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USE OF A COLORIMETRIC PROTEIN PHOSPHATASE INHIBITION ASSAY AND ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE STUDY OF MICROCYSTINS AND NODULARINS

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J. AN and W. W. CARMICHAEL. Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. *Toxicon* 32, 1495–1507, 1994.—Microcystins and nodularins are cyclic peptide hepatotoxins and tumor promoters produced by several genera of cyanobacteria. Using a rabbit anti-microcystin-LR polyclonal antibody preparation, the cross-reactivity with 18 microcystin and nodularin variants was tested. A hydrophobic amino acid, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4(E),6(E)-dienoic acid (Adda), which has the (E) form at the C-6 double bond in both microcystin and nodularin, was found essential for these toxins to express antibody specificity. Modification of -COOH in glutamic acid of microcystin and nodularin did not alter their antigenicity. Antibody cross-reactivity of these toxins was compared with their ability to inhibit protein phosphatase type 1 (PP1). Detection of PP1 inhibition was done by measuring the inhibition effect of the toxins on *p*-nitrophenol phosphate activity toward PP1. PP1 was obtained as recombinant PP1 expressed in *E. coli*. The inhibition effect of five microcystins and two nodularins on recombinant PP1 activity toward *p*-nitrophenol phosphate was measured in a microwell plate reader. The concentration of microcystin-LR causing 50% inhibition of recombinant PP1 activity (IC_{50}) was about 0.3 nM, while that of two modified microcystins had a significantly higher IC_{50}. Microcystin-LR and nodularin with the (z) form of Adda at the C-6 double bond or having the monoester of glutamic acid did not inhibit PP1. These three toxins were also nontoxic in the mouse bioassay. These results show the importance of Adda and glutamic acid in toxicity of these cyclic peptides and that PPI inhibition is related to the toxins' mechanism of action.

INTRODUCTION

MICROCYSTINS are monocyclic heptapeptide liver toxins produced by species of cyanobacteria within the genera *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc* (CARMICHAEL, 1992). The toxins contain two variable L-amino acids plus three D-amino acids plus the unusual amino acids, *N*-methyldehydroalanine and 3-amino-9-methoxy-10-phenyl-2,6,8-

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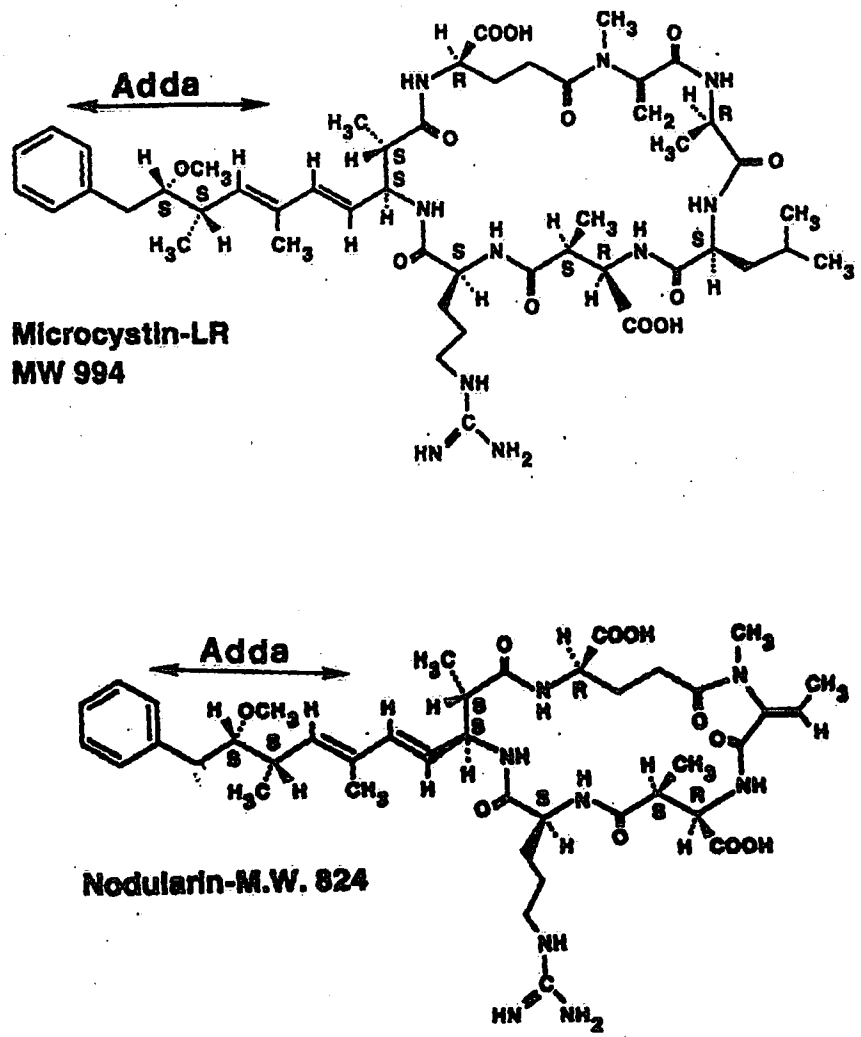

FIG. 1. CHEMICAL STRUCTURE OF MICROCYSTIN (TOP), WHICH CONTAINS TWO VARIABLE L-AMINO ACIDS, TWO D-AMINO ACIDS, ALANINE AND GLUTAMIC ACID PLUS THE UNUSUAL AMINO ACIDS, METHYLASPARTIC ACID (MeAsp) AND METHYLDEHYDROALANINE (MDha) AND 3-AMINO-9-METHOXY-10-PHENYL-2,6,8-TRIMETHYLDECA-4,6-DIENOIC ACID (Adda).
Chemical structure of nodularin (bottom), which contains L-arginine and D-glutamic acid, methylaspartic acid and methyldehydrobutyrine and Adda.

trimethyl-deca-4(E)6(E)-dienoic acid (Adda) (RINEHART *et al.*, 1988) (Fig. 1). There are now over 50 different microcystins which differ primarily in the two L-amino acids plus methylation and demethylation on the two unusual amino acids (CARMICHAEL, 1994; RINEHART *et al.*, 1994). These microcystins all contain Adda, which is essential for expression of their biological activity (DAHLEM, Ph.D. Thesis, University of Illinois, 1989). Nodularins are monocyclic pentapeptide liver toxins produced by the brackish water cyanobacterium *Nodularia* (RINEHART *et al.*, 1988), which have also recently been found in the marine sponge *Theonella swinhoei* (DE SILVA *et al.*, 1992). Nodularins contain Adda but lack one of the L- and D-amino acids found in the microcystins (Fig. 1).

Recently, both microcystin and nodularin have been found to be potent inhibitors of protein phosphatase types 1 and 2A (YOSHIZAWA *et al.*, 1990) as well as tumor promoters in laboratory animals (NISHIWAKI-MATSUSHIMA *et al.*, 1992*b*; FALCONER, 1991). They are also suspected to be involved with promotion of primary liver cancer in humans exposed to long-term low doses of these cyclic peptide toxins through drinking water (CARMICHAEL, 1994; YU, 1989). It is therefore important to develop sensitive and reliable detection methods for these toxins. Using polyclonal antibodies developed against microcystin-LR (MCYST-LR), CHU *et al.* (1989) developed a direct competitive enzyme linked immunosorbent assay (ELISA) with nM sensitivity (CHU *et al.*, 1990). In order to evaluate the effectiveness of the anti-MCYST-LR antibodies for the detection of other microcystins and nodularins, we chose 18 different microcystins and nodularins for testing in this study. Results of these comparisons will provide valuable information on the ability of this ELISA to detect different toxins in this group, plus useful information on the functional groups against which the antibodies were raised.

Since microcystins and nodularins inhibit protein phosphatase 1 (PP1), the potential for using this enzyme to develop a sensitive toxin detection method has been explored (BOLAND *et al.*, 1993; LAMBERT *et al.*, 1994). In this paper, we report the use of a colorimetric PP1 inhibition assay for microcystins adapted from TAKAI and MIESKES (1991) and used on a microtiter plate reader. Using this colorimetric PP1 inhibition assay, five microcystins and two nodularins were tested for their inhibitory effect on PP1. Results of the PP1 inhibition are compared with the ELISA results to investigate the relationship between structure and function for the microcystins and nodularins tested.

MATERIALS AND METHODS

Direct competitive enzyme linked immunosorbent assay (ELISA) for detection of microcystins and nodularins

Reagents used for ELISA. Phosphate buffered saline (PBS): sodium phosphate buffer, 0.01 M containing 0.15 M NaCl, pH 7.5. Washing buffer–Tween–PBS: 0.1% (v/v) Tween 20 (Sigma, St. Louis, U.S.A.) in PBS. Blocking buffer: 0.1 g bovine serum albumin (BSA) (Sigma) in 100 ml PBS. Substrate buffer: 0.05 M citric acid plus 0.1 M NaH_2PO_4, pH 5.0. Enzyme substrate solution: 40 mg *O*-phenylenediamine (OPD) (Sigma) plus 0.04 ml 30% H_2O_2 in 100 ml of substrate buffer. This solution was used within 60 min after preparation. Stopping reagent: 1 N HCl.

Apparatus used in the ELISA. The ELISA plates used were NUNC polystyrene high binding microwell modules (Cat. no. 4-69914) (A/S NUNC, Roshilde, Denmark). The ELISA plate reader was a Bio-Tek kinetic microplate reader (Bio-Tek Instruments, Inc., Highland Park, Winooshi, VT, U.S.A.). The ELISA washer was a Corning 12 channel manual microplate washer.

Source of the polyclonal antibodies against microcystin-LR. The polyclonal antibodies against MCYST-LR were supplied by Prof. FUN SUN CHU (Food Research Institute, Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, U.S.A.). These antibodies were raised in rabbits by immunizing with MCYST-LR linked to BSA. Linkage of MCYST-LR to BSA was achieved using the water-soluble carbodimide (EDPC) method of CHU *et al.* (1989).

Making the microcystin-LR–horseradish peroxidase conjugate. Conjugation of MCYST-LR to horseradish peroxidase (HRP) was done using the water-soluble carbodimide (EDPC) method. MCYST-LR (0.5 mg) was dissolved in 1 ml of 25% ethanol and mixed with 15 mg of EDPC. One milliliter of HRP solution (1.12 mg of HRP in 1 ml 25% ethanol) was added to this mixture, followed by the addition of 15 mg of solid EDPC. The reaction mixture was stirred at room temperature for 30 min and then stirred in the cold room (6°C) overnight. Separation of unconjugated MCYST-LR from conjugated MCYST-LR was achieved by dialyzing the reaction mixture against 2 liters of 0.01 M ammonium bicarbonate (pH 7.1) at 6°C for 48 hr, with the buffer being changed twice daily. The dialysate was stored at −20°C until used.

HOARE and KOSHLAND (1967) reported that linkage of two protein molecules using the water-soluble carbodimide method could be enhanced by lowering the pH of the reaction buffer. Therefore in this study, the chemical reactions were carried out at pH = 6 using 10 mM phosphate buffer.

Purification and preparation of microcystin-LR standards for ELISA. MCYST-LR was purified from toxic blooms of *Microcystis aeruginosa* using a modified method of KRISHNAMURTHY *et al.* (1986). Freeze-dried *Microcystis aeruginosa* cells were extracted with water:methanol:butanol (75:20:5) overnight at room temperature (22°C) with stirring. The cell extract was either centrifuged (20,000 × *g*) for 30 to 60 min or filtered over glass wool and then centrifuged. The supernatant was air dried at 25–30°C overnight to remove methanol and butanol and the remaining aqueous solution was applied to Bond Elut (Analytichem) C-18 reverse phase columns. The columns were washed with water, 20% methanol, and then 80% methanol which eluted the MCYST-LR. The 80% methanol wash fraction was dried overnight at 20–25°C. Preparative HPLC using a Waters Prep Pak C-18 cartridge (47 × 300 mm, 55–105 μm particle size, 125 Å pore size) was used to purify MCYST-LR further. Mobile phase solvents used were 28% acetonitrile and 72% 20 mM NH_4OAc, pH 5.0. The eluent was monitored at 238 nm at a flow rate of 30 ml/min. Fractions containing the toxin were air-dried overnight to remove the acetonitrile. MCYST-LR was then desalted on another Waters Prep Pak C-18 column. This was followed by Gel chromatography on Toyo Pearl HW-40 F using 100% methanol for separation of MCYST-LR from the remaining pigments. The purity of MCYST-LR was estimated at 95–98% by analytical HPLC (55% methanol/45% 55 mM Na_2SO_4 pH 6.8) and Pico Tag amino acid analysis.

MCYST-LR (0.5 mg) was dissolved in 1 ml methanol and diluted to working concentrations of 0.5, 1, 5, 10 and 50 ng/ml with 0.01 M phosphate buffer saline (PBS), pH 7.4. The final methanol concentration in the standards was less than 1%.

Procedures for the direct competitive ELISA to detect microcystin and nodularin. The procedure for the direct competitive ELISA used in this study is as described by CHU *et al.* (1990).

Source of the microcystin and nodularin variants. Microcystin analogs isolated from natural water bloom samples were kindly provided by Dr M. NAMIKOSHI and Prof. K. L. RINEHART (Department of Chemistry, University of Illinois, U.S.A.). The microcystin variants used are listed in Table 1.

TABLE 1. AFFINITY OF DIFFERENT MICROCYSTINS AND NODULARINS WITH ANTIBODIES AGAINST MICROCYSTIN-LR

Toxin	LD_{50} (μg/kg, i.p. mouse)	ELISA reaction (IC_{50} ng/ml)
[DMAdda^3]NODLN	150*	—§
[6(*z*)Adda^3]NODLN	nontoxic*	—§
[D-Asp^1]NODLN	75*	35
MCYST-FR	250*	10
MCYST-AR	250*	30
MCYST-WR	150–200*	10
[D-Asp^3]MCYST-LR	200–500†	8
[DMAdda^5]MCYST-LR	90–100*	—§
[D-Glu-OCH$_3^6$]MYCST-LR	nontoxic*	3.1
[6(*z*)Adda^5]MCYST-LR	nontoxic*	60
[Dha^7]MCYST-LR	200–250*	30
MCYST-(H_4)YR	300–400*	8
[L-MeSer^7]MCYST-LR	150*	5
MCYST-HilR	100*	3.4
Dimethylester NODLN (Methylester at Glu and MeAsp)	nontoxic‡	15
MCYST-YR	70*	10
MCYST-LR	50*	3.1
NODLN	50*	12

*RINEHART *et al.* (1994); †HARADA *et al.* (1991); ‡NAMIKOSHI *et al.* (unpublished data).

§No inhibition at a concentration up to 1 μg/ml.

Dha, Dehydroalanine; Hil, homoisoleucine; (H_4)Y, 1,2,3,4tetrahydro-tyrosine; MCYST, microcystin; NODLN, nodularin.

Nodularin variants were isolated from *Nodularia spumigena* strain L575 (RINEHART *et al.*, 1994).

Protein phosphatase inhibition assay

Source of protein phosphatase 1. The catalytic subunit of rabbit skeletal muscle protein phosphatase 1 as expressed in *E. coli* was kindly provided by Prof. E. Y. C. LEE (Department of Biochemistry and Molecular Biology, University of Miami, U.S.A.) (ZHANG *et al.*, 1992).

Procedure for the protein phosphatase inhibition assay using microcystin-LR. PP1 activity was determined by measuring the rate of color production from the liberation of *p*-nitrophenol from *p*-nitrophenol phosphate (Sigma) using the microtiter plate reader. The assay was carried out at 37°C.

The PP1 was diluted in 50 mM Tris–HCl, pH 7.4, 1 mg/ml BSA, 1 mM $MnCl_2$ and 2 mM Dithiothreitol (DTT). Reaction of enzyme and substrate was done in a reaction buffer containing 50 mM Tris–HCl, pH 8.1, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5 mg/ml BSA. Different concentrations of MCYST-LR ranging from 0.5 to 100 ng/ml were incubated with the PP1 for 4 min at room temperature and the reaction was started by the addition of substrate (pNPP = 5 mM) and reaction buffer. The plate was read at 405 nm with a microplate reader.

IC_{50} determination for microcystin-LR using different concentrations of protein phosphatase 1. Recombinant PP1 was dissolved in the enzyme dilution buffer to give concentrations of 2.5, 5 and 10 μg/ml. An estimation of the MCYST-LR IC_{50} for these three different enzyme concentrations was obtained by using MCYST-LR concentrations of 0.005, 0.025, 0.25, 2.5 nM.

Testing the inhibition effect of some microcystin and nodularin variants on protein phosphatase 1. Microcystin and nodularin variants were first dissolved in 1 ml methanol, and then diluted to nM concentrations using distilled water. All toxin concentrations were run in triplicate.

RESULTS

ELISA for microcystin and nodularin detection

ELISA standard curve for microcystin and nodularin detection. The standard curve was created by plotting the MCYST-LR concentration (ng/ml) on a log scale vs % binding (B/B_0); (B_0 = maximum rate of color production when no MCYST-LR was added; B = rate of color production observed when various concentrations of MCYST-LR were added) of MCYST-LR–HRP to the antibodies (Fig. 2). The linear response for binding inhibition of MCYST-LR–HRP to the polyclonal antibodies by different concentrations of MCYST-LR was found to be in the range of 0.5–50 ng/ml. The concentration of MCYST-LR that caused a 50% inhibition of MCYST-LR–HRP binding to antibodies (IC_{50}) was calculated to be 3.1 ng/ml using the standard curve created from different MCYST-LR concentrations ranging from 0.5 to 100 ng/ml.

Cross-reactivity of different microcystins and nodularins with rabbit polyclonal antibodies against microcystin-LR. The direct competitive ELISA, in which MCYST-LR conjugated to HRP as the marker, was used to determine specificity of the anti-MCYST-LR rabbit antibodies. Various concentrations of each microcystin and nodularin were added to the microtiter plates together with MCYST-LR–HRP conjugate. The concentration of each microcystin and nodularin variant causing 50% inhibition of MCYST-LR–HRP binding to the antibodies is shown in Table 1. [DMAdda^5]NODLN, [6(z)-Adda^5]NODLN and [DMAdda^5]MCYST-LR did not cross-react with the polyclonal anti-MCYST-LR antibodies at a concentration up to 1 μg/ml. [6(z)-Adda^5]MCYST-LR had an IC_{50} about 20 times higher than that of MCYST-LR. The monoester of glutamic acid MCYST-LR had

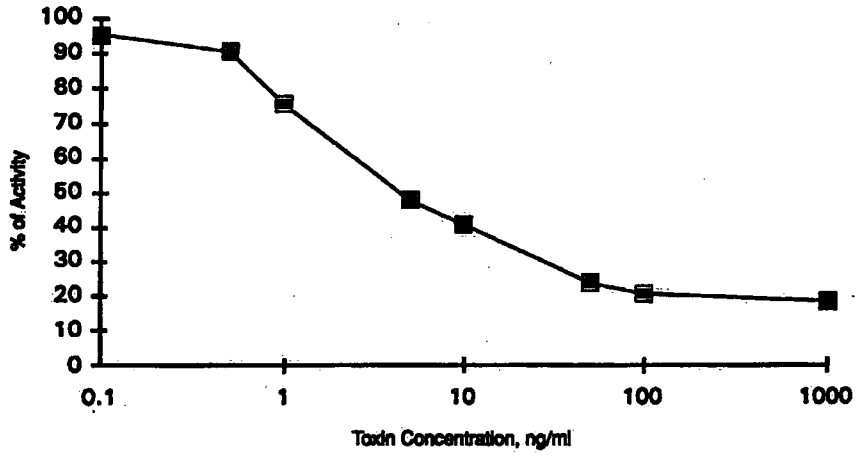

FIG. 2. STANDARD CURVE FOR THE DIRECT COMPETITIVE ELISA USING MICROCYSTIN-LR.
Fifty microliters of MCYST-LR-HRP conjugate and 50 μl of different concentrations of standard MCYST-LR in PBS were used in each assay. Results are shown as percentage of control activity in the absence of toxins. Concentrations shown on the x-axis use the log scale. All data are means $\pm$ 0.04 ($n = 3$).

an IC_{50} about 3.1 ng/ml which is the same as that of MCYST-LR. MCYST-FR, WR and YR had an IC_{50} of about 10 ng/ml. Figure 3 shows the cross-reactivity of some microcystin and nodularin variants with the antibodies by plotting the toxin concentration on a log scale vs % of the maximum binding for MCYST-LR–HRP conjugate to the antibodies.

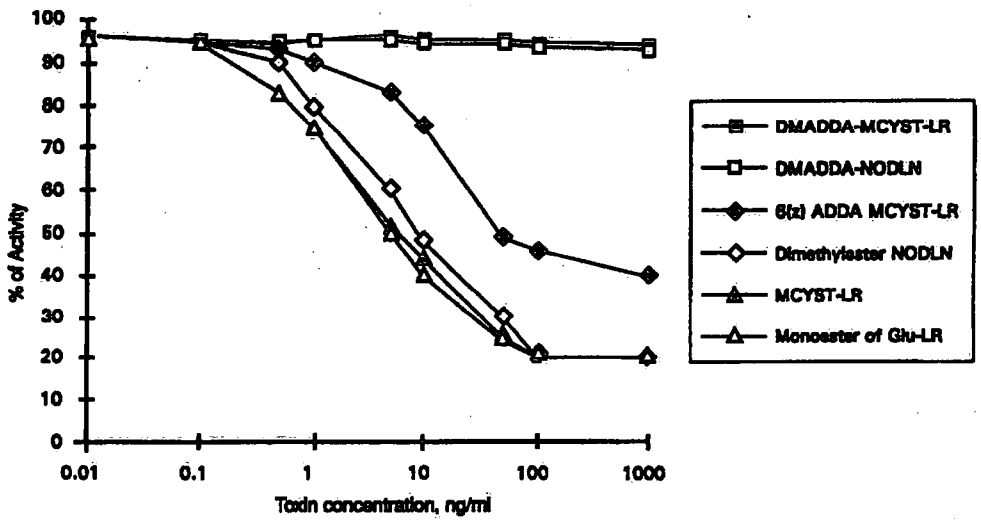

FIG. 3. AFFINITY OF FOUR MICROCYSTINS AND TWO NODULARINS TO ANTIBODIES AGAINST MICROCYSTIN-LR IN THE DIRECT COMPETITIVE ELISA.
Fifty microliters of 0.01 ng/ml to 1 μg/ml of four microcystins and two nodularins was tested to find their cross-reactivity with the anti-MCYST-LR antibody. Results are shown as percentage of control activity in the absence of toxins. All data are means $\pm$ 0.03 ($n = 3$).

The inhibition effect of three microcystins and two nodularins on recombinant protein phosphatase 1 *expressed in* E. coli

Inhibition effect of microcystin-LR on protein phosphatase 1. The PP1 enzyme was diluted with buffer to three different concentrations (2.5, 5, 10 μg/ml) and incubated with MCYST-LR at concentrations ranging from 0.005 to 2.5 nM. The curve used to estimate the IC_{50} of MCYST-LR on PP1 was generated by plotting the concentration of MCYST-LR vs B/B_0 (Fig. 4). The IC_{50} of MCYST-LR using 10 μg/ml of PP1 is about 0.6 nM, while the IC_{50} of MCYST-LR using 5 and 2.5 μg/ml of PP1 is about 0.25 nM.

The standard curve generated by the protein phosphatase 1 *inhibition assay using* 5 μg/ml *protein phosphatase* 1. Figure 5 shows the standard curve obtained by plotting MCYST-LR concentration on a log scale vs % of maximum enzyme activity determined by the rate of color production. The linear response of PP1 inhibition from different concentrations of MCYST-LR is between 0.05 and 1 nM.

Concentration of some microcystin and nodularin analogs causing 50% *inhibition of protein phosphatase activity* (IC_{50}). Three microcystins and two nodularins were tested to find their inhibition effect on PP1 activity towards PNPP. The IC_{50} for each toxin is listed in Table 2. The IC_{50} was obtained from the curve by plotting different concentrations of each toxin vs the inhibition effect (B/B_0) (Fig. 6). [6(z)Adda^3]NODLN,

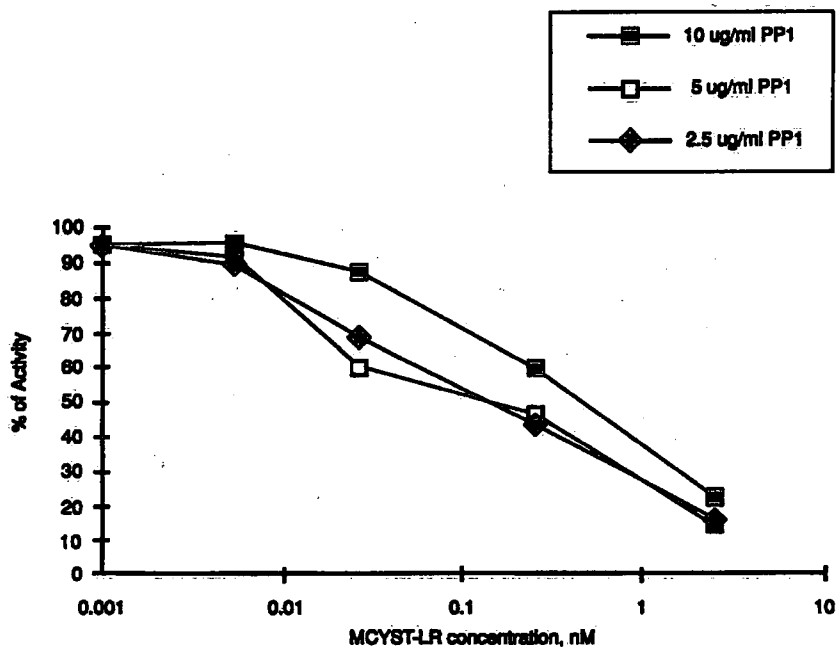

FIG. 4. INHIBITION EFFECT OF MICROCYSTIN-LR USING THREE CONCENTRATIONS OF RECOMBINANT PROTEIN PHOSPHATASE 1.
Ten microliters of 0.005 nM to 2.5 nM of MCYST-LR was tested to find their inhibition effect on 10 μl of three concentrations of recombinant PP1 (2.5, 5 and 10 μg/ml). Results are shown as percentage of control activity in the absence of toxins. All data are means $\pm$ 0.03 ($n = 3$).

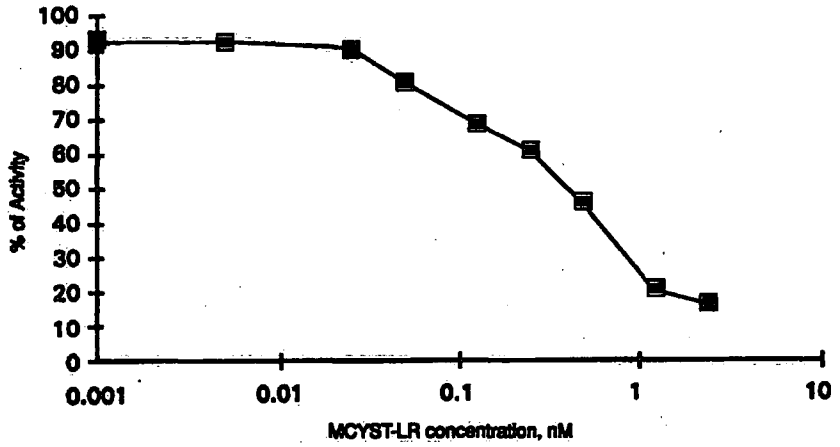

FIG. 5. STANDARD CURVE OF PROTEIN PHOSPHATASE 1 INHIBITION USING DIFFERENT CONCENTRATIONS OF MICROCYSTIN-LR.
Ten microliters of 0.005 nM to 2.5 nM MCYST-LR and 10 μl of PP1 was used. The assay was done in a 96-well microtiter plate at 37°C. The plate was read at 5 min intervals for 40 min at 405 nm. The color production rate of each well was calculated using Kineticalc computer software. All data are means $\pm$ 0.05. ($n = 3$).

[6(*z*)Adda^5]MCYST-LR and the monoester of glutamic acid-MCYST-LR had no inhibition effect on PP1 at concentrations up to 0.1 μM. [DMAdda^5]MCYST-LR had an IC_{50} near 1.5 nM, and both [DMAdda^3]NODLN and [Dha^7]MCYST-LR had an IC_{50} near 5 nM.

DISCUSSION

Cross-reactivity of microcystin variants and nodularin analogs with polyclonal antibodies against microcystin-LR

A direct competitive enzyme linked immunosorbent assay (ELISA) using polyclonal antibodies raised in the rabbit against MCYST-LR was first developed by CHU *et al.* (1989, 1990). This direct competitive ELISA was used in our study for the purpose of testing cross-reactivity of different microcystins and nodularins against three antibodies. In CHU's study, the antibodies were found to have a good cross-reactivity with MCYST-RR ($IC_{50} = 1.75$ ng/ml), MCYST-YR ($IC_{50} = 3.4$ ng/ml) and nodularin ($IC_{50} = 4.6$ ng/ml), but

TABLE 2. INHIBITION EFFECT OF FIVE MICROCYSTINS AND TWO NODULARINS ON RECOMBINANT PROTEIN PHOSPHATASE 1

Toxin	IC_{50} (nM)
MCYST-LR	0.3
[DMAdda^5]MCYST-LR	1.5
[Dha^7]MCYST-LR	5
[6(*z*)Adda^5]MCYST-LR	—
[D-Glu-OCH$_3^6$]MCYST-LR	—
[6(*z*)Adda^3]NODLN	—
[DMAdda^3]NODLN	5

—, no inhibition at a concentration up to 0.1 μM.
Abbreviations as in Table 1.

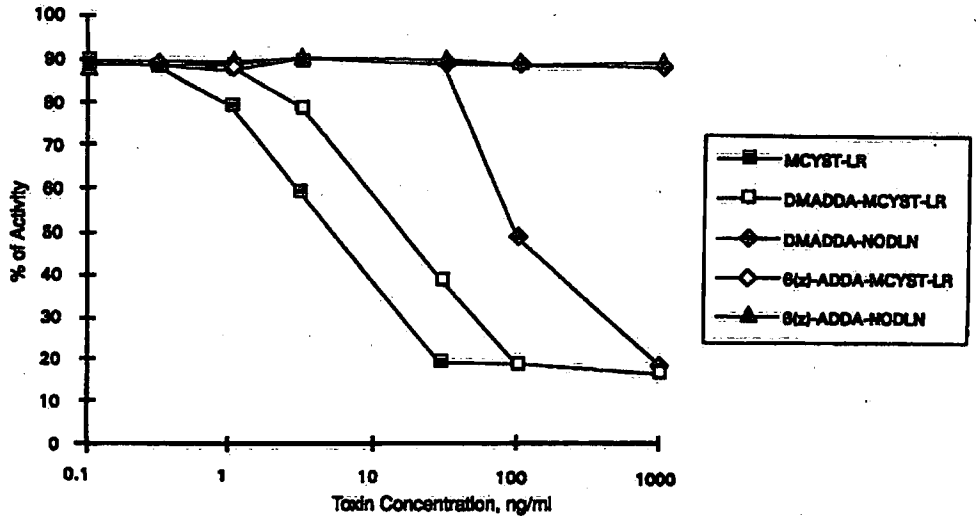

FIG. 6. INHIBITION EFFECT OF THREE MICROCYSTINS AND TWO NODULARINS ON RECOMBINANT PROTEIN PHOSPHATASE 1.
Ten microliters of 0.005 nM to 50 nM (0.1–1000 ng/ml; diluted 20 ×) for three microcystins and two nodularins was tested to find their inhibition effect on 10 μl of PP1 (5 μg/ml). Results are shown as percentage of control activity in the absence of toxins. All data are means of there sets of experiments.

less so with MCYST-LY ($IC_{50} = 50$ ng/ml) and MCYST-LA ($IC_{50} = 114$ ng/ml). They concluded that the three amino acids: erythro-β-methyl-*d*-isoaspartate, arginine and Adda play a dominant role in expressing antibody specificity.

In our study, 18 different microcystins and nodularins were used to test the cross-reactivity with the polyclonal anti-MCYST-LR antibodies. An estimate of the IC_{50} for each toxin is shown in Table 1. [6(*z*)Adda^5]NODLN (stereoisomer at C-6 double bond in Addas) showed no cross-reactivity with these antibodies when tested at concentrations up to 1 μg/ml. [6(*z*)Adda^5]MCYST-LR (stereoisomer at C-6 double bond in Adda) had an IC_{50} around 60 ng/ml, which is 20 times higher than that of MCYST-LR. This indicates that the configuration of the double bond in Adda is important for expressing the antibodies' specificity. It appears that microcystins and nodularins which contain the (E) form at the C-6 position in Adda are recognized better by these antibodies.

The (E) form at the C-6 double bond position in Adda for both microcystin and nodularin is also essential for toxicity of the toxins. Demethylation in the Adda of both MCYST-LR and nodularin results in a structural change that these antibodies fail to recognize even though the mouse toxicity of these two compounds is still retained.

The monoester of glutamic acid in MCYST-LR is nontoxic in mice but still shows a good reactivity with the antibodies. This is not surprising because the carboxyl group in the glutamic acid in MCYST-LR was used to link BSA in the water-soluble carbodimide method before MCYST-LR was injected into the rabbits.

Results in our study (Table 1) also show that the arginine-containing MCYST-FR and MCYST-WR have good cross-reactivity with anti-MCYST-LR antibodies. This gives further support to the finding of CHU *et al.* (1989) that Adda and arginine are essential for expressing the antibodies' specificity.

Colorimetric protein phosphatase 1 inhibition assay

Since microcystin and nodularin are known to be potent inhibitors of protein phosphatase type 1 and type 2A, a method of using recombinant PP1 expressed in *E. coli* (ZHANG *et al.*, 1992) for detecting these cyanobacteria liver toxins was developed in our study. The colorimetric method was chosen in our study for assaying the inhibition effect of microcystin and nodularin on recombinant PP1 because it is more convenient and less expensive than the radioisotope method.

The recombinant PP1 used in our studies has three different properties compared to that of authentic PP1 enzyme isolated from animal tissues (ZHANG *et al.*, 1992). First, this recombinant enzyme requires the presence of μM Mn^{2+} for full expression of its activity toward both pNPP and phosphorylase a, the two commonly used substrates for testing PP1 activity. Second, this recombinant enzyme is less sensitive to the okadaic acid class compounds with their IC_{50} being two to three times lower than that obtained using the authentic enzyme. Third, the values obtained for the PP1 activity toward pNPP were close to that obtained with ^{32}P phosphorylase a as the substrate. With the authentic enzyme, the activity toward pNPP was normally one-third less than that obtained with phosphorylase a. This provides an advantage for using the colorimetric over the radioisotope method for assaying enzyme activity.

Determining protein phosphatase 1 activity and estimating the IC_{50} of microcystin-LR. The enzyme kinetic study of protein phosphatase activity on pNPP by TAKAI and MIESKES (1991) showed that the okadaic acid class compounds act as noncompetitive, tightly binding inhibitors on PP1 and PP2A. When the concentration of PP1 or PP2A is greater than that of MCYST-LR, the toxin is titrated from the assay apparently by its binding to the catalytic subunits of these phosphatases. Therefore an accurate IC_{50} can only be estimated by using the enzyme when its concentration drops below the titration end point (the dilution after which no change in IC_{50} is observed with subsequent dilution) (HONKANEN *et al.*, 1990). For this reason, the recombinant PP1 used in our study was diluted to three different concentrations. The IC_{50} of MCYST-LR using 10 μg/ml of PP1 is 2.5 times higher than that obtained by using 5 μg/ml and 2.5 μg/ml of the enzyme. Therefore, 5 μg/ml of PP1 was used to create a standard inhibition curve with different concentrations of MCYST-LR (Fig. 4). The linear response of PP1 inhibition from different concentrations of MCYST-LR was between 0.05 and 1 nM (Fig. 5) (equal to 1–25 ng/ml in the tested solution). This means that the detection range of this PP1 inhibition assay is very close to that of the direct competitive ELISA used in this study.

The inhibition effect of some microcystins and nodularins on recombinant protein phosphatase 1. [DMAdda^5]MCYST-LR, [DMAdda^3]NODLN, [6(*z*)Adda^5]MCYST-LR, [6(*z*)Adda^3] nodularin, the monoester of Glu-MCYST-LR and [Dha^7]MCYST-LR were chosen for testing their activity on PP1 because some of these toxins are nontoxic in mice and show no cross-reactivity with antibodies against MYCST-LR. Others are toxic in mice but show no cross-reactivity with those antibodies. A PP1 enzyme concentration of 5 μg/ml was chosen to make the estimation of IC_{50} for these five toxins.

It was interesting to find that [DMAdda^5]MCYST-LR and [DMAdda^3]NODLN, which have no cross-reactivity with the antibodies, still inhibit PP1 and are also toxic in mice. The monoester of glutamic acid MCYST-LR, which is nontoxic to mice, also shows no inhibition effect on PP1 at concentrations up to 0.1 μM. [6(*z*)Adda^3]NODLN and [6(*z*)

Adda^5]MCYST-LR, which are nontoxic in mice, also show no inhibition effect on PP1 (Table 2).

These data show that the configuration at the C-6 double bond in Adda and the acid group in glutamic acid are essential for retaining microcystin and nodularin toxicity in addition to their inhibition effect on PP1. These data also support the recent finding of QUINN *et al.* (1993), who studied binding domain models for the okadaic acid class compounds of protein phosphatase 1 and 2A using computer modeling. They found that the pharmacophore model of these compounds consists of a central core containing one conserved acidic group and two potential hydrogen binding sites and a nonpolar side chain. In the case of microcystin and nodularin, the acidic group is the COOH in glutamic acid. The two hydrogen binding sites are on Adda at the C-4 and C-6 position and the nonpolar side chain is Adda.

Data from these studies also support studies indicating that animal toxicity is mediated through inhibition of protein phosphatase since the nontoxic microcystins and nodularins used also have no inhibition effect on PP1.

A comparison of the protein phosphatase 1 *inhibition effect of different toxins to the cross-reactivity of these toxins with antibodies against microcystin-LR.* Table 3 shows a summary of the PP1 inhibition effect, cross-reactivity with the anti-MCYST-LR antibodies and toxicity of some microcystin and nodularin variants used in the study. It shows that the epitope of the MCYST-LR which determines the antibodies specificity includes the entire Adda group plus the arginine. This supports the finding of CHU *et al.* (1989) that the arginine residue is essential for expressing the polyclonal antibodies specificity.

For the protein phosphatase inhibition activity of these different microcystins and nodularins, an acidic group (-COOH) in the glutamic acid and an Adda group, which has the (E) form instead of the (*Z*) form at the C-6 position, are important for the molecules to retain their protein phosphatase inhibition effect and also show toxicity in the mouse assay.

All our findings indicate that the configuration of the double bond in Adda is critical for the toxin's ability to express antibody binding specificity and also inhibit protein phosphatase 1 (PP1). Other studies (CHU *et al.*, 1989; NISHIWAKI-MATSUSHIMA *et al.*, 1992*a*) have shown that the arginine is microcystin and nodularin is essential for expressing antibody specificity but not essential for the toxin to express its inhibition effect on PP1. Instead of arginine, which is critical for recognition by the antibodies, the -COOH in glutamic acid is important for expression of a PP1 inhibition effect.

As stated in the Introduction, it is very important to have a sensitive method to monitor the quality of the drinking water sample for the presence of microcystin activity. In our

TABLE 3. CROSS-REACTIVITY OF SOME MICROCYSTINS AND NODULARINS WITH ANTI-MICROCYSTIN-LR ANTIBODIES AND THEIR INHIBITION EFFECT ON RECOMBINANT PROTEIN PHOSPHATASE 1

Toxin	ELISA	PP1 inhibition	Toxicity
[DMAdda^5]MCYST-LR	no	yes	yes
[DMAdda^3]NODLN	no	yes	yes
[6(*z*)Adda^3]NODLN	no	no	no
[6(*z*)Adda^5]MCYST-LR	*	no	no
[D-Glu-OCH$_3^6$]MCYST-LR	yes	no	no

*IC_{50} is 20 times higher than that of MCYST-LR.
Abbreviations as in Table 1.

study, a colorimetric PP1 inhibition assay was developed for this purpose. This colorimetric method was found to be very sensitive and able to detect the bioactive microcystins used in the study. The major drawback of the protein phosphatase method is its possible reaction with nonspecific phosphatases in the sample or its reaction with endogenous protein phosphatases that will lead to an underestimate of the toxins (SIM and MUDGE, 1993). We believe that a combination of an immunoassay such as ELISA and the colorimetric PP1 inhibition assay will prove very useful in detecting many of the microcystins and nodularins in environmental samples.

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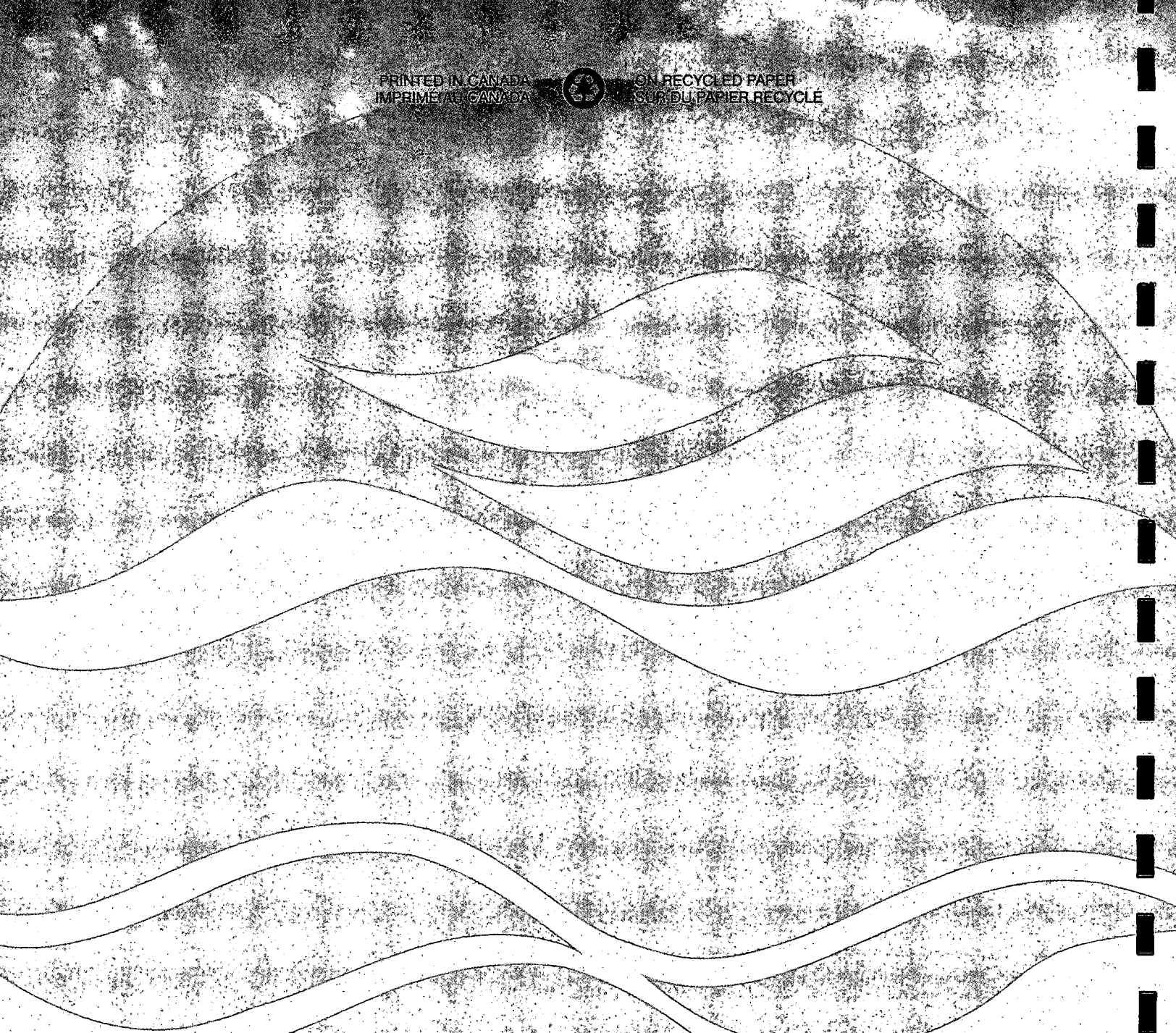

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