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High-performance liquid chromatography with chemiluminescence detection of derivatized microcystins

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Abstract

Microcystins, hepatotoxic peptides produced by cyanobacteria, strongly inhibit protein phosphatases 1 and 2A in a manner similar to okadaic acid and possess tumor-promoting activity. Low-dose constant exposure to microcystins is being suspected of incidence of primary liver cancer. We tried to establish a chemical method for individual detection and determination of trace amounts of microcystins in the low femtomole range using peroxyoxalate chemiluminescence (PO-CL) detection. In order to detect microcystins with CL, dansyl-cysteine adducts of microcystins RR, YR and LR (Dns-Cys-RR, -YR and -LR) were prepared. The Dns-Cys adducts of microcystins are based on nucleophilic addition of the thiol group in cysteine to the α,β-unsaturated carbonyl of the N-methyldehydroalanine moiety. A HPLC system with three pumps was constructed, in which one pump is used for delivering the mobile phase, and the other two pumps deliver the imidazole–nitrate buffer and PO-CL reagent, respectively. The optimization of the imidazole–nitrate buffer and PO-CL reagent was investigated. The detection limits for Dns-Cys-RR, -YR and -LR were less than 15 fmol (S/N 10), and the relationship between the peak area and concentration of Dns-Cys-RR, -YR and -LR was linear in the range 15–1670 fmol (Dns-Cys-RR, $r = 0.994$; Dns-Cys-YR, $r = 0.997$; Dns-Cys-LR; $r = 0.999$).

1. Introduction

The occurrence of toxic freshwater blooms of cyanobacteria (blue-green algae) has been reported in many countries. These toxic water blooms have caused deaths of domestic animals and wildlife [1]. Toxins produced by cyanobacteria include hepatotoxins such as microcystins, nodularins and cylindrospermopsin, and neurotoxins such as anatoxin-a, anatoxin-a(s) and aphantoxins [2]. Much attention has been paid to microcystins because of their biological activities and wide distribution all over the world. They have been produced by *Microcystis*, *Oscillatoria*, *Anabaena* and *Nostoc* [2] and have a common moiety composed of 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda), N-methyldehydroalanine (Mdha), D-alanine, β-linked D-*erythro*-β-methylaspartic acid, and γ-linked D-glutamic acid and two L-amino acids as variants [1]. Over 50 microcystins have been isolated so far.

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Microcystins strongly inhibit protein phosphatases 1 and 2A in a manner similar to okadaic acid and show tumor-promoting activity [3–7]. Although human deaths by cyanobacteria toxicosis have not been reported, low-dose constant exposure of microcystins is suspected of incidence of primary liver cancer (PLC). Yu [8] has reported that there are about 100 000 deaths annually from PLC caused by various factors in China. The higher incidence of PLC in Qidong and Haimen counties near Shanghai does not correlate with PLC-causing agents such as aflatoxin and hepatitis B virus, but people who drank pond and ditch water had a higher risk of PLC than people who drank well water [8]. This may suggest that the incidence of PLC is related to microcystins in the drinking water.

In our preliminary experiments in Japan, microcystins were detected in a eutrophicated lake at concentration of at most μg/l levels. To precisely analyze microcystins in a complex matrix, lower femtomole quantities have to be detected. Although HPLC–UV detection has been widely used for this purpose [9], its detection limit was estimated to be at most at picomole level. There have been several attempts to detect microcystins sensitively using HPLC with fluorescence detection for an oxidation product of microcystins [10], enzyme-linked immunosorbent assay (ELISA) [11,12] and HPLC-linked protein phosphatase bioassay [13,14]. Although these methods are useful for sensitive screening of microcystins, they could not separately determine microcystins. It was reported that protein phosphatases are also inhibited by compounds other than microcystins in cyanobacteria [15].

It is desirable to establish a chemical method for individual detection and determination of trace amounts of microcystins in the low femtomole range. Recently, peroxyoxalate chemiluminescence (PO-CL) detection has been investigated for highly sensitive detection of fluorophores [16,17]. Thus, we tried to apply this detection method to establish a sensitive analysis method for microcystins. However, there is no appropriate fluorophore in the molecules for PO-CL detection. In this paper, we describe a suitable derivatization method for the detection, optimization of operation conditions including chromatography and an evaluation of the established analysis method with PO-CL detection.

2. Experimental

2.1. Chemicals

Bis[4 - nitro - 2 - (3,6,9 - trioxadesyloxycarbonyl)phenyl]oxalate (TDPO), dansyl chloride (Dns-Cl) and hydrogen peroxide (30%) were purchased from Wako (Osaka, Japan), imidazole from Tokyo Chemical Industry (Tokyo, Japan), acetonitrile, phosphoric acid, potassium dihydrogenphosphate from Nacalai Tesque (Kyoto, Japan), and cysteine and Dns-Ala from Sigma (St. Louis, MO, USA). Distilled water was purified with a Barnsted E-pure system (Boston, MA, USA). Microcystins RR and LR were purified from water blooms of Lake Suwa in Japan and microcystin YR from the culture strain *Microcystis aeruginosa* M-228 according to Ref. [18].

2.2. Preparation of Dns-Cys adducts of microcystins

A mixture of microcystin LR (4.5 mg) and cysteine (5.5 mg) in 5% potassium carbonate aqueous solution was stirred for 1 h at room temperature. The reaction mixture was neutralized with 0.2 *M* hydrochloric acid and applied to an ODS cartridge (Baker, NJ, USA). The cartridge was washed with 10 ml of water and then eluted with 15 ml of methanol–water (90:10) to give 6.2 mg of the reaction product. The reaction product was reacted with 1.5 ml of Dns-Cl acetone solution (10 mg/ml) in 2.0 ml of 0.2 *M* sodium hydrogencarbonate for 1 h at 40°C. After evaporation of acetone, 10 ml of water were added to the solution, which was then applied to an ODS cartridge. The cartridge was washed with 10 ml of water and then eluted with 15 ml of methanol–water (90:10). The eluate was evaporated to dryness and the reaction product was purified by preparative HPLC to give 1.4 mg of Dns-Cys-LR. Dns-Cys-YR and -RR were simi-

larly prepared. They were confirmed by fast atom bombardment (FAB) mass spectral analysis: Dns-Cys-LR, m/z 1348 $(M+H)^+$; Dns-Cys-YR, m/z 1399 $(M+H)^+$; Dns-Cys-RR, m/z 1392 $(M+H)^+$.

2.3. Apparatus

The HPLC system consisted of three Shimadzu (Kyoto, Japan) LC-9A high-performance pumps, a Rheodyne (CA, USA) 7125 injector with a loop of 5 μl, a Tosoh (Tokyo, Japan) CO-8010 column oven, a Jasco (Tokyo, Japan) 825-CL chemiluminescence detector (spiral flow cell of 150 μl), a separation column (150 mm × 4.6 mm I.D.) packed with Tosoh TSKgel ODS-80Ts, a mixing coil (400 mm × 0.25 mm I.D.), a Jasco rotating flow mixing device and a Shimadzu C-R6A integrator. A schematic diagram of the system is shown in Fig. 2. A Jasco UV-970 and a Jasco FR-210 were used as ultraviolet and florescence detectors, respectively. The stainless-steel pipes between the rotating flow mixing device [19] and spiral flow cell in the detector were 400 mm × 0.25 mm I.D. in size.

2.4. Optimized conditions

Acetonitrile–0.05% trifluoroacetic acid (TFA) in water (40:60) was used as the mobile phase and the flow-rate was set at 1.0 ml/min. A solution of 75 m*M* imidazole–nitrate buffer (pH 7.0) in acetonitrile–water (50:50) was used for pH adjustment of the mobile phase and the flow-rate was set at 0.2 ml/min. PO-CL reagent (0.5 m*M* TDPO + 50 m*M* hydrogen peroxide in 100% acetonitrile) was pumped at 2.0 ml/min. The column, mixing coil, and rotating flow mixing device were placed in the column oven, which was set at 40°C.

3. Results and discussion

3.1. Preparation of a fluorescent derivative of microcystin

In order to detect microcystins with PO-CL, a fluorescent-active group has to be introduced into the molecules, because intact microcystins have no fluorophore. Microcystins unfortunately do not possess an appropriate target functional group such as an amino, keto or hydroxy group, which is easily available for fluorescent labeling. It was considered that the two functional groups, the carboxylic acids of the acidic amino acid residues and the α,β-unsaturated carbonyl group of Mdha, would be available for the labeling. Although various labeling reagents were tried to react with the carboxylic groups, no reproducible results were obtained probably due to steric hindrance around these functional groups. However, the α,β-unsaturated carbonyl group is available for a Michael addition acceptor. Actually, this functional group has already been used for preparation of glutathione adducts of microcystins [20]. In the present study, we planned to prepare Dns-Cys adducts of microcystins (Fig. 1) instead of the Dns-glutathione adducts. Initially, we tried to prepare the desired compounds by reaction of microcystins with Dns-Cys, but it was difficult to obtain the pure reagent, because the resulting reagent was easily converted into its oxidized form. The desired adducts, therefore, could be formed by two-step reactions, in which

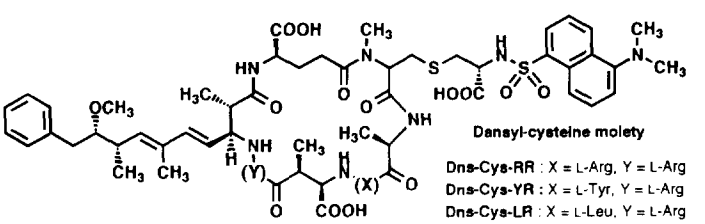

Fig. 1. Structures of dansyl cysteine adduct of microcystins.

the toxins reacted with cysteine to give the Cys adducts under slightly basic conditions in the first step, and then the adducts were further converted to the Dns-Cys adducts by the usual dansylation reaction in the second step. Finally, three Dns-Cys adducts of microcystins RR, YR and LR were smoothly prepared and used in subsequent studies.

3.2. Parameters for PO-CL detection conditions

PO-CL is known to be one of the most sensitive detection methods in HPLC, and it shows a detection limit in the low femtomole range for many compounds. The proposed mechanism of the PO-CL reaction consists of: (1) the production of a key chemical intermediate such as 1,2-dioxetanedione; (2) the conversion of the chemical energy into electron energy; (3) the emission of the fluorophore. Aryl oxalate and hydrogen peroxide generated PO-CL in the presence of fluorescent compounds. In HPLC using PO-CL detection, imidazole–nitrate buffer has been frequently added to the mobile phase for production efficiency, because it not only adjusts the pH of the mobile phase to around neutral but also serves as a catalyst for the production [21].

Because microcystins and their Dns-Cys adducts are well separated by HPLC using acidic aqueous methanol as a mobile phase such as methanol–0.05 *M* phosphate buffer (pH 3) or methanol–0.05% TFA in water [9], it was not desirable to use a mobile phase including imidazole–nitrate buffer in our case. We constructed a HPLC system with three pumps, in which pump 1 is used for delivering the mobile phase, and pumps 2 and 3 deliver the imidazole–nitrate buffer and PO-CL reagent, respectively, as shown in Fig. 2 [22]. The mobile phase, acetonitrile–0.05% TFA in water (40:60), was used in the present study, because PO-CL intensity in acetonitrile was significantly higher than that in methanol [23,24] and the mobile phase including acetonitrile resulted in good separation for Dns-Cys-LR, -YR and -RR as shown later.

Acetonitrile–water (50:50) and 100% acetonitrile were used as the dissolving solvents of the imidazole–nitrate buffer and PO-CL reagent because of smooth mixing with the mobile phase and the stability of PO-CL reagent, respectively [26]. Because the solubility of TDPO in acetonitrile is higher than that of bis(2,4,6-trichlorophenyl)oxalate (TCPO), which has been widely used as an aryl oxalate derivative, and TDPO gives higher PO-CL intensity than that given with TCPO [25], TDPO was selected as the aryl oxalate for the CL reagent. Thus, the optimization of the imidazole–nitrate buffer (pH, concentration of imidazole and flow-rate) and the PO-CL reagent (concentrations of TDPO and hydrogen peroxide, and flow-rate) was mainly investigated for sensitive PO-CL detection.

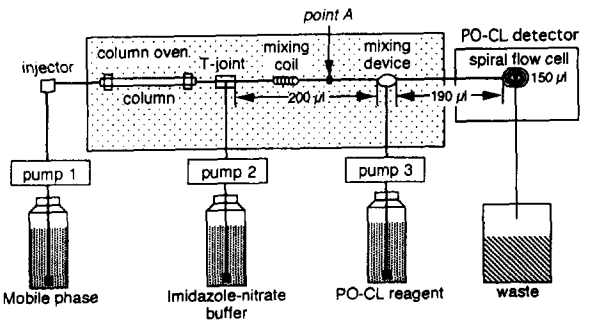

Fig. 2. Schematic diagram of HPLC system for PO-CL detection.

3.3. Optimization of operating conditions

Firstly, the effect of the pH of the imidazole–nitrate buffer on PO-CL intensity was examined using Dns-Ala as a model compound. Although the maximum PO-CL intensity was observed around pH 7.0 of the imidazole–nitrate buffer, it was essential to adjust the mobile phase at point A (Fig. 2) to pH 6.4 as shown in Fig. 3. The pH at point A was influenced by the concentration of imidazole and the flow-rate of imidazole–nitrate buffer (Table 1). Therefore, we carried out the following experiments for the imidazole–nitrate buffer: (1) imidazole-nitrate buffers (pH 7.0) in acetonitrile–water (50:50) with various concentrations of imidazole were prepared; (2) flow-rates of imidazole–nitrate buffers were de-

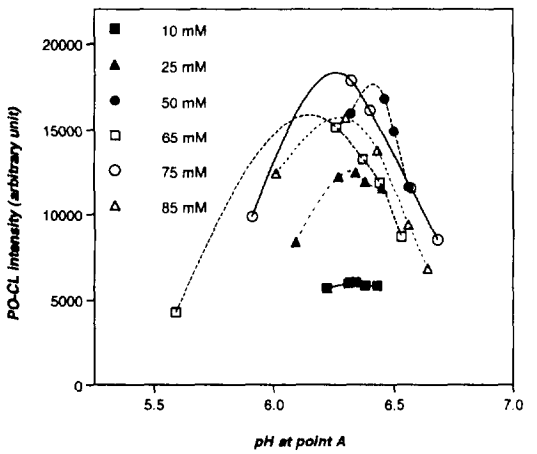

Fig. 3. Effect of imidazole concentration on PO-CL intensity. Mobile phase: acetonitrile–0.05% TFA in water (40:60), 1.0 ml/min; 10–85 m*M* imidazole–nitrate buffer (pH 7.0) in acetonitrile–water (50:50); flow-rate; see Table 1; PO-CL reagent: 0.25 m*M* TDPO + 12.5 m*M* hydrogen peroxide in acetonitrile, 1.5 ml/min. Sample: Dns-Ala 100 pg/μl, 2-μl injection.

termined to adjust the solution at point A to pH 6.4; (3) PO-CL intensities were measured under the conditions mentioned above (Fig. 3). The PO-CL intensity increased with increasing imidazole concentration and reached a maximum at 75 m*M*. Consequently, a concentration of 75 m*M* and a flow-rate of 0.2 ml/min for imidazole–nitrate buffer (pH 7.0) were chosen as the optimal conditions.

The effects of TDPO and hydrogen peroxide concentrations on the PO-CL intensity are shown in Fig. 4a and b, respectively. A maximum of the PO-CL intensity was obtained at 0.5 m*M* TDPO. Although the PO-CL intensity increased with increasing concentration of hydrogen peroxide, it did not change when the concentration exceeded 50 m*M*. Fig. 4c also shows the relationship between PO-CL intensity and flow-rate of the PO-CL reagent. The PO-CL intensity increased rapidly with increasing flow-rate in the range of 1.0–2.0 ml/min and then remained nearly constant up to 3.0 ml/min, but it decreased rapidly in the range of 3.0–4.0 ml/min. The decrement in the PO-CL intensity at higher flow-rates may be due to a time lag for emission, the volume (190 μl) between the rotating flow mixing device and the spiral flow cell and the total flow-rate of pumps 1, 2 and 3. It was reported that the time lag of emission is 5 to 10 s in PO-CL detection [26]. When the PO-

Table 1
Relationship between flow-rate and concentration of imidazole–nitrate buffer to adjust to pH 6.4 at point A

Imidazole concentration (m*M*)		Run				
		1	2	3	4	5
10	ml/min	0.90	1.00	1.10	1.20	1.30
	pH	6.22	6.31	6.34	6.38	6.43
25	ml/min	0.30	0.40	0.45	0.50	0.60
	pH	6.09	6.27	6.34	6.38	6.45
50	ml/min	0.10	0.20	0.25	0.30	0.40
	pH	4.26	6.32	6.46	6.50	6.56
65	ml/min	0.10	0.20	0.25	0.30	0.40
	pH	5.59	6.26	6.37	6.44	6.53
75	ml/min	0.10	0.17	0.20	0.30	0.40
	pH	5.91	6.32	6.40	6.57	6.68
85	ml/min	0.10	0.15	0.20	0.30	0.40
	pH	6.01	6.30	6.43	6.56	6.64

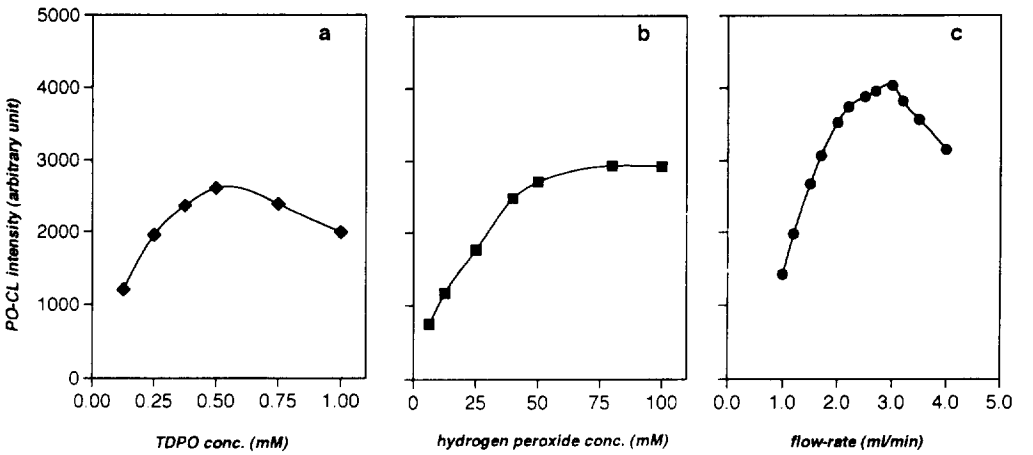

Fig. 4. Effect of (a) TDPO and (b) hydrogen peroxide concentration, and (c) flow-rate of PO-CL reagent on PO-CL intensity. Mobile phase: acetonitrile–0.05% TFA in water (40:60), 1.0 ml/min; 75 m*M* imidazole–nitrate buffer in acetonitrile–water (50:50) (pH 7.0), 0.2 ml/min; PO-CL reagent: (a) 0.125–1.0 m*M* TDPO + 100 m*M* hydrogen peroxide in acetonitrile, 1.5 ml/min, (B) 0.5 m*M* TDPO + 6.25–100 m*M* hydrogen peroxide in acetonitrile, 1.5 ml/min, (c) 0.5 m*M* TDPO + 50 m*M* hydrogen peroxide in acetonitrile, 1.0–4.0 ml/min. Sample: Dns-Ala 100 pg/μl, 2-μl injection.

CL reagent was delivered at over 3.0 ml/min, a fluorescent compound was mainly emitted out of the spiral flow cell in our apparatus, leading to the conclusion that it is most effective to deliver the PO-CL reagent at 2.0 ml/min. Therefore, 0.5 m*M* TDPO + 50 m*M* hydrogen peroxide in acetonitrile (flow-rate 2.0 ml/min) was optimized as the PO-CL reagent. In addition, PO-CL reagent in 100% acetonitrile was found to be stable for 48 h.

3.4. HPLC separation with PO-CL detection

As a result of extensive experiments, the measuring conditions for the Dns-Cys adduct of microcystins were established as follows. Acetonitrile–0.05% TFA in water (40:60) was used as the mobile phase and the flow-rate was set at 1.0 ml/min. The eluent from the column was mixed with 75 m*M* imidazole–nitrate buffer (pH 7.0) in acetonitrile–water (50:50), whose flow-rate was 0.2 ml/min, and the pH value of the resulting solution was adjusted to 6.4. Then the PO-CL reaction was performed by mixing 0.5 m*M* TDPO + 50 m*M* hydrogen peroxide in acetonitrile. The flow-rate of the CL reagent was set at 2.0 ml/min.

The HPLC profile of Dns-Cys-RR, -YR, and -LR with PO-CL detection is shown in Fig. 5, and the three components are clearly separated from one another. The detection limits for Dns-Cys-RR, -YR, and LR were less than 15 fmol (S/N 10), and the relationship between the peak area and the concentration of the microcystin Dns-Cys adducts was linear in the range 15–1670 fmol (Dns-Cys-RR, $r = 0.994$; Dns-Cys-YR, $r = 0.997$; Dns-Cys-LR, $r = 0.999$). Finally, the sen-

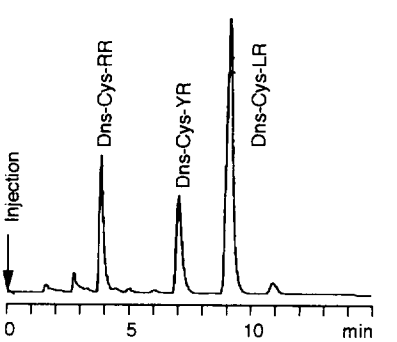

Fig. 5. HPLC separation of Dns-Cys-RR, Dns-Cys-YR and Dns-Cys-LR with PO-CL detection. Mobile phase: acetonitrile–0.05% TFA in water (40:60), 1.0 ml/min; 75 m*M* imidazole–nitrate buffer in acetonitrile–water (50:50) (pH 7.0), 0.2 ml/min; PO-CL reagent: 0.5 m*M* TDPO + 50 m*M* hydrogen peroxide in acetonitrile, 2.0 ml/min.

sitivities of Dns-Cys-LR with UV, FL and CL detection were compared. Although the sensitivity of Dns-Cys-LR with FL was similar to that with UV, the sensitivity with PO-CL was 200 times greater than that with UV and FL detection.

In order to detect microcystins more sensitively, HPLC with PO-CL detection was established. In our system, Dns-Cys adducts of microcystins can be detected in the femtomole range, which was as sensitive as previous methods, such as phosphatase bioassay, ELISA and HPLC–FL for oxydative degradation product of microcystins. While the previous studies could not detect microcystins separately, our method can detect individual microcystins with high sensitivity. Furthermore, the established method also provides the additional advantage that any microcystin can be specifically detected based on nucleophilic addition of a thiol group to the α,β-unsaturated carbonyl of the Mdha moiety followed by dansylation, because the moiety is common with all microcystins isolated.

For an actual analysis of trace amounts of microcystins in a complicated matrix such as lake water, we have to optimize the following three steps: (1) clean-up step for microcystins in environmental samples; (2) derivatization step for CL detection and preparation of an internal standard; (3) analysis step for highly sensitive detection with CL-detection. In the present paper we describe a suitable derivatization for CL detection and its optimization of operation conditions including LC, which corresponds to the third step. Recently, Tsuji et al. [27] reported a clean-up method for analysis of trace amounts of microcystins in lake water, which can be applied to our method. Consequently, we have to establish the second step. The reactivity of the fluorescence derivatization and preparation of an internal standard for the quantitative analysis are being examined.

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