Toxicon 52 (2008) 745–753

Contents lists available at ScienceDirect

Toxicon

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

# Assessment of protein phosphatase in a re-usable rapid assay format in detecting microcystins and okadaic acid as a precursor to biosensor development

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ARTICLE INFO

*Article history:*
Received 12 June 2008
Accepted 20 August 2008
Available online 5 September 2008

*Keywords:*
Protein phosphatase
Biosensor
Microcystin
Okadaic acid

ABSTRACT

The feasibility of developing an immobilised protein phosphatase (PP) biosensor was tested by immobilising PP onto CNBr-activated Sepharose beads placed in Millipore microfilter plate wells. Under optimised immobilised enzyme assay conditions, okadaic acid (OA) and microcystin LR (MC-LR) inhibited Upstate Biotechnology PP (PP-2A), with $IC_{50}$ values of 12.5 and 11 nM respectively. Similarly, immobilised recombinant PP type 1 (rec PP-1) was inhibited by MC-LR and OA, with $IC_{50}$ values of 150 and >1000 nM respectively. The $IC_{50}$ values for free PP-2A against OA and MC-LR were 2.5 and 3.5 nM, and 0.7 nM and 200 nM for rec PP-1 against the same substrates respectively. For free and immobilised *Neptunea arthritic* PP (PP-$2A_{na}$) against OA the $IC_{50}$ values were 0.45 and >1000 nM respectively. Of the three immobilised enzyme systems, PP-2A showed greatest sensitivity to OA and MC-LR followed by rec PP-1 and PP-$2A_{na}$. In assessments for re-usability (determined by removal of ≥70% OA or MC-LR inhibition of PP-2A by washing), <50% of the original activity remained after 20 washings. Including 1 M NaCl in the wash buffer did not increase enzyme activity with wash frequency, but rather "salted in" the inhibitor. The LoD of immobilised PP-2A to MC-LR meets the WHO guideline of 1 $\mu g\,l^{-1}$ for drinking water, and the sensitivity to OA (3.5 $\mu g\,l^{-1}$) would allow detection of DSP during the peak of some phytoplankton blooms.



## 1. Introduction

The current methods for the determination of micro-algal toxins are "in house" techniques such as LC-MS, ELISA and mouse bioassay (e.g. Toyofuku, 2006). These methods are conducted in a central purpose built laboratory facility and often require a high technical capability to operate. The concept of measuring aquatic toxins using a biosensor device requires a technological leap from centrally based detection systems to those that can be used for field monitoring of aquatic toxins. Most of the recently described assay methods for detection of marine toxins include the use of alkaline or acid phosphatase or labelled antibodies incorporated into immunosensors in a re-usable or disposable format using a chemiluminescent, electrode or microcrystalline balance system for detection (Croci et al., 2001; Kreuzer et al., 2002; Marquette et al., 1999; Tang et al., 2002). Campas et al. (2005) developed a sensor

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doi:10.1016/j.toxicon.2008.08.010

method to detect freshwater toxins (e.g. MC) using various support matrices with colorimetric or electrochemical detection formats.

However, of the methods developed to date, to our knowledge none have been accepted for use by regulatory authorities. Most have the disadvantage in that they utilise complex reaction systems (Croci et al., 2001), lack sensitivity (Tang et al., 2002) or employ enzyme systems like alkaline and acid phosphatase, that are non-specific to aquatic toxins such as OA and/or MC-LR. In contrast, the enzyme systems described by Campas et al. (2005) who selected protein phosphatases (PP), are highly sensitive and have high specificity towards MC-LR (Bialojan and Takai, 1988). They investigated the sensitivity of two PP enzymes towards MC-RR and LR on different support matrices, in which the assay mixture was transferred from the test system to a microplate for fixed point analysis by colorimetry. Using this procedure, sensitivities of their enzymes towards MC-LR and microcystin RR (MC-RR) expressed as $IC_{50}$, ranged from 3 to 8 nM respectively. However, the authors expressed concern over the uncertainty of their results due to the need to transfer the assay matrices to another plate for analysis, and because diffusion barriers were created for reaction products from some supports (e.g. soft gel matrix). Additionally, inaccuracies may have occurred through the lack of sensitivity of the colorimetric assay system and the use of fixed point assay. Therefore, an improved assay approach was necessary before immobilised enzyme inhibition systems could be fully appraised for biosensor applications.

In this context we have envisaged an optical fluorometric biosensor based on PP for the detection of MC or diarrhetic shellfish toxins (responsible for the syndrome known as diarrhetic shellfish poisoning [DSP]). Linked to our work on a fluorescent microsphere and nanosphere 96-well plate format (Ruedas-Rama & Hall, 2006), we have looked at enzyme microspheres, using three different PP enzymes housed in 96-well microfilter plates with activity determined by fluorescence measurement. The activities of the free form of these enzymes towards DSP toxins and/or MCs have been previously described (Fontal et al., 1999; Mountfort et al., 1999, 2001, 2005; Sato et al., 2003; Tubaro et al., 1996; Zhang et al., 1994). The immobilised format described herein is used to evaluate the potential to translate PP into a prototype biosensor suitable as a regulatory assay allowing faster throughput than a solution assay and in particular, in assessments of sensitivity and reusability.

## 2. Methods

### 2.1. Materials

Okadaic acid (purity grade 99%) was obtained from the National Research Council Certified Material Programme, Halifax, Canada. Microcystin LR (purity grade 97%) was obtained from DHI Water and Environment, Hoersholm, Denmark. Protein phosphatase (type PP-2A), obtained from Upstate Biotechnology Inc (New York, USA), was isolated from human red blood cells as a heterodimer of 60 kDa and 36 kDa subunits. The enzyme is stable for 1 year at −18 °C in its dilution buffer: 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.5, 150 mM NaCl, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $MnCl_2$, 10% glycerol and 0.1 mg $ml^{-1}$ serum albumin. Recombinant PP-1 was obtained from New England Biolabs (Massachusetts, USA) isolated from *Escherichia coli* that carries the catalytic subunit of the $\alpha$ isoform of PP-1 from rabbit skeletal muscle. Protein phosphatase (type PP-$2A_{na}$) isolated from the marine snail *Neptunea arthritic* was provided as a gift from Nissui Pharmaceuticals Co., Ltd., Japan. CNBR-activated Sepharose™ (6 MB) was obtained from Amersham Biosciences, Sweden. All other chemicals were of reagent grade and obtained from commercial sources.

### 2.2. Analysis of microcystins and DSP by protein phosphatase inhibition assay (free enzyme)

The protein phosphatase inhibition assay was carried out in 96-well plates based on a method previously described for the detection of shellfish toxins (Mountfort et al., 1999, 2001). The assay system (200 μl) contained 50 μl of 50 mM Tris buffer (pH 7.0) and 0.1 mM $CaCl_2$, 120 μl of buffer with 1.7 mM fluorescent substrate, 4-methylumbelliferyl phosphate (MUP), 5 μl of 40 mM $NiCl_2$, 5 μl of BSA (1 mg $ml^{-1}$ in distilled water) and 10 μl enzyme (0.02 U per assay, final concentration, 1.5 nM) together with 5 or 10 μl of standard OA, MC-LR, or sample. The reaction was initiated by automatic injection of the substrate, which was pre-warmed together with the reaction mix at 37 °C, and measurements ($E_x$, 360; $E_m$, 460 nm) were by real time kinetics in a microplate reader (BMG Lab Technologies) over a period of 1 h. Activity was determined by linear regression of the reaction curves to give slope $min^{-1}$.

### 2.3. Enzyme immobilisation procedure

The method followed that described previously for a free-flow biosensor (Hatz & Hall, 2006) with some modifications. In brief, 100 mg of freeze-dried activated CN-Br Sepharose beads (particle size range, 200–300 μm) were washed 15 times in distilled water (1 ml) in microfuge tubes to remove additives. Washing was carried out by inverted mixing and pulse centrifugation (2000 × g) and the supernatant was removed between washes by Pasteur pipette. Protein phosphatase (100 μl containing approximately 1 U of enzyme) was mixed with 1 ml of standard coupling buffer (0.1 M Na phosphate containing 0.05 M KCL and 0.2 mM ascorbate phosphate, final pH 7.0) and added to 100 mg of washed beads. The enzyme-bead complex was spun overnight at 4 °C in microfuge tubes on a rotating disc (20 revs $min^{-1}$). The beads were washed twice with 50 mM Tris buffer (pH 7) containing 0.1 mM $CaCl_2$, and were then stored at 2 °C.

### 2.4. Transfer of immobilised enzyme-beads complex to microfilter plates

Enzyme-bead complex, equivalent to 50 mg of beads, was transferred to wells of a 96-well Multiscreen™ filterplate (Millipore, MA, USA) in approximately 350 μl of

buffer. The microplate was vacuum filtered using a vacuum manifold (Millipore, MA, USA) attached to an air-cadet® vacuum pressure station (Biolab Scientific Ltd., USA) to remove liquid from the enzyme-bead complex. The beads were washed twice by successive buffer additions (150 μl) and stored in buffer (200 μl) at 2 °C before use.

### 2.5. Assay of immobilised protein phosphatase

Prior to the assay, beads were washed twice with successive 150 μl buffer additions and the liquid content was evacuated to the top of the bead volume but not to dryness. In the standard immobilised assay system, each well contained 0.5 U of PP immobilised onto 50 mg of Sepharose beads, 55 μl of 50 mM Tris assay buffer (pH as specified in individual experiments) containing 0.1 mM $CaCl_2$, 120 μl of buffer containing substrate (0.42 mM MUP) and 5 μl of 40 mM $NiCl_2$. The total volume of the immobilised system (bead + liquid) was 250 μl (200 μl liquid volume). Controls contained either no beads, or contained beads but no substrate, with Tris assay buffer used to make up to the final volume. The assay was carried out as described in Section 2.2.

### 2.6. Generation of dose–response curves for MC-LR and OA

For the three PP enzymes in both unimmobilised and immobilised formats, standard curves were generated with ≥8 concentrations of MC-LR or OA in the range 0–1000 nM. The assay working range was 20–80% of the reference standard value. Inhibitor concentration giving 50% inhibition ($IC_{50}$) against the reference value (no toxin standard present) was determined from dose–response activity curves versus the log of concentration. The $IC_{50}$ values were verified using Hill plots in which the intersection of $v/(V_{max}-v) = 1$ was taken as the $IC_{50}$ value in which $V_{max}$ is 100% activity and $v$ is percent activity at any given concentration of inhibitor. Standard curves were checked using QC standards set at concentrations to give approximately 25 and 50% inhibition of the enzymes. Limits of detection (LoD were determined as the mean of the control (0 nM)) – (3 times the SD of the control).

### 2.7. Re-usability of immobilised enzyme following toxin addition

In this procedure, toxin (MC-LR or OA) QC standards were added to the assay mix to inhibit the enzymes at >70%. Between assays, wells were washed several times either with washing buffer (50 mM Tris, pH 8) or with washing buffer containing 1 M NaCl in sets of 5 washes. After each wash sequence, the enzyme activity was re-measured without further toxin addition.

### 2.8. Quality checks on immobilised enzyme in determining OA and MC-LR in spiked samples

Inhibitors were added at two different concentrations to standard assay buffer made up using freshwater (MC-LR) and standard assay buffer made up using filter and UV sterilised seawater (OA). Microcystin LR or OA concentration was determined from fitted curves (Sigma Plot) using the Microsoft Excel look-up function. Amount of inhibitor determined by the curves was compared to the amount of toxin added to the assay system.

## 3. Results and discussion

### 3.1. Rationalisation of assay system

Okadaic acid (OA) and dinophysistoxin-1 (3,5-methylokadaic acid) are major toxins involved in diarrhetic shellfish poisoning (DSPs) in humans. They have been shown to be potent tumor promoters in rodents, with the toxic effects of the DSP toxins appearing to originate from their inhibitory activity against a family of structurally related serine/threonine protein phosphatases (PP). In particular, the inhibition of essential PPs (e.g. PP-1 and PP-2A) has serious consequences in most eukaryotic cells. Exploiting this potent inhibitory property of the DSP toxins a solution based assay system for PP-2A activity determination has been previously optimised (Mountfort et al., 2001). The availability of a recombinant PP-1 and PP-2A enzyme now offers some potential to develop a useful DSP biosensor. However, the first assays of free rec PP-1 showed extremely low rates of activity using the assay buffer supplied by the manufacturers. This necessitated revisiting and optimisation of the assay system for this enzyme including determining whether the co-factor combination used for PP-2A was appropriate for rec PP-1. The activity of rec PP-1 was enhanced when $MnCl_2$ was used instead of $MgCl_2$ ($V_{max}$ 0.9 nmol ml$^{-1}$ min$^{-1}$ cf 0.5 nmol ml$^{-1}$ min$^{-1}$) and the reaction rate further improved by the addition of BSA ($V_{max}$ 1.2 nmol ml$^{-1}$ min$^{-1}$). These results mirror the earlier findings of Zhang et al. (1994) and Chu et al. (1996) that suggest that there are separate metal binding sites for the enzyme with Mn giving the most active conformation. Based on these results, $MnCl_2$ replaced $MgCl_2$ in the assay mixture and was used at 4 mM together with BSA at 0.25 mg ml$^{-1}$ for subsequent experiments with free rec PP-1 enzyme. The role of BSA in this optimisation is now fully elucidated, but our previous work has shown that elevated BSA reduced matrix effects in the PP-2A assay (Mountfort et al., 2003) and a similar effect may be valuable for PP-1 in solution.

### 3.2. Optimisation of immobilisation

The optimal amount of rec PP-1 and PP-2A required for immobilisation was determined by immobilising different quantities of enzyme onto 50 mg beads and measuring the activity once the assay was stabilised post immobilisation. The initial rapid reduction in enzyme activity over ~100 h is probably due to desorption of non-chemically bonded protein, since at longer times a stable enzyme activity is recorded. Based on these data, only ~25% of the initially adsorbed protein becomes attached to the beads, with the residual activity for PP-1 an order of magnitude lower than for the same number of units of PP-2A. For rec PP-1 and PP-2A, the levels of loaded enzyme producing satisfactory activities upon immobilisation were 12.5 and 0.5 units of designated activity per 50 mg of beads giving reaction rates

for methylumbelliferone (MUM) production of near 1 nmol ml$^{-1}$ min$^{-1}$ (Fig. 1). Enhancing the amount of enzyme did not result in proportional increases in activity. Consequently, the above amounts were used in subsequent experiments.

The optimal coupling buffer pH values were determined using standard coupling buffer over the range, pH 7 and 8. With both rec PP-1 and PP-2A increasing the pH of the coupling buffer only slightly enhanced the activity (by ≤5%).

The effects of various permutations of the coupling buffer were tested on the activity after stabilisation of immobilised of rec PP-1 and PP-2A (see Fig. 1). Table 1 demonstrates the effects of changing the composition of the immobilisation buffer. Within the two buffer groups, for both enzymes there was no significant difference detected with the various combinations (e.g. with or without KCl and/or ascorbic acid ($p > 0.05$)); one exception to this was the value for Tris–KCl in the Tris buffer series for PP-2A which was significantly different from the other combinations ($p < 0.05$). The coupling buffer conditions originally selected for immobilisation utilising phosphate buffer/KCl/ascorbate phosphate was therefore used in subsequent immobilisations for both enzymes.

The recoveries of the rec PP-1 and PP-2A immobilised enzyme activity after stabilisation, are shown in Table 2.

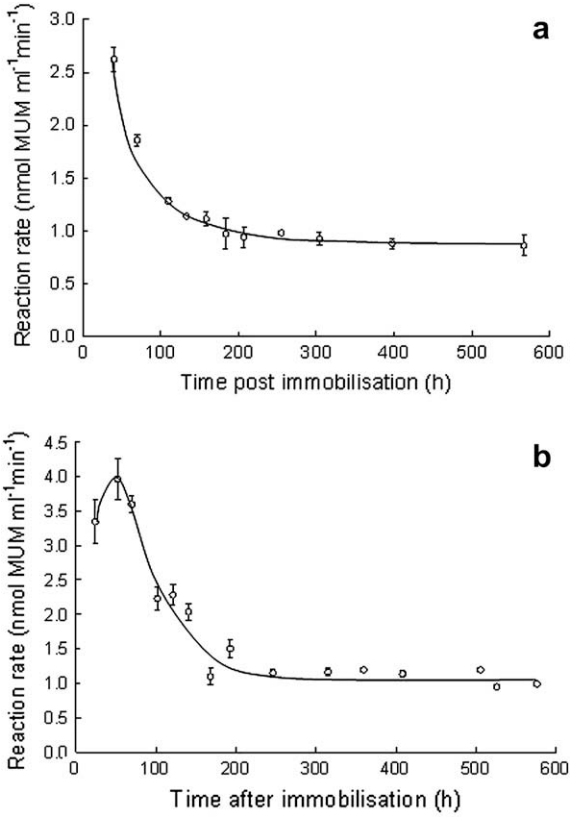


**Fig. 1.** Immobilised rec PP-1 (a) and PP-2A (b) activity versus time (post immobilisation) according to the standard protocol. The concentrations of loaded PP-2A and rec PP-1 in the microplate wells were 0.5 and 12.5 designated units respectively. Values are means of triplicate determinations ± 1SE.

**Table 1**
Effects of coupling buffer on the activity of immobilised enzyme

| Buffer matrix[a] | Activity rec PP-1 (nmol MUM ml$^{-1}$ min$^{-1}$)[b] | Activity PP-2A (nmol MUM ml$^{-1}$ min$^{-1}$)[c] |
|---|---|---|
| Na phosphate/KCl/ Ascorbate P | $0.94 \pm 0.1^{d}$ | $0.64 \pm 0.03^{f}$ |
| Na phosphate/ Ascorbate P | $0.83 \pm 0.1^{d}$ | $0.62 \pm 0.02^{f}$ |
| Na phosphate/KCl | $0.74 \pm 0.1^{d}$ | $0.58 \pm 0.08^{f}$ |
| Na phosphate | $0.80 \pm 0.1^{d}$ | $0.64 \pm 0.1^{f}$ |
| Tris/KCl/ Ascorbate P | $0.44 \pm 0.12^{e}$ | $0.14 \pm 0.01^{g}$ |
| Tris/Ascorbate P | $0.47 \pm 0.06^{e}$ | $0.16 \pm 0.02^{g}$ |
| Tris/KCl | $0.43 \pm 0.04^{e}$ | $0.09 \pm 0.02^{h}$ |
| Tris | $0.47 \pm 0.01^{e}$ | $0.18 \pm 0.03^{g}$ |

No statistical difference between values denoted within each superscript ($p > 0.05$). Values denoted by 'd' and 'e' are significantly different ($p < 0.05$).
No statistical differences between values within 'f' or 'g' ($p > 0.05$). Values are statistically different between those represented by different superscripts ($p < 0.05$).
[a] Buffer consisted of 50 mM phosphate or Tris buffer in combination with 0.05 M KCl and/or 0.2 mM ascorbate phosphate (see also Section 2.3).
[b] Determined after 570 h post immobilisation (see Fig. 1a). Values are means of duplicate determinations ± SE.
[c] Determined after 583 h post immobilisation (see Fig. 1b). Values are means of duplicate determinations ± SE.

For both rec PP-1 and PP-2A immobilised forms, the recoveries were 3–4%, of the free form when activity was expressed as equivalent to 100 mg beads added. Thus, most of the enzyme added was lost during the immobilisation and perhaps during the washing process as suggested by Fig. 1. Taking into account that Fig. 1 suggests a loss of at least 75% of the enzyme activity by simple desorption of non-covalently attached enzyme, during the first 100 h after immobilisation, the maximum residual activity represents a much higher retention of activity.

### 3.3. Selection of conditions for optimal activity of immobilised enzyme

A desirable feature in enzyme based biosensors is to have the assay matrix as simple as possible. Unnecessary co-factors add additional expense and unnecessary complexity to the assay system. Additionally, the requirements for the immobilised enzymes may change post immobilisation. Therefore the requirement for specific assay components was tested by omitting them from the assay mix with the immobilised enzyme.

The immobilised rec PP-1 and PP-2A enzyme activities are presented in Table 3. The results showed that removal of BSA from the assay mix resulted in a slight decrease in rec PP-1 and PP-2A activities whereas removal of metal ion co-factor decreased the activity substantially. The role of the metal ion-cofactor is consistent with the earlier discussion on the solution based enzyme performance, whereas the proposed matrix effect induced by BSA no longer appears to induce a beneficial effect for the immobilised enzyme and thus, in subsequent experiments with the biosensor system, BSA was not employed in the assay matrix.

To establish the optimum pH for immobilised rec PP-1 and PP-2A, initial assays were carried out at pH values

**Table 2**
Recovery of enzyme activity post immobilisation[a]

| Enzyme | Activity of enzyme (nmol MUM ml$^{-1}$ min$^{-1}$) | | Activity equivalent to that added to 100 mg beads (nmol MUM ml$^{-1}$ min$^{-1}$) | | % Recovery of enzyme |
|---|---|---|---|---|---|
| | Immobilised[b] | Free[c] | Immobilised[d] | Free[e] | |
| PP-2A | 0.45 ± 0.05 | 0.63 ± 0.02 | 0.99 ± 0.1 | 31.65 ± 1.05 | 3.15 |
| rec PP-1 | 1.52 ± 0.06 | 1.55 ± 0.05 | 3.03 ± 0.12 | 77.45 ± 2.55 | 3.61 |

[a] Determined under standard assay conditions for free and immobilised PP-1 and PP-2A. Values are means ± SE.
[b] Observed activity in assay system containing 0.5 and 12.5 U of PP-2A and rec PP-1 per 50 mg beads.
[c] Observed activity for free enzyme (0.02 and 0.5 U of PP-2A and rec PP-1 respectively).
[d] Activity amended for 100 mg beads.
[e] Activity predicted for amount of enzyme added to immobilisation system (1 and 25 specified units of PP-2A and rec PP-1 respectively per 100 mg beads (column 3 × 50)).

ranging from 5.5 to 9. For both enzymes, the optimum pH was 8. Therefore, in subsequent experiments the standard protocol was amended to this pH.

### 3.4. Inhibition of immobilised protein phosphatase by okadaic acid

Fig. 2a shows representative dose–response curves for OA inhibition of free and immobilised PP-2A ($n = 3$). Although the shape of the curves was similar, the curve for the immobilised system was shifted to the right giving an $IC_{50}$ value (12.5 nM), 5-fold higher than its free enzyme counterpart (Table 4). Additionally, the activity change for the immobilised enzyme occurred over 1 decade concentration range (10–100 nM OA) compared to 2 decades for the free enzyme (1–100 nM OA) (Hill plot slope, 0.8 [free enzyme]; 2.3 [immobilised]). The activity of free rec PP-1 increased in the range of OA concentrations 1–25 nM followed by a decrease at higher increases in toxin concentrations (Fig. 2b). When the enzyme was immobilised, the increase in activity over mid-range OA concentrations was diminished, and as in the case of PP-2A, was less sensitive to OA than the free enzyme. However, the immobilised PP-2A was far more sensitive to OA than rec PP-1 as determined by $IC_{50}$ (Table 4) and LoD (Table 5, Fig. 2). An investigation of PP-$2A_{na}$ immobilised by the same procedure as PP-2A indicated that like rec PP-1 an $IC_{50}$ for OA could not be achieved within the concentration range used (0–1000 nM OA): the maximal inhibition was only 20% of the control.

**Table 3**
Assessment of components for assay of immobilised enzyme[a]

| Enzyme | Omissions | Activity (nmol MUM ml$^{-1}$ min$^{-1}$)[b] |
|---|---|---|
| PP-2A | None | 0.64 ± 0.02[d] |
| | $NiCl_2$ | 0.21 ± 0.01[c] |
| | BSA and $NiCl_2$ | 0.28 ± 0.01[c] |
| | BSA | 0.60 ± 0.1[d] |
| rec PP-1 | None | 1.27 ± 0.12[f] |
| | $MnCl_2$ | 0.34 ± 0.03[e] |
| | BSA and $MnCl_2$ | 0.35 ± 0.07[e] |
| | BSA | 1.16 ± 0.13[f] |

Paired values for 'c' or 'd' not statistically different ($p > 0.05$) but 'c' and 'd' are statistically different ($p < 0.05$).
Paired values for 'e' or 'f' not statistically different ($p > 0.05$) but 'e' and 'f' are statistically different ($p < 0.05$).
[a] Assays were carried out under standard conditions for immobilised but with omissions as indicated.
[b] Values are means of triplicate determinations ± 1 SD.

### 3.5. Inhibition of immobilised protein phosphatase by microcystin LR (MC-LR)

Fig. 3a shows representative dose–response curves for immobilised PP-2A and the free enzyme ($n = 3$). The shapes of the two dose–response curves were similar but with the immobilised enzyme, there was a shift in the curve to the

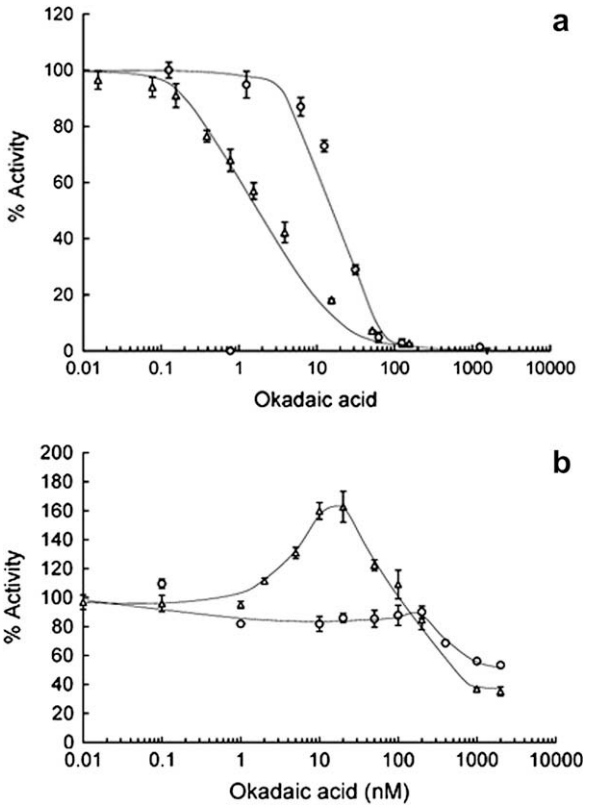


**Fig. 2.** Representative dose–response curves ($n = 3$) for free and immobilised PP-2A (a) and rec PP-1 (b) against OA. Symbols: ○, immobilised; △, free enzyme. Values are means of triplicate determinations ± SE. Limits of detection for immobilised enzymes (a) and (b) were 3 and >100 nM respectively.

**Table 4**
$IC_{50}$ values for inhibition of immobilised and free PP-2A and rec PP-1 by OA and MC-LR

| Enzyme | Enzyme status | $IC_{50}$ (nM) | |
|---|---|---|---|
| | | OA | MC-LR |
| PP-2A | Free | 2.5 (0.8)[a] | 3.5 (1.6)[b] |
| | Immobilised | 12.5 (2.3)[a] | 11.0 (1.3)[b] |
| Rec PP-1 | Free | 220 (0.74)[c] | 0.7 (1.8)[b] |
| | Immobilised | 2100 (0.68)[c] | 150 (0.6)[b] |
| $PP\text{-}2A_{na}$[d] | Free | 0.43 | ND[e] |
| | Immobilised | >1000 | ND[e] |

[a] Determined from Fig. 2a and confirmed with Hill plots (log $v/(V_{max}-v)$ versus log [OA]) (not shown). Values in brackets refer to the slope obtained from Hill plots.
[b] Determined from Fig. 3 and confirmed in Hill plots (log $v/(V_{max}-v)$ versus log [MC-LR]) not shown. Values in brackets refer to the slope (Hill plots).
[c] Determined from Hill plots (log $v/(V_{max}-v)$ versus log [OA]).
[d] PP-2A from *Neptunea arthritic*.
[e] ND, not determined.

right indicating decreased sensitivity ($IC_{50}$ MC-LR, 11 nM cf 3.5 nM for free enzyme [Table 4]). The LoD for immobilised PP-2A against MC-LR was 1 nM (equivalent to 1 $\mu g\,l^{-1}$) (Table 5). The $IC_{50}$ for MC-LR for the free rec PP-1 enzyme was lower than for PP-2A ($IC_{50}$, 0.7 nM), but when the rec PP-1 enzyme was immobilised there was a substantial decrease in its sensitivity by over 200-fold (Fig. 3b, Table 4). Because immobilised $PP\text{-}2A_{na}$ showed little or no sensitivity to OA (Section 3.4) this enzyme was not tested against MC-LR.

### 3.6. Comparisons of free and immobilised PP-2A affinity towards MUP

One possibility for the differences in $IC_{50}$ observed between the free and immobilised forms of PP towards OA and MC-LR (see Table 4) was that when the enzymes were immobilised, a diffusion barrier was created to this compounds by the bead matrix. To test this possibility the affinity of free and immobilised PP-2A towards the reaction substrate, MUP was examined (Fig. 4). The results showed that the Km for both immobilised and free PP-2A determined by Hanes plots was 0.05 mM.

### 3.7. Recovery of enzyme activity after toxin challenge

Immobilised PP-2A was tested for re-usability after treatment with OA. This was carried out by the addition of sufficient OA to produce >50% inhibition, washing the immobilised enzyme preparation with washing buffer, 1 M NaCl in buffer followed by buffer, or by repeated washing sequences with buffer containing 1 M NaCl. The results (Fig. 5) show that by increasing the number of wash sequences, a small but noticeable increase in activity occurred. However, with buffer containing NaCl, though there was a small increase in activity initially after washing, further washing produced a decrease in activity which was only restored when washed with buffer only (final wash sequence). A similar effect was seen when MC-LR was substituted for OA. These data show that salt does not improve the activity with washing, but may be "salting in" the inhibitor via changes in electrostatic interactions linked to conformational change in the protein structure as has been observed in related enzymes (Tougu & Kesvatera, 2001). The inability of any of the buffer systems tested to release inhibition by either OA or MC-LR is consistent with earlier findings that OA and MC-LR bind tightly by non-competitive inhibition to protein phosphatase (Takai & Mieskes, 1991; Takai et al., 1995).

### 3.8. Determination of MC-LR and OA in spiked samples

Known quantities of MC-LR and OA added to standard buffer made up with freshwater or seawater were determined from Sigma plots generated from the same data as used in the curves in Figs. 2a and 3a for immobilised PP-2A. Table 6 shows that the recoveries of the toxins were ≥96%.

## 4. Conclusions

The present work is the first to evaluate different immobilised protein phosphatases for biosensor application in the detection of both MC-LR and OA. Of the protein phosphatases studied, PP-2A is the most suitable of the three enzymes tested for biosensor development, mainly because of its sensitivity to the two toxin classes, OA and

**Table 5**
LoD for immobilised PP-2A against MC-LR and OA and comparisons with published values for sensors

| Biosensor system | LoD (MC-LR) (nM or $\mu g\,l^{-1}$) | LoD (OA) (nM) | LoD (OA) ($\mu g\,l^{-1}$) | Reference |
|---|---|---|---|---|
| PP-2A | 1[a] | 3[a] | 3.5[a] | This paper |
| PP-2A | 3 | ND[b] | ND | Campas et al., 2005[c] |
| Screen printed electrode immunosensor | ND | 1.9 (6)[d] | 2.3 (7.2)[d] | Kreuzer et al., 2002 |
| Acid phosphatase bioelectrode | ND | 6[e] | 7.2[e] | Croci et al., 2001 |
| Quartz crystal microbalance immunosensor | ND | >100 | >120 | Tang et al., 2002 |
| Semi-automated membrane chemiluminescence | ND | 2[f] | 2.4[f] | Marquette et al., 1999 |

[a] LoD was determined as the mean of the control (0 nM) – (3 times the SD of the control).
[b] ND, not determined.
[c] Polyvinylchloride – SbQ immobilised enzyme on bottom of Maxisorp microtitre plate wells.
[d] Value in bracket represents the LoD re-calculated based on the average SD of the test samples.
[e] No calculations of LoD presented but determined from a sensitivity value of 0.002 mg $g^{-1}$ hepatopancreas.
[f] Based on equivalence of 0.4 $\mu g\,ml^{-1}$ OA to 40 $\mu g$ OA per 100 g mussel tissue and a LoD of 0.2 $\mu g$ OA per 100 g mussel tissue.

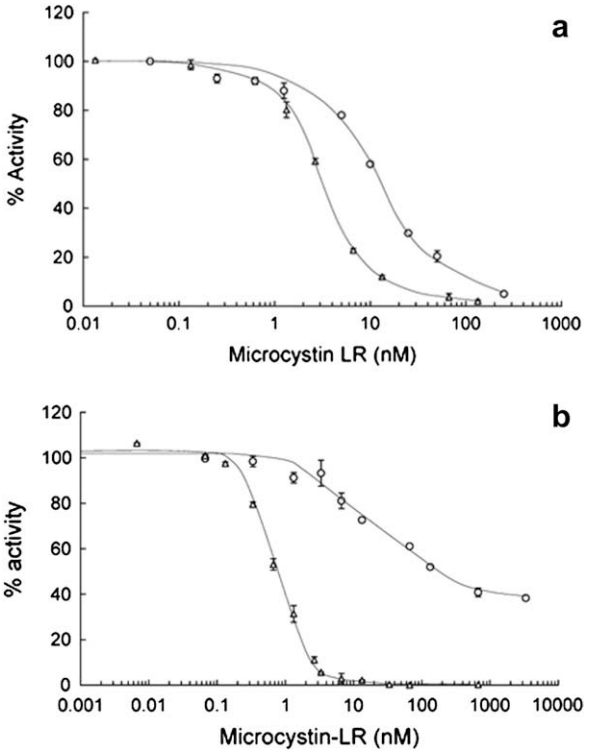


**Fig. 3.** Representative dose–response curves ($n = 3$) for free and immobilised PP-2A (a) and rec PP-1 (b) against MC-LR. Symbols: ○, immobilised; △, free enzyme. Values are means of triplicate determinations ± SE. Limits of detection for immobilised enzymes (a) and (b) were 1 and 2 nM respectively.

MC. The advances in fully evaluating the performance of the enzymes were made possible through the use of a rapid multiple format testing platform, namely the Millipore multiscreen filterplate. Although the detection limit of the immobilised system applied to MC-LR meets the WHO regulatory limit of 1 $\mu g\,l^{-1}$ for drinking water, the sensitivity limit of 3 nM (3.5 $\mu g\,l^{-1}$) towards OA was less clear, since there is no regulatory limit for DSP toxins in the field. Blooms of DSP-producing dinoflagellates can reach levels of

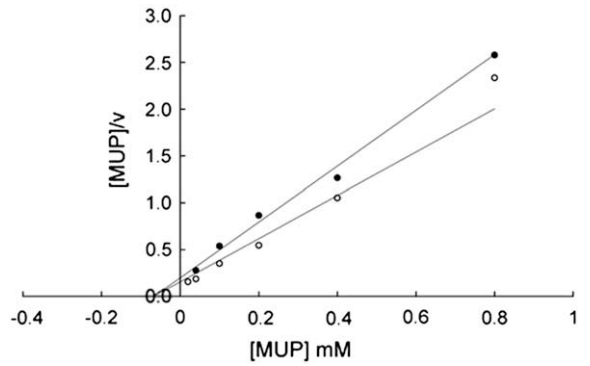


**Fig. 4.** Hane plots of [MUP]/v versus [MUP] for PP-2A in which units for v are nmol $ml^{-1}\,min^{-1}$. Symbols: ○, free enzyme; ●, immobilised. Values are means of at least duplicate determinations.

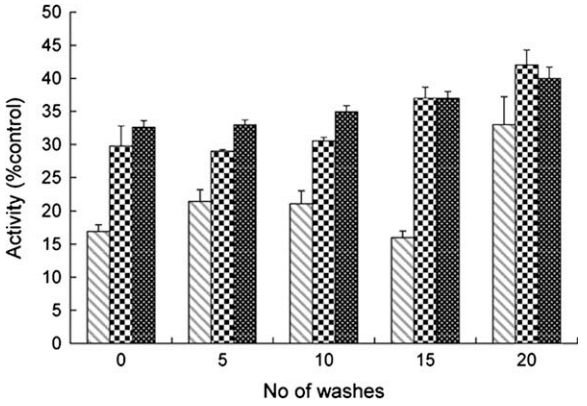


**Fig. 5.** Recovery of enzyme activity versus number of washes after treatment of PP-2A with OA. Bars: speckled with black background, with washing buffer only; chequered, single washing with buffer + 1 M NaCl followed by washings with buffer only; cross-hatched buffer + 1 M NaCl but last wash sequence switched to buffer only. Values are means of duplicate determinations ± SE.

30,000 organisms $l^{-1}$ (Gladstone, 2003) and total DSP can reach as high as 200 pg $cell^{-1}$ (Suzuki et al., 1997) which is equivalent to about 6 $\mu g\,l^{-1}$. However, approximately 20% of toxins are usually released from the cell. Therefore, although the assay in its present format could detect DSP toxin in peak blooms, it is unlikely to have the sensitivity to detect toxins that would accompany the onset of a bloom. In such a case, a sensitivity of around 0.1 $\mu g\,l^{-1}$ would be required.

The sensitivity of our PP-2A system is comparable to other sensors developed for detection of OA, and represents an improvement over others for MC detection (see Table 5). Immuno type sensors have been developed for detection of OA with LoD's in the range 2.4 $\mu g\,l^{-1}$ (Marquette et al., 1999) to >120 $\mu g\,l^{-1}$ (Tang et al., 2002). An immunosensor developed by Kreuzer et al. (2002) using a disposable screen printed carbon electrode coupled with amperometric detection of *p*-aminophenol, was claimed to have an LoD of 2.3 $\mu g\,l^{-1}$ but review of their data suggests at best it to be nearer to 7 $\mu g\,l^{-1}$. Also, there was no information to indicate whether or not their sensor was OA specific, and no data points were presented for $B/B_o$ ([test value/control]

**Table 6**
Application of immobilised PP-2A sensor for the detection of MC-LR and OA in "spiked" samples

| Assay system | Concentration of added toxin ($\mu g\,l^{-1}$) in assay system | Toxin recovered in assay system ($\mu g\,l^{-1}$)[a] | % Recovery |
|---|---|---|---|
| Standard buffer (SB) | | <0.1 | |
| SB buffered seawater (SBSW) | | <0.1 | |
| SB + MC-LR | 5.5 | 5.3 ± 0.1 | 96 |
| SB + MC-LR | 11.0 | 10.8 ± 0.2 | 98 |
| SBSW + OA | 6.0 | 6.5 ± 0.1 | 108 |
| SBSW + OA | 12.0 | 11.5 ± 0.1 | 96 |

Assays using only SB and SBSW were carried out using standard assay buffer (amended to pH 8) made up with freshwater (MC-LR) or seawater (OA).

[a] Values are means of triplicate determinations ± SE.

100) below 50%. Non-immunological sensors such as the enzyme inhibition bioelectrode developed by Croci et al. (2001) are based on inhibition of acid phosphatase linked to glucose oxidase in a complex reaction producing hydrogen peroxide. However, this complex system had a LoD of near 7 $\mu g\,l^{-1}$. Campas et al. (2005) demonstrated using various matrices for immobilisation that good sensitivities of PP-2A to MC-LR and MC-RR could be achieved, but transferring the assay matrices to a microwell plate for reaction product analysis and the use of fixed point determinations posed experimental limitations in addition to difficulties in interpretation and reproducibility of their results. In the method reported here, these constraints were overcome by conducting product analysis in the reaction matrix and by determining the reaction rate by real time kinetics using fluorescence measurement as well as comparing the performance of the immobilised and free forms of PP-2A. As a result, our technical modifications contributed mostly to good reproducibility and improved assay sensitivity.

The biosensor pro format, as reported here, is also quite stable over time with only about 5% loss in activity after 3 months of storage at 4 °C, suggesting that the immobilised PP-2A enzyme system would meet the required robustness needed for incorporation into a regulatory biosensor. The performance of the assay in terms of accuracy was demonstrated from experiments in which freshwater and seawater were spiked with MC-LR or OA respectively. The immobilised assay format is simple and includes only a metal ion co-factor and MUP substrate.

The losses in sensitivity of PP-2A to MC-LR and OA upon immobilisation were 4 to 5-fold. This compares favourably to losses obtained by other workers for this enzyme of 8–16 fold against MC-LR using immobilised matrices such as sol–gel entrapment, and immobilisation with glutaraldehyde or photocrosslinkable poly (vinyl alcohol) bearing styrylpyridinium groups (PVA-SbQ) (e.g. Campas et al., 2005). Our experiments were designed so that immobilised enzyme gave similar assay activities to the free enzyme. Since both OA and MC-LR bind to the same binding site of protein phosphatase (e.g. Holmes et al., 2002) and have comparable $IC_{50}$ values with free or immobilised enzyme PP-2A, it was possible that changes in binding affinity upon immobilisation might explain the decreased sensitivity. The similarities in the apparent Km observed between the free and immobilised enzyme for MUP suggests that a diffusion barrier is unlikely to be the reason for somewhat lower sensitivities to the inhibitors observed for the immobilised preparation. Additionally, although an attempt was made to keep the assay activities of the immobilised system similar to the free enzyme, in the event of small differences occurring, this would be unlikely to be the cause of a consistent decrease in sensitivities observed for both MC-LR and OA as our previous studies showed that the $IC_{50}$ is unaffected by changes in immobilised [PP-2A] over 10-fold concentration range (Mountfort et al., 1999). Taken together, these data suggest that somewhat decreased sensitivities of immobilised PP-2A to OA and MC-LR are consistent with a shift in inhibitor binding site conformation and inhibitor binding affinity.

The relatively large decreases in sensitivity observed for immobilised rec PP-1 and PP-$2A_{na}$ towards the inhibitors and the differences in sensitivities between OA and MC-LR in the case of rec PP-1 appear to be linked to decreased slopes of Hill plots (Table 4) suggesting absence of co-operativity between inhibitor binding sites to be the most likely explanation. The slight enhancement of rec PP-1 activities at lower concentrations of OA (e.g. 1–25 nM) is unexpected and does not have any obvious explanation.

A key requirement for translating an immobilisation system to a re-usable biosensor format such as the "fill flow" sensor described by Gooding and Hall (1998) or the prototype alkaline phosphatase fill flow sensor described by Hatz and Hall (2006), is the ability for re-use of the immobilised component. Our results showed that using a variety of washing conditions, does not return activity to near initial levels before the addition of toxin, though no decline in activity was observed after repeated washing of controls never exposed to toxin. This suggests potential for re-use in the event of a negative result, however the potential for re-use after a positive is recorded is limited. Therefore should translation to biosensor format be considered, it would need to be in a device in which either, the biological component is disposable, or the whole device is disposable.

A protein phosphatase based biosensor has the advantage that specificity of protein phosphatases towards MC and DSP is already known (Takai et al., 1987; Bialojan and Takai, 1988; MacKintosh et al., 1990; Matsushima et al., 1990). However, because the assay is unlikely to detect DSP toxins during the onset of a bloom, further improvements to sensitivity will be required, prior to any prototype biosensor being developed. We suggest that priority should now be given to understanding the causes for sensitivity loss of protein phosphatase to inhibitors upon immobilisation. Such decreases were substantial particularly for the more sensitive rec PP-1 and PP-$2A_{na}$ systems. With the availability of new generation highly sensitive protein phosphatases (Ikehara et al., 2008), the focus of new research should be on translating such enzymes into biosensor systems that would achieve sensitivities that would either enable detection of MC's below the WHO regulatory limit, or detection of DSP toxins at the onset of phytoplankton blooms. Based on the current study, one of the key towards achieving this goal would be selection of an immobilisation matrix as well as procedure that would minimize conformational changes in structure including subunit interactions that lead to decreased affinities towards these inhibitors.

## Acknowledgements

We gratefully acknowledge technical support initiating this project at the Institute of Biotechnology, University of Cambridge. This work was supported by the New Zealand Foundation for Research, Science and Technology under the New Economic Research Fund (NERF) contract number, LVL X0205, and a grant from the Engineering and Physical Sciences Research Fund in the UK.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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