Talanta 85 (2011) 2498–2503

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a colorimetric inhibition assay for microcystin-LR detection: Comparison of the sensitivity of different protein phosphatases

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ARTICLE INFO

Article history:
Received 29 March 2011
Received in revised form 28 July 2011
Accepted 29 July 2011
Available online 5 August 2011

Keywords:
Microcystin-LR
Colorimetric assays
Protein phosphatases
Photopolymer
Agarose gel

ABSTRACT

A colorimetric protein phosphatase (PP) inhibition test for the detection of microcystin-LR (MC-LR) has been developed. Three PP2As, one recombinant and two natural versions, as well as one PP1 produced by molecular engineering, were tested. First, assays were performed using the enzymes in solution to compare their sensitivity to MC-LR. The PP2A purchased from ZEU Immunotec and PP1 appeared more sensitive to the toxin than the other enzymes. With PP2A from ZEU Immunotec, the colorimetric test showed a detection limit of 0.0039 $\mu g\,L^{-1}$ and an IC_{50} value of 0.21 $\mu g\,L^{-1}$. With PP1, the assay gave a detection limit of 0.05 $\mu g\,L^{-1}$ and an IC_{50} value of 0.56 $\mu g\,L^{-1}$. Therefore, this assay allowed the detection of lower microcystin-LR (MC-LR) concentrations than the maximum level (1 $\mu g\,L^{-1}$) recommended by the World Health Organisation (WHO).

The main drawback of this PP-based approach in solution is poor enzyme stabilisation. To overcome this problem, enzymes were entrapped within either a photopolymer or an agarose gel. PP2A from ZEU Immunotec and PP1 were immobilised at the bottom of microwells. The agarose-based tests performed better than the photopolymer-based assay for all of the enzymes. Therefore, the agarose gel is a good candidate to replace the photopolymer, which is generally used in PP-immobilising membranes. The assays based on enzyme-entrapping agarose gels showed detection limits equal to 0.17 $\mu g\,L^{-1}$ and 0.29 $\mu g\,L^{-1}$ with immobilised PP2A from ZEU and PP1, respectively. In view of these performances, these tests can potentially be used for monitoring water quality.

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1. Introduction

Water blooms of toxic cyanobacteria (blue-green algae) represent a serious problem because of the potent toxins that can be released by these algae [1]. Toxin-producing microalgae species have a negative influence on the environment, food safety and health [2]. Microcystins (MCs) are a group of cyanobacterial toxins that are mainly produced by microcystis, which appear in lake, ponds, reservoirs and rivers with low turbidity flow regimes. More than 70 structural variants of MCs are known, and each one shows different toxicity levels. These toxins are cyclic heptapeptides with the general structure cyclo-(D-Ala-X-D-MeAsp-Y-Adda-Adda-D-Glu-N-methyldehydro-Ala), where X and Y represent variable L-amino acid residues. The amino acid Adda (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid) is considered to be responsible for the MC hepatotoxicity [1]. MC-LR (L and R designating leucine and arginine, respectively) was the first MC chemically identified, and it is the most toxic and most frequently found.

After ingestion, MC can penetrate into hepatocytes. Within the hepatocytes, MCs irreversibly inhibit protein phosphatases type 2A (PP2A) and 1 (PP1) [1]. External signs of poisoning, which include weakness, pallor, heavy breathing, vomiting and diarrhoea, are then observed. MCs are potent tumour promoters, causing disruption of liver structure and function, haemorrhaging into the liver and death by respiratory arrest [3]. Several cases of animal and human intoxication due to MCs have been reported. For example, in 1996, patients at a Brazilian haemodialysis centre using municipal water contaminated with cyanotoxins were exposed to lethal levels of MCs. One hundred of the 131 patients developed acute liver failure and 52 of these victims are died due to hepatotoxin poisoning [4,5]. In 2009, water pollution in Yancheng China affected the water supply system, which was closed for three days [6].

The toxicity and ubiquity of MCs necessitate the development of fast, sensitive and reliable methods to detect them. To guarantee water quality and to minimise the potential risk to human health, the World Health Organisation (WHO) has recommended a maximum level of 1 $\mu g\,L^{-1}$ of MC-LR in drinking water [7]. Accordingly,

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doi:10.1016/j.talanta.2011.07.101

detection systems must be sensitive to MC concentrations below the limit established by the WHO. The simplest screening method is the mouse bioassay, which suffers from low sensitivity, specificity and ethical problems due to animal experimentation. In vitro cytotoxicity assays, based on morphological changes in cells after exposure to toxins, have been developed to provide a substitute for the mouse bioassay [8,9]. These assays are easy to perform and economical but they are also subjective, time-consuming and confusing results may appear in the presence of toxin mixtures [10]. Other cytotoxicity tests are based on the simple and sensitive analysis of the toxin effect on cells by measuring changes in O_2 consumption by optical oxygen sensing technique [11]. MCs are routinely analysed using high-performance liquid chromatography (HPLC) and mass spectrometry. These techniques allow for highly selective identification and sensitive quantification of the different toxins present in a sample. However, they require expensive equipment, complex procedures, lengthy analysis times and trained personnel [12,13]. The ability of MCs to inhibit PPs has allowed the development of enzymatic assays [14–18] and biosensors [19–21].

Protein-phosphatase inhibition assays are considered as a good alternate to the mouse bioassay. In this work, an efficient protein phosphatase-based colorimetric test has been developed to detect microcystin. The enzymatic method is based on the protein phosphatase inhibition by microcystin. Colorimetry is the most commonly used method due to the simplicity, cost effectiveness and sufficient sensitivity. In the first part of this work, four different protein phosphatases were used to detect MC-LR: one PP2A produced by molecular engineering (GTP Technology), two commercially produced PP2As (Millipore and ZEU Immunotec) and one PP1 produced by molecular engineering. Assays were performed using enzymes in solution to compare the sensitivities of different protein phosphatases to MC-LR. PP2A purchased from ZEU Immunotec and the recombinant PP1 were used for the first time to develop colorimetric inhibition assays for microcystin detection.

Most protein phosphatase-based colorimetric tests for MC have been developed using enzyme in solution. A main drawback of the enzymatic approach is that fast PP inactivation occurs when it is not stored under specific conditions. Protein phosphatase immobilization contributing to stabilise the enzyme activity, PP2A and PP1 were entrapped within several matrices: photopolymer and agarose gels. In a previous work, a colorimetric inhibition assay was developed by immobilising PP2A from GTP Technology within a photopolymer. In this paper, different PP2As and PP1 were immobilised not only in the photopolymer but also in an agarose gel. This article described the first agarose-based colorimetric assays allowing efficient microcystin-LR detection.

2. Materiel and methods

2.1. Reagents

MC-LR standards were purchased from Alexis (San Diego, USA). Microcystin solutions were prepared in methanol and subsequently diluted in distilled water.

Agarose (type IX) and p-nitrophenyl phosphate (pNPP) were purchased from Sigma.

Photocrosslinkable poly(vinyl alcohol) bearing methylpyridinium groups (PVA-super porous hydrogel, PVA-SPH) (solid content 10.2 wt.%, pH 6.7) was provided by Toyo Gosei Kogyo Co. (Chiba, Japan).

All solutions were prepared with Milli-Q water.

2.2. Enzymes

2.2.1. PP2A

Three batches of PP2A, two natural and one recombinant, were used.

- Enzyme purchased from Millipore (NY, USA) was isolated as a heterodimer of 60 kDa (A) and 36 kDa (C) subunits from human red blood cells.
- PP2A from ZEU Immunotec (Zaragoza, Spain) was isolated from human red blood cells. ZEU produces a lyophilised form of PP2A with excellent storage stability.
- GTP Technology (Toulouse, France) produced a genetically engineered PP2A that consists of a 39 kDa (His-tag included) human catalytic (C) subunit of the α-isoform isolated from SF9 insect cells infected by baculovirus.

2.2.2. PP1

PP1 was purchased from CRITT (Toulouse, France).

Synthetic gene coding for rabbit protein phosphatise 1α (PP1α, emb XO7798) was constructed according to Denis-Quanquin et al. [22]. Three mutations were introduced to increase the production and solubility (C127S, C38G, C105V), and a histidine tail was added at the N-terminus to facilitate purification. A green fluorescent-protein (GFP) tag was introduced in the C terminal end to facilitate synthetic gene construction and protein purification. The construction was introduced into the pETG vector downstream of the T7 promoter and the *lac* operator. The *Escherichia coli* BL21(DE3) strain transformed with the plasmid including this construction was grown in a rich medium containing 1 mM $MnCl_2$ until an OD of 1 was reached. T7 RNA polymerase-mediated transcription was induced with 1 mM isopropyl-β-thiogalactopyranoside, and the culture temperature was decreased to 25 °C. The cells were harvested by centrifugation and lysed by sonication. The purification of protein was achieved by affinity chromatography using nickel as the ligand. Purified extracts were found to be devoid of any contaminant, as assessed by overloaded SDS-PAGE.

2.3. Apparatus

The colorimetric measurements were performed with a Labsystems Multiskan EX microtiter plate reader (Thermo Life Sciences, Cergy Pontoise, France). Maxisorp microtiter plates were obtained from Nunc (Roskilde, Denmark).

2.4. Protein-phosphatase inhibition assays

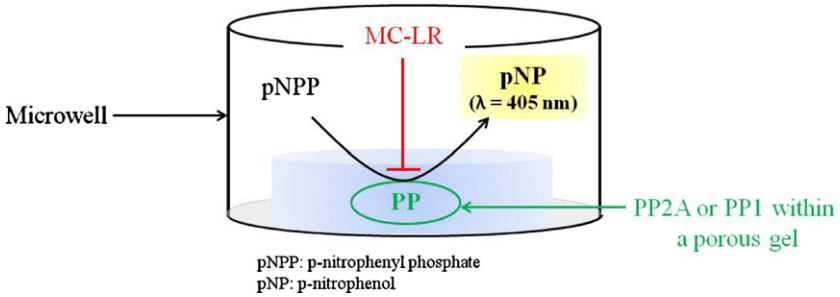
2.4.1. Enzyme immobilisation

PP2A provided by ZEU Immunotec and PP1 purchased by CRITT were entrapped within different matrices: photopolymer and agarose gels. In all cases, the amount of immobilised enzyme was calculated to be 3 mU.

2.4.1.1. Photopolymerisation. This method has been previously used for enzyme immobilisation [14,23–30].

An enzyme solution was mixed with PVA-SPH in a 1:1 ratio (v/v). After homogenisation, 10 μL of this solution was spread on the bottom of a Maxisorp well. The support was then exposed to neon light (two 15 W lamps) for 3 h at 4 °C to allow entrapment of the enzymes by photopolymerisation. The supports were dried for 24 h at 4 °C.

CRITT lyophilised the PP1 using ammonium persulfate. However, this molecule prevents the photopolymerisation process. Therefore, for this enzyme, a preliminary dialysis step was performed using Slide-A-Lyzer® Mini Dialysis Units (Thermo Scientific).



Scheme 1. Principle of the colorimetric PP inhibition assay.

2.4.1.2. Entrapment within an agarose gel. A 2% (w/v) agarose solution was prepared by heating the agarose powder in Tris–HCl buffer at 60 °C for 5 min. When the agarose solution temperature cooled to 27 °C, the diluted PP2A was added to the agarose solution in a 1:1 volume ratio. After homogenisation, 10 μL of the mixture was deposited at the bottom of a Maxisorp well and dried for 4 h at 4 °C. The agarose gel formed when the temperature was decreased.

2.4.2. Colorimetric detection

The activity measurements are based on the production of yellow-coloured p-nitrophenol (p-NP) by the enzymatic reaction between PP2A and the colourless p-NPP substrate. MC-LR inhibits PP2A and PP1 and causes the loss of production of the yellow pNP (Scheme 1). The PP-inhibition assays were performed by adding 20 μL of MC-LR solutions at different concentrations into microtiter plates containing 20 μL of 100 mM pNPP, PP-immobilising gel (10 μL) or enzyme in solution (50 μL) and Tris–HCl buffer (to obtain a final volume of 200 μL). After 1 h at 37 °C, the absorbance was measured at λ = 405 nm using the microtiter plate reader. Controls without enzyme and/or without MC-LR were always performed. The assays were performed in triplicate.

3. Results

The method based on PP inhibition gives an indication of the toxicity of a sample. In this work, four enzymes were tested to develop a suitable PP-based colorimetric test for MC detection.

First, the assays were developed using PPs in solution to study the sensitivity of different enzymes to MC-LR. These tests suffered from fast enzyme inactivation, and the enzyme immobilisation process stabilises the PP enzymatic activity. Therefore, the PPs were immobilised to the bottom of a microtiter plate using two entrapment matrices: a photopolymer and an agarose gel. Direct coating of the surface with a mixture of enzyme and pre-polymer is a straightforward process, and it does not need complex reaction processes, such as covalent linkages.

Because MC-LR inhibits phosphatase activity, colorimetric inhibition assays were performed for biotoxin detection. The test takes advantage of the ability of PP2A to dephosphorylate a colourless substrate (p-NPP) to a yellow product (p-NP). Phosphatase activity is inhibited by MC-LR. This inhibition induces a reduction in the rate of production of p-NP (Scheme 1). For PPs in solution or immobilised within a gel, calibration curves were determined. The curves were fit with a sigmoidal logistic four-parameter equation (OriginPro 8):

$$y = A2 + \frac{A1 - A2}{1 + (x/x_0)p}$$

where $A1$ and $A2$ are the asymptomatic minimum and maximum values, respectively. x_0 is the x value at the inflection point. p is the slope at the inflection point.

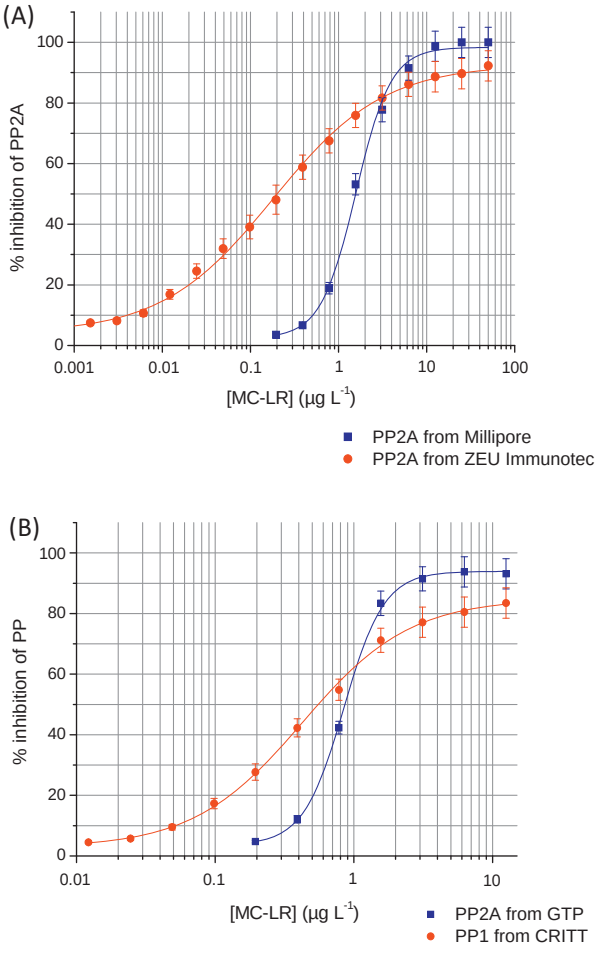


Fig. 1. Standard curves for the inhibition of PP2As in solution by MC-LR. Inhibition is expressed as a percentage of the control (no microcystin). (A) Commercial PP (PP2A from Millipore and ZEU Immunotec). (B) PPs produced by molecular engineering (PP2A from GTP Technology and PP1 from CRITT).

3.1. Sensitivity of different PPs to MC-LR

The different PP2As and the PP1 were used to develop efficient colorimetric phosphatase-inhibition tests for MC detection. The assays were performed using enzymes in solution to compare the sensitivities of different PPs to MC-LR. Fig. 1A and B shows the standard curves for the inhibition of commercial PPs (PP2As from Millipore and ZEU Immunotec) and enzymes produced by molecular engineering (PP2A from GTP Technology and PP1 from CRITT). The calibration curves for the inhibition of PPs by different concentrations of MC-LR showed typical sigmoidal responses. Table 1 summarises the curve parameters derived from the regression curves: the detection limits corresponding to the MC concentration

Table 1
The curve parameters derived from the sigmoidal logistic four-parameter fitting for the inhibition of PPs from different sources in solution by MC-LR.

Enzyme		Detection limit ($\mu g\,L^{-1}$)	IC_{50} ($\mu g\,L^{-1}$)	Sigmoidal logistic equation	R
PP2A	Millipore	0.48	1.55	$y = 99.84 + \frac{-99.4}{1+(x/1.56)^{1.91}}$	0.997
	ZEU	0.0039	0.21	$y = 95.01 + \frac{-92.42}{1+(x/0.18)^{0.62}}$	0.999
	GTP	0.38	0.85	$y = 93.7 + \frac{-92.52}{1+(x/0.83)^{3.02}}$	0.998
PP1	CRITT	0.05	0.56	$y = 85.67 + \frac{-83.21}{1+(x/0.43)^{1.07}}$	0.998

that induces a 10% inhibition, the 50% inhibition coefficient (IC_{50}) and the correlation coefficient (R).

The performances of the colorimetric tests obtained with the two commercial enzymes are drastically different. The detection limit of PP2A from ZEU Immunotec is 123-fold lower than the value obtained with the enzyme from Millipore (0.0039 $\mu g\,L^{-1}$ versus 0.48 $\mu g\,L^{-1}$). The IC_{50} value of PP2A from ZEU Immunotec is 7.4-fold lower than the value observed with the other enzyme (0.21 $\mu g\,L^{-1}$ versus 1.55 $\mu g\,L^{-1}$). This level of difference is surprising because both of these enzymes are natural and isolated from human blood cells. However, the experiments were repeated, and the results are reproducible. The plots are linear in the MC concentration range for PP2A from Millipore between 0.78 $\mu g\,L^{-1}$ and 3.125 $\mu g\,L^{-1}$ ($y = 42.4 \ln x + 31.07$, $R^2 = 0.991$) and between 0.0245 $\mu g\,L^{-1}$ and 1.563 $\mu g\,L^{-1}$ ($12.64 \ln x + 70.07$, $R^2 = 0.997$) for PP2A from ZEU Immunotec.

Comparing the curves obtained with the PPs produced by molecular engineering, PP1 appears more sensitive to MC-LR than PP2A from GTP Technology with a 7.6-fold lower detection limit (0.05 $\mu g\,L^{-1}$ versus 0.38 $\mu g\,L^{-1}$) and a 1.5-fold lower IC_{50} (0.56 $\mu g\,L^{-1}$ versus 0.85 $\mu g\,L^{-1}$). The plots are linear in the MC concentration range 0.37–1.563 $\mu g\,L^{-1}$ ($y = 51.35 \ln x + 58.66$, $R^2 = 0.993$) for PP2 from GTP Technology and 0.098–1.563 $\mu g\,L^{-1}$ ($y = 19.48 \ln x + 60.97$, $R^2 = 0.995$) for PP1.

From the results, PP2A from ZEU Immunotec is more sensitive to the toxin than the other enzymes. The calibration curves show the suitability of the colorimetric test to detect MC concentrations (linear between 0.0245 and 1.563 $\mu g\,L^{-1}$) at lower levels than what are recommended by the WHO (1 $\mu g\,L^{-1}$).

The differences in the performances of the PP inhibition tests are likely due to structural properties. PP2A from GTP is a catalytic subunit, whereas the enzymes from Millipore and ZEU Immunotec are heterodimers. The differences between the performances obtained with the enzymes supplied by ZEU Immunotec and Millipore are more difficult to explain because these enzymes are both natural and isolated from human blood cells. ZEU Immunotec produced lyophilised PP2A with an excellent sensitivity to MC-LR. Possible mutations in the sequence of the genetically engineered enzyme from GTP and CRITT could also modify the performance of the colorimetric test.

Table 2 is a compilation of colorimetric phosphatase-inhibition tests for MC detection found in the literature. These enzymatic assays used PPs from different sources in solution. Our test based on PP2A from ZEU Immunotec performed better than all of the previously reported assays in term of IC_{50} values and detection limits. The detection limit obtained with our colorimetric test is 12.8-fold lower than the value obtained by Rivasseau et al. using PP2A from Millipore [17].

3.2. Enzyme immobilisation to develop colorimetric PP inhibition assays

The next step in the assay development explored immobilisation strategies to stabilise the PP activity. The most MC-LR-sensitive enzymes were chosen for further experiments. In view of the previously reported results, PP2A from ZEU Immunotec and PP1 from CRITT were selected to develop colorimetric PP inhibition assays based on enzyme-entrapped gel. These enzymes were entrapped within a photopolymer and an agarose gel at the bottom of a microwell.

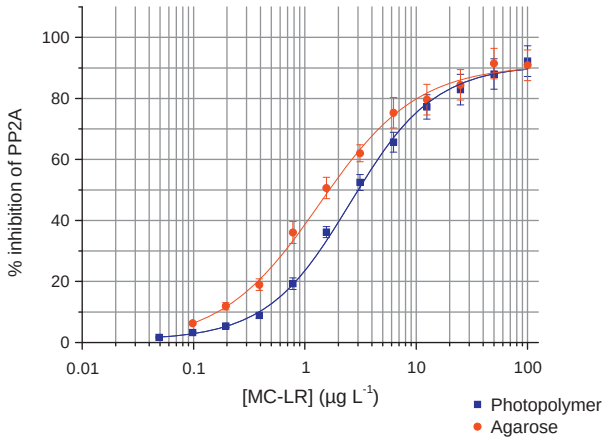


Fig. 2. Standard curves for the inhibition of PP2A from ZEU Immunotec immobilised within a gel by MC-LR.

Fig. 2 shows the standard curves for the inhibition of PP2A from ZEU Immunotec immobilised within a membrane by MC-LR. The experimental data derived from the regression curves are summarised in Table 3. The detection limits and the IC_{50} values indicate that the test based on PP2A from ZEU Immunotec immobilised within an agarose gel is more efficient than the photopolymer-based test. The detection limit obtained with the agarose-based assay is two-fold lower than the value observed with the photopolymer-based test (0.17 $\mu g\,L^{-1}$ versus 0.36 $\mu g\,L^{-1}$). The linear detection regime ranges from 0.39 to 12.5 $\mu g\,L^{-1}$ ($y = 20.48 \ln x + 27.06$, $R^2 = 0.995$) and from 0.195 to 6.25 $\mu g\,L^{-1}$ ($y = 18.99 \ln x + 40.60$, $R^2 = 0.993$) for the photopolymer and agarose-based tests, respectively.

Comparing the inhibition assays based on the immobilised PP1 (Fig. 3 and Table 3), the results show that PP1 immobilised within an agarose gel is more sensitive to the presence of toxins than enzyme entrapped by the photopolymerisation process. The IC_{50} value obtained with the PP1 immobilised within an agarose gel is 9.5-fold lower than the IC_{50} obtained with the photopolymer-based test. The linear detection regime ranges from 1.56 $\mu g\,L^{-1}$ to 8.67 $\mu g\,L^{-1}$ ($y = 18.4 \ln x + 0.046$, $R^2 = 0.992$) and 0.78 $\mu g\,L^{-1}$ to 3.125 $\mu g\,L^{-1}$ ($y = 34.83 \ln x + 38.16$, $R^2 = 0.991$) for the photopolymer and agarose-based tests, respectively.

The best performances were observed with the assays using an agarose gel to immobilise PP2A from ZEU Immunotec. Our group previously described a colorimetric test using PP2A from GTP Technology entrapped at the bottom of a microwell within a photopolymer [14]. In this case, the IC_{50} value was 8.31 $\mu g\,L^{-1}$, whereas we obtained a 5.2-fold lower IC_{50} with the colorimetric test described in this work. Therefore, the test based on PP2A

Table 2
Performances of colorimetric PP inhibition assays based on PP2A or PP1 in solution for MC-LR detection.

PP types	Detection limit ($\mu g\,L^{-1}$)	Linearity range ($\mu g\,L^{-1}$)	IC_{50} ($\mu g\,L^{-1}$)	Reference
PP2A	–	1–10	5.06	[31]
PP2A from Promega (36–38 kDa catalytic subunit from rabbit skeletal muscle)	–	0.2–1	0.5	[16]
PP2A from Millipore	0.25	–	–	[18]
PP2A from Millipore	0.05	0.1–0.4	0.21	[17]
PP2A from Millipore	0.3	0.3–0.75	0.5	[14]
PP2A from Millipore	0.48	0.78–3.13	1.55	In this work
PP2A from ZEU Immunotec	0.0039	0.0245–1.563	0.21	In this work
PP2A from GTP Technology	0.9	0.9–2.25	1.4	[14]
PP2A from GTP Technology	0.38	0.37–1.56	0.85	In this work
Catalytic subunit of PP1 (rabbit muscle) from Calbiochem	0.01–0.05	0.1–10	0.5	[32]
Recombinant PP1 from CRITT	0.05	0.098–1.56	0.56	In this work

Table 3
Experimental data obtained from the standard curves for the inhibition of PP2A from ZEU Immunotec and PP1 from CRITT immobilised within a photopolymer or an agarose gel by MC-LR.

Enzyme	Immobilisation matrix	Detection limit ($\mu g\,L^{-1}$)	IC_{50} ($\mu g\,L^{-1}$)	Sigmoidal logistic equation	R
PP2A from ZEU	Photopolymer	0.36	2.99	$y = 92.15 + \frac{-92.67}{1+(x/2.51)1.05}$	0.998
	Agarose gel	0.17	1.6	$y = 92.75 + \frac{-96.79}{1+(x/1.24)^{0.88}}$	0.996
PP1 from CRITT	Photopolymer	1.62	12.1	$y = 96.91 + \frac{-96.65}{1+(x/11.54)^{1.13}}$	0.994
	Agarose gel	0.28	1.31	$y = 93.45 + \frac{-90.6}{1+(x/1.22)^{1.67}}$	0.997

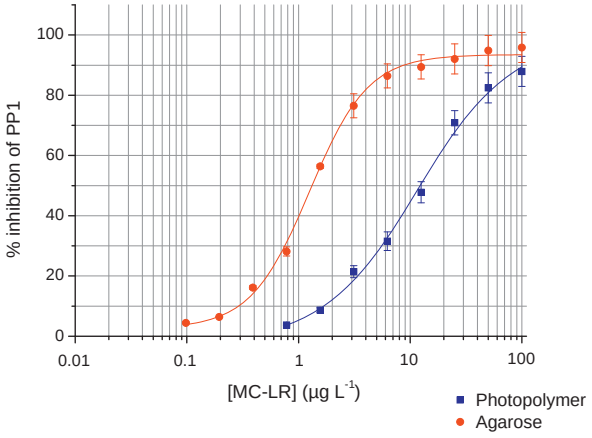


Fig. 3. Standard curves for the inhibition of PP1 from CRITT immobilised within a gel by MC-LR.

from ZEU Immunotec entrapped within an agarose gel is the most efficient for the detection of MC-LR in tainted water.

4. Conclusion

In this work, a colorimetric PP inhibition assay for the detection of OA was developed. For this purpose, one PP2A produced by molecular engineering, two commercial PP2As and one recombinant PP1 were tested. The enzyme from ZEU Immunotec in solution had a higher sensitivity to MC-LR than the other enzymes.

The major problem of PPs is their poor enzymatic stability. Enzyme immobilisation can be used stabilise the PP activity; therefore, a photopolymer and an agarose gel were separately used to entrap the enzymes. Tests based on PP2A from ZEU Immunotec immobilised within an agarose gel showed the best performances with a detection limit of $0.17\,\mu g\,L^{-1}$. The assay based on PP1 immobilised within the same membrane gave a detection limit of $0.28\,\mu g\,L^{-1}$ and an IC_{50} value of $1.31\,\mu g\,L^{-1}$. These results demonstrated the efficiency of the test to detect lower concentrations of MC-LR than recommended by the WHO.

These colorimetric tests were used to detect MC-LR. This paper focused on the detection of MC-LR because of its high toxicity and its ubiquity. We will soon conduct experiments to test whether the colorimetric inhibition assays are able to detect efficiency other microcystin variants (e.g., MC-RR and MC-YR).

The advantages of immobilisation and the simplicity of the colorimetric detection make the use of this colorimetric test very attractive to detect MC-LR in contaminated water. Currently, water samples from different areas in Spain are being tested using our colorimetric protein phosphatase-inhibition assay. The effect of the matrix composition (e.g., a sandy area, water rich in organic matter) [17] on the enzyme activity will be evaluated.

Acknowledgement

This work has been funded through INTERREG SUDOE IVB and FEDER through the SOE1/P1/E129 ALARMTOX project.

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