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Verification of the Role of MlrC in Microcystin Biodegradation by Studies Using a Heterologously Expressed Enzyme

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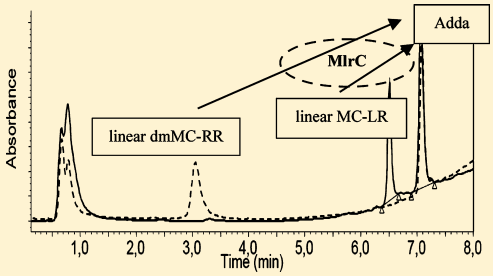
Supporting Information

ABSTRACT: The MlrC protein from *Sphingomonas* ACM-3962 strain was heterologously expressed in *Escherichia coli* strain BL21(DE3) and purified to investigate participation of this enzyme in the biodegradation of two microcystin variants. In contrast with previous reports, our results indicated that MlrC cleaves linear microcystins, thus shedding new light on the role of MlrC enzyme in microcystin biodegradation.



Enzymatic degradation of microcystins (MCs) has usually been associated with members of the Proteobacteria family.[1] The known MC degradation pathway of *Sphingomonas* strains was described as a linear, three-step process catalyzed by three enzymes, named MlrA, MlrB, and MlrC.[2] The gene cluster involved in biosynthesis of the enzymes was also characterized.[3] The cloning of the total genomic DNA of *Sphingomonas* sp. enabled identification of a 5.8 kb sequence that includes three genes, called mlrA, mlrB, and mlrC, which encode proteins exhibiting enzymatic activity, and a fourth gene, called mlrD, which encodes a transporter protein.

This scheme was repeated by several authors[1,4−6] that assumed sequential activity of the three enzymes and dependence of MlrC on MlrB following the production of tetrapeptides from linear MCs. Here, we describe how expression of the MlrC enzyme in the heterologous host, *Escherichia coli* BL21(DE3), enabled us to verify the order of action of three enzymes involved in the degradation of MCs. Additionally, we identified a main product of MlrC and evaluated its toxicity.

Linear MCs used in the experiments were obtained using heterologous MlrA, as described by Dziga et al.[7] Whereas MC-LR was purified from a culture of *Microcystis aeruginosa* strain PCC 7813,[8] dmMC-RR was purified from *Microcystis aeruginosa* strain NIES 107.[9]

The sequence coding for MlrC was amplified by PCR, using *Sphingomonas* sp. ACM-3962 genomic DNA as the template. The amplified fragments were inserted into the pTZ57R/T cloning vector (Fermentas), and the sequence was verified. Subsequently, the fragments were cut out using the *Nde*I and *Not*I restriction enzymes, and inserted into the expression vector pET21a (Novagen). The resulting construct, named pET21-*mlrC*, was transformed into *E. coli* BL21(DE3).

Recombinant MlrC was purified using an NiTNA (Qiagen) column under denaturing conditions and refolded by dialysis. Enzyme preparation was used to estimate the activity against linear MCs, and the products of its activity were analyzed using HPLC and/or mass spectrometry (MS)-based methods.

Our HPLC analyses were performed using a Waters HPLC system. The MCs and degradation products were quantified and isolated using a Purospher STAR RP-18 endcapped column as described by Meriluoto and Spoof.[10] MS analyses were performed using a Bruker Daltonics HCT Ultra Ion Trap with an electrospray (ESI) ion source. The toxicity of the MlrC product obtained after HPLC was established by the PP1 inhibition assay described by An and Carmichael.[11]

To date, most experiments involving enzymes responsible for degradation of MCs required the use of crude extracts of *Sphingomonas* cells. The only exception, the work of Bourne et al.,[3] involved cloning the total genomic DNA of *Sphingomonas* sp., identifying a 5.8-kb gene cluster involved in the biosynthesis of protein exhibiting proteolytic activity toward MCs, and then characterizing this cluster by nucleotide sequencing. The heterologous expression of Mlr enzymes seems to be the best way to control any particular step of interest in MC degradation.

In contrast to MlrA,[7] recombinant MlrC can be overexpressed in *E. coli*. However, given that the protein was exclusively deposited in inclusion bodies, we purified the

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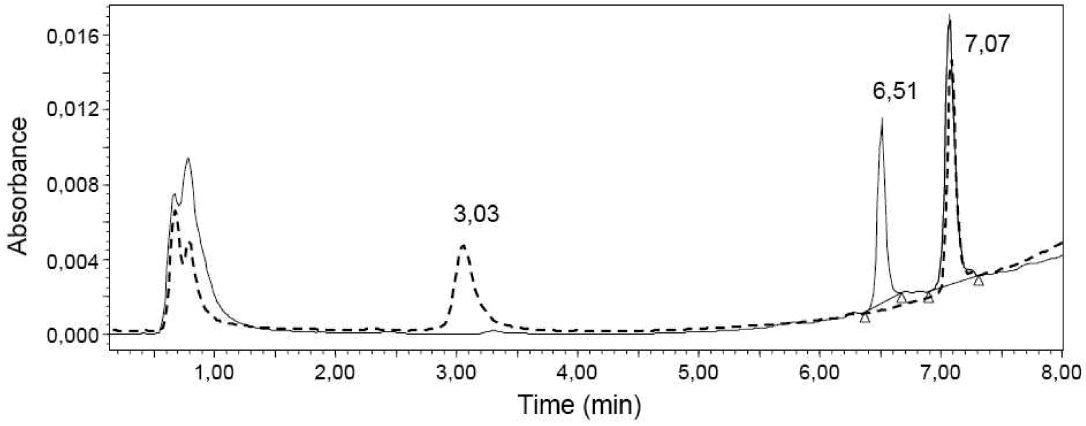

Figure 1. HPLC chromatographic profile showing MlrC activity expressed in *E. coli* BL21 (DE3) toward the linear MC-LR (solid line) and the linear dmMC-RR (dashed line) variants. Peaks with retention times of approximately 3.0 and 6.5 min correspond to linear dm-MC-RR and MC-LR, respectively, whereas the peak with an approximate retention time of 7.1 min corresponds to the products of MlrC activity (Adda molecules).

recombinant protein under denaturing conditions, taking advantage of a C-terminal histag. After purification, recombinant MlrC was refolded using a dialysis-based method. Although rates of protein recovery were low, the remaining activity was sufficient to unambiguously indicate the role of MlrC in the degradation of MCs. Cyclic MC molecules were not hydrolyzed, whereas linear MC-LR and linear dmMC-RR were degraded by MlrC, with the formation of degradation products visible at 238 nm (Figure 1). The retention time (7.1 min) and UV spectrum typical for Adda diene ((2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid) were identical for both MC variants used.

Samples that corresponded to peaks with a 7.1 min retention time were collected manually and analyzed using LC-MS. The isolated products had *m*/*z* ratios of 663 (Figure 1SA,B, Supporting Information) and mass spectra typical for coupled Adda molecules (2 M + H^+), as described previously.[4,5] The MSn fragmentation pattern of the 663 ion indicated peaks typical for Adda (Figure 1SC, Supporting Information) and similar to those obtained by Imanishi et al.[5] This enabled us to confirm that MlrC hydrolyzes the Adda-Glu peptide bond of the two linear MCs, MC-LR and dmMC-RR.

Our findings shed new light on the degradation of MCs. Although Bourne et al.[2] documented the sequential accumulation of linear MCs and tetrapeptide, they did not identify Adda as a degradation product. A subsequent publication[3] mentioned that MlrC is active toward both linear MC-LR and tetrapeptide. The authors suggested that MlrC may cut the Adda-Glu bond or diene bond of Adda amino acid.[3] However, they found no degradation products indicative of an MlrC activity directed toward linear MCs. All subsequent papers that concern the detection of degradation products of MCs have assumed the sequential action of Mlr enzymes. The isolation of Adda was also performed previously by way of two intermediates, linear MC-LR and the tetrapeptide.[4]

The experiments with crude extracts of *Sphingomonas* strains (ACM and B-9) require the use of protease inhibitors to accumulate sequential degradation products. Given that both MlrA and MlrC are metalloproteases, use of ethylene diamine tetraacetic acid (EDTA) or phenatroline to inhibit MlrA inhibits MlrC activity. Whereas addition of phenylmethylsulfonyl fluoride (PMSF) promotes the accumulation of linear MC, addition of EDTA promotes accumulation of the tetrapeptide. The inhibition of MlrB should result in the accumulation of Adda, following the sequential action of MlrA and MlrC. Nonetheless, this product has never been recognized as the molecule released following MlrC activity. In the work of Imanishi et al.,[5] the presented LC-MS analyses of MC-RR degradation indicated simultaneous accumulation of ions with *m*/*z* 332.2 (Adda) and *m*/*z* 615.3 (tetrapeptide). Nonetheless, these products were not interpreted as the result of simultaneous activities of MlrB and MlrC toward linear MC-RR. The almost complete scheme of MC degradation was published in 2009.[6] Using the advanced Marfey method (detection of the products without typical UV spectra), the authors identified Adda as a product of hydrolysis of either thetetrapeptide Adda-Glu-Mdha-Ala or the tripeptide Adda-Glu-Mdha. This method indicated the accumulation of several intermediates within 96 h of degradation. In contrast to all other compounds, Adda concentration increased continuously and uniformly during the experiment. This suggests that its production was not dependent on the appearance of other intermediates (Adda-Glu-Mdha or Adda-Glu-Mdha-Ala) but was the consequence of enzymatic activity (MlrC in our opinion) toward linear MC-LR.

The results of Jiang et al.[12] are also consistent with a role for MlrC role in the degradation of MCs. That study reported a gene cluster encoding MC-degrading enzymes in *Novosphingobium* sp. THN1. Interestingly, a frame shift in the gene that encodes *mlrB* generates a premature stop codon, causing it to be transcriptionally silent. The observation that the bacteria were able to degrade MC-LR suggests that MlrA and MlrC in this strain may hydrolyze MC via an alternative pathway that does not involve MlrB. This statement is congruent with our results; nonetheless, it requires experimental confirmation using purified enzymes from Novosphingobium sp. THN1.

Although Adda plays a key role in the inhibition of protein phosphatase PP1 and PP2A, the biological activities of MCs derive not only from Adda but also depend on other components of MC molecules.[13] Previous papers[4,5,14] reported that Adda is not toxic. In the current study, we found that the calculated IC_{50} of Adda molecule was 0.975 ± 0.168 $\mu g\ mL^{-1}$ (Figure 2), a value that suggests toxicity similar to that of linear MC-LR.[7] This means that the linear MC-LR and Adda derivatives are not toxic in practice.

In conclusion, it has been documented for the first time that MlrC from *Sphingomonas* ACM-3962 strain cleaves the Adda-Glu peptide bond of linear heptapetides. Our results do not

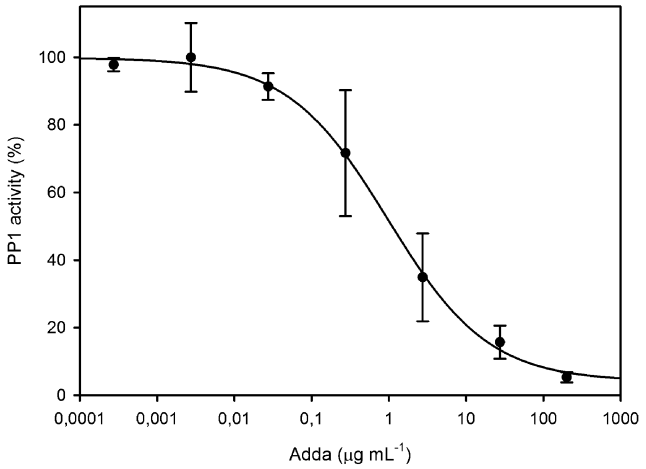

Figure 2. Inhibition pattern of PP1A catalytic subunit by Adda. Bars represent standard deviations, $n = 3$.

exclude the possibility that MlrC is active toward other peptide bonds, resulting in the production of different peptides or/and amino acids. Further studies are needed to confirm or exclude such potential activities.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and MS spectra of the Adda molecule and its fragmentation pattern. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MlrC, microcystinase C; MlrA, microcystinase A; MCs, microcystins; Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid

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