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Comprehensive analysis system using liquid chromatography–mass spectrometry for the biosynthetic study of peptides produced by cyanobacteria

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Abstract

Microcystins are hepatotoxic heptapeptides and general tumor promoters produced by several species of the genera *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc*. They are non-ribosomally synthesized via a mixed polyketide synthase/non-ribosomal peptide synthetase system called microcystin synthetase. We have carried out the detection, isolation and structural determination of non-toxic peptides produced together with microcystins by toxic cyanobacteria, which are classified into several groups on the basis of their structures and some of these non-toxic peptides are also non-ribosomally synthesized as well as microcystins. In the present study, we tried to correlate the secondary metabolic peptides produced by the hepatotoxic cyanobacteria with the corresponding peptide synthetase genes. An analytical method using LC–electroscopy ionization MS and photodiode array detection was developed for the exhaustive screening of cyanobacterial peptides in Japanese strains and it was successfully applied to the peptide fractions extracted from these strains. The established method was advantageous over conventional ones using the usual HPLC and matrix-assisted laser desorption ionization time-of-flight MS, because more structural information could be obtained and it is easier to distinguish microcystins from other peptides using this method. Small amounts of other peptides could also be detected by this method. The established method will contribute to the investigation of the relationship between genes encoding the peptide synthetase and secondary metabolic peptides.

Keywords: Bacteria; Peptides; Microcystins

1. Introduction

Cyanobacteria (blue–green algae) are known to produce structurally diverse peptides as the secondary metabolites [1]. A serious environmental problem is caused by the production of cyclic hepatotoxins, microcystins, in water blooms. Microcystins are hepatotoxic heptapeptides and general tumor promoters produced by several species of the genera *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc*, of which *Microcystis* is the most harmful freshwater bloom-forming cyanobacteria [2]. For regulating the production of microcystins by toxic cyanobacteria, we investigated their biosynthesis. Microcystins are non-ribosomally synthesized *via* peptide synthetases, polyketide synthases, and an additional modifying enzyme system called microcystin synthetase [3]. Recently, microcystin synthetase (*mcy*) genes were cloned from a hepatotoxic cyanobacterium, *Microcystis aeruginosa* K-139 [4,5], which produces 3-demethyl- and 3,7-didemethylmicrocystins-LR [6]. The production of microcystins was knocked out by insertional inactivation of the peptide synthetase gene, demonstrating that microcystins are formed by the peptide synthetase system [7]. In our previous investigations, we carried out the detection, isolation and structural determination of non-toxic peptides produced together with microcystins by toxic cyanobacteria [7–10]. The isolated non-toxic peptides were classified into several groups on basis of their structural skeletons [1]. Interestingly, it was found that one of these non-toxic peptides is also non-ribosomally synthesized like the microcystins [11].

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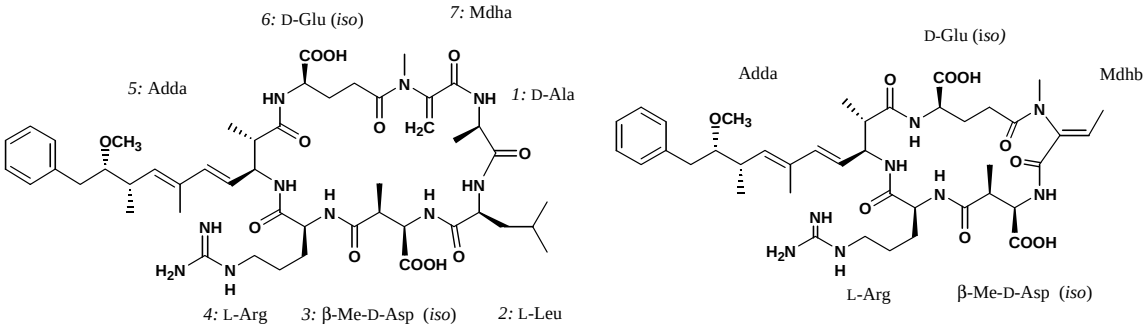

Fig. 1. Structures of toxic peptides, microcystin-LR and nodularin-R isolated from cyanobacteria.

As mentioned above, freshwater cyanobacteria produce a variety of toxic and non-toxic peptides. The most commonly detected toxic peptides are the microcystins, cyclic heptapeptides with the general structure of cyclo-(D-alanine^1–*X*^{2}–D-MeAsp^3–*Z*^{4}–Adda^5–D-glutamate^6–Mdha^7) in which *X* and *Z* are variables: L-amino acids, D-MeAsp^3 is D-*erythro*-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and Adda is an unusual C_{20} amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid. The structure of microcystin-LR is shown in Fig. 1. About 60 microcystin variants have been isolated to date from water bloom samples and strains of cyanobacteria [12]. Nodularin is also hepatotoxic and a pentapeptide composed of D-Glu, D-*erythro*-β-methylaspartic acid, L-X, Adda and Mdhb [2-(methylamino)-2(*Z*)-dehydrobutyric acid] produced by *Nodularia spumigena* (X = Arg for nodularin-R, X = Val for nodularin-V) (Fig. 1) [12].

Non-toxic peptides are mainly grouped into two categories, cyclic and acyclic ones and the former is further classified into several groups according to their structural characteristics. The first group of the former is a 19-membered depsipeptide with Ahp (3-amino-6-hydroxy-2-piperidone), which are named aeruginopeptin, anabaenopeptilide, micropeptin, oscillapeptilide, etc. They have the same skeleton composed of only L-amino acids and approximately 60 components have already been isolated [1]. The second group is also a 19-membered cyclic peptide possessing an ureido linkage; they are designated as anabaenopeptins, nodulapeptins and oscillamide [1]. Among the constituent amino acids in these compounds, Lys has only the D-configuration [8]. The third group includes a β-amino acid in the molecule such as nostophycin which is a 22-membered cyclic peptide isolated from *Nostoc* spp. The structure is similar to those of microcystins because they have in common a β-amino acid and D-amino acids [9]. Cyanobacteria produce simple cyclic peptides that include kawaguchipeptin A, oscillacyclin, etc. [13]. They belong to the fourth group and normally are constructed with only L-amino acids. Microcyclamide is also a cyclic peptide but it has one oxazole and two thiazoles in the molecule [14]. Microviridins (fifth group) are tricyclic peptides composed of 13 or 14 L-amino acids and have been produced by both toxic and non-toxic cyanobacteria [15]. Representative structures of each group are shown in Fig. 2.

Linear peptides are classified into two groups with or without a β-amino acid. Aeruginosins and spumigins are linear peptides composed of three amino acid residues including arginine and related amino acids as the C-terminus [16]. Linear tripeptides possessing a β-amino acid are designated as microginis or oscillaginis, in which the N-terminus is blocked with the constituent β-amino acid (Fig. 3) [17].

During the course of our biosynthetic study of cyanobacterial peptides including microcystins, we tried to establish a comprehensive analysis method for cyanobacterial peptides using LC–electrospray ionization (ESI) MS and photodiode array detection for the elucidation of the correlation between the peptide synthetase genes and metabolic peptides. Finally, the method was satisfactorily applied to three toxic cyanobacteria.

2. Experimental

2.1. Materials

M. aeruginosa K-139 and *M. viridis* S-70 (isolation and strain history described in [6] and [18], respectively) were isolated from Lake Kasumigaura and purified. *M. aeruginosa* NIES-298 was obtained from the National Institute for Environmental Studies, Japan (NIES). These strains were cultivated in the defined inorganic nutrient culture medium, called MA, under continuous illumination at 2500 lx at 25 °C and 70 rpm [19]. Cells were harvested after 2 weeks of cultivation and freeze–dried.

2.2. Extraction of peptide fraction for HPLC and LC–ESI-MS analysis

Each dried cell (100 mg) was extracted three times with 5% AcOH(aq.) (10 ml) for 40 min while stirring. The combined extracts were centrifuged at 5000 rpm for 10 min, and the supernatant was applied to a preconditioned octadecyl

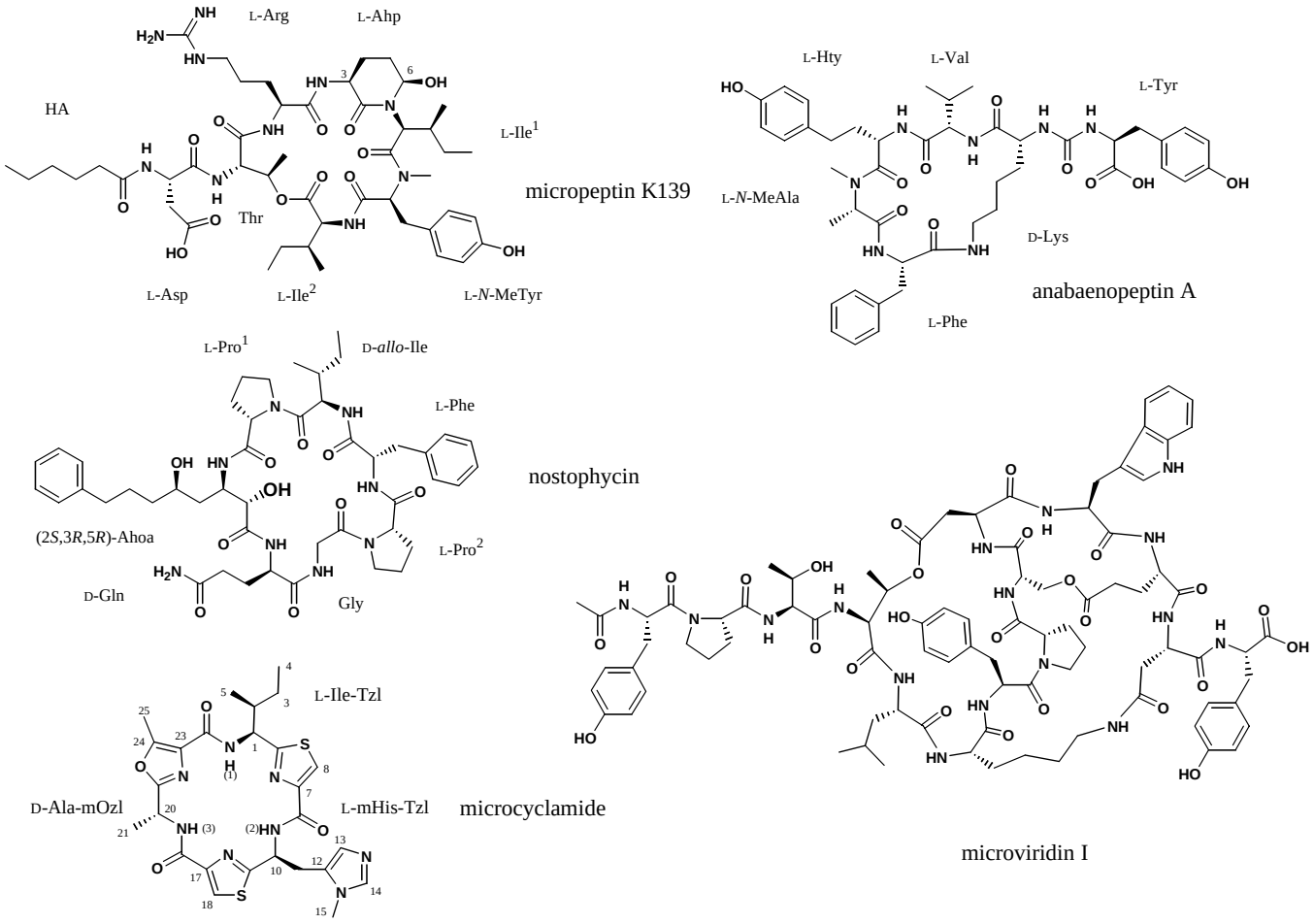

Fig. 2. Structures of non-toxic cyclic peptides, micropeptin K-139, anabaenopeptin A, nostophycin, microcyclaamide and microviridin I isolated from cyanobacteria.

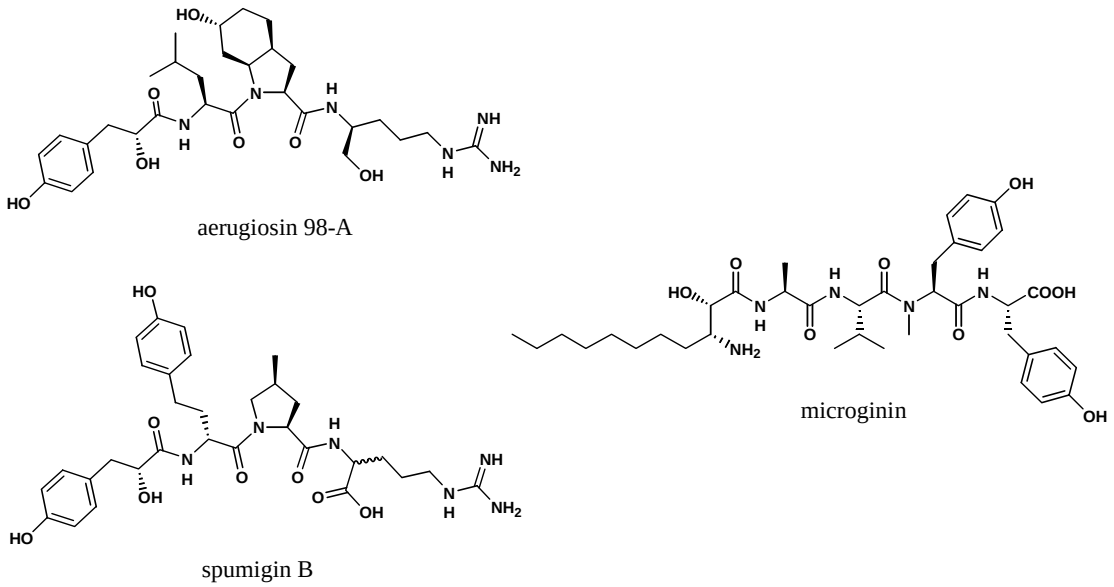

Fig. 3. Structures of non-toxic linear peptides, aeruginosin 98-A, spumigin B and microginin isolated from cyanobacteria.

silyl (ODS) silica gel cartridge (0.5 g, Varian Bond Elut C_{18}) after filtration through a glass microfiber filter (GF/C). The cartridge was rinsed with water (5 ml) and 20% MeOH(aq.) (5 ml) and then eluted with 90% MeOH(aq.) and MeOH (each 5 ml) to give the desired peptide fraction. After dilution with 400 μl of the mobile phase for the LC–ESI-MS analysis, 1 μl of the extracted samples was analyzed by HPLC and LC–ESI-MS.

2.3. HPLC and LC–ESI-MS conditions for the extracted peptide fraction

The separation of the peptides was performed on a TSK gel ODS-80Ts (150 mm × 2.0 mm i.d., Tosoh) column maintained at 40 °C using an HP1100 HPLC system (Agilent) at 238 and 280 nm as the detection. Methanol–water containing 0.1% formic acid was used as the mobile phase at a flow rate of 0.2 ml/min under the following gradient elution mode: methanol, 30% (0 min) → 35% (5 min) → 55% (15 min) → 65% (40 min). The mass spectrometer used was an API Qstar Pulsar-*i* (MDS Sciex, Toronto, Canada). All mass spectra were acquired using time-of-flight (TOF) MS. A mass range of *m*/*z* 300–2000 was covered with a scan time of 1 s, and data were collected in the positive ion mode. The high performance liquid chromatograph and mass spectrometer were interfaced with a laboratory-made flow splitter and an IonSpray ion source (MDS Sciex). The effluent from the HPLC was split at a ratio of 1:40, and a smaller portion of the effluent was introduced into the ion source at the flow rate of 5 μl/min. The IonSpray voltage was 5 kV with the nebulizer gas air pressure and curtain gas nitrogen pressure set at 20 and 25 psi, respectively (1 psi = 6894.76 Pa).

3. Results

3.1. Establishment of an analysis method

In order to correlate peptides from cyanobacteria to the corresponding peptide synthetase genes, a comprehensive analysis method is required, which can detect all secondary metabolic peptides in cells. As shown above, a

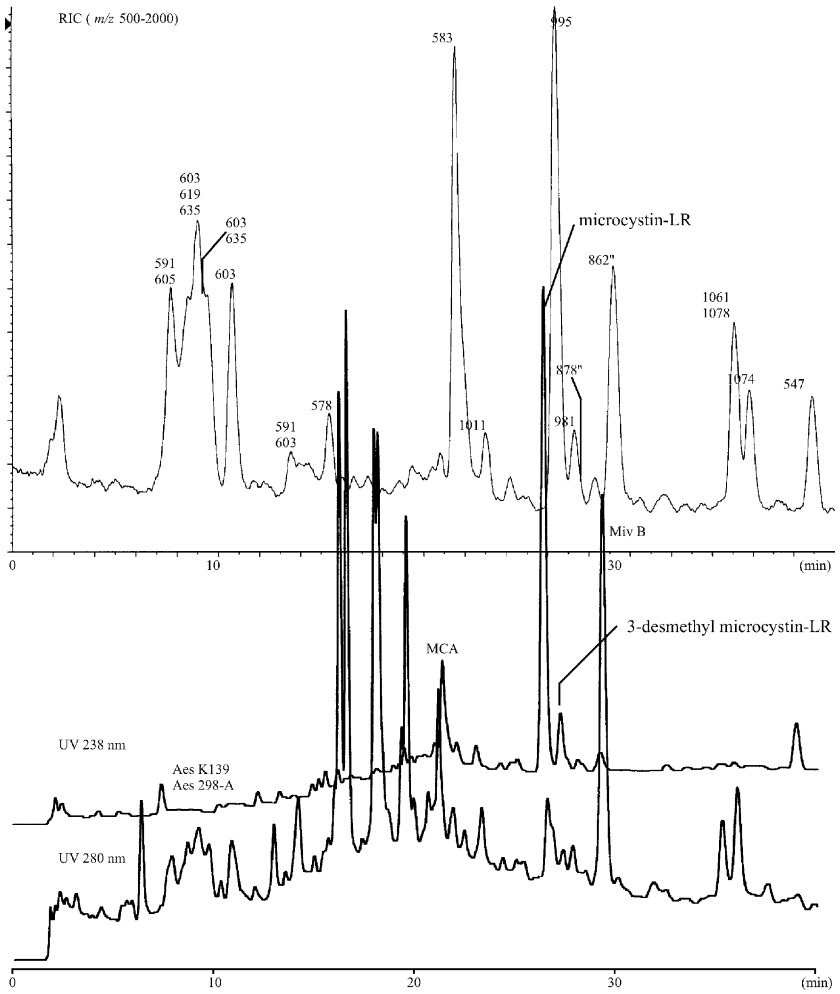

Fig. 4. HPLC chromatograms monitored by UV at 238 and 280 nm, and RIC chromatogram (*m*/*z* 500–2000) by LC–ESI-MS analysis of the extract from *M. aeruginosa* NIES-298. For analysis conditions, see Section 2. Aes: aeruginosin, MCY: microcyclamide, Miv: microviridin.

variety of peptides is produced by cyanobacteria, whose physico-chemical properties are diverse and no suitable method has been reported for the screening of these peptides. In a previous study, we isolated several peptides from Finnish toxic cyanobacteria under guidance using a screening method composed of LC–ESI-MS and a photodiode array [20]. This screening method consisted of 5% acetic acid extraction and ODS cartridge treatment followed by LC–MS analysis, in which a linear gradient elution mode (methanol, 45–65%, 40 min) was used to separate the microcystins, anabaenopeptins and anabaenopeptilides. They were detected using UV irradiation at 238 and 280 nm, and mass spectrometry. The analysis time was within 40 min [20].

In the present study, the previous method was applied without modification and satisfactory results were obtained except for the following point that the aeruginosins were eluted around 2 min, whereas other peptides were eluted at 30–35 min. This result indicated that aeruginosins must be eluted much more slowly and the resolution among the other peptides must be better. Based on the results of many trials, the following gradient elution mode [methanol, 30% (0 min) → 35% (5 min) → 55% (15 min) → 65% (40 min)] was optimized. The method using the optimized analysis conditions was applied to a cyanobacterium, *M. aeruginosa* NIES-298. Fig. 4 shows the HPLC chromatograms monitored by UV at 238 nm for the microcystins and at 280 nm for the other peptides, and the reconstructed ion chromatogram (RIC, *m/z* 500–2000) of the extract from this strain. In the chromatogram, the molecular related ions (mostly protonated molecules) obtained by this LC–MS method are indicated in the peaks and the doubly charged ions are shown by the double prime notation (″).

It is known that *M. aeruginosa* NIES-298 produces microcystin-LR and 3-demethylmicrocystins-LR as the toxin and aeruginosins and microcyclaminde as the non-toxic peptides [14]. The microcystins monitored by UV at 238 nm could be identified from the LC–ESI-MS spectra. Many peptides other than the microcystins could be clearly detected by the HPLC chromatogram monitored by UV at 280 nm and the RIC. Aeruginosins appear at 8–10 min but the resolution is not good among these components. Two of them were identified as aeruginosins K-139 and 298-A [21]. Although it is difficult to detect microcyclamide using UV at 280 nm, the peak of this peptide is clearly observed in the RIC. Two doubly charged ion peaks (*m/z* 878 and 862) are also found around 30 min in the RIC, one of which corresponds to microviridin B [22,23]. Although several peptides such as the three peaks between 30 and 35 min appear in the RIC, they could not be identified.

The method was further applied to another hepatotoxic cyanobacterium, *M. aeruginosa* K-139 [6], which is known to produce four types of peptides, microcystins, micropeptin, microviridins and aeruginosins. Although 7-demicrocystin-LR was easily detected by UV (280 nm) and LC–MS (Fig. 5), it was impossible to detect the minor component, 3,7-didemicrocystin-LR. There are several aeruginosins around 10 min in the RIC, two of which were identified as aeruginosins K-139 and 298-A [21]. The microviridins was easily found because they appeared as the doubly charged ions. In this sample, at least two components were identified as microviridins B and C [22,23]. Additionally, the established method clarified the existence of other types of peptides, micropeptin K-139 at 22 min.

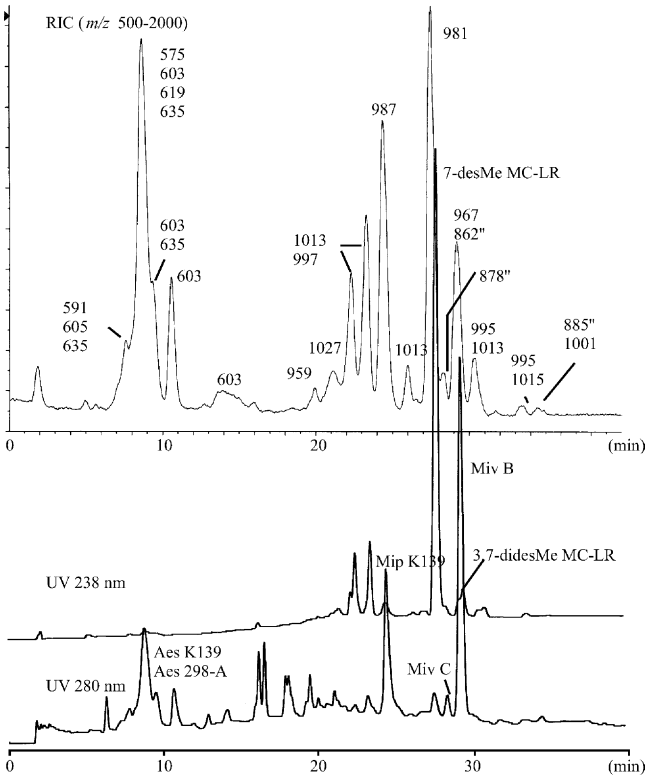

Fig. 5. HPLC chromatograms monitored by UV at 238 and 280 nm, and RIC chromatogram (*m/z* 500–2000) by LC–ESI-MS analysis of the extract from *M. aeruginosa* K-139. For analysis conditions, see Section 2. Aes: aeruginosin, Mip: micropeptin, Miv: microviridin, 7-desMe MC-LR: 7-demethylmicrocystin-LR.

3.2. Application of the established method

Gene disruption is a useful technique to clarify which cloned synthetase is responsible for the biosynthesis of the metabolites. In order to definitively verify the relationship between the synthetase gene and corresponding metabolite, the established method was applied to the wild and gene disrupted *M. vididis* S-70 strains [18]. The chromatogram of the wild strain is shown in Fig. 6a and no peaks of the aeruginosins appear around 10 min. There are several peaks assigned as microcystins in the RIC, three of which were identified as microcystin-LR, microcystin-YR and microcystin-RR based on their molecular weights. Because microcystin-RR has two arginine residues, it is observed as the doubly charged ion at *m/z* 519. Furthermore, there is an intense peak around 30 min in the RIC but this peak

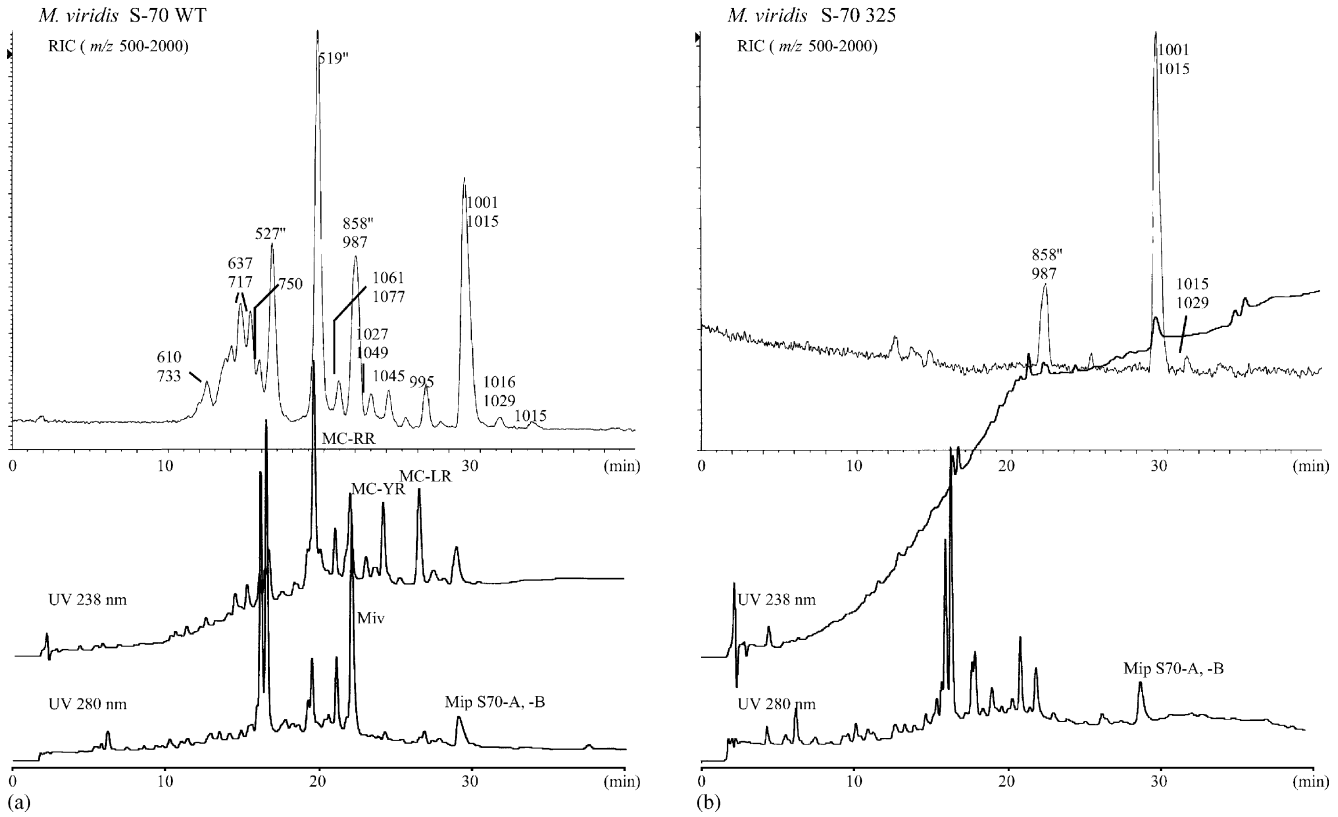

Fig. 6. HPLC chromatograms monitored by UV at 238 and 280 nm, and RIC chromatogram (*m*/*z* 500–2000) by LC–ESI-MS analysis of the extract from (a) *M. viridis* S-70 wild strain and (b) the corresponding mutant strain. For analysis conditions, see Section 2. MC-RR: microcystin-RR, MC-YR: microcystin-YR, MC-LR: microcystin-LR, Miv: microviridin, Mip: micropeptin.

contains two compounds. After isolation and characterization they were identified as micropeptins S-70A and S-70B. Although a component of the microviridins was also found at 22 min, its structure is still unknown.

Fig. 6b shows the chromatograms of the mutant of the *M. vididis* S-70 strain that was disrupted at microcystin synthetase gene (*mcy*) D. The peaks with *m*/*z* 858, 987, 1001 and 1015 are only observed and other peaks are completely lost, demonstrating that micropeptins and microviridin remained in the mutant. Although two peaks detected at 16 min strongly appear in the chromatogram at 280 nm, these peaks could not be detected in the RIC. These peaks were also found in the chromatograms of *M. aeruginosa* NIES-298 and *M. aeruginosa* K-139 as shown above. These results suggested that the protonated molecules of the two peaks are out of the range of *m*/*z* 500–2000 or they are not peptides.

4. Discussion

In order to analysis exhaustively the cyanobacterial peptides, a suitable method is required. Fastner et al. proposed an analytical method using matrix-assisted laser desorption ionization (MALDI) TOF-MS and used successfully their method for the typing of a single colony of a producing strain [24]. Although we also used their method for our objective, it was difficult to obtain reproducible results because it relies only on the molecular ion species of the analytes. Therefore, we improved our previously proposed LC–MS method including the photodiode array, and the established method under the specific elution conditions was satisfactorily applied to a few Japanese cyanobacterial strains. The established method was advantageous over conventional ones using usual HPLC and MALDI-TOF-MS [24], because more structural information such as retention behavior, molecular weight and ion intensity could be obtained and it is easier to distinguish microcystins from other peptides using this method.

The obtained structural information for each class of peptide by this method is summarized as follows: because microcystins show the characteristic absorption maximum at 238 nm, it is much easier to differentiate them from other types of peptides. The molecular weight information from mass spectrometry can distinguish known from unknown microcystins. Tricyclic peptides, microviridins, show the characteristic mass spectrometric behavior that one of their molecular ion species appears as the doubly charged ion. This is very useful for the differentiation of microviridins from other types of peptides. One of the linear peptides, the aeruginosins, has an extremely poor retention power on ODS silica gel compared with other peptides. Indeed, they appear within 10 min under the optimized conditions,

indicating that this is available for the differentiation of aeruginosins from other types of peptides. Naturally, molecular ion species of other types of peptides can be also obtained. Using the structural information summarized here, it is possible to characterize the types of cyanobacterial peptides.

Cyanobacteria produce diverse secondary metabolic peptides, which are formed through non-ribosomal peptide synthesis using the multifunctional thiotemplate mechanism. While the peptide synthetase genes for microcystin and micropeptin have been already cloned [3–5,11,25], the genetic analysis for the remaining peptides is now being carried out. In order to complete the understanding of the biosynthesis mechanism of cyanobacterial peptides, not only the genetic analysis but also the exhaustive analysis of metabolic peptides are required. Particularly, it is important to know the correlation of the peptides produced by cyanobacteria to the corresponding peptide synthetase genes. The established analysis method will contribute to the investigation of these correlations. In the very near future, the exact biosynthesis mechanism for cyanobacterial peptides will be elucidated, which will be useful for the regulation of the production of microcystins and related peptides in a lake.

Acknowledgements

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