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Short communication

New values of molecular extinction coefficient and specific rotation for cyanobacterial toxin cylindrospermopsin ☆

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Abstract

The molecular extinction coefficient ε of cylindrospermopsin (CYN) purified by the anion exchange and the normal-phase HPLC procedures was determined to be 9800 at 262 nm. This ε is significantly higher than those (ε, 5800–6250) reported previously. In order to determine CYN concentrations in solutions using UV absorption, the ε-value of CYN should be corrected from 5800 to 9800. Further, the $[\alpha]_D$ value of CYN should be corrected from +12.5° to +17.0°.

Keywords: Cyanotoxin; Cylindrospermopsin; Molecular extinction coefficient (ε); Specific rotation $[\alpha]_D$; ERETIC method

Cylindrospermopsin (CYN) (**1** in Fig. 1) is a water-soluble hepatotoxin isolated from *Cylindrospermopsis raciborskii*, *Anabaena bergii*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Umezakia natans*, and *Raphidiopsis curvata* that seriously threatens the water quality of drinking water reservoirs (Welker et al., 2004). 7-Epi CYN (**2** in Fig. 1) and deoxy CYN (**3** in Fig. 1) have been identified as its toxic and non-toxic analogs, respectively.

The LD_{50} of CYN in mice was reported to be 2.1 mg kg^{-1} after 24 h and 0.2 mg kg^{-1} after 5–6 days after an intraperitoneal injection (Ohtani et al., 1992). A guideline value for CYN is being determined by the WHO (Welker et al., 2004), and has been proposed to be 1 μg L^{-1} (Humpage and Falconer, 2003) and 10 μg L^{-1} (Gijsbertsen-Abrahamse et al., 2006). The concentration of CYN has been determined using the molecular extinction coefficient (ε). However, the value of ε has been variously reported as 5800 (Ohtani et al., 1992), 5900 (Banker et al., 1997), 6100 (Harada et al., 1994), and 6250 (Banker et al., 2000), and two different $[\alpha]_D$ values of CYN have also been reported as +12.5° (*c*, 0.6, H_2O) (Banker et al., 2000) and +8.0° (*c*, 0.05, H_2O) (Looper et al., 2006). In 2006, the certified reference material (CRM) for CYN has been released from the National Research Council Institute for Marine Biosciences, Halifax, Canada. In the CRM catalogue (NRC CRM-CYN Catalogue, 2006), the ε-value for CRM at 262 nm has been given as 9800 ± 300; however, that for $[\alpha]_D$ is not given.

☆ *Ethical statement*: We did not use any animals in the experiments.

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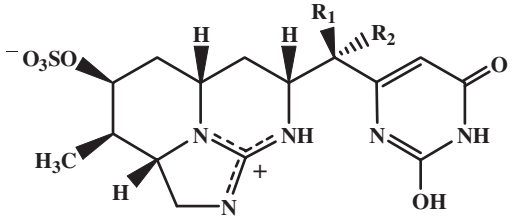
In order to reconfirm the value of ε and $[\alpha]_D$ for CYN, the toxin was produced by the cultivation of a CYN-producing cyanobacterium, and was purified using the anion exchange cartridge method (Kubo et al., 2005) and by rechromatography with a hydrophilic interaction chromatography column such as an Amide-80 column (Toso, Tokyo, Japan) (Kikuchi et al., 2007).

The CYN-producing *C. raciborskii* AWT 205 was cultured in 10 L culture bottles with Jaworski medium having the following composition: $Ca(NO_3)_2 \cdot 4H_2O$, 4.0 g; KH_2PO_4, 2.48 g; $MgSO_4 \cdot 7H_2O$, 10.0 g; $NaHCO_3$, 3.18 g; EDTA Fe Na, 0.4 g; EDTA···2Na, 0.45 g; H_3BO_3, 0.496 g; $MnCl_2 \cdot 4H_2O$, 0.248 g; cyanocobalamin, 0.8 mg; thiamine···HCl, 8 mg; biotin, 8 mg; $NaNO_3$, 16.0 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 g; and $NaHPO_4 \cdot 12H_2O$, 7.2 g; these were mixed in 10 L of distilled water, pH 8.5. The cells were grown isothermally at 20 °C (light intensity, less than 250 μmol photons m^{-2} s^{-1}; aeration rate, 1.5 L min^{-1}). After 4 weeks, the cells were harvested by centrifugation and freeze-dried. The average yields of lyophilized cells were 0.11 g L^{-1}.

CYN was extracted from the lyophilized cells (5.0 g) with 5% aqueous acetic acid using ultra sonication for 5 min. After centrifugation, the supernatant was collected and condensed under reduced pressure. The remaining residue was suspended with 100 mL of 0.1 M potassium carbonate buffer (pH 10.5). The solution was passed through a styrene polymer cartridge (InertSep, RP-1, GL Science Corp., Tokyo, Japan). The eluted solution was further passed through an anion exchange cartridge (Oasis MAX, Waters, USA).



	R1	R2
1	OH	H
2	H	OH
3	H	H

Fig. 1. Structure of cylindrospermopsin (CYN) analogs. (**1**) CYN; (**2**) 7-epi CYN; and (**3**) deoxy CYN.

After washing with water, CYN was eluted from the anion exchange cartridge with 20 mL of 50% MeOH containing a 1.0% formic acid aqueous solution. The eluted solution was dried up under reduced pressure. The CYN-containing residue was dissolved with 5.0 mL of MeOH aqueous solution for purification. The CYN fraction was applied to reverse-phase HPLC on C18 with 5% methanol in water to obtain a white amorphous solid of CYN. After the amorphous CYN was dissolved in water, the solution was applied to normal-phase HPLC on Amide-80 with MeCN/water (7:3, v/v). The CYN-containing eluent was collected, and condensed under a nitrogen stream at 40 °C, and then dried under reduced pressure at room temperature. The purified CYN comprised white microcrystals, and 35 mg of it was obtained from 5.0 g of lyophilized cells.

The purified CYN ($C_{15}H_{21}N_5O_7S$; positive ion HRFABMS, glycerol matrix: $[M+H]^+$ m/z 416.1235, $\Delta = 0.3$ mmu) was obtained with 0.7% yield. The structure of CYN was confirmed by the analysis of the 500 MHz ^1H and 125 MHz ^{13}C NMR spectra in D_2O by the two-dimensional COSY, HMQC, and HMBC experiments.

The purified CYN (4.15 mg) was accurately weighed, and was dissolved in 200 mL of pure water, 0.1 M HCl and 0.1 M NaOH. The ε-values for CYN in various solutions were determined using a spectrophotometer (Shimadzu UV-2450) at λ_{max}. CYN exhibited UV(H_2O) λ_{max} 262 nm (ε, 9800), UV(0.1 M NaOH) λ_{max} 284 nm (ε, 9500), and UV(0.1 M HCl) λ_{max} 263 nm (ε, 9800). These ε-values were the corrected values by the absolute concentration determined using the ERETIC method with caffeine as the external reference (Akoka et al., 1999). Before the correction, the CYN gave UV (H_2O) λ_{max} 262 nm (ε, 9750). The ε was increased about 0.5% by the application of the ERETIC method. Probably, the increase in the ε was due to moisture content in the purified CYN.

The specific rotation of CYN was $[\alpha]_D^{28°}$ +16.9 (*c*, 0.01, H_2O). By the application of the ERETIC method, the $[\alpha]_D$ was corrected to +17.0°. Previously, Banker et al. (2000) showed $[\alpha]_D$ of CYN and 7-epi CYN as +12.5° and −20.5°, respectively.

Before the rechromatography of normal-phase HPLC on Amide-80, the ε-value of the CYN fraction was 8400. After the rechromatography, it increased to 9750, and the $[\alpha]_D$ was showed as +16.9° at 28 °C without the application of the ERETIC method. The ε and $[\alpha]_D$ values were

significantly higher than those in published papers (Ohtani et al., 1992, Harada et al., 1994, Banker et al., 1997, Looper et al., 2006). In the purification procedure of CYN, the use of the anion exchange cartridge (Oasis MAX) at pH 10.5 (Kubo et al., 2005) was very effective for separation from other extracted compounds. At this pH value, CYN, CYN analogs, and other acidic compounds should be adsorbed on the cartridge. CYN and the adsorbed compounds were released from the cartridge by 50% methanol containing a 1.0% formic acid aqueous solution. Under these conditions, the guanidino group in CYN and CYN analogs should form a salt with formic acid, and the sulfate of CYN and the acidic groups of acidic compounds should be free. By the treatment of reverse-phase HPLC on C18, acidic compounds were removed from the CYN fraction. An intermolecular salt should be formed between the sulfate and guanidino groups of CYN by the rechromatography of the CYN fraction using normal-phase HPLC on Amide-80 with MeCN/water (7:3, v/v).

In 1992, Ohtani et al. isolated CYN from *C. raciborskii* cells, and purified it by gel filtration using Toyopearl HW40F and HPLC on C18. The purified CYN exhibited UV (H_2O) λ_{max} 262 (ε, 5800) and $[\alpha]_D$ -31° (*c*,0.1, H_2O). Later, the identified compound was renamed 7-epi CYN. These are the first data on CYN analogs. Subsequently, many ε-values of CYN have been reported, and these have ranged from 5800 to 6250. However, these data were obtained from CYN prepared according to the method of Ohtani et al. (1992). Their CYN preparation procedures did not consider the pH shift, and CYN salts such as calcium and/or phosphoric acid salts.

The UV absorption of CYN is due to the uracil moiety (ring D). The UV λ_{max} of uracil [2,4(1H,3H) pyrimidinone] is 260 nm, and the ε is 8200 (O'Neil et al., 2001). On this basis, the ε-value (9800) of purified CYN appears reasonable.

In this report, we found that the ε and $[\alpha]_D$ values of CYN were increased by the treatment using the anion exchange cartridge at pH 10.5. These results suggested that the inorganic compounds attached to CYN were released by the treatment using the anion exchange cartridge at pH 10.5 and by elution from the cartridge with 1.0% formic acid.

The ε-value of CYN has been used as 5800 at 262 nm for determining its concentration in the solution (Törökné et al., 2004). In order to estimate the concentration of CYN by UV absorption, the ε-value of CYN should be corrected from 5800 to 9800.

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