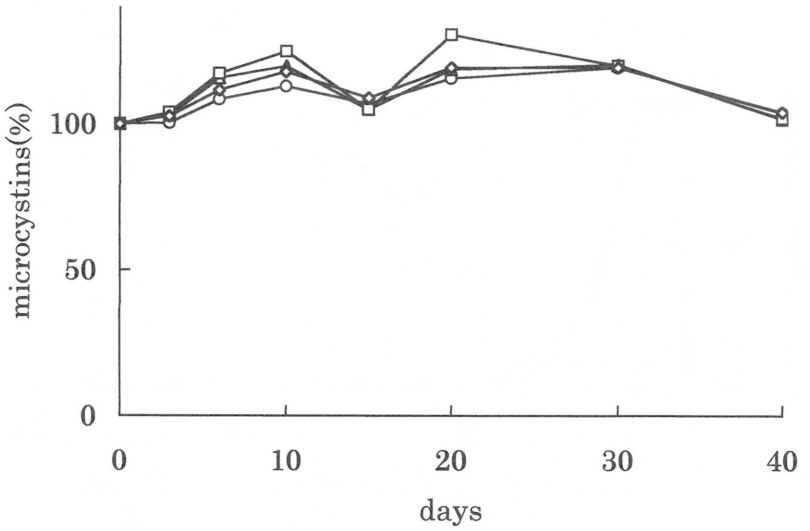

Fig. 4. Degradation of microcystins in distilled water in the dark. The ratio of microcystin-LR(○), -LY(△), -LW(□), and -LF(◇) at each point to those at time 0 was expressed as percentages.

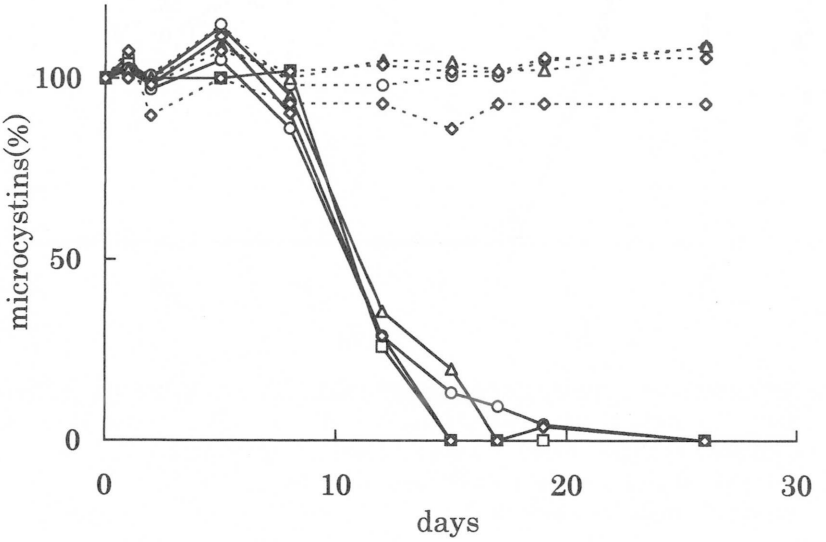

Fig. 5. Degradation of microcystins in water (solid line) and boiled water (dotted line) from Lake Suwa. The ratio of microcystin-LR(○), -LY(△), -LW (□), and -LF(◇) at each point to those at time 0 was expressed as percentages.

rapidly decreased in the next 14 days (Fig. 3(a)). Figure 3(b) shows the changes in the concentrations of hydrophilic microcystin-LR and hydrophobic microcystin-LY, -LW, and

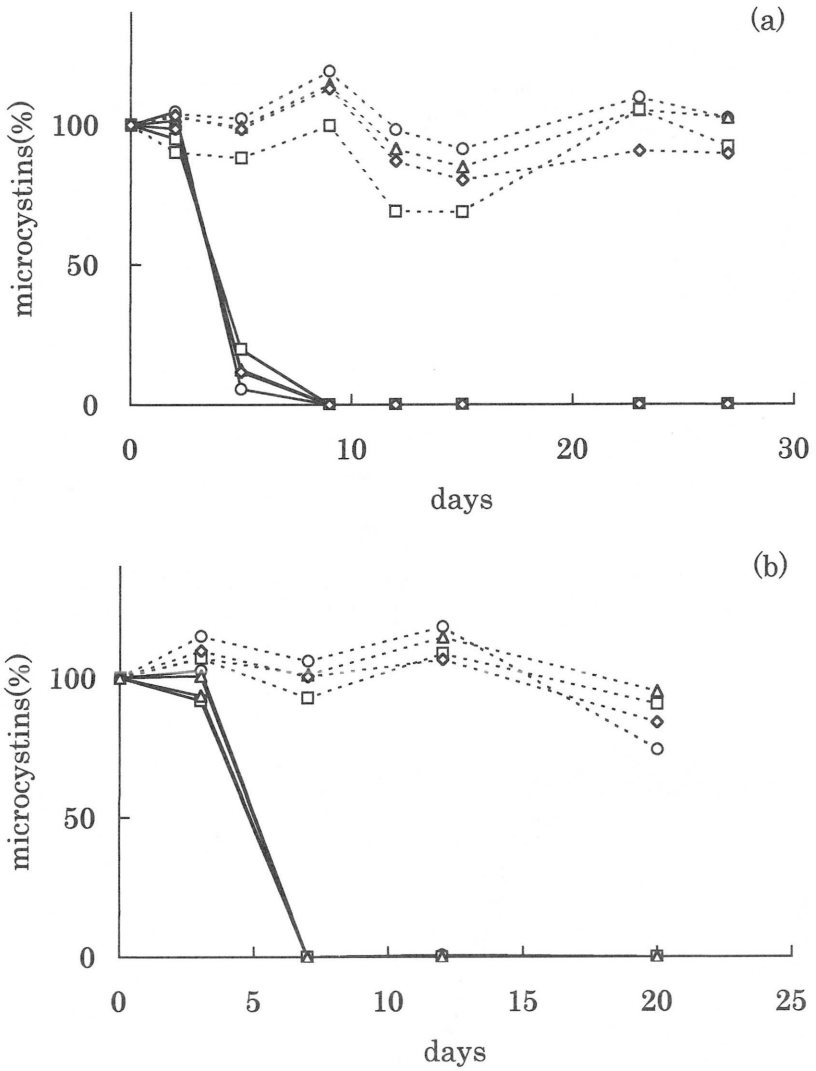

Fig. 6. Degradation of microcystins in water (solid line) and boiled water (dotted line). The ratio of microcystin-LR(○), -LY(△), -LW(□), and -LF(◇) at each point to those at time 0 was expressed as percentages.
(a) bed sediment sample from Funakoshi Oh-ike
(b) mud sample from rice field

-LF inside the cells and in the medium during growth. The intracellular toxin concentrations increased along with A_{720} (Fig. 3(b)). When the lights were turned off, intracellular toxin concentrations rapidly decreased (Fig. 3(b)). The toxin concentration in the medium increased immediately after cell lysis (Fig. 3(b)) and the toxin persisted in the medium in the dark (Fig. 3(b)). The profile of change in concentration of hydrophobic microcystin-LY, -LW, and -LF was almost the same as that of hydrophilic microcystin-LR (Fig. 3(b)).

Figure 4 shows the change in microcystin concentrations in distilled water. Little change was observed in all microcystin throughout the experiment (Fig. 4), indicating the toxins remained intact in distilled water for 40 days under the experimental condition.

Time-dependent degradation of microcystins was investigated in surface water sample from Lake Suwa. Figure 5 shows the degradation of microcystin-LR, -LY, -LW, and -LF in filtrated lake water and in the boiled one. In the filtrated water, all microcystins began to decompose at the 8th day, and degraded almost completely at the 15th day except microcystin-LR and -LY (Fig. 5). Microcystin concentrations in boiled lake water were kept constant (Fig. 5), indicating that the toxins were not subject to degradation in the experiment.

Figure 6 shows the degradation of microcystins in boiled sample water and filtrated water of both bed sediment sample from Oh-ike (Fig. 6(a)) and mud sample from rice field (Fig. 6(b)), Shimizu, Japan. In the filtrated water, it is shown that sharp degradation of all microcystins began in 2 or 3 days and degraded almost completely at 9th or 7th day, in bed sediment sample water and mud sample water, respectively. Little degradation of microcystins occurred during 20 days in both boiled sample waters.

Discussion

Microcystis cells possess hydrophilic and hydrophobic toxins. The release of hydrophilic microcystin-RR, -YR, and -LR from *Microcystis* cells has been extensively investigated in natural environment and laboratory conditions (PARK et al., 1998; WATANABE et al., 1992). There have been, however, few reports on hydrophobic microcystin-LY, -LW, and -LF which were recently detected in Japanese *Microcystis* spp. (KURISU et al., 1998).

BURY et al. (1996) reported that the hydrophobic fraction of toxins extracted from *Microcystis* cells inhibited Ca^{2+} transport in tilapia gills more than the hydrophilic fraction, suggesting that hydrophobic microcystins should be more toxic *in vivo* than hydrophilic ones. Hydrophobic toxins are possibly more permeable to the lipid bilayer of cytoplasmic membrane than hydrophilic ones. Although most *Microcystis* cells synthesize hydrophobic microcystin such as microcystin-LY, -LW, and -LF much less than hydrophilic microcystins, these water-insoluble microcystins would be much more hazardous to health of animals and humans. The behavior of hydrophobic toxins in nature should be investigated in more detail.

In the present study, the intra- and extra-cellular distribution of hydrophilic and hydrophobic microcystins was evaluated during growth. Both microcystins persisted within the cells when cells grew linearly, but rapid release of them was observed when cells began to lyse (Figs. 2 and 3). WATANABE et al. (1983; 1989) reported that both the toxicity and

the concentration of microcystin-LR showed higher at late exponential phase than those at any other growth phases, suggesting that the toxicity and/or the concentration of hydrophobic microcystins might be variable during growth of *Microcystis* cells. In fact, the concentration of microcystin-(LY + LW + LF) was at its most in the end of active growth, and the time-dependent pattern of intra-and extra-cellular microcystin-(LY + LW + LF) concentration was similar to that of microcystin-LR (Figs. 2 and 3). It is reported that both productivity and toxicity of microcystin-RR were changeable in response to environmental factors (SIVONEN, 1990). In particular, light intensity obviously affects on the toxicity and the productivity (UTKILEN and GJOLME, 1992). Release of microcystins into medium occurred in laboratory condition after lysis of *Microcystis* cells (Figs. 2(b) and 3(b)). The similar result has been obtained by WATANABE et al. (1992), in which the hydrophilic toxins in unialgal *Microcystis* cells were released in the dark. Free microcystins have been detected in water samples of Lake Suwa and Funakoshi Oh-ike (KURISU et al., 1998). Although purified microcystins are stable under sunlight, microcystin was decomposed by the addition of pigment extracted from blue-green algae (TSUJI et al., 1994). The extracellular toxins other than microcystin-LR completely degraded at the end of experiment (Fig. 2(b)), suggesting that the degradation of released hydrophobic microcystins would occur in the presence of pigments from *Microcystis* cells, which is consistent with the result reported by TSUJI et al. (1994).

Aquatic organisms are liable to decompose by the activity of microorganism. Blue-green algae-lysing bacteria have been also reported (STEWART and DAFF, 1997). Although cyclic peptide toxins are relatively resistant to chemical treatments, the released toxins are reduced in the environmental water in early autumn. The contribution of aquatic microorganism to the degradation of microcystin was studied with lake water, mud, and bed sediment. The profile of decrease in hydrophobic microcystins extracted from *Microcystis* PCC7820 was similar to that in hydrophilic toxins (Figs. 5 and 6), and no degradation of microcystins occurred in the distilled water (Fig. 4), indicating that degradation of toxins probably occurred via microorganism(s) in natural surroundings.

The experiments on biodegradation of microcystins had been carried out with water bloom-forming surface lake waters (JONES et al., 1994; COUSINS et al., 1996). In this study, the experiments were done with samples collected from bed sediment (Oh-ike) and mud (rice field). More prominent degradation of microcystins was observed in the samples collected from local sources than in water sample from Lake Suwa (Figs. 5 and 6). It is possible that microcystin degradation by bacteria should be much more active in mud and bed sediment than that in Lake Suwa.

BOURNE et al. (1996) described that degradation of microcystin-LR was mediated by hydrolytic enzymes in an isolated bacteria. The bacterial enzymes acted at the Adda-

arginine peptide bond in a cyclic peptide to form a linearized microcystin. The degradation products were less active on protein phosphatase assay than intact microcystin-LR and still showed a maximal absorption at 238nm owing to the conjugated double bond in Adda moiety. HARADA et al. (1990) demonstrated that a geometrical isomers in the Adda moiety showed no toxicity on protein phosphatase assay. In the present study, the degradation of microcystins was evaluated and confirmed by the observation of a loss of an absorbance at 238nm on HPLC. Any product derived from microcystins was never observed, suggesting that conjugated double bond responsible for microcystin toxicity in Adda moiety might be isomerised or cleaved. Since toxicity of microcystin is probably related to Adda moiety modification (HARADA et al., 1990), the decomposed products in this study might be less toxic or non-toxic. Although HPLC analysis is time-saving and useful for detection of microcystins, an attempt to estimate the toxicity to mice and the inhibitory effect of protein phosphatses should be made.

It is concluded that behavior of hydrophobic microcystins in eutrophic fresh water-bodies is a matter of great importance to human and animal health. Isolation and characterization of bacteria which actively detoxify microcystins, as well as evaluation of toxicity and inhibition of protein phosphatases are now in progress.

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[要　旨]

Microcystis 細胞から放出されるミクロシスチンとその生物分解

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日本においては富栄養化した湖沼に肝臓毒素ミクロシスチンを生産するラン藻 *Microcystis* が優占的に水の華を形成する．親水性および疎水性のミクロシスチンを生産する *Microcystis* PCC7820の生育にともなうミクロシスチンの放出を検討したところ，全てのミクロシスチンは細胞の死滅後に細胞外へ放出され，培地中のミクロシスチンLR濃度は最大で5.5mg/*l* に達した．また暗黒下においては全ての毒素は分解を受けずに培地中に残存した．しかし，自然の水界においては微量のミクロシスチンしか検出されないため，ミクロシスチンは湖水中では微生物によって分解されるものと考えられる．諏訪湖水を添加した場合，ミクロシスチンは8日目から分解が始まり，26日間で完全に消失した．夫池の底砂の懸濁液と田の泥水を用いた場合、ミクロシスチンは実験開始後2または3日目に分解が始まり，9日目には完全に試水中から消滅した．これらミクロシスチン分解能を持つ微生物は，動物や人間が飲料水として使用している水域から効果的にミクロシスチンを取り除くものと期待される．