การทำบริสุทธิ์สารพิษจากไซยาโนแบคทีเรีย ไมโครซิสตินโดยดีอีเออี และสตราต้า-เอ๊กซ์ โครมาโตกราฟี

Purification of cyanobacterial toxin, microcystins, by DEAE and Strata-X SPE chromatography

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บทคัดย่อ

เซลล์แห้ง (20 กรัม) ของไซยาโนแบคทีเรียที่เจริญอย่างมากมายจากบึงหนองโคตร จังหวัดขอนแก่น ถูกสกัดและทำบริสุทธิ์โดยสารผสมของสารพิษไมโครซิสตินหลายชนิดถูกระบุชนิด สารพิษถูกทำให้บริสุทธิ์โดยดีอีเออี และสตราต้า-เอ๊กซ์ โครมาโทกราฟี และระบุชนิดโดยใช้เครื่องโครมาโทกราฟีของเหลวสมรรถนะสูงที่มีโฟโตไดโอดอาร์เรย์เป็นตัวตรวจวัด และใช้เครื่องโครมาโทกราฟีของเหลวสมรรถนะสูงที่มีแมสสเปกโทรมิเตอร์เป็นตัวตรวจวิเคราะห์มวล จากการศึกษาองค์ประกอบทางเคมีของเซลล์แห้งพบไมโครซิสติน 3 ชนิด ประกอบด้วย ไมโครซิสตินชนิดอาร์-อาร์ ชนิดเอฟ-อาร์ และชนิดแอล-อาร์ที่มีกรดอะมิโนในตำแหน่งที่ 7 เป็น Dha ซึ่งชนิดแอล-อาร์ชนิดดังกล่าวเป็นสารพิษชนิดหลักที่พบ มีความบริสุทธิ์ 93 เปอร์เซ็นต์ และได้ผลผลิตจำนวน 25.38 มิลลิกรัม โดยไมโครซิสตินแอล-อาร์ชนิดที่มีกรดอะมิโนในตำแหน่งที่ 7 เป็น Dha จัดเป็นไมโครซิสตินชนิดแอล-อาร์ชนิดย่อยชนิดหนึ่ง ที่เกิดการขาดหมู่เมทิลของกรดอะมิโน Mdha ทำให้เกิดเป็น Dha ซึ่งเป็นชนิดหลักที่พบในบึงหนองโคตร แทนที่จะเป็นชนิดแอล-อาร์อย่างที่พบในทะเลสาบหลายแห่งในต่างประเทศ จากผลการตรวจพบไมโครซิสตินนี้อาจเชื่อมโยงกับรายงานการตายของสัตว์ และการเจ็บป่วยของมนุษย์ เนื่องจากเป็นสารพิษที่มีความคงตัวสูง และอาจคงอยู่ในน้ำได้หลายสัปดาห์ ความรู้ของการตรวจพบชนิดของไมโครซิสตินในแหล่งน้ำจะนำสู่การกำจัดสารพิษต่อไป

ABSTRACT

The lyophilized cells (20 grams) of cyanobacterial bloom from Bueng Nong Khot, Khon Kaen province was extracted and purified and a mixture of microcystins (MCs) were identified. The toxins were purified with DEAE and Strata-X SPE cartridge chromatography and identified by reversed-phase HPLC with photodiode-array UV detection (UVD) and liquid chromatography-tandem mass spectrometry (LC-MS). Chemical characterization of the lyophilized cells revealed three variants of MCs, including MC-RR; MC-FR and [Dha^7]MC-LR) of which [Dha^7]MC-LR was the major variant extracted with a purity of 93% and yield of 25.38 mg. [Dha^7]MC-LR is MC-LR which is the

loss of a methyl group which occurs at the amino acid, Mdha, resulting in [Dha^{7}]MC-LR The main variant appears to be [Dha^{7}]MC-LR not MC-LR as seen in lakes overseas. These toxins have been implicated in animal deaths and human illness. They are extremely stable and may persist in water bodies for several weeks. Knowledge of the MC-variants present in the water bodies will help in further investigations in eliminating them.

คำสำคัญ: ไซยาโนแบคทีเรียที่เจริญอย่างมากมาย การทำบริสุทธิ์ ไมโครซิสติน บึงหนองโคตร
Keywords: Cyanobacterial bloom, Purification, Microcystin, Bueng Nong Khot

INTRODUCTION

Microcystins (MCs) are a family of hepta-peptide hepatotoxins synthesized by planktonic cyanobacteria, belonging to a diverse range of species from the genera *Microcystis, Anabaena*, *Planktothrix, Oscillatoria,* and *Nostoc* (Sivonen and Jones, 1999; Zyska and Jasik-**Ślę**za, 2014). Their occurrence is well documented in eutrophic brackish and fresh water throughout the world. They have caused the death of wild and domestic animals worldwide, and are recently recognized as a potential threat to human health (Carmichael and Falconer, 1993; Zurawell et al., 2005). Risk may occur through acute exposure resulting in hepatic injury, which may prove fatal. One such incident occurred recently and resulted in the death of 50 dialysis patients in Brazil due to the use of MC contaminated water in their treatment (Jochimsen et al., 1998; Pyo and Lee, 2002). Chronic exposure can occur due to the presence of MCs in drinking water and is thought to be a contributing factor in primary liver cancer through the known tumor-promoting activities of these compounds (Nishiwaki-Matsushima et al., 1992; Trevino-Garrison et al., 2015).

The general structure of MCs includes three D-amino acids (D-alanine, D-glutamic acid and D-b-methylaspartic acid), two unusual amino acids (N-methyl dehydroalanine and 2S, 3S, 8S, 9S-3-amino-9-methoxy-2, 6, 8-tri-methyl-10-phenyldeca-4E, 6E-dienoic acid [Adda]), and two variable L-amino acids (Carmichael, 1992; Barco et al., 2005).

MCs, being cyclic peptides, are extremely stable and resistant to chemical hydrolysis or oxidation at near neutral pH. The toxins can remain potent even after boiling (Carmichael and Falconer, 1993). In natural waters and in the dark, MCs may persist for months or years (Sivonen and Jones, 1999; Somdee et., 2014). At 40°C and at high or low pH, slow hydrolysis was observed, with the time for greater than 90 % breakdown being approximately 10 weeks at pH 1 and greater than 12 weeks at pH 9 (Harada et al., 1996). Rapid chemical hydrolysis can occur only in conditions that are unlikely to be attained outside the laboratory, e.g. 6M HCl at high temperatures (Sivonen and Jones, 1999; Shang et al., 2018).

Botes et al. (1982) were the first research group to purify and characterize MCs. Since then, many different techniques have been developed. Typically, cyanobacterial cells are extracted, the resultant extract concentrated and the MCs purified by a range of sample separation techniques (Lawton and Edwards, 2001). However, the number of steps and methods employed vary greatly (Lawton et al., 1994; Edwards et al., 1996a; Edwards et al., 1996b; Fastner et al., 1998; Lawton and Edwards, 2001; Pyo and Lee, 2002; Aranda-Rodriguez et al., 2003; Barco et al., 2005; Somdee et., 2016). To date, there is no consensus on the most efficient method for MC purification, and ultimately what methods are used may depend on what the MC variants will be used for. For instance, where the aim is to purify substantial quantities of one

or two key MCs the approach will tend to be different from that employed to purify and identify all MCs present in a sample. It is therefore important to have a clear idea of required outcome prior to beginning purification.

In our laboratory, significant quantities of MCs were required for testing the effectiveness of biodegradation as a means of purifying drinking water. However, we also interested in the variety of variants present in Thailand lakes such as Bueng Nong Khot, Khon Kaen, which was known to produce *Microcystis* blooms, containing high potential of producing a wide variants of MCs. The purification method that suited our purpose was modified from Saito et al. (2002), which employed anion exchange chromatography as the first purification step. However, a different type of secondary purification (Strata-X cartridges) was used. The toxins were further characterized by reversed-phase HPLC and LC-MS.

MATERIALS AND METHODS

2.1 Cyanobacterial cells

A large mass of *Microcystis aeruginosa* cell material was harvested from the littoral zone of Bueng Nong Khot reservoir, Khon Kaen, with a 200-µm plankton-net from between February and May 2015. The material was lyophilized using a freeze drier and the lyophilized material was stored at -20°C prior to extraction.

2.2 Extraction and purification with DEAE anion exchange chromatography

MCs were extracted and purified using the method reported by Saito et al. (2002), with some modification. Briefly, 20 g of the freeze-dried material was extracted 3 times with 70% methanol (MeOH)-0.1% trifluoroacetic acid (TFA) and then precipitated with 55% saturated ammonium sulfate. Ten millilitres of the extract (250 mg/ml) dissolved in 0.05 M 2-morpholinoethanesulfonic acid (MES)–potassium hydroxide (KOH) (pH .5) – 20% (v/v) ethanol (EtOH) (solution A) were added to a column (4x30 cm i.d.) packed with a Toyopearl DEAE-650M resin. The toxins were separated by a gradient elution of 0.05 M MES-KOH (pH 5.5)-20% EtOH (solution A) and 0.05 M MES-KOH (pH 5.5)-20% EtOH-1M sodium chloride (solution B), starting at 100% of solution A for 80 min, and then solution B was linearly increased from 0 to 100% at 140 min. A flow rate was controlled at 1 ml/min by a peristaltic pump (Minipuls 3, Gilson). Ten ml fractions were collected by a fraction collector and pooled according to absorbance at 238 nm.

2.3 Solid phase extraction (SPE)

A final step of desalting using SPE cartridges was employed when the toxins were contaminated with salts (i.e. NaCl in this study) in the mobile phase of column chromatography. The pooled fractions obtained from anion exchange chromatography were passed through the Strata-X SPE cartridges which were conditioned with 90% and 20% MeOH. Impurities were successively washed with 30% (v/v) aqueous MeOH and the toxins were eluted with 70% MeOH at a flow rate of 1.0 ml/min under the compression module. The eluted toxins were collected and analyzed individually by HPLC/UVD and LC-MS as described in Sections 2.4 and 2.5.

2.4 Primary identification by reversed-phase HPLC

The toxins were analyzed and identified by a modular Shimadzu LC-10AD HPLC system comprised of a degasser unit (GT-104), solvent pump (LC-10AT pumps), autoinjector (SIL-10A), column oven (CTO1-10A), diode-array detector (SPD-M10A), and CBM-10A communication controller system, and LC-10 class software. The analytical column was a TSK-GEL ODS-80Ts column (150 x 4.6 mm, Tosoh), maintained at

30°C under water/acetonitrile gradient conditions with 0.05% TFA. The flow rate was at 1.0 ml/min. UV-spectra were acquired from 200 to 300 nm and MCs were identified by their characteristic absorption spectra (UV_{max} 238 nm).

2.5. Secondary identification by LC-MS

The MC variants were performed with a coupled liquid chromatography-tandem mass spectrometry system consisting of an alliance 2790 liquid chromatography (Waters) and a Quattro Ultima (Micromass) tandem mass spectrometer. A Luna C-18 column (150 mm x 2 mm i.d., 5 µm) (Phenomenex) was used for LC separation with the column oven at 30°C. The gradient mobile phase was water/acetonitrile with acidic buffer (1.7 mM ammonium formate and 24 mM formic acid) at the flow rate of 0.2 ml/min. The mass spectrometer was operated in positive mode electrospray ionization (ESI^+) for parent ion spectrum and daughter ion spectrum for MS-MS channels set up. The nebulizing, desolvation and cone gas were supplied with nitrogen. The instrument was calibrated with authentic standards of MC-RR and MC-LR and gave highly linear calibration curves for concentrations in the range of 5–200 ng/ml. The response factors for MC-LR were applied to the other related toxins for which no pure analytical standards were available.

RESULTS

3.1 Extraction and first purification with anion exchange chromatography

The lyophilized cyanobacterial bloom material (20 g) provided the successive extractive values of 3.52 g (17.6% yields by weight from the lyophilized cyanobacterial material) after acidic-methanol extraction and precipitation with ammonium sulfate. The crude extract loaded onto a Toyopearl DEAE-650M column gave the elution profile as shown in Figure 1. The chromatographic profile shows three maxima at 238 nm (3 fractions), designated MC-1 to MC-3.

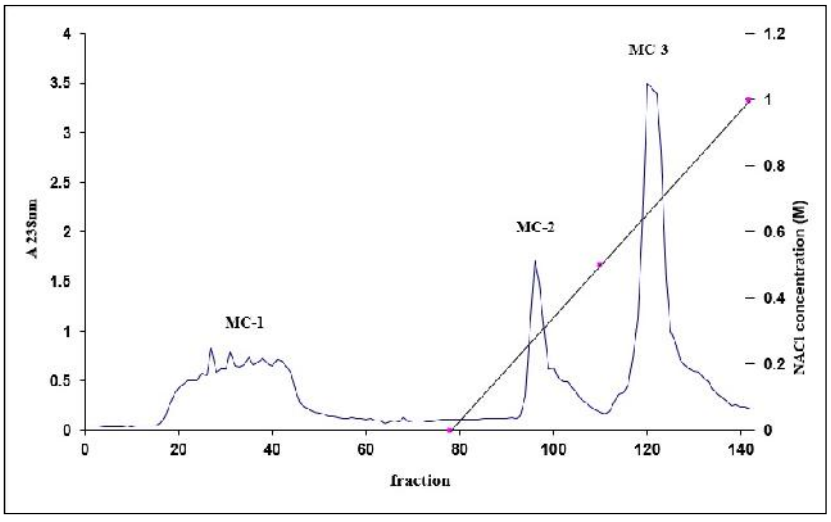

Figure 1. Chromatograms of DEAE measured at 238 nm. Broken lines show concentrations of NaCl. The toxins were categorized into three fraction groups (designated by MC-1 to MC-3).

3.2 Identification of purified MCs with HPLC and LC-MS

The compounds from MC-1 to MC-3 fractions were identified using LC-MS at Cawthron Institute, Nelson, New Zealand.

The MC-1 fraction contained MC-RR. MC-RR exhibited both $[M+2H]^{2+}$ and $[M+H]^{+}$ ions at *m/z* 520 and 1038, respectively (Figure 2a) (the molecular weight of MC-RR = 1037). The 1027 *m/z* ion confirmed MC-FR in the MC-2 fraction (Figure 2b).

Fraction MC-3 contained the most toxin material and was identified as [Dha^{7}]MC-LR from the MS spectrum with a *m/z* of 981.75 in ESI^{+} (Figure 2c). [Dha^{7}]MC-LR is 14 mass units less than MC-LR due to the loss of a methyl group which occurs at Mdha. Therefore, the toxin has Dha (dehydroalanine) instead of Mdha (*N*-Methyldehydroalanine).

3.3 The yields and purity of MCs

Three MC variants, MC-RR, MC-FR and [Dha^{7}]MC-LR were purified from the samples extracted from Bueng Nong Khot. The major variant of the MCs extracted and identified was [Dha^{7}]MC-LR, which was presented in the M3 fraction (Table 1). The MC-1 fraction contained predominantly MC-RR (92%), giving a total yield of 21.63 mg; whereas MC-2 was mainly MC-FR (91%) total yield of 19.45 mg and MC-3 mainly [Dha^{7}]MC-LR (93%) with total yield of 25.38 mg.

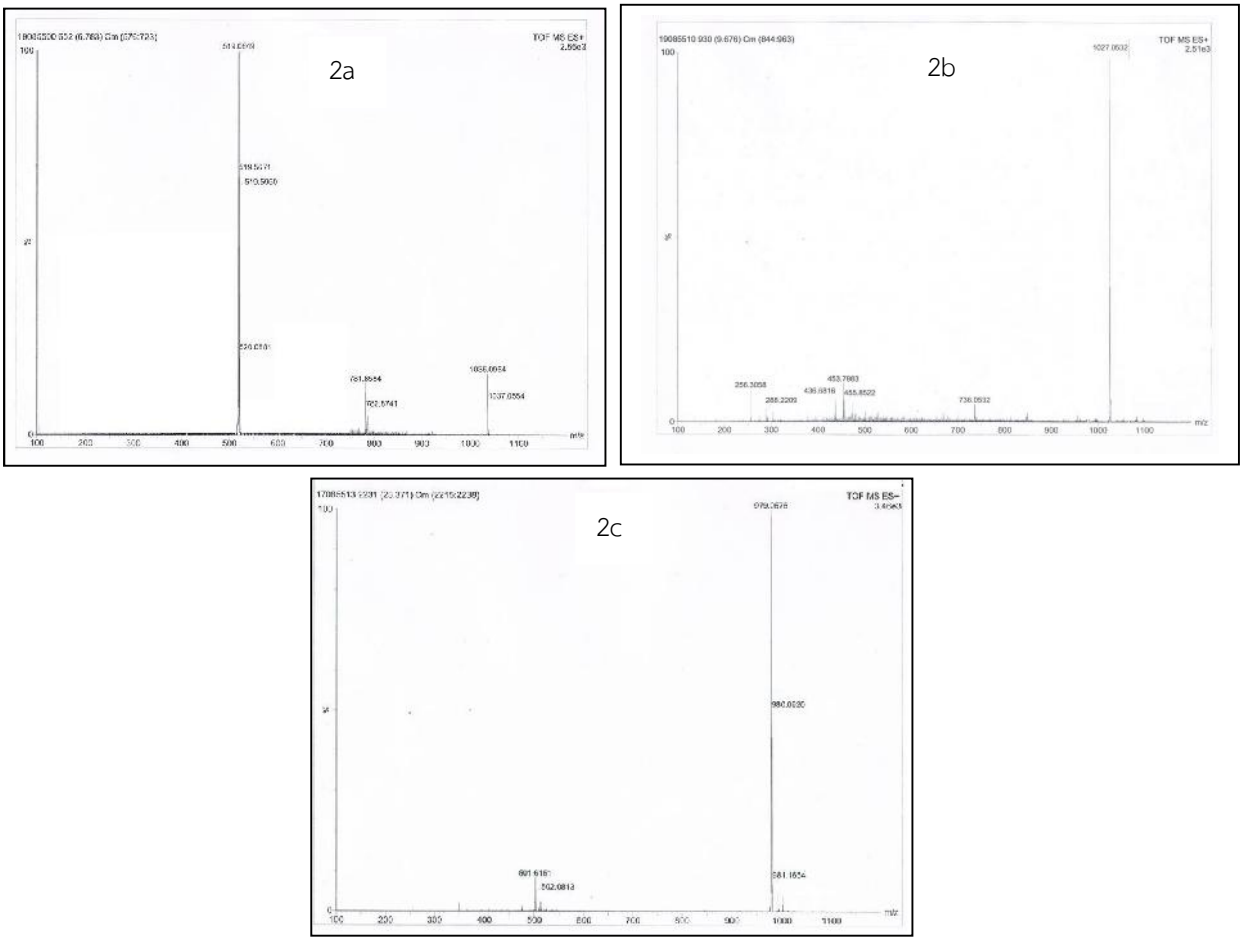

Figure 2. MS spectra of MC-RR (a); MC-FR (b); [Dha^{7}]MC-LR (c)

Table 1. Results of MCs purification

Fraction	Number of fractions from DEAE column	Total volume of the fractions (ml)	Yield (mg)	% Purity	Pure MCs in fractions
MC-1	17-41	24	21.63	92	MC-RR
MC-2	93-109	17	19.45	91	MC-FR
MC-3	113-135	25	25.38	93	[Dha^7]MC-LR

DISCUSSION

To obtain significant amounts of MC variants for biodegradation experiments, extraction, and purification of the toxins from field collected bloom samples was the best solution, rather than purchase expensive commercial grade MCs. Normally MC-LR is the most common and the most toxic variant detected in cyanobacterial blooms worldwide (Gupta et al., 2003). Interestingly, the cyanobacterial bloom in Bueng Nong Khot contains [Dha^7]MC-LR and MC-FR, which are moderately toxic variants with the LD_{50} (ip) of 250 µg/kg in mice, whereas MC-RR is lowly toxic variants with the LD_{50} (ip) of 500-800 µg/kg in mice (Zurawell et al., 2005). Given the presence of the MC variants in significant quantities and their known toxicity, Bueng Nong Khot water is a potential health risk for people who use the lake for recreation.

Preliminary data about MC variants and concentration of each in the lyophilized material should be determined for the feasibility of using samples from Bueng Nong Khot as a source of MCs. Methods of MC extraction and purification have been extensively studied (Edwards et al., 1996a; Edwards et al., 1996b; Lawton et al., 1999; Ramanan et al., 2000; Saito et al., 2002). Several studies indicated that aqueous MeOH at the concentration of 70-75% is optimal for MC extraction from lyophilised cyanobacteria (Ward et al., 1997; Fastner et al., 1998; Hyenstrand et al., 2001) and addition of TFA to aqueous MeOH improves MC extraction (Meriluoto, 1997). In this study, we found that the best extraction of MCs from lyophilized cells was obtained using 70% MeOH-0.1%TFA solution.

A wide range of chromatographic techniques has been used to purify large numbers as well as large quantities of MC variants, such as size exclusion, typically Sephadex LH-20 from Pharmacia, as a primary separation method to remove pigments and large interfering molecules. However, ion exchange has also been used as a preliminary step in the purification of MCs and shown to be very effective for cleanup, enabling simple and rapid further purification of MC-LR and [D-Asp] MC-LR (Lawton and Edwards, 2001). We used DEAE anion exchange chromatography as the first purification in this study and the toxins in the column were eluted with linear gradient of sodium chloride.

A secondary clean-up step is strongly recommended for elimination of trace impurities as well as desalting where fractions contain salts that were used in the mobile phase such as sodium chloride in this study (Tsuji et al., 1994; Lawton and Edwards, 2001). In this study, the Strata-X cartridges were used for the second clean-up process which proved to be effective as similar as Sep-pak C18 reported by Saito et al. (2002). One MC fraction was passed through the Strata-X cartridge using different concentrations of methanol to establish optimal elution. It revealed that the aqueous methanol, less than 30%, was proved to be a suitable washing solution since it did not strip any MCs from the cartridge, whereas the solution containing methanol of higher than 50% could eluted large amounts of MC

variants. Therefore, our recommendation is that the 30% aqueous methanol is used as the washing solution and 70 % aqueous methanol is appropriate for eluting MCs from Strata-X cartridges.

CONCLUSION

This study reported for the first time that a combination of the anion exchange DEAE chromatography and the Strata-X SPE cartridges were used and proved to be effective for cleaning up the cyanobacterial samples for MC purification. [Dha^7]MC-LR was the major MC variant extracted.

It is obvious from this study that DEAE and the Strata-X SPE column chromatography is effective to extract and purify MCs for further use and the cyanobacterial bloom in Bueng Nong Khot, contained significant quantities of the toxic variants, [Dha^7]MC-LR, MC-RR, and MC-FR (Zurawell et al., 2005). Given the presence of the MC variants in significant quantities and their known toxicity, Bueng Nong Khot water is a potential health risk for people who use the lake for recreation.

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