Further Investigation of the Enzymatic Pathway for Microcystins Biodegradation by *Sphingopyxis* sp. USTB-05

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Keywords: Microcystin-LR, Enzymatic pathway, *Sphingopyxis* sp. USTB-05, Biodegradation.

Abstract. Based on the isolation of a promising bacterial strain of *Sphingopyxis* sp. USTB-05 for biodegrading MCs, the genes USTB-05-A (HM245411), USTB-05-B (KC513423) and USTB-05-C (KC573527) that are responsible for the biodegradation of MCs were cloned and expressed for the first time. After purification, the MC-degrading enzymes encoded by these genes were used for the catalytic degradation of MC-LR. The results demonstrated that the second enzyme encoded by USTB-05-B could convert linear MC-LR to a tetrapeptide by breaking the Ala–Arg bond. The third enzyme encoded by USTB-05-C could cleave Adda-Glu peptide bonds of both linear MC-LR and the tetrapeptide of Adda-Glu-Mdh-A, producing Adda as their common product. These findings will help better understand the biodegradation mechanism of MCs by *Sphingopyxis* sp. USTB-05.

Introduction

With increased wastewater discharge containing nitrogen and phosphorus into rivers and lakes, harmful cyanobacterial blooms have become more frequent worldwide. This has led to the destruction of the natural ecological system and production of cyanobacterial toxins such as microcystins (MCs).

More than 70 microcystin isoforms are found in part due to the variable L-amino acids X and Z. Microcystin-LR (MC-LR) is one of the most frequent and studied variant. In 1998, the World Health Organization established a guideline value of 1 µg/L as the maximum concentration of MC-LR in drinking water.

Microcystin-LR is chemically stable compounds and conventional drinking water treatments has limited efficacy in removing MC-LR [1,2], while it can be readily biodegraded by the strain of *Sphingomonas* sp. USTB-05 [3]. And three enzymes, encoded by the microcystin-degrading gene USTB-05-A (HM245411), USTB-05-B (KC513423) and USTB-05-C (KC573527), respectively, were found to be involved in sequentially biodegrading MC-LR [3,4]. Previous analysis showed that the enzyme USTB-05-A can cleave the Adda-Arg peptide bond in MC-LR, and convert cyclic MC-LR to linear MC-LR (H-HN-Adda-D-Glu-Mdh-A-Ala- L-Arg-D-Masp-L-Arg-OH) (m/z 1056.5) as its first degradation product. While the second and third steps on the catalytic degradation of MC-LR by the USTB-05-A and USTB-05-B were still unknown. The objective of this study was to obtain direct evidence of the second and third steps on the biodegradation pathway of MC-LR by *Sphingopyxis* sp. USTB-05, and to lay a foundation for constructing a genetically engineered bacterium to remove MCs efficiently using the gene of *Sphingopyxis* sp. USTB-05 involved in MCs biodegradation.

Materials and Methods

Degradation Enzyme Preparation

The recombinant enzyme B (encoded by USTB-05-B) and enzyme C (encoded by USTB-05-C) were prepared like the methods shown in the reference [5].

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Enzyme Activity Assays

To detect the activity of recombinant enzyme B (encoded by *USTB-05-B*) and enzyme C (encoded by *USTB-05-C*), MC-LR was used as the target to degradation. LB liquid (1 L) medium supplemented with ampicillin (50 µg mL⁻¹) was inoculated with 25 mL overnight *E. coli* BL21 (DE3) containing recombinant plasmid culture. The inoculated culture was grown with agitation at 37 °C, 200 rpm to an OD600nm of approximately 0.6, induced with isopropyl b-D-thiogalactoside at final concentration of 0.1mM and grown constantly at 30 °C, 200 rpm for 3 h. Cell free extracts (CE) of recombinant *USTB-05-A* were used to produce the first biodegradation product of MC-LR (linear MC-LR) [5]. The catalytic degradation of linear MC-LR by enzyme B and enzyme C respectively were shown in Figure 1. All samples were centrifuged at 12,000 rpm for 10 min, and then used for high performance liquid chromatography (HPLC). After purified and concentrated using a C18 solid-phase extraction cartridge (OASISTM HLB, Waters Corporation, Milford, MA), the samples were used for liquid chromatography tandem mass spectrometry (LC-MS). The experimental conditions of HPLC and LC-MS were adopted to the reference [5].

![Figure 1. Catalytic degradation roadmap of linear MC-LR by enzyme B and enzyme C respectively.](image)

Results and Discussion

Enzymatic Activity Detection of Recombinant *USTB-05-B* and *USTB-05-C*

To confirm the enzymatic activity of the expressed protease, CE of recombinant *E. coli* DH5α was prepared and used to biodegrade linear MC-LR and the products respectively. As shown in Fig. 2 and Fig. 3, the area of the targets to be degraded decreased with time course. In contrast, a new peak (product A, product B, product C) appeared and increased with time course. The absorbance profiles of all the products were very similar to that of MC-LR and the maximum absorbance for both was at 239 nm or so. This indicates that the expressed protease encoded by *USTB-05-B* and *USTB-05-C* are thus capable of biodegrading linear MC-LR and its products respectively. While the enzyme B has no enzymatic activity to product C as while as the enzyme B to product B.

LC-MS Analysis of Biodegradation Product

The liquid chromatogram-mass spectrum (LC-MS) was used to measure the mass/charge ratios of product A, product B and product C (Fig. 4). As shown in Fig. 4A, the protonated molecular ion of the product A was detected at m/z 615.3 [M+H]⁺ resulting from the loss of Tyr-MeAsp-Arg from linear MC-LR. The peak at m/z 464.2 [M+H-151]⁺ corresponded to the loss of the terminal phenylethylmethoxy group (MW: 135) and the amino NH₂ group (MW: 16) from Adda [6], and the ions at m/z 509.3 were caused by the loss of the Adda amino group from the proposed parent compound. So, it was confirmed that the structure of the second product of MC-LR was tetrapeptide Adda-Glu-Mdha-Ala.
Figure 4B shows that mass-to-charge ratios of the product B and product C is at \( m/z \) 332.2, \( m/z \) 315.2 and \( m/z \) 135.1, which relate to M+H, the loss of the amino \( \text{NH}_2 \) group (MW: 16) from Adda and the PhCH\(_2\)CHOMe part of Adda, respectively. Therefore, it was inferred that the third product was Adda. This result is in agreement with the report by [7].

Figure 2. High performance liquid chromatography (HPLC) profiles for the biodegradation of linear MC-LR by enzyme B with time course (from top to down): (a) 0 min; (b) 10 min; (c) 30 min; (d) 60 min.

Figure 3. High performance liquid chromatography (HPLC) profiles for the biodegradation of product A (A) and linear MC-LR (B) by enzyme C with time course respectively (from top to down): (a) 0 min; (b) 5 min; (c) 10 min.
Thus, we can conclude that the enzyme B is capable of degrading linear MC-LR and producing the product tetrapeptide Adda-Glu-Mdha-Ala, while the enzyme C is not only capable of degrading tetrapeptide but also linear MC-LR producing the product Adda (Fig. 5).

Figure 4. Liquid chromatogram-mass spectrum (LC-MS) profiles. (A) mass spectrum (MS) for product A; (B) MS spectrum for product B and product C.

Figure 5. The second and third steps involved in the enzymatic pathway for the biodegradation of MC-LR by *Sphingopyxis* sp. USTB-05. The big arrows indicate the biodegradation route and the small arrows indicate the addition of the enzymes.

**Conclusions**

We cloned and expressed the second (*USTB-05-B*, 1626 bp) and third (*USTB-05-C*, 1587 bp) genes involved in the biodegradation of MC-LR by *Sphingopyxis* sp. USTB-05. We found that the enzyme encoded by *USTB-05-B* could convert the linear MC-LR to a tetrapeptide. The enzyme encoded by
USTB-05-C could cleave Adda–Glu peptide bonds of both linear MC-LR and the tetrapeptide Adda–Glu–Mdha–Ala. Adda was produced as their common product. This study is not only a significant for understanding the biodegradation pathway of MCs, but also provides insight into an important mechanism to remove harmful MCs from drinking water sources.

Acknowledgement

This research was financially supported by the National Natural Science Foundation of China (No. 21467009, 21677011).

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