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Direct Colorimetric Assay of Microcystin Using Protein Phosphatase

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Abstract A new direct colorimetric assay of microcystin in water and algal samples is proposed consisting of two procedures as follows: 1) the elimination of phosphorus in the sample and concentration of microcystin using a C_{18} cartridge, 2) the detection of the released phosphorus by the ascorbic acid method and determination of protein phosphatase (PP) inhibition by microcystin. The optimum amounts of phosphorylase α and PP-1 in 50 μL concentrated sample were 50 μg/50 μL buffer and 1.0 unit/50 μL buffer, respectively, for the best assay. The pH for the maximum activity of PP-1 was 8. The minimum detectable concentration for this method was about 0.02 μg/L, which is sufficient to meet the proposed guideline level of 1 μg microcystin/L in drinking water. Consequently, it would seem that the proposed direct colorimetric assay using PP is a rapid, easy, and convenient method for the detection of microcystin in water and algal samples.

Keywords: ascorbic acid method, direct colorimetric assay, microcystin, protein phosphatase inhibition

The bloom of cyanobacterium, *Microcystis aeruginosa*, is a ubiquitous phenomenon in eutrophic lakes and reservoirs in many countries of the world. Many strains of *Microcystis* are known to produce cyanobacterial hepatotoxins called microcystins. This toxin, a soluble peptide, is lethal to many kinds of aquatic organisms and damages zooplankton, fish [1], and the liver of higher animals [2,3].

Normally, an HPLC analysis is used for the detection and qualification of microcystins in water [4-7]. However, this method has certain weaknesses in that it usually requires a complex process and is only feasible in a laboratory equipped with an HPLC system. The protein phosphatase (PP) inhibition assay for microcystins includes measuring the release of acid-soluble ^{32}P from [^{32}P]glycogen phosphorylase [8]. A colorimetric version utilizing the ability of PP-1 to dephosphorylate *p*-nitrophenyl phosphate has also been introduced [9,10]. The colorimetric PP-1 inhibition assay was compared with the HPLC method in German lakes [11].

Although some research has been carried out to compare the results from the above two methods [11], a clear relationship between them has not as yet been established. However, the development of a simple and sensitive method to estimate microcystin concentrations is still needed to manage water quality. Accordingly, an attempt was made to develop a rapid, easy, and convenient method for the detection of microcystins in water and algal samples.

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The proposed direct colorimetric assay of microcystin is composed of two procedures: phosphorus (P)-elimination and P-determination. The overall procedure of the proposed method is summarized in Fig. 1. Water samples were passed through a Sep-Pak C_{18} cartridge (Waters, USA) in order to remove any other impurities and to concentrate the microcystin. The cartridge was then washed with 10 mL of double distilled water and 10% of methanol solutions (v/v), and eluted with 10 mL of methanol. Thereafter, the eluate was evaporated under reduced pressure below at 40°C, dissolved with 1 mL of methanol, and diluted approximately before the assay.

To measure the PP-1 activity, the method described by An and Carmichael [9] was employed with certain modifications. The catalytic subunit PP-1, α-isoform, from rabbit muscle was diluted in ice-cold 50 mM Tris-HCl, pH 7.6, 1 mM Na_2EDTA, 1 mM $MnCl_2$, 20 mM $MgCl_2$, 0.1% β-mercaptoethanol (v/v), and 1 mg/mL BSA prior to each assay. In the assays, 50 μL of diluted extract was incubated with 50 μL of a reaction buffer (50 mM Tris-HCl, pH 7.0, 0.2 mM Na_2EDTA, 0.2 mM $MnCl_2$, 2.0 mM $MgCl_2$, 0.1% β-mercaptoethanol (v/v), 0.5 mg/mL BSA, and 10 mg/mL phosphorylase a) for 5 min at 30°C. To measure 100% PP-1 activity, 50 μL of methanol (50%, v/v) was used instead of the extracts. The reaction was performed by the addition of 1.0 unit of PP-1 in 50 μL of a dilution buffer (unit definition: one unit will hydrolyze 1.0 nmol of *p*-nitrophenyl phosphate per min at 30 °C) for 2 h at 30°C. To stop the dephosphorylation reaction, 250 μL of 20% TCA (v/v) was added.

The orthophosphate was determined using the ascor-

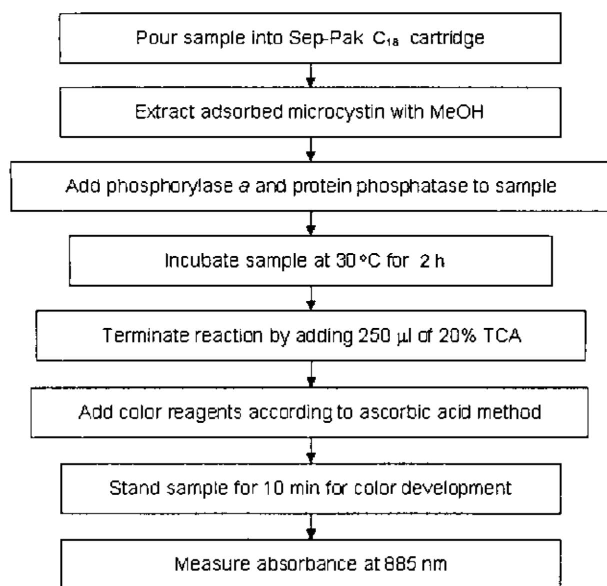

Fig. 1. Experimental procedure of proposed direct colorimetric method for the detection of microcystin in water and algal samples.

bic acid method [12]. 500 µL of a mixed-color reagent was added for the blue color-formation of the inorganic phosphorus released from phosphorylase *a*. The mixed-color reagent was composed of 0.8% ammonium molybdate (w/v), 4.0% sulfuric acid (v/v), 0.34% potassium antimonyl tartrate (w/v), and 0.35% ascorbic acid (w/v). After 10 min, the PP-1 activity was measured at 885 nm using a spectrophotometer (Shimadzu, Japan). For the 0% activity control, a dilution buffer without PP-1 was added. The assay was run in triplicate for each dilution step of the extract. Because the degree of PP-1 inhibition depends on the PP-1 concentration, standard concentrations of microcystin-RR (Sigma, USA) were tested in parallel in each assay to account for variations in the activity of different PP-1 preparations. The minimum detectable concentration of microcystin was the lowest microcystin concentration at which the treatment showed a difference in the PP-1 activity when compared with the control.

Microcystin-RR has been previously identified as the main component of the microcystin variants in Korean freshwater [13] and in *Microcystis aeruginosa* cultured in a P-limited chemostat [7]. Therefore, in the current study microcystin-RR was selected as the experimental representative from over 60 microcystin variants for developing a new method to determine the concentration of microcystin in water and algal samples.

The proposed method uses the ability of microcystins to specifically inhibit the catalytic subunits of serine/threonine PP-1 and PP-2A [11]. Phosphorylase *a* was used as the substrate. The effects of the phosphorylase *a* concentration on the minimum detectable concentration of microcystin are shown in Fig. 2. The minimum detectable concentration of microcystin was lowered

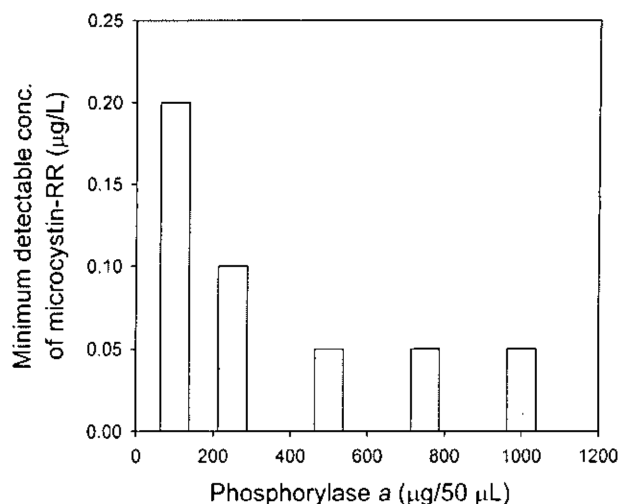

Fig. 2. Effect of phosphorylase *a* concentration on minimum detectable concentration of microcystin-RR.

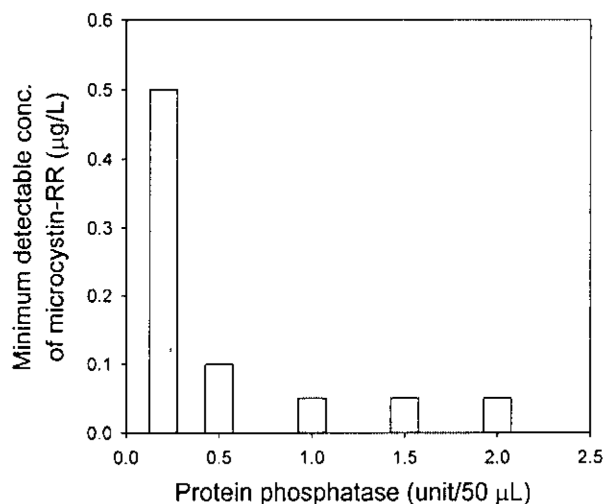

Fig. 3. Effect of protein phosphatase concentration on minimum detectable concentration of microcystin-RR.

with an increasing substrate concentration up to 500 µg/50 µL buffer in which the microcystin solution was 50 µL. PP-1 was used as an enzyme in order to catalyze the dephosphorylation of phosphorylase *a*. The minimum detectable concentration of microcystin was reduced by increasing the concentration of PP-1 up to the level of 1.0 unit/50 µL buffer (Fig. 3). From these results, the substrate and enzyme concentrations could be determined to optimize the reaction, thereby reducing the minimum detectable concentration of microcystin. The minimum detectable concentration of microcystin varied depending on the pH within a range of 4–10 (Fig. 4). In a weak alkaline condition, pH 8, the minimum detectable concentration of microcystin was lowest at 0.05 µg/L.

The inhibition of PP-1 by microcystin-RR was also investigated (Fig. 5). Microcystin-RR was tested within

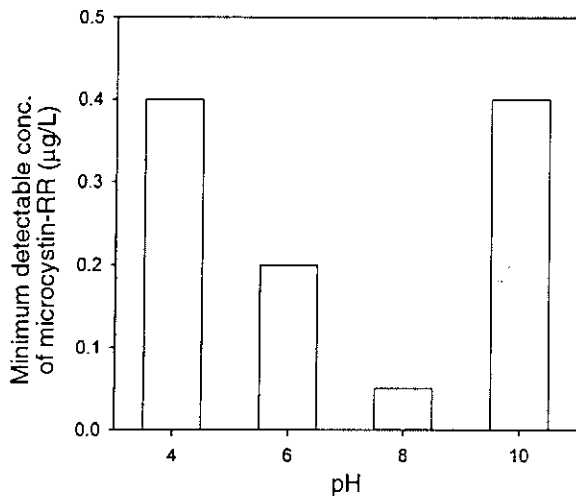

Fig. 4. Effect of pH on minimum detectable concentration of microcystin-RR.

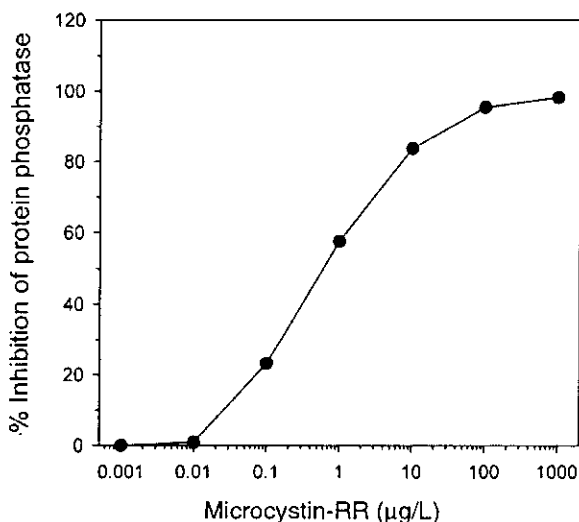

Fig. 5. Standard curve for inhibition of protein phosphatase activity by microcystin-RR.

a range of 0.001–1,000 μg/L. The minimum detectable microcystin-RR was about 0.02 μg/L at which the treatment showed a difference in the PP-1 activity when compared with the control. Ward *et al.* [10] reported that the limit of detection for microcystin-LR by the colorimetric PP-1 inhibition assay using *p*-nitrophenyl phosphate as a substrate was 0.1–0.2 ng/mL when 100 mL of water sample was analyzed. Thus the proposed direct colorimetric method using phosphorylase *a* as a substrate is about 5–10 times more sensitive than by the colorimetric PP-1 inhibition assay.

Water safety guidelines suggested 1 μg/L cyanobacterial peptide toxins as the maximum concentration in drinking water, and 20,000 cells/mL of cyanobacteria as the maximum for the safe use of recreational waters [14]. Therefore, the proposed direct colorimetric method is sensitive enough to meet the recommended guideline level of 1 μg microcystin per liter in drinking water.

The colorimetric PP-1 inhibition assay introduced by An and Carmichael [9] would seem to be useful for the preliminary screening of cyanobacterial field samples as it requires little equipment and allows for rapid detection and a rough estimation of the microcystin content [11]. However, it has certain weaknesses including the following: 1) there is the possibility of a discrepancy between the real reaction of phosphorylase *a* and the substituted reaction of *p*-nitrophenyl phosphate, 2) the color of the produced *p*-nitrophenol is a weak yellow, which is difficult to detect, and 3) *p*-nitrophenol is known as one of 129 EPA Priority Pollutants which are unsuitable as an experimental compound.

However, the proposed direct colorimetric method that uses phosphorylase *a* as the substrate may solve the above-mentioned problems of the traditional colorimetric method and is almost as sensitive as a colorimetric PP-1 inhibition assay. Furthermore, the proposed direct colorimetric assay using PP is a quick, easy, and convenient method for screening many water samples for microcystins. For example, about three hundred samples can be analyzed within one day.

Microcystin analysis is a necessary process to determine the safety of drinking water and water resources, however, it is a somewhat complex process when using an HPLC or isotopes and requires much time. As a result, the development of a more simple and convenient method for estimating the microcystin concentration in water is of great importance. Accordingly, the proposed direct colorimetric assay of microcystin can be effectively used in the monitoring step for the management of water quality. Based on this initial process, a further analysis with an HPLC or isotopes can then be carried out with selected samples to gain more detailed information. This strategy would seem to be more profitable for field samples.

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