Pergamon

Chemosphere, Vol. 38, No. 5, pp. 1113–1122, 1999

0045-6535/99/ $ - see front matter

PII: S0045-6535(98)00354-3

A COLORIMETRIC ASSAY FOR SCREENING MICROCYSTIN CLASS COMPOUNDS IN AQUATIC SYSTEMS

Bryan S.F. Wong[1], Paul K.S. Lam[1]*, Lihong Xu[2], Yongyuan Zhang[2] and Bruce J. Richardson[1]

[1]*Centre for Environmental Science & Technology and Department of Biology & Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong and*
[2]*Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.*

(Received in Germany 12 February 1998; accepted 9 June 1998)

ABSTRACT

Secondary metabolites produced by water-blooming cyanobacteria in eutrophic waters include some potent hepatotoxins. These compounds also have tumour-promoting properties, attributable to their inhibition and activation of protein phosphatases and kinases respectively. The inhibitory effect of these toxins on protein phosphatases have been employed in a commonly used radiometric assay, involving the use of a ^{32}P-labeled substrate, for the detection and quantitation of these compounds. This paper investigates and describes a colorimetric method in which the activity of protein phosphatase 2A is determined by measuring the rate of colour production from the release of yellow *p*-nitrophenol using *p*-nitrophenyl phosphate as the substrate. Results of this study suggest that the colorimetric protein phosphatase inhibition assay is a simple, inexpensive tool for screening substances that may have tumour-promoting characteristics in aquatic systems. The detection limit of the colorimetric method is comparable to the radiometric assay.

INTRODUCTION

The blooming of cyanobacteria and dinoflagellates in eutrophic waters may result in the production of toxic secondary metabolites such as okadaic acid, microcystins and nodularins, often collectively referred to as microcystin class compounds (MCYSTs). Such chemicals, apart from being acutely toxic to humans and domestic animals [1,2], are notorious for their tumour-promoting characteristics due to their strong inhibitory effects on protein phosphatases [3]. Indeed, the threat of these tumour-promoting toxins has already been recognized as a world-wide public health problem, particularly due to the risks associated with public drinking water supplies and consumption of contaminated aquatic foods [4]. Despite the important health implications of these powerful toxins, information on their effects at low concentrations is

scanty. Clearly, there is a need to develop sensitive assay systems capable of detecting these tumour-promoting toxins at low levels so that the ecological integrity of valuable aquatic resources can be effectively protected, and risks to human and animal life minimized [5]. Currently, at least two biological methods are available for the determination of MCYSTs in aquatic systems: (1) a ^{32}P-labeled protein phosphatase inhibition test [6,7]; and (2) an enzyme-linked immunosorbent assay (ELISA) protocol [8,9]. The principle of the radiometric protein phosphatase inhibition assay relies on the inhibitory effect of MCYSTs on protein phosphatases, which are important enzymes catalyzing the reverse action of protein kinases. The phosphatases are responsible for the dephosphorylation of serine and threonine residues in the cytoplasmic and nuclear compartments of eukaryotic cells. An inhibition of the activity of these enzymes will disrupt normal cell structure and function, contributing to the formation of tumours. It is important to note that a demonstration of the existence of compounds that inhibit PP2A activity does not unequivocally imply the presence of MCYSTs. Notwithstanding, the presence of protein phosphatase inhibitors in water is a legitimate cause for concern.

Protein phosphatase type 2A (PP2A) is a serine/threonine phosphatase. These enzymes exhibit high phosphatase activity for *p*-nitrophenyl phosphate (*p*-NPP), giving rise to a yellow product, *p*-nitrophenol (*p*-NP). Indeed, it has been suggested that *p*-NPP phosphatase activity is intrinsic to PP2A as compared to the activities of type 1 and type 2C phosphatases [10].

In this work, the inhibitory effect of a typical microcystin class compound (purified microcystin-LR) on the enzymatic activity of protein phosphatase 2A is investigated by using the rate of production of yellow *p*-NP from *p*-NPP as a measure of the enzyme activity. This investigation allows the development of a colorimetric assay for detecting low levels of MCYSTs in natural waters. Specifically, this paper describes (1) a colorimetric protein phosphatase inhibition assay for screening MCYSTs in water, and (2) the sensitivity and effectiveness of this colorimetric technique in comparison with the conventional radiometric assay method.

MATERIALS AND METHODS

All chemicals and reagents were of analytical grade, and purchased from Sigma™ Chemical Co. (St. Louis, MO, USA). Microcystin-LR was isolated and purified from *Microcystis aeruginosa*, from the East Lake, Wuhan, China during an algal bloom [11].

Isolation of protein phosphatases 1 and 2A

A solution (Buffer A) containing 500 mM Tris/HCl, 2 mM EDTA, 2 mM EGTA, 0.2 mM phenylmethyl sulfonylflouride (in I-propanol), 0.2 mM Leupeptin, 10 % glycerol and 2 mM mercaptoethanol was prepared and adjusted to pH 7.4. Using a glass homogenizer placed in a salted ice bath, ten fresh mouse brains were homogenized in buffer A with 0.25 M sucrose. The homogenate was then centrifuged at 6,000g for 30 minutes at 4 °C. The supernatant was further centrifuged at 100,000 g for 60 minutes at 4 °C. The supernatant was then loaded on a DEAE-cellulose (Whatman DE-52) chromatography column (2.5 X 20 cm) connected to a Fast Liquid Chromatography Workstation (Biologic, Bio-rad™). Protein phosphatases 1 and 2A (as peaks 1 and 2 respectively) were collected by eluting, in turn, with 0.1 M NaCl and 0.2 M NaCl. The activity of PP2A (corresponding to peak 2) was confirmed by the radiometric ^{32}P assay (see below) and only fractions with relative high PP2A activities were collected. The purity of the enzyme preparations were further determined by SDS-Polyacrylamide gel electrophoresis. The enzyme extract was concentrated by a process of ultrafiltration (PLHK ultrafiltration kit, Millipore). The enzyme preparations were packed in small vials and stored at -80°C.

Radiometric ^{32}P assay

^{32}P-labeled phosphorylase **a** was prepared by the phosphorylation of phosphorylase **b** using phosphorylase kinase. Inhibition of PP2A was determined by using the radioassay ([γ-^{32}P]ATP) method, as described previously [12].

Colorimetric PP2A inhibition assay

In this method, activity of PP2A is measured by the rate of production of *p*-NP from *p*-NPP. The product *p*-NP has a yellow colour at alkaline pH, whilst acidic solutions of *p*-NP, and both acidic and alkaline solutions of *p*-NPP are colourless. On this basis, one unit of protein phosphatase activity can be defined as the amount of enzyme that will hydrolyze 1.0 ηmol *p*-NPP per minute at 25 °C in a pH 8.1 system.

The assay was carried out at 25±1°C in a reaction buffer TKMD containing 40 mM Tris HCl (at pH 8.1), 20 mM KCl, 30 mM $MgCl_2$, 2 mM Dithiothreitol (DTT) to which was added Bovine Serum Albumin, BSA (1 $mgmL^{-1}$). PP2A enzyme preparations were first diluted with TKMD buffer at pH 7.4 into the required concentrations [13]. A MCYST-LR standard solution (1 $mgmL^{-1}$) was serially diluted to provide a range of concentrations from 0.01 to 100 $ngmL^{-1}$. PP2A enzymes were pre-incubated at 20±1°C with various concentrations of MCYST-LR in individual microwells of standard 96-well microplates for 5 minutes. The reaction was started by the addition of *p*-NPP substrate. Changes in absorbance at 405nm were measured for 60 minutes by a microtiter plate reader (SpectraMAX® 340, Molecular Device).

Selection of optimal assay conditions for colorimetric protein phosphatase inhibition assay

In order to identify the optimal assay conditions, the above procedure was repeated by varying the test conditions with respect to the following specific components:

Concentration of p-*NPP substrate.*

It is generally believed that enzyme assays should be optimal at high substrate concentrations ([s]>>K_m). However, as only a small amount of the substrate actually takes part in the reaction, the use of excessive substrate could be wasteful and costly for routine monitoring situations. Moreover, color production in enzyme-free blanks may be magnified at high substrate concentrations due to natural breakdown of *p*-NPP into *p*-NP, resulting in a higher noise to signal ratio especially at high concentrations of MCYST-LR. A series of substrate concentrations (*viz.* 1.25, 2.5, 5.0, 10.0, 50.0 mM) was tested to identify the optimal substrate concentration for the assay.

Concentrations of $MnCl_2$

Previous studies have found that Mn^{2+} significantly affects the activity of PP2A. Indeed, a wide range of Mn^{2+} concentrations have been previously employed, e.g., 1 mM, [10]; 0.2 mM [13]. In this study, the effect of different Mn^{2+} concentrations (0 to 1 mM) on the activity of PP2A was investigated with a view to determine the optimal level of Mn^{2+}.

Establishing standard inhibition curves for comparison between methods

Following the establishment of optimal conditions for the colorimetric PP2A inhibition assay, standard inhibition curves were constructed to compare and contrast the sensitivity and effectiveness of the colorimetric assay with the radiometric assay using microcystin-LR as the standard protein phosphatase inhibitor.

RESULTS

Estimation of specific enzyme activity

One unit of enzyme activity is defined as that which catalyses the formation of 1 ηmol of product (*p*-NP) in one minute at 25°C, while specific activity is expressed as activity per mg protein. In order to convert the observed optical density (absorbance) to absolute quantity of *p*-NP for calculating enzyme activity, a standard curve was constructed by measuring the absorbance of known concentrations (range 0.005 to 0.03 mM) of *p*-NP in the buffered medium using the least-squares linear regression method.

The protein content of the PP2A extract was determined as 0.851 mg mL^{-1} using the Bio-Rad™ microassay kit for BSA content. On this basis, the specific activity of the enzyme extract was estimated as 217 ηmol of *p*-NP per minute per mg protein.

Enzyme kinetics

The reaction of PP2A and *p*-NPP was fitted to the one-site binding Michaelis-Menten equation, $V_O = V_{max}$ [s]/(K_m + [s]) (Fig. 1), where *Vo* is the initial velocity; V_{max}, the velocity at the saturation concentration of the substrate; [s] is the substrate concentration, and K_m the concentration of substrate required to reach one-half of V_{max}.

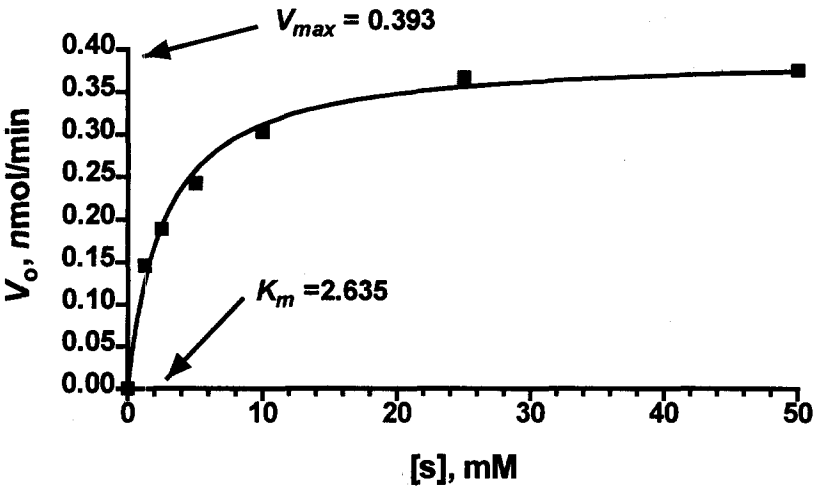

Fig. 1 Plot of initial velocity, V_O, against *p*-NPP concentration, [s]. $R^2 = 0.9922$.

Calculation of the relative activity of PP2A

The percent relative inhibition at each dose level of MCYST-LR is calculated by the equation:

$$\% \text{ PP2A inhibition} = \left[1 - \frac{\text{(Sample - Blank)}}{\text{(Positive control - Blank)}} \right] \times 100\%$$

For the radiometric method, positive control CPM represents the maximum value of PP2A activity, i.e. 100% activity, in which MCYST-LR has been replaced by ultra pure water; and blank (background noise) CPM is the soluble fraction of ^{32}P-labeled phosphate from the phosphorylase a solution [14].

Selection of optimal assay conditions for colorimetric protein phosphatase inhibition assay

Concentration of p-*NPP substrate.*

In theory, the optimal substrate concentration is the maximum value of V_0^2/[s] (i.e. 1.25mM). However, in this case, a value of 5 mM rather than 1.25 mM was selected due to the relatively low colour intensity developed at 1.25 mM of *p*-NPP (Fig. 1).

Effect of $MnCl_2$ concentration.

A two-way analysis of variance, with Mn^{2+} and PP2A levels as the two factors, revealed that an increase in Mn^{2+} concentration can promote the enzymatic conversion of *p*-NPP to *p*-NP ($P < 0.01$; Fig. 2). A *post hoc* test revealed that a complete exclusion of Mn^{2+} would cause a significant suppression of PP2A activity, while differences among other Mn^{2+} concentrations (0.25, 0.5, 0.75 mM) were not significant. The interaction between Mn^{2+} and enzyme levels was not significant ($P = 0.476$). In this study, a Mn^{2+} concentration of 0.5 mM, giving a reaction rate equivalent to 95% of the maximum, was selected.

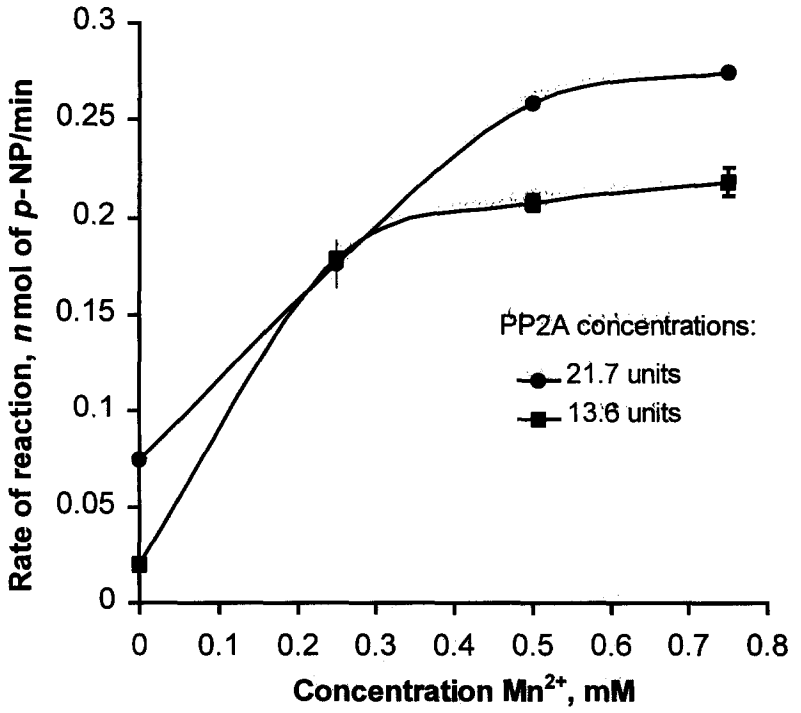

Fig. 2 Effect of Mn^{2+} on the activity of protein phosphatase 2A

Comparison of colorimetric and radiometric PP2A inhibition assays

Although both these methods are based on the inhibition of enzyme activity by MCYST-LR, there appears to be some difference in their dose-response relationships (Fig. 3). The curve representing the colorimetric method (*p*-NPP curve) has a shorter linear region and higher EC_{50} value (5.06 $ngmL^{-1}$ of MCYST-LR) compared with the radiometric curve (EC_{50} value = 3.13 $ngmL^{-1}$). In addition, the *p*-NPP curve shows a maximum inhibition of about 80%, which is lower than the 100% inhibition evident in the radiometric curve (Fig. 3). It is conceivable that other types of protein phosphatases exist in the reaction mixture, which are not amenable to specific inhibition by MCYST-LR.

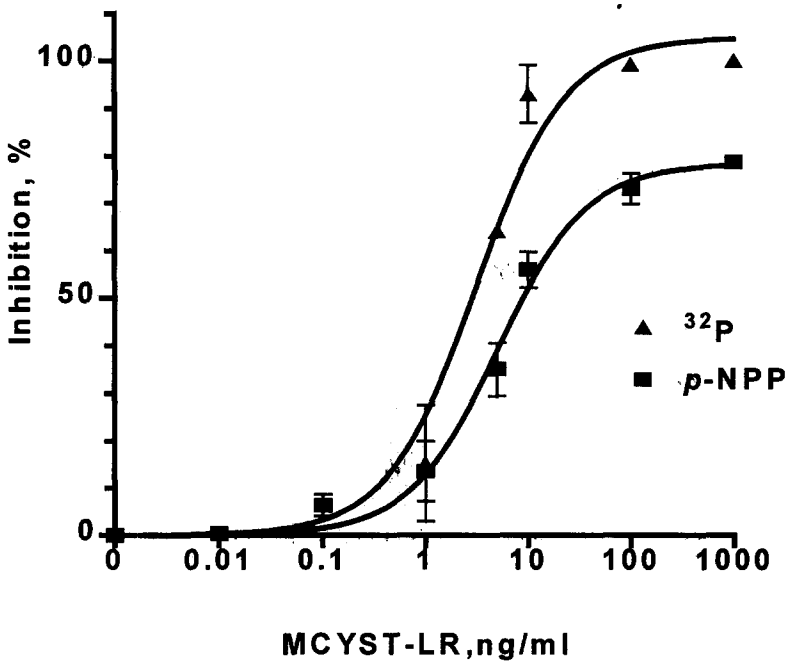

Fig. 3 Standard curves of PP2A inhibition based on radiometric (^{32}P) and colorimetric (*p*-NPP) methods at different concentrations of MCYST-LR. Vertical lines are means + 1 S.D..

DISCUSSION

In China, statistics have shown that the incidence of liver cancer for populations drinking deep well water was 117 per million; for those drinking pond water 141.4; and for ditch water drinkers, 723 per million [15,16]. To date, a guideline intake (no adverse effect level as seen by direct liver injury) of 0.28 $\mu g\ kg^{-1}\ day^{-1}$ with an application of an uncertainty factor of 1000 is adopted, i.e. it is considered unsafe for a 60 kg adult to drink 2 L water containing over 8.4 μg toxins per litre per day. Applying an additional safety factor of 10, the safe level proposed is 0.84 μg toxins L^{-1} (or approximately 1 μgL^{-1} = ~5000 cells per mL) [17]. Also of relevance is the report of microcystin contamination in shellfish consumed in southern Europe and eastern Canada [18,19], indicating that these toxins may pose a direct health hazard to humans *via* the food chain. Thus, the epidemiological importance of these highly toxic compounds should not be underestimated. These findings underline the direct and indirect risks of tumour-promoting toxins to humans, and call for a simple, cost-effective means of detecting these toxins, especially at trace levels.

A commonly used detection method for MCYSTs involves the use of radioactive labeled substrates for the phosphatases. This method, though sensitive, has a number of disadvantages: pre-labeling of protein substrates with ^{32}P is time consuming; the labeled substrate must be prepared fresh and made repeatedly; and there are problems associated with handling radioactive wastes. Moreover, the procedure requires very skilled operators, and all these factors limit the possibility of large-scale applications of the method for routine screening of water and aquatic foods. An alternative method for determining protein phosphatase inhibitory factors is the enzyme-linked immunosorbent assay (ELISA) which has high specificity for particular strains of microcystins. Thus, it has been applied in the identification and immunochemical analysis of *in vivo* samples of hepatotoxin-poisoned animals [8,9]. Despite this, due to the large variety of protein phosphatase inhibitory factors (over 50 different structural variants in the case of microcystins alone), the usefulness of the ELISA technique will depend on the availability of a correspondingly wide spectrum of toxin antibodies to allow toxin detection and identification in aquatic environments [2].

The results of the colorimetric protein phosphatase inhibition method outlined in this paper are promising, and the procedure has similar detection limits as the radiometric assay. Notwithstanding, the colorimetric method can be further refined as the highest percentage inhibition of the protein phosphatase activity for the colorimetric assay was only around 80%, while 100% inhibition is normally achieved in the assay involving radioactive labeled substrate. The possibility of achieving higher percentage inhibition, by obtaining a more pure enzyme extract using more sophisticated enzyme purification procedures, should also be explored [20]. Nonetheless, the colorimetric protein phosphatase inhibition assay does offer a simple, inexpensive tool for large-scale screening of protein phosphatase inhibitors in fresh, estuarine and marine waters. However, it

should be noted that neither the radiometric nor colorimetric protein phosphatase inhibition assays could provide detailed information on the chemical identity of the inhibitors present in the water samples.

The inhibitory effects of these compounds on protein phosphatases suggest that they are of potential concern as tumour promoting agents. The use of a screening tool will greatly reduce the number of water samples that may require further analyses by other more expensive and elaborate methods (e.g. HPLC, ELISA techniques). Of course, whether further analyses are required will depend on the nature of the monitoring work involved and the type of management questions asked.

The authors acknowledge the support of research grants from the City University of Hong Kong and the Hong Kong Research Grants Council (No: CityU1057/97M).

REFERENCES

1. W.W. Carmichael, C.L.A. Jones, N.A. Mahmood and W.C. Theiss, Algal toxins and water-based diseases. *CRC Critical Reviews in Environmental Control* **15**, 275 313 (1985).
2. G.A. Codd, S.G. Bell and W.P. Brooks, Cyanobacterial toxins in water. *Water Science and Technology* **21**, 1-13 (1989).
3. R. Nishiwaki-Matsushima, T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W.W. Carmichael and H. Fujiki, Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *Journal of Cancer Research Clinical Oncology* **118**, 420-424 (1992).
4. I.R. Falconer, Effects on human health of some toxic cyanobacteria (blue-green algae) in reservoirs, lakes and rivers. *Toxicity Assessment* **4**, 175-184 (1989).
5. S.G. Bell and G.A. Codd, Cyanobacterial toxins and human health. *Reviews in Medical Microbiology* **5**, 256-264 (1994).
6. D.L. Brautigan and C.L. Shriner, Methods to distinguish various types of protein phosphatase activity. *Methods in Enzymology* **159**, 339-347 (1988).
7. T. Sassa, W.W. Richter, N. Uda, M. Suganuma, H. Suguri, S. Yoshizawa, M. Hirota and H. Fujiki, Apparent "activation" of protein kinases by okadaic acid class tumor promoters. *Biochemical and Biophysical Research Communications* **159**, 939-944 (1989).
8. J.-R. Lin and F.S. Chu, Kinetics of distribution of microcystin LR in serum and liver cytosol of mice: an immunochemical analysis. *Journal of Agricultural and Food Chemistry* **42**, 1035-1040 (1994).

9. S. Nagata, H. Soutome, T. Tsutsumi, A. Hasegawa, M. Sekijima, M. Sugamuta, K. Harada, M. Suganuma and Y. Ueno, Novel monoclonal antibodies against microcystin and their protective activity for hepatotoxicity. *Natural Toxins* **3**, 78-86 (1995).
10. A. Takai and G. Mieskes, Inhibitory effect of okadaic acid on the *p*-nitrophenyl phosphate phosphatase activity of protein phosphatases. *Biochemical Journal* **275,** 233-239 (1991).
11. K. Harada, M. Suzuki, A.M. Dahlem, V.R. Beasley, W.W. Carmichael and K.L. Rinehart, Improved method for purification of toxic peptides produced by cyanobacteria. *Toxicon* **26**, 433-439 (1988).
12. G.J. Jones and P.T. Orr, Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. Water Research **28**, 871-876 (1994).
13. J.S. 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).
14. T.W. Lambert, M.P. Boland, C.F.B. Holmes and S.E. Hrudey, Quantitation of the microcystin hepatotoxins in water at environmentally relevant concentrations with the protein phosphatase bioassay. *Environmental Science and Technology* **28**(4):753-755 (1994).
15. S. -Z. Yu, Drinking water and primary liver cancer. In *Primary Liver Cancer* (Edited by Z.Y. Tung, M.-C. Wu and S.-S. Xia), pp.30-37. China Academic Publishers, Beijing, Springer-Verlag, Berlin, Germany (1989).
16. K. Harada, M. Oshikata, M., H. Uchida, M. Suzuki, F. Kondo, K. Sato, Y. Ueno, S. Yu, G. Chen and G. Chen, Detection and identification of microcystins in the drinking water of Haimen City, China. *Natural Toxins* **4**:277-283 (1996).
17. I.R. Falconer, M.D. Burch, D.A. Steffensen, M. Choice and O.R. Coverdale, Toxicity of the blue-green alga (cyanobacterium) *microcystis aeruginosa* in drinking water to growing pigs, as an animal model for human injury and risk assessment. *Environmental Toxicology and Water Quality* **9**, 131-139 (1994).
18. E. Fattorusso, P. Chiminiello, V. Costantino, S. Magno, A. Mangoni, A. Milandri, R. Poletti, M. Pompei and R. Viviani, Okadaic acid in mussels of Adriatic Sea. *Marine Pollution Bulletin* **24**, 234-237 (1992).
19. D.Z.X. Chen, M.P. Blan, M.A. Smillie, H. Klix, C. Ptak, R.J. Anderson and C.F.B. Holmes, Identification of protein phophatase inhibitors of the microcystin class in the marine environment. *Toxicon* **31**, 1407-1414 (1993).
20. S. Nishiwaki, H. Fujiki, M. Suganuma, R. Nishiwaki-Matsushima and T. Sugimura, Rapid purification of protein phosphatase 2A' from mouse brain by microcystin-affinity chromatography. *Federation of European Biochemical Societies* **279**, 115-118 (1991).