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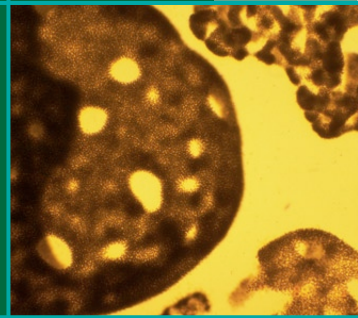
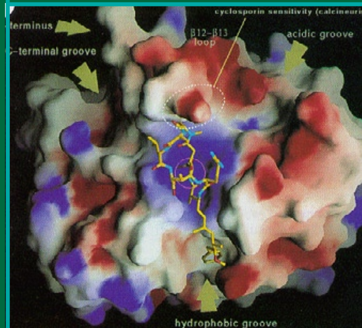
Determination of Cyanobacterial Microcystins and Nodularin by a Colorimetric Protein Phosphatase Inhibition Assay using Phosvitin as Substrate





Research Report 1

Research Report

Determination of Cyanobacterial Microcystins and Nodularin by a Colorimetric Protein Phosphatase Inhibition Assay using Phosvitin as Substrate

Tamila Heresztyn and Brenton C. Nicholson

Cooperative Research Centre for Water Quality and Treatment

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FOREWORD

Determination of Cyanobacterial Microcystins and Nodularin by a Colorimetric Protein Phosphatase Inhibition Assay Using Phosvitin as Substrate

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Research Node: Australian Water Quality Centre

CRC for Water Quality and Treatment Project No. 2.3.1.1 - Development of Analytical Methods for Rapid Detection of Toxins in Water

EXECUTIVE SUMMARY

A colorimetric protein phosphatase inhibition assay based on the dephosphorylation of phosvitin by recombinant protein phosphatase 1 was developed for analysis of waters for cyanobacterial hepatotoxins. The phosphate released in the assay was determined using a malachite green reagent. Good agreement with toxin concentrations determined by HPLC was obtained. The assay was optimised and was capable of determining these toxins at concentrations around 1 ug/L with high precision and without sample concentration. This is of considerable benefit as the World Health Organisation specifies a provisional guideline of 1 ug/L for microcystin-LR. There was evidence, however, that the sample matrix might affect quantification and further work is required to resolve this question if phosphatase inhibition assays are used directly for measuring toxin levels in water, especially if this information is used to check compliance with water quality guidelines.

The inhibition assay using phosvitin as a substrate was found to be of comparable sensitivity to other assays using PP1, even radiolabelled assays. The inhibition assay using *p*-nitrophenyl phosphate as substrate may have some advantages over the phosvitin assay in that it is of comparable sensitivity but requires one less step. As it may have some advantages, it should be pursued as the method of choice. However, it is important to determine whether the matrix effects observed with phosvitin also apply to this substrate.

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1. INTRODUCTION

1.1 Project objectives

Protein phosphatase inhibition assays are an important and useful indicator of cyanobacterial toxicity as they are a rapid alternative to the mouse bioassay, and can provide a more accurate estimation of toxicity than High Performance Liquid Chromatography (HPLC) which measures concentration of toxic and non-toxic hepatotoxins. Assays currently available for determining hepatotoxins based on inhibition of protein phosphatase activity include using ^{32}P radiolabelled phosphorylated protein substrates (Honkanen et al. 1990, 1994, MacKintosh et al. 1990, Sim and Mudge 1993) and a colorimetric assay based on measurement of para-nitrophenol (An and Carmichael 1994, Ash et al. 1995, Ward et al. 1997). A drawback of radioactive assays is that ^{32}P isotopes only have a half-life of about 14 days. Therefore the labelled substrate protein for the assay has to be prepared regularly, the laboratory has to be set up to carry out radioactive determinations, and the substrate proteins have to be prepared by fairly complex biochemical extraction and purification procedures as they are not commercially available.

Early work on colorimetric alkaline and protein phosphatase assays has been described by Baykov et al. (1988), Geladopolous et al. (1991), and Takai and Mieskes (1991). Colorimetric determination of phosphate released from a substrate by protein phosphatases can be used to measure inhibitors such as microcystins. An assay based on this feature would be simpler and cheaper than the radioactive assay and also has more potential for development as a field kit.

The initial aim of this part of Project 2.3.1.1 was to assess a colorimetric phosphatase inhibition assay for hepatotoxins developed by Wheldrake et al. (1996) based on the above criteria. Initial attempts to implement this assay at the Australian Water Quality Centre were unsuccessful with commercially available protein phosphatase Type 1. Several problems had to be overcome in order to obtain enzyme activity toward the substrate. This study therefore encompassed the following objectives:

- Assess the production of colour by the malachite green reagent with inorganic phosphate to determine that this part of the assay worked.
- Develop a buffer system which would allow commercially available PP1 to dephosphorylate the chosen substrate, phosvitin.
- Optimise the buffer with respect to pH and component concentration to maximise enzyme activity towards the substrate, thereby making the assay less expensive.
- Examine the inhibition of PP1 by microcystin with the optimized enzyme assay.
- Quantify microcystin in a range of water and algal samples and compare the values obtained principally with High Performance Liquid Chromatography (HPLC) but also with the protein phosphatase inhibition assay using para-nitrophenol phosphate as substrate, Capillary Electrophoresis (CE) and Enzyme Linked Immunosorbent Assay (ELISA) as part of the collaboration between Australian Water Quality Centre (Adelaide) and AWT EnSight (Sydney).

1.2 Background to protein phosphatase enzymes

Microcystins and nodularin were first reported to inhibit protein phosphatases by Fujiki and coworkers at the 1988 Gordon Conference on Marine Natural Products. Since then, various workers (MacKintosh et al. 1990, Yoshizawa et al. 1990, Honkanen et al. 1990 and 1994, Eriksson et al. 1990, An and Carmichael 1994) have studied the inhibition of serine/threonine phosphatases by microcystins and nodularin (Figure 1). This class of enzymes is ubiquitous in nature and is responsible for dephosphorylation of intracellular proteins. They are so named because they dephosphorylate the serine and threonine residues in proteins in both the cytoplasmic and nuclear compartments of eukaryotic cells (MacKintosh 1993). Protein phosphorylation, carried out by protein kinases, regulates many metabolic and cell functions. The phosphatases are important in allowing these reactions to be reversible (Lee, 1995). One of the best-studied phosphorylation cycles occurs in the metabolism of glycogen in skeletal muscle and liver.

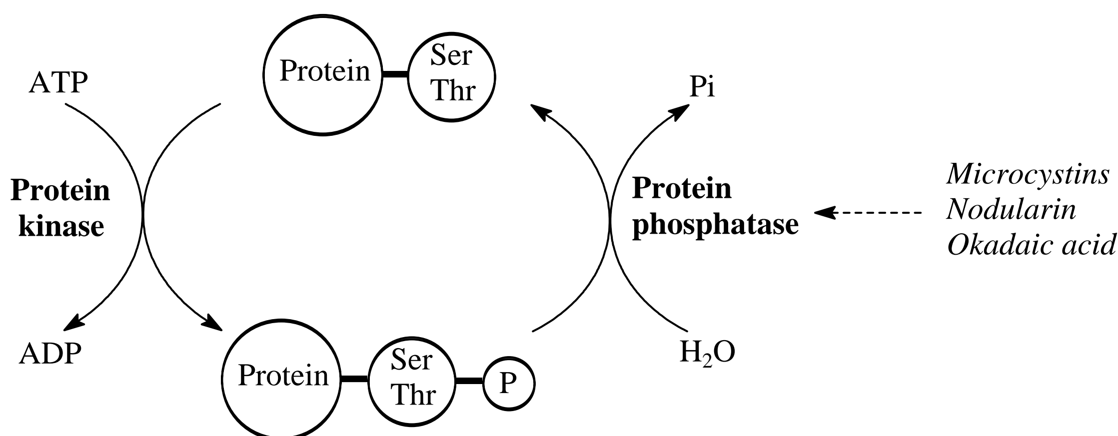


Figure 1. Phosphorylation and dephosphorylation of intracellular phosphoproteins

The protein phosphatases most inhibited by microcystins are Type 1 and Type 2A. These enzymes can be classified into 2 groups, Type 1 (PP1) and Type 2 (PP2A, PP2B, PP2C). *Some* of the properties of these enzymes which allow them to be distinguished biochemically are outlined in Table 1. A more comprehensive summary is detailed by MacKintosh (1993).

Carmichael reports the requirement of Mg^{++} for optimum PP2A activity (personal communication). The toxin okadaic acid, found in marine dinoflagellates and a major cause of diarrhetic shellfish poisoning, also inhibits PP1 and PP2A.

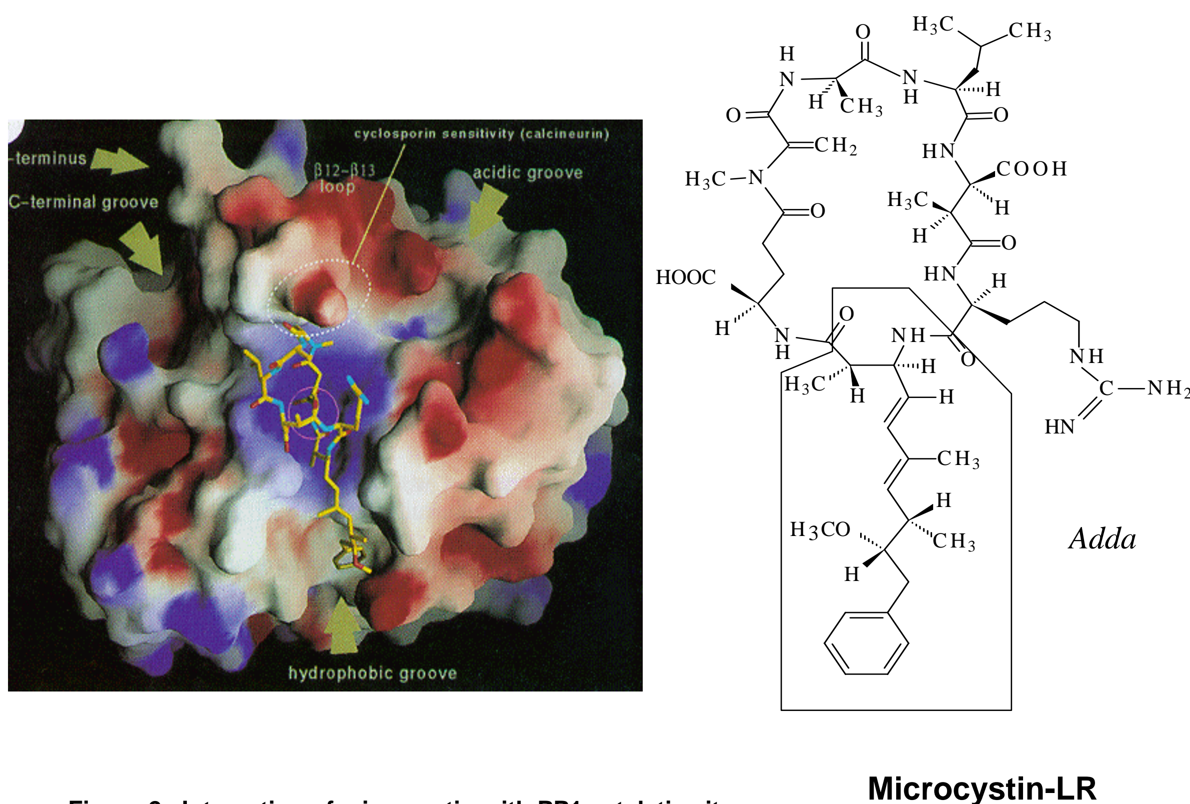
Table 1. Biochemical characteristics of eukaryotic protein phosphatases (MacKintosh1993)

PROPERTY	PP1	PP2A	PP2B	PP2C
Inhibition by microcystin-LR	Yes	Yes	Weak	No
Inhibition by okadaic acid	Yes (IC_{50}=10-20nM)	Yes (IC_{50}=0.1nM)	Weak	No
Inhibition by Inhibitor 1 and	Yes	No	No	No
Absolute requirement for	No*	No*	Yes (Ca^{++})	Yes (Mg^{++})
Phosphorylase phosphatase	High	High	Very low	Very low

*These enzymes can become Mn^{++} dependent.

PP1 is a 37 kDa protein which exists in several isoforms. The catalytic subunit (the α-isoform) is complexed to regulatory proteins (inhibitor-1 and inhibitor-2) to form a larger holoenzyme *in vivo* which targets such subcellular locations as glycogen particles and myofibrils in skeletal muscle. Hence, rabbit muscle has been one source for the extraction of these enzymes. The holoenzyme is the inactive form of protein phosphatase and the complexing of the catalytic subunit with inhibitor-1 and inhibitor-2 is the regulatory mechanism of this enzyme (Lee 1995). The enzyme is activated when inhibitor-1 and inhibitor-2 are cleaved from the holoenzyme to release the catalytic subunit.

Zhang et al. (1992) first successfully expressed the catalytic subunit of a mammalian protein phosphatase 1 in *Escherichia coli* in an active form. They used the gene coding sequence for the enzyme in rabbit muscle and incorporated it into a vector in *E. coli* which led to expression of the enzyme in the bacterial cells. They found this recombinant enzyme had an almost absolute requirement for Mn^{++}, otherwise activity was very low, (<3%). In other respects its enzymatic properties were comparable with those of PP1 prepared from rabbit skeletal muscle i.e. it was active towards phosphorylase a and was inhibited by microcystin-LR (mLR), okadaic acid and inhibitor-2.



Microcystin-LR

Figure 2. Interaction of microcystin with PP1 catalytic site

Figure 2 illustrates the molecular surface of the PP1 enzyme's catalytic site (Goldberg et al. 1995). The microcystin molecule binds to this unique part of the enzyme blocking the entry of a phosphorylated substrate. The Adda group fits into a hydrophobic pocket next to the catalytic site. Two of the carboxylic acid groups in the peptide ring of the toxin molecule (γ-Glu and Masp) can hydrogen bond with amino acid residues in the catalytic site and covalent bonding occurs with the carbon of the CH_3 group of methyldehydroalanine although this bond is not essential for inhibition.

Goldberg et al. (1995) detail a model of the binding of phosphate from a substrate molecule to the catalytic site of the enzyme. The phosphate group binds to the same area as the peptide ring in the hepatotoxin molecule. Even though the Adda group is situated adjacent to this site, its configuration is important for the toxicity of the molecule. Microcystin-LR (mLR) and nodularin containing the (Z) form of the Adda group at the C-6 double bond are not toxic to mice and were found to show no inhibition of recombinant PP1 by An and Carmichael (1994). Goldberg et al. (1995) describe the position of the Adda group in the hydrophobic pocket as a "snug fit". Taylor et al. (1996) synthesized several linear and cyclic mLR analogues. They found PP2A inhibition by these analogues was at least 10^6 times lower than for mLR which they believed to be due to the absence of the hydrophobic Adda group. However, the fact there was some inhibitory activity to PP2A suggested the cyclic amino acid ring also plays an important role in the interaction of microcystins with the catalytic subunit of PP2A. Nishiwaki-Matsushima et al. (1991) also demonstrated that the Adda group of mLR and mRR is important in the interaction of these toxins with the binding site of PP2A. Their 6(Z)-Adda forms were found to inhibit PP2A about 100 times less strongly than 6(E)-Adda form.

The levels of methylation of various functional groups in the toxin molecule also influence its toxicity. An and Carmichael (1994) found the methyl ester on the glutamic acid residue of mLR at concentrations up to 1 μM did not inhibit PP1, further emphasizing the role its carboxylic acid group plays in binding to the catalytic site of the enzyme. They also found that [$DMAdda^5$] mLR and [$DMAdda^3$] nodularin still inhibited PP1 and are toxic to mice. DMAdda (desmethyl Adda) refers to the Adda side chain with a methyl group missing.

Fujiki et al. (1996) report that mLR and okadaic acid do not inhibit protein tyrosine phosphatase 1. Goldberg et al. (1995) comment that the protein tyrosine phosphatases have different amino acid sequences from the serine/threonine phosphatases. By preincubating mLR, mYR, mRR and nodularin with cytosolic fractions of mouse liver, Fujiki et al. (1996) were able to inhibit the binding of okadaic

acid. This suggests that microcystins and nodularin bind to same receptors on PP1 and PP2A as okadaic acid, even though they do not bind to exactly the same site in the catalytic subunit.

1.3 Colorimetric assay developed by Wheldrake et al. (1996)

The essence of the assay developed by Wheldrake et al. (1996) is summarised in Figure 3.

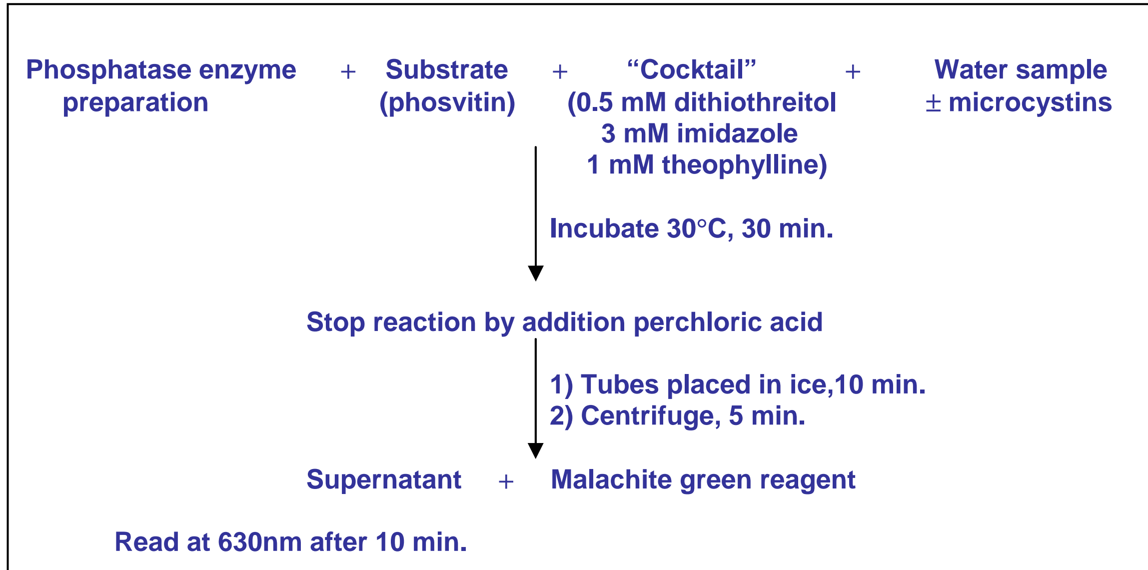


Figure 3. Non-radioactive protein phosphatase inhibition assay developed by Wheldrake et al. (1996)

The assay is carried out in two stages. The first part involves the enzymatic hydrolysis of the phosvitin substrate, followed by the measurement of liberated inorganic phosphate. If PP1 inhibitors are present in the sample, the activity of the phosphatase enzyme is reduced, resulting in less inorganic phosphate being liberated. Phosvitin refers to the most highly phosphorylated protein fraction of egg yolk and represents about 7% of egg yolk protein, so it is not a pure protein (Taborsky, 1974). In hen egg yolk it has a molecular weight of ~35,500. It is very hydrophilic and quite heat resistant. 54% of its amino acid residues are serine and it consists of about 10% phosphorous; probably all of the phosphoryl groups are esterified as phosphoserine residues. Figure 4 is a schematic representation of the assay.

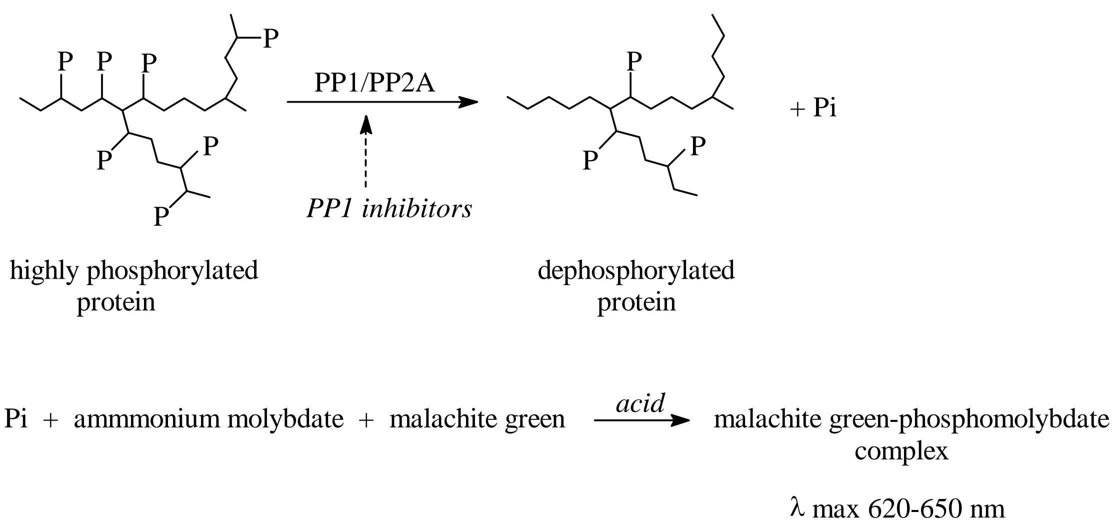


Figure 4. Schematic diagram of the principle behind the assay developed by Wheldrake et al. (1996)

The measurement of inorganic phosphate (Pi) has been studied by various groups (Itaya and Ui 1966, Van Veldhoven and Mannaerts 1987) based on the principle that malachite green at lower pH forms a complex with phosphomolybdate resulting in a shift in the absorption maximum. Van Veldhoven and Mannaerts (1987) calculated a molar absorption coefficient (ε) of 110,620 ±1430 $M^{-1}cm^{-1}$ for the malachite green complex and found this method superior to other colorimetric assays on the basis of sensitivity and ease of use. They found rhodamine B was even more sensitive (ε=330,000 $M^{-1}cm^{-1}$), however the method was more tedious to use.

Baykov et al. (1988) and Geladopoulos et al. (1991) used the detection of phosphate with malachite green as a basis for the determination of Pi enzymatically released from various substrates. Baykov et al. (1988) used *p*-nitrophenyl phosphate (*p*NPP) and pyrophosphate as substrates. They found the absorption coefficient of *p*-nitrophenol (ε=15,000 $M^{-1}cm^{-1}$) at 400 nm about six times lower than that for the malachite green-phosphomolybdate complex. Absorbance values obtained by measuring Pi release from either substrate were higher (four times) than by measuring *p*NPP. They felt the sensitivity of the phosphatase assay based on measuring Pi approached that of the fluorogenic assay using methylumbelliferyl phosphate. Their assay did have a drawback, however, in that it could not continuously monitor the hydrolytic reaction if they so wished. They found pyrophosphate much more stable and cheaper than *p*NPP which spontaneously hydrolyses. The malachite green method also produces an easily perceptible colour change which they felt was more useful in developing an analysis not requiring spectrophotometric measurement. Following on from this work, Geladopoulos et al. (1991) improved the method slightly by increasing the acidity of the dye solution and combining all reagents in one concentrated solution. They found the determination of phosphorylase phosphatase activity in bovine brain extracts, with phosvitin as substrate, to be comparable with the radioisotopic phosphatase assay using ^{32}P-labelled phosphorylase a. The marginally lower sensitivity of the colorimetric assay was offset by its being cheaper and easier to set up. This is the basis on which Wheldrake et al. (1996) developed their procedure.

1.4 Outline of other protein phosphatase assays

Colorimetric assay using p-nitrophenyl phosphate as substrate

Apart from the radiolabelled phosphatase assay which will be discussed in the next section, the most commonly used method of detecting phosphatase activity is by the conversion of *p*-nitrophenyl phosphate (*p*NPP) to *p*-nitrophenol which shows an absorbance maximum at 405 nm (Figure 5).

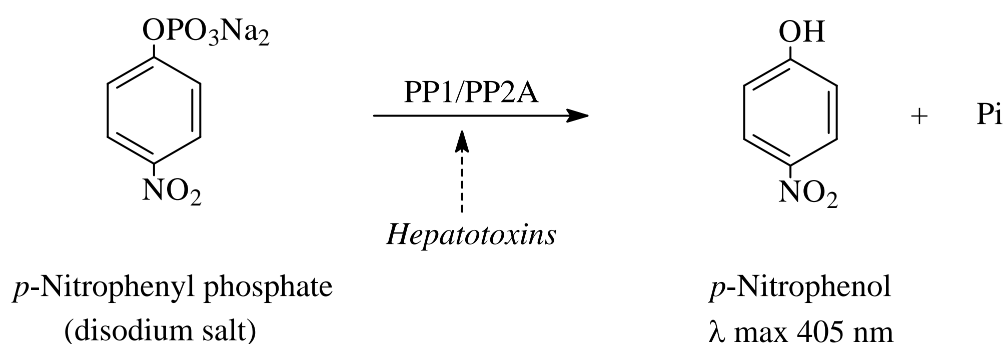


Figure 5. Hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol by protein phosphatase and its inhibition by hepatotoxins

This assay is commonly used in the field of Clinical Chemistry to determine alkaline phosphatase in human serum (Sigma Chemical Company). As already mentioned in Section 1.3, Baykov et al. (1988) used *p*NPP as a substrate but decided a more sensitive assay could be developed by measuring Pi release. Takai and Mieskes (1991) studied the phosphatase activity of purified extracts of PP1, PP2A and PP2C towards *p*NPP, rather than the crude mixtures of alkaline phosphatase used by Baykov et al. (1988). Although their interest centred on the inhibitory effect of okadaic acid on these phosphatases, they found PP2A had a very high level of activity towards *p*NPP compared with PP1

and PP2C. This work provided the basis for the assay An and Carmichael (1994) developed in which they examined the inhibitory effects of 5 microcystins and 2 nodularins on recombinant PP1 activity towards *p*NPP. They measured the rate of colour production by conversion of *p*NPP to *p*-nitrophenol at 37°C in microtitre plates. AWT EnSight in Sydney have adopted this assay as part of the collaboration with AWQC to evaluate HPLC, phosphatase, ELISA and CE for the detection and quantitation of hepatotoxins.

Radioactive inhibition assay for hepatotoxins using radiolabelled glycogen-metabolising enzymes as substrates

Glycogen metabolism has been one of the most extensively studied biochemical systems and provides useful substrates for studying protein phosphatases and their inhibition. MacKintosh (1993) details procedures in which the skeletal muscle glycogen-metabolising enzymes phosphorylase a and phosphorylase kinase are used as protein phosphatase substrates. Sim and Mudge (1993) have adopted the phosphorylase a procedure in which ^{32}P-labelled ATP is used to prepare ^{32}P-labelled glycogen phosphorylase a with phosphorylase kinase. The ^{32}P-labelled glycogen phosphorylase a is then used as a substrate for determining protein phosphatase activity with ^{32}Pi measured in a liquid scintillation counter (Figure 6). They used crude chicken forebrain extracts as their source of PP1 and PP2A to study the effects of endogenous phosphatases in cyanobacteria. If microcystins were present, then less ^{32}Pi was measured, which is the same principle as some of the colorimetric assays. However, when endogenous phosphatases were present, as found in their study, more ^{32}Pi was formed, resulting in an under-estimation of microcystin concentration. ^{32}P-labelled phosphorylase kinase can also be used as a substrate for PP1, PP2A, PP2B, and PP2C (MacKintosh, 1993) however, inhibition constants for hepatotoxins with this substrate have yet to be reported.

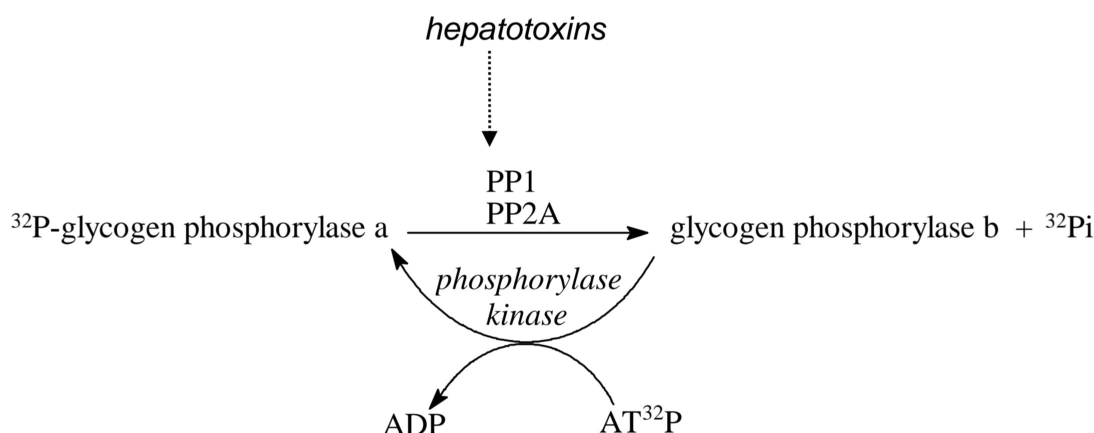


Figure 6. Inhibition of protein phosphatases catalysing dephosphorylation of ^{32}P-labelled glycogen phosphorylase a

Non-radioactive assay for PP1 and PP2A using 4-methylumbelliferyl phosphate

Figure 7 details a fluorescent inhibition assay based on the hydrolysis of 4-methyl-umbelliferyl phosphate to its fluorescent product 4-methylumbelliferone. To date this method has been applied for the determination of okadaic acid (Shimizu et al. 1997, Vieytes et al. 1997). However, as microcystins inhibit protein phosphatases as effectively as this toxin (MacKintosh 1993), this procedure could be applied for the determination of hepatotoxins. The advantage of this assay over the commonly used *p*NPP assay is its greater sensitivity and it is easier to use compared with assays involving radiolabelled substrates. Vieytes et al. (1997) report a detection limit for okadaic acid 50 times lower than when using *p*NPP as substrate. The assay can be carried out in 96 well microtitre plates with a scanning fluorescent plate reader.

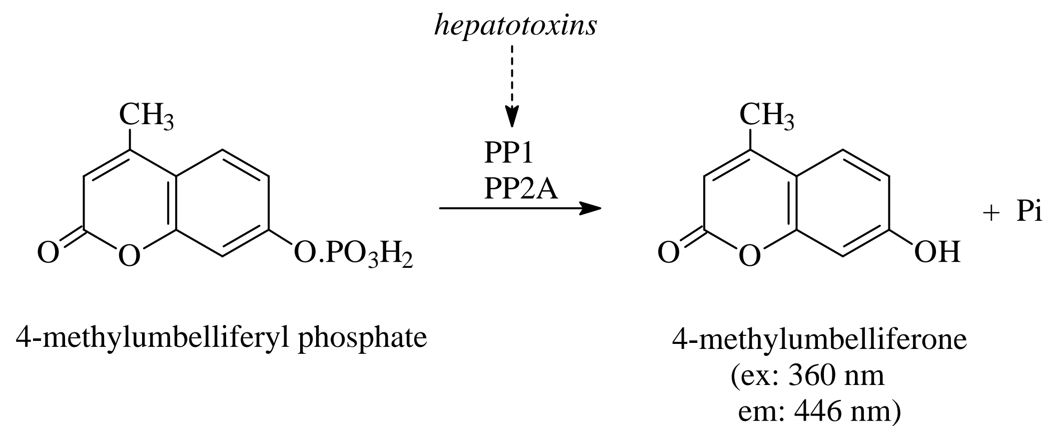


Figure 7. Fluorescent phosphatase inhibition assay by hydrolysis of 4-methyl-umbelliferyl phosphate to a fluorescent product, 4-methylumbelliferone

Other phosphatase assays

Some other assays which have been used to determine hepatotoxins based on the inhibition of phosphatase activity include:

- Dye-tagged peptide substrate assay (Sim and Rostas, 1996).
- Assay using ^{32}P-myosin light chain filaments (Eriksson et al. 1990).
- Radioactive assay using ^{32}P-phosphohistone (Honkanen et al. 1990).
- Radioactive assay using ^{33}P-phosphorylase a as substrate (Mez et al. 1996).
- Firefly bioluminescence system (Sugiyama et al. 1996).

2. MATERIALS AND METHODS

2.1 Colorimetric assay of inorganic phosphate

The procedure used to quantitate inorganic phosphate (Pi) released from the dephosphorylation of the substrate by the enzyme is essentially the same as developed by Wheldrake et al. (1996). Acidified malachite green, ammonium molybdate and Tween 20 are prepared in one concentrated solution (malachite green reagent mix) and reacted with the supernatant from the enzyme assay (second stage of assay depicted in Figure 3, Section 1.3). The resulting absorbance of the malachite green-phosphomolybdate complex is measured at 630 nm using a microtitre plate reader.

Acidified malachite green solution

60 mL of concentrated sulphuric acid (1.84 g/L) is added to 300 mL of Milli-Q water (Millipore Australia). Malachite green oxalate (0.44g) (Sigma Aldrich) is added to this solution resulting in 0.122% malachite green in 6N sulphuric acid.

Ammonium molybdate solution

A 7.5% (w/v) solution of ammonium molybdate tetrahydrate (Sigma Aldrich) is prepared in 6N sulphuric acid.

Tween 20 solution

Tween 20 (Sigma Aldrich) is made to 11% (v/v) with Milli-Q water.

Malachite green reagent mix

5 mL of malachite green (0.122%), 3.4 mL ammonium molybdate (7.5%) and 0.2 mL Tween 20 (11%) are combined to form the reagent mix. This solution is prepared fresh before the assay.

Inorganic phosphate standard curve

Standard solutions of KH_2PO_4 ranging from 1 to 100 μM Pi were prepared in Milli-Q water. The KH_2PO_4 (Sigma Aldrich) was dried for 2 hours at 115°C prior to weighing.

200 μL of Pi standard and 75 μL of malachite green reagent were added to the wells of a 96 well microtitre plate. The solutions were mixed in the wells and after 10 minutes at room temperature the absorbance at 630 nm was measured on a Dynex MRX microtitre plate reader with Revelation v. 3.04 software. The plate reader has a six position filter wheel with the following filters: 340, 400, 450, 490, 550, 630 nm.

Even though the procedure of Wheldrake et al (1996) suggests 70 μL, the Finnpipette stepper used for repeat dispensing can only dispense 75 μL so this volume was chosen for convenience. A comparison was made with a range of Pi standards using these two volumes, and it was found that the higher volume of dye reagent gave a slightly higher absorbance even though the higher volume dilutes the Pi slightly.

UV-Vis spectrum of malachite green-phosphomolybdate complex

UV-Vis spectra of the malachite green reagent mix before and after addition of Pi to illustrate the spectral shift upon formation of the malachite green-phosphomolybdate complex were determined using a Cary 1 UV-Visible Spectrophotometer (Varian) with Cary software.

2.2 Enzyme assay developed by Wheldrake et al. (1996)

Phosphatase activity of PP1 with phosvitin as substrate was carried out under the conditions described by Wheldrake et al. (1996) except that they prepared their own protein phosphatase from skeletal rabbit muscle. 200 μL of phosvitin (5 mg/mL), 200 μL of enzyme solution and 80 μL of the "cocktail" mix were combined in 1.5 mL microfuge tubes and incubated at 30°C for 30 minutes. The reaction was stopped by the addition of perchloric acid (70%, 10 μL) (Sigma Aldrich), the tubes mixed on a vortex mixer and placed on ice for 10 minutes. The precipitated protein was removed by centrifugation in a Hettich EBA 12 centrifuge for 7 minutes at 14,000 rpm and the supernatant assayed for Pi by the colorimetric assay detailed in Section 2.1.

Preparation of phosvitin solution

Phosvitin (Sigma Aldrich) was made up in 50 mM Tris-HCl (Sigma Aldrich), 4 mM EGTA (Sigma Aldrich) at pH 7.5 to a concentration of 5 mg/mL. As the purchased solid contains free phosphate which will contribute to the absorbance of the assay, phosvitin solutions must be dialysed or ultrafiltered to remove background Pi. The solution was ultrafiltered through a Centriprep 10 concentrator (Amicon) with 10,000 MW cutoff and the retentate made to volume in 50 mM Tris-HCl, 0.1 mM EGTA, pH 7.3.

PP1 enzyme solution

As these laboratories do not have facilities to extract and purify rabbit muscle protein phosphatase, commercial PP1 was purchased from Calbiochem (α-isoform, rabbit muscle, recombinant, *E. coli*). The enzyme was diluted in 50 mM Tris-HCl, pH 7.4 (4^oC) containing 0.1 mM EGTA, 10% (v/v) glycerol prior to assay. The concentrations of the enzyme stock solutions are described in Section 3.4.2.

Preparation of "cocktail" solution

A solution containing 0.5 mM dithiothreitol (Sigma Aldrich), 3 mM imidazole (Calbiochem-Novabiochem Pty Ltd) and 1 mM theophylline (Sigma Aldrich) was adjusted to pH 7.2.

2.3 Improved buffer system for enzymatic dephosphorylation of phosvitin

The optimised enzyme assay has been altered from the procedure of Wheldrake et al. (1996) in terms of final assay volume, substrate concentration, assay pH, buffer components and their concentration, and incubation time. The enzyme activity towards the substrate is now determined by incubating 100 μL of PP1 in buffer with 200 μL of phosvitin (1 mg/mL) in 1.5 mL microfuge tubes. The mixture is incubated for 2 hours at 30^oC, after which the reaction is stopped by the addition of 10 μL perchloric acid (70%). The tubes are placed on ice for 10 minutes and centrifuged at 14,000 rpm for 7 minutes. The supernatant (200 μL) is combined with 75 μL of freshly prepared malachite green reagent in microtitre wells and analysed as described in Section 2.1. A drawback of this part of the assay is that each well must be mixed with a fine stirring rod. Pasteur pipettes which had been sealed off with a flame were used for this. The malachite green reagent is dense and viscous, and when it is added to the wells containing the solutions to be assayed for Pi, it remains as a discrete layer at the bottom of each well. The shaker facility on the plate reader is unable to mix the two layers of solution. If the solutions are added to the wells in reverse order, mixing occurs more easily however, it is preferable to add the assay supernatants first as this step is time consuming, and then start the colour reaction by adding the dye mix with a Finnpipette stepper. A series of Pi standards can be included at this stage as well.

Protein Phosphatase 1 buffer

100 mM Tris, pH 7.5, 4 mM dithiothreitol (DTT), 0.2 mM EDTA, 2 mM $MnCl_2$ tetrahydrate (Sigma Aldrich), 0.4 mg/mL bovine serum albumin (BSA) (Sigma Aldrich). The DTT and BSA are added to the buffer prior to the enzyme analysis from stock solutions stored in the freezer. All solutions are kept on ice until the assay is started.

Phosvitin preparation

Phosvitin is prepared as a stock solution of 5 mg/mL in 50 mM Tris-HCl, 4 mM EGTA, pH 7.5 and ultrafiltered in the Amicon units as described above. The small amount of retentate (~ <0.5 mL) is made to volume in 50 mM Tris, pH 7.5, 0.1 mM EDTA and stored in the freezer as a stock solution. When required, the phosvitin is diluted to 1 mg/mL in 50 mM Tris, pH 7.5, 0.1 mM EDTA to which BSA is added to a concentration of 0.2 mg/mL prior to analysis.

2.4 Hepatotoxin inhibition of PP1

Standard inhibition curve

Four hepatotoxins were chosen for inhibition studies of PP1 enzyme with phosvitin as substrate. Microcystin-LR (Sapphire Bioscience) and nodularin (Calbiochem) were purchased commercially. The toxin from Sapphire Bioscience was chosen in preference to material from Calbiochem because of impurities in the Calbiochem standard. Microcystins YR and RR were a gift from K. Harada (Meijo University, Nagoya, Japan). Working solutions ranging from 0.01 to 300 ppb were prepared by dilution of 5 ppm stock solutions in Milli-Q water. A Milli-Q water control was included, and the microcystin

inhibition curve plotted as % inhibition of PP1 by microcystin relative to the positive control versus microcystin concentration. Inhibition curves in the literature are expressed in this way so that the IC_{50} can be calculated, and the degree of inhibition of the enzyme by different methods can be compared.

Inhibition of protein phosphatase was studied by combining 100 μL of PP1 with 100 μL of microcystin solution in 1.5 mL microfuge tubes. After 5 minutes at room temperature the enzyme assay was started by adding 200 μL of phosvitin substrate as described in Section 2.3.

Environmental samples

The procedure was the same for environmental samples and their dilutions except that the lower dilutions included a negative control in which enzyme was not added to the assay. This allowed matrix contribution to the background to be subtracted. Traces of inorganic phosphate and anything else that absorbs at 630 nm in the sample would contribute to the absorbance of the sample causing an under-estimation of microcystin. The presence of endogenous phosphatases (Sim and Mudge, 1993) also needs to be considered but should be compensated for in the controls.

2.5 Removal of phosphate from environmental samples and HPLC conditions

The presence of inorganic phosphate in environmental samples needs to be considered as high levels will contribute to the background absorbance once the extract is analysed for Pi release with malachite green reagent. Generally, most raw waters will not contain sufficient phosphate to cause an off-scale reading (see Discussion). However, algal extracts and their low dilutions (~1/100) were found to result in a high or off-scale absorbance reading. A C18 cleanup and concentration step was employed to cleanup the algal samples for HPLC (Section 2.5) and phosphatase assay instead of the ion exchange assay developed by Wheldrake et al. (1996). Their phosphate removal step was considered just as time consuming as the C18 procedure which has already been validated in this laboratory.

The C18 cleanup procedure and the HPLC conditions used by this laboratory are based on the method described by Lawton et al. (1994).

C18 cleanup

1. A C18 Sep-Pak cartridge (500 mg, 3 cc, Waters Chromatography) is primed with methanol (10 mL) followed by Milli-Q water (10 mL).
2. The water or algal sample that has been prefiltered through a Whatman GF/C glass microfibre filter is passed through the Sep-Pak cartridge at a flow rate of less than 10 mL/min.
3. The cartridge is washed in succession with Milli-Q water (10 mL), 10 % methanol (10 mL), and 20 % methanol (10 mL). The cartridge should not be allowed to run dry until the end of this step.
4. The cartridge is air dried by vacuum for approximately 2 minutes.
5. The toxin is eluted with 3 bed volumes of 90 % methanol.
6. The methanol eluent is evaporated to dryness at 45°C under high purity nitrogen gas and reconstituted to 1 mL. The laboratory method has been changed at this point in that the extract is reconstituted to 20 % methanol rather than 50 % methanol. The toxin in the tube is first dissolved in 0.2 mL 100 % methanol followed by 0.8 mL Milli-Q water.

HPLC conditions

Analysis of toxins by HPLC was carried out with a Waters Symmetry C18 column (3.9 x 150 mm) using the following mobile phase conditions at a flow rate of 1 mL/minute.

Table 2. HPLC gradient conditions

TIME (min.)	SOLVENT A	SOLVENT B
0	100	0
25	0	100
35	0	100
40	100	0
50	100	0

- Solvent A is 30 % acetonitrile in Milli-Q water containing 0.05 % trifluoroacetic acid (TFA) (Sigma Aldrich).
- Solvent B is 55 % acetonitrile in Milli-Q water containing 0.05 % TFA.

Detection was via a Waters 996 photodiode array detector connected to Millennium 2010 Chromatography Manager. The injection volume was 50 μL and all samples had been filtered through a 0.45 μm PVDF low protein binding membrane filter (Gelman Sciences) prior to analysis. Quantitation of microcystins and nodularin was against a 5 ppm toxin standard. Linearity was established from 0.0625 to 5 ppm and 0.0625 ppm was very close to the limit of detection of the instrument.

2.6 Extraction of hepatotoxins from environmental samples

Water and bloom material containing low numbers of cyanobacteria

Water samples which have experienced a bloom but no longer contain intact cells are filtered through GF/C filters to remove turbidity. If the sample is to be concentrated for HPLC analysis, 250 mL is passed through a C18 Sep-Pak as described in Section 2.5. This removes organic material and concentrates the toxin resulting in a 250-fold concentration factor following elution, drying and reconstitution.

Samples containing relatively low numbers of cyanobacterial cells (< 100,000 cells/mL) can be analysed for intracellular and extracellular toxin. The cells are collected on GF/C filters which are then resuspended in methanol in the fridge overnight. The methanol is decanted into another test tube with as much of the solvent squeezed out of the filters as possible. This extract is then blown down to dryness at 45°C under a stream of nitrogen gas and the toxin redissolved in 0.5 mL of methanol followed by 0.5 mL water or, 0.2 mL of methanol/0.8 mL water. The sample is filtered through a 0.45 μm PVDF acrodisc into a 1 mL HPLC vial.

Freeze-dried material

Algal scum material containing large numbers of cells may be freeze-dried for long term preservation or if mouse bioassay is required. In this case the scum is frozen and freeze-dried under vacuum. Freeze-dried material is generally extracted at a concentration of 50 mg dry weight per 10 mL solvent. If mouse bioassay and/or C18 cleanup is required the algal material is extracted into 2 x 5 mL Milli-Q water. The dried cells (50 mg) are resuspended in the first 5 mL of solvent and sonicated on ice for 2 minutes with an ultrasonicating probe with microtip. The suspension is centrifuged at 4000 rpm for 20 minutes, and the supernatant decanted. The pellet is resuspended in the second 5 mL aliquot, vortexed and sonicated a second time. The suspension is again centrifuged and the two supernatants combined and filtered through a 0.45 μm acrodisc.

2.7 Study of matrix effect on phosphatase assay

Various matrices were prepared to examine the effect the matrix has on the accuracy of quantitation by the inhibition assay. Microcystin-LR was spiked in to the matrices at the following concentrations: 0.1, 0.2, 0.3, 0.6, 1, 1.5, 2, 3, 10 ppb.

Humic acid solution

A concentrated humic acid solution estimated to be about 30 g/L dissolved organic carbon (DOC) was used to study the effect of natural organic matter on microcystin inhibition of PP1. The concentrate was obtained by adsorbing organics from water on to an anion exchange resin and desorbing them with NaCl. The solution was then passed through an RO unit to remove the salt to about 2 M. The solution was diluted to approximately 10 mg/L DOC and was analysed on a SK12 Organic Carbon Analyser (Skalar Pacific) and found to contain 9 mg/L organic carbon. The total dissolved solids (TDS) of the diluted solution was 21 mg/L.

Phosphate-free algal scum, ultrafiltered farm dam

An algal matrix that did not contain interfering inorganic phosphate was obtained by ultrafiltering an environmental sample. A thick green algal scum from a farm dam at Balhannah was received at the laboratory on 29th December 1997 and contained a large amount of *Anabaena circinalis*; a lesser amount of coiled and straight *Anabaena* and *Botryococcus*, and a small amount of *Microcystis flos-aquae*. The scum sample was freeze-thawed to break open the algae to simulate the procedure used for extraction of toxin from algal material. It was then filtered through Whatman GF/C glass microfibre filters prior to ultrafiltration through Centriprep 10 ultrafiltration units. The retentate was made up to the

original volume with Milli-Q water. The DOC and conductivity of the scum sample was measured before and after ultrafiltration (UF).

Environmental samples

A raw water and a diluted algal extract (1/100) were included in the range of samples representing typical matrices that are likely to contain hepatotoxins. The raw water chosen was a sample from Hindmarsh River taken at Hindmarsh Weir on 4th February 1998. The algal extract was obtained by extracting 112 mg freeze-dried *Microcystis sp*. that contained no toxins by HPLC into 20 mL of Milli-Q water as described in Section 2.6. A 1/100 dilution of this material contained concentrations of Pi unlikely to interfere with the enzyme assay.

Table 3 DOC and conductivity of ultrafiltered farm dam scum sample

TREATMENT	DOC (mg/L)	CONDUCTIVITY (μSiemens/cm)
Dam water before UF	104	808
Dam water after UF	54	25

3. RESULTS AND DISCUSSION

3.1 Colorimetric assay

The enzyme assay developed by Wheldrake et al. (1996) (Figure 3, Section 1.3) initially did not work when tried in this laboratory. Our source of enzyme was different and there was no literature reference to dephosphorylation of highly phosphorylated proteins such as phosvitin by protein phosphatases. Harada et al. (1981) have demonstrated phosphatase activity of bovine intestinal phosphatase towards phosvitin, but found no protein phosphatase activity in alkaline phosphatases from bovine liver and pulp. Huang et al. (1976) also demonstrated phosphoprotein-phosphatase activity with human placental alkaline phosphatase with phosvitin. Given that Wheldrake et al. (1996) demonstrated protein phosphatase activity towards phosvitin, there was no clear explanation as to why the assay was not working. It was therefore decided to test the assay in stages, starting from the last step and working back up the procedure.

Firstly, aspects of the colorimetric reaction were examined; the "survival" of inorganic phosphate (Pi) into a "dummy" enzyme assay with and without phosvitin was determined; PP1 activity towards phosvitin was then optimised; factors influencing the inhibition of PP1 by hepatotoxin were examined; quantitation of microcystin in environmental samples was carried out.

3.1.1 UV-visible spectrum of malachite green-phosphomolybdate complex

Figure 8 illustrates the UV-Vis spectrum of the malachite green reagent mixture detailed in Section 2.1 and the resulting spectrum when Pi reacts with this reagent to form the malachite green-phosphomolybdate complex. The complex does not form if ammonium molybdate is not present in the reagent mix. As previous workers have found, the addition of Pi results in an absorbance shift to the right and an intense absorption band at 620-650 nm (Van Veldhoven and Mannaerts 1987, Baykov et al. 1988). This is easily visualised as a colour change from orange to deep green which Wheldrake et al. (1996) believed made it amenable for use as a visual test.

3.1.2 Phosphate standard curve and effect of final acidity on sensitivity

Standard solutions of KH_2PO_4, oven dried for 2 hours, were assayed with the malachite green reagent mix (detailed in Section 2.1) and the absorbance measured at 620 nm. The MRX plate reader sent off to the United States for repair had a wavelength filter for 620 nm, whereas the one that was returned to the Laboratories had a filter for 630 nm. This explains why the latter part of this report will detail absorbance readings at 630 nm. The phosphate standard curve obtained over a wide concentration range is illustrated in Figure 9. The assay is linear up to almost 100 μM Pi and also over an absorbance range of about 3 units. The linear regression (R) for the data at 0.9 N is 0.9919, and the R value at 1.5 N is 0.9988. Dynex specifications quote a linearity of -0.100-2.000 OD $\pm$ 1.0% with precision of <1% CV and 2.001-3.500 OD $\pm$ 1.5% with precision of <1.5% CV for the MRX plate reader. The assay can vary in sensitivity from day to day. On some days the curve starts to plateau out at 80 μM PO_4 and this data point is not included in the quantitation. Since the amount of Pi liberated from the enzyme assay was later found to be considerably less than this concentration, this variability was not considered to be a problem.

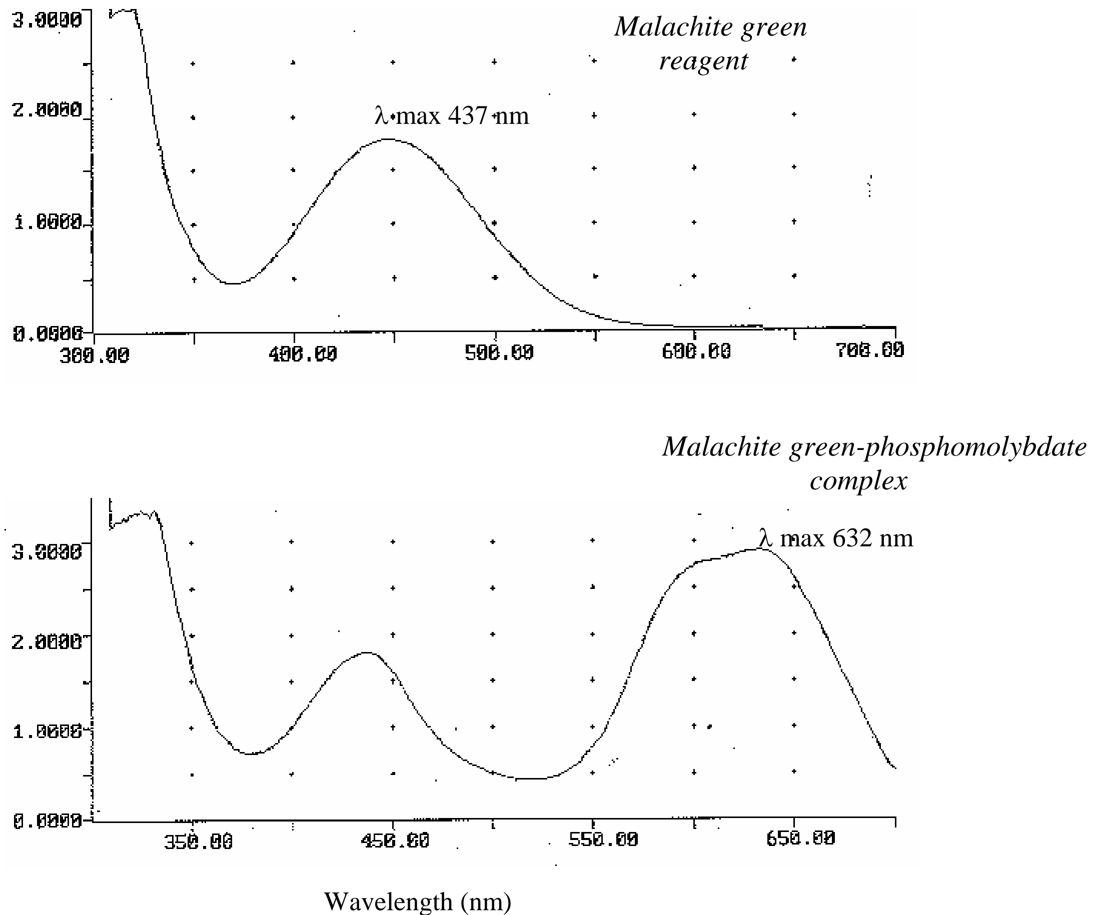


Figure 8. UV-Vis spectra of the malachite green reagent before addition of inorganic phosphate and after formation of the malachite green-phosphomolybdate complex (Absorbance versus Wavelength)

Some of the literature is a little unclear as to whether the ammonium molybdate is made up in H_2O or 6N H_2SO_4 and different final concentrations of acidity in the assay are quoted. The assay was therefore checked at a series of final concentrations of H_2SO_4 (0.9, 1.0, 1.1, 1.2, 1.3, 1.5N) and two sets of data have been included in the following graph. The assay was more sensitive at 0.9 N but the working range for phosphate quantitation was wider at 1.5N H_2SO_4. It was decided to keep the conditions described by Wheldrake et al. (1996) in which the ammonium molybdate is prepared in 6N H_2SO_4, resulting in a concentration of 1.5N in the final assay.

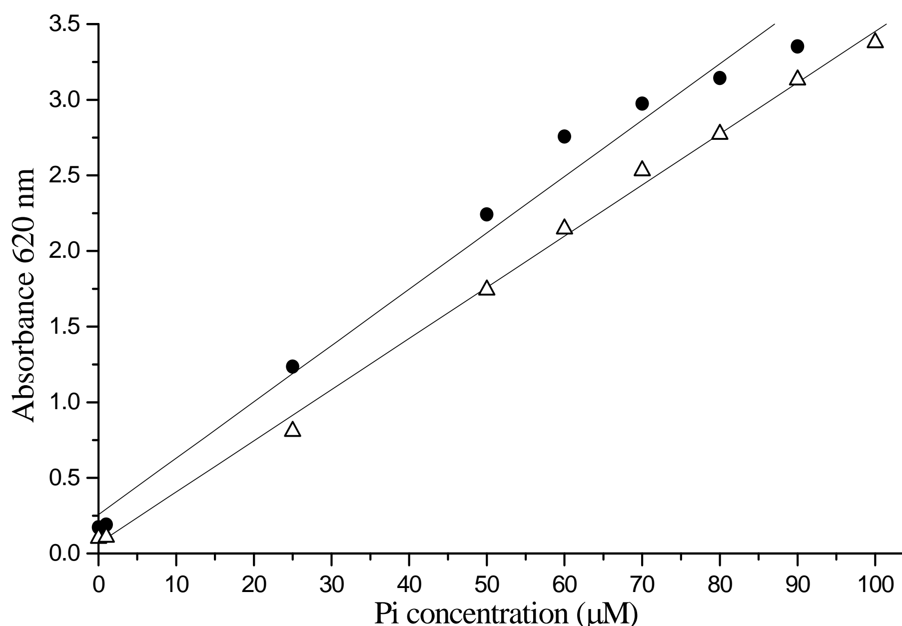


Figure 9. Phosphate standard curve obtained with colorimetric assay using malachite green reagent at two levels of acidity. Final normality of assay is 0.9 N (!) and 1.5 N (Δ)

The rate of colour formation of the malachite green-phosphomolybdate complex was examined at the same range of acidities since the MRX plate reader has the capacity to monitor kinetic assays. The complex forms at different rates depending on the acidity of the assay. However there is not a large difference in the final absorbance values (just over 0.2 absorbance units). Figure 10 illustrates the rate of colour production between 0.9 and 1.5N. As the wells need to be mixed, the very beginning of the reaction (about 20-30 seconds) was not measured. The reaction proceeded faster at 0.9N than 1.5N and was more sensitive, but both graphs level out at 10-12 minutes. The final absorbance was directly related to acidity of the assay. As was shown in Figure 9, the increase in sensitivity has to be weighed up against the shorter working range of the assay. The reason the T=0 absorbance at 1.5N is much lower than for 0.9N may be that the dye complex is more soluble under more acidic conditions. The lower normality dye mixes were more likely to be cloudy and this may have contributed to the initial absorbance.

3.1.3 Effect of Tween 20 on stability of malachite green-phosphomolybdate complex

Van Veldhoven and Mannaerts (1987) discuss different compounds that stabilise the dye-phosphomolybdate complex and prevent it precipitating. Different concentrations of Tween 20 were examined to determine their effect on the stability of the complex. Figure 11 illustrates that in the absence of Tween, the complex is unstable at higher phosphate concentrations. The levelling off of the graph reflects decay of the complex and precipitation which agrees with the work of Baykov et al.(1988). 22% Tween marginally extends the linear range of the assay at 1.5N acidity, but it does so at the expense of sensitivity. It was therefore concluded that 11% Tween was the optimum concentration to stabilise the complex and extend the linear range of the assay.

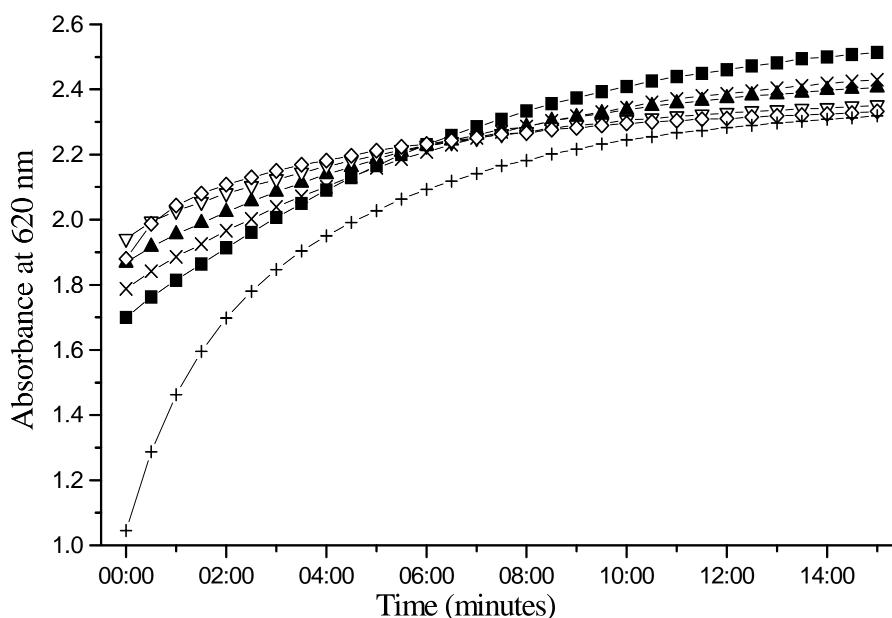


Figure 10. Formation of malachite green-phosphomolybdate complex. Absorbance of complex at 620 nm versus time at different final normality of assay. Final N = 0.9 (#), 1.0 (x), 1.1 (σ), 1.2 (∇), 1.3 (◊), 1.5 (+)

10 to 12 minutes was considered optimum for colour development. The phosphate concentration in the assay is calculated from the absorbance reading at 620 nm after 10 minutes by which time the graph is levelling off and the rate of colour development is at a minimum.

3.1.4 Effect of methanol on colorimetric assay

The presence of methanol in algal and water extracts cleaned up by C18 cartridges in preparation for HPLC, and its effect on microcystin quantitation by the PP1 assay was examined once the enzyme assay was optimised (Section 3.4.4). It was unclear from the results in Section 3.4.4 however, whether the PP1 assay itself was affected by the presence of methanol or whether the colorimetric stage of the assay was affected. Ward et al. (1997) showed that in their assay using *p*-nitrophenyl phosphate as substrate, enzyme activity towards the substrate and microcystin inhibition of PP1 was unaffected by up to 70 % methanol in their mLR solutions. The sample (10 μL) in their assay was added to a total assay volume of 200 μL. Therefore, the effect of methanol on the colorimetric measurement of Pi was determined in the phosvitin assay after the data presented in Section 3.4.4 was obtained.

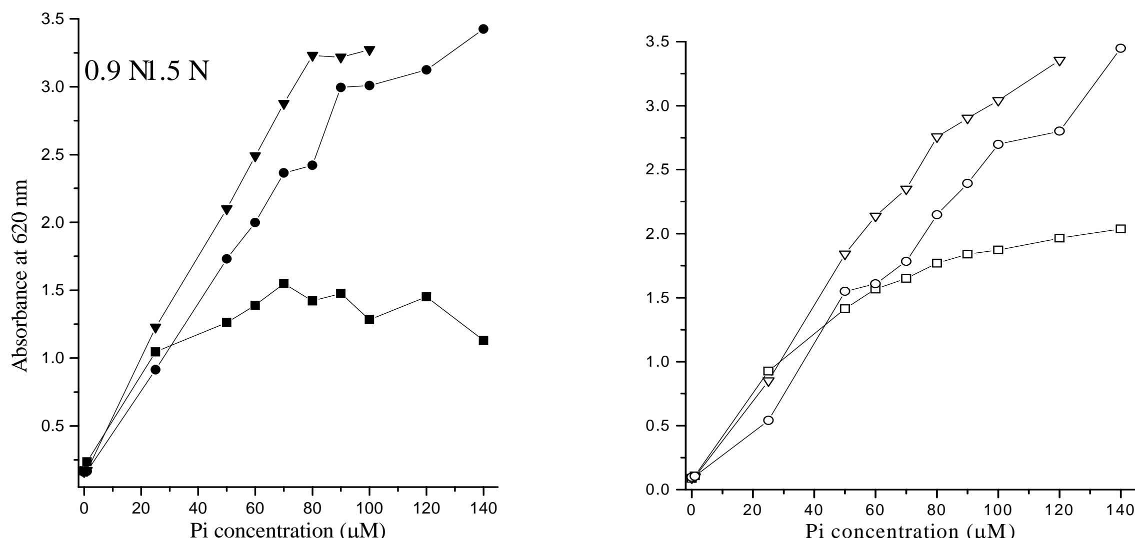


Figure 11. Effect of Tween 20 on stability of malachite green-phosphomolybdate complex. Comparison of colorimetric assay at 0.9N ((#) 0%, (τ) 11%, (!) 22% Tween) and 1.5N ((Θ) 0%, (∇) 11%, (∀) 22% Tween) final acidity

A series of orthophosphate standards (25, 50, 60, 70, 80 µM) made up in Milli-Q water, 20 % and 50 % methanol (final concentration) were compared for maximum absorbance with the malachite green reagent as described in Section 2.1. Figure 12 illustrates the influence of methanol in the Pi matrix on the colorimetric assay. It is clear that the presence of methanol results in a diminished absorbance of the malachite green-phosphomolybdate complex at 630 nm. It is therefore highly likely that the diminished absorbance response seen in the microcystin inhibition assay was the result of the colorimetric assay being affected by methanol rather than the PP1 enzyme activity on phosvitin. The use of 20 % methanol was chosen as the preferred solvent for sample extracts purified by C18 Sep-Pak cleanup.

The effect of methanol on the *formation* of the malachite green-phosphomolybdate complex or *absorbance* reading of the complex once it has been formed was then examined. A 100 µM solution of KH_2PO_4 was combined with malachite green reagent to form the dye-phosphate complex. This complex was then diluted 1:1 with Milli-Q water, 20% and 50% methanol and the absorbance read at 630 nm at 1, 10 and 20 minutes. The absorbance of these solutions immediately after dilution was not significantly different.

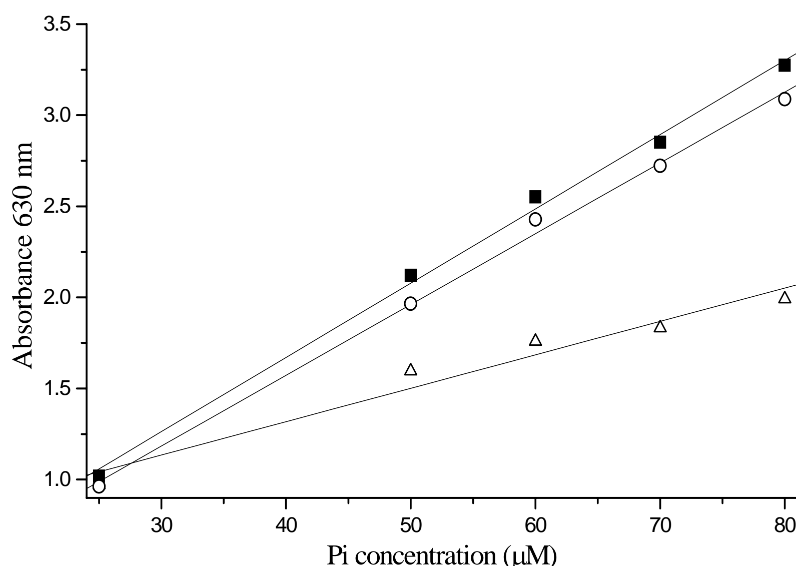


Figure 12. Effect of solvent on reaction of inorganic phosphate with malachite green reagent. Pi made up in Milli-Q water (#), 20% methanol (∀), 50% methanol (∆)

However, as Table 4 shows, after a further 10 and 20 minutes at room temperature the absorbance of the complex in the presence of the higher concentration of methanol had decreased while the absorbance of the control (dilution in Milli-Q water) had increased slightly as expected (Section 3.1.2). This result along with the data in Figure 12 suggests that the presence of methanol in a sample to be assayed for Pi with the malachite green reagent mix does not actually interfere with the formation of the phosphomolybdate complex nor its absorbance at 630 nm. However, methanol causes the absorbance of the complex to decrease with time. This could be due to precipitation of the complex, however this was not investigated further as 50% methanol was avoided as a solvent for reconstituting sample extracts.

Table 4. Effect of methanol on the absorbance of the phosphomolybdate complex at 630 nm.

TIME (minutes)	1:1 MQ water	1:1 20% MeOH	1:1 50% MeOH
1	1.736	1.699	1.765
10	1.795	1.762	1.665
20	1.823	1.787	1.357

3.1.5 Replicate studies on colorimetric assay of inorganic phosphate

Replicate assays were conducted on both sections of the assay, the enzymic dephosphorylation of substrate and the colorimetric determination of the released inorganic phosphate. The variation between four replicates in the latter part of the assay was examined by measuring Pi concentration in a series of Pi standards (10, 25, 50, 60, 70, 80 μM) arranged in three ways in the microtitre plate (Figure 13). The first variation placed each concentration of standard as close to one another as possible. In the second variation, the standards were placed in sequence, but still as close to one another as possible. The third variation placed the sequence of standards on alternate rows of the microplate to simulate replicates situated far apart from one another. The data is presented as absorbance of replicate wells at 620 nm.

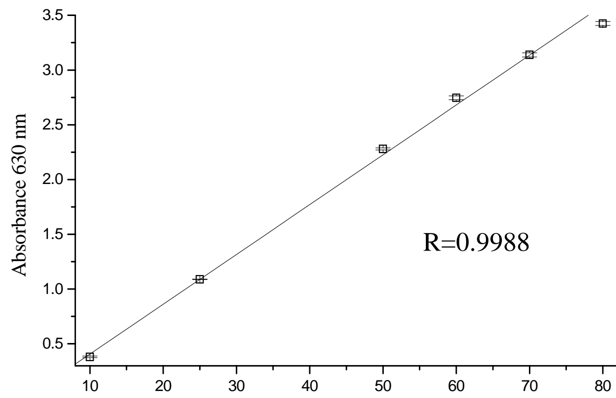
The 3 sets of replicates showed good agreement visually and this was supported by statistical data in Table 5 which details the standard deviation and coefficient of variation for each set. The coefficient of variation was lowest over the range of Pi standards when the replicate wells were situated next to one another as in Set 1. The degree of error increased when the replicate wells were situated further apart however, the error was still less than 2.2% even when the replicates were placed as far apart as possible. All three sets of data were similar in that the lowest concentration of Pi (10 μM) produced the largest experimental error. This was also reflected in the replicate study on the PP1 dephosphorylation of substrate described in Section 3.4.4 when very low quantities of enzyme were used.

3.1.6 Effect of matrix on colorimetric assay

As part of the investigation into the effect of sample matrix on the inhibition of PP1 by mLR, two “problem” matrices were spiked with Pi at 25, 50, 60, 70 and 80 μM KH_2PO_4. The matrices examined were a diluted algal extract (1/100) and an ultrafiltered farm dam extract described in Section 2.7. Milli-Q water was used as the control matrix. The sample matrix spiked with Milli-Q water was used as the blank. Figure 14 illustrates that there is a linear relationship between concentration of Pi and absorbance between 25 and 70 μM Pi for both matrices. The curve starts to plateau at 60-70 μM Pi in dilute algal extract as the absorbance readings prior to the blank being subtracted are close to the limit of the working range of the plate reader. The linear regression for the data in Milli-Q water over the 25-70 μM range is 0.999; in dilute algal extract R=0.993; and in the ultrafiltered farm dam extract R=0.999. The problems encountered later with inaccuracies in mLR quantitation are therefore unlikely to be caused by the effect of sample matrix on the colorimetric part of the assay.

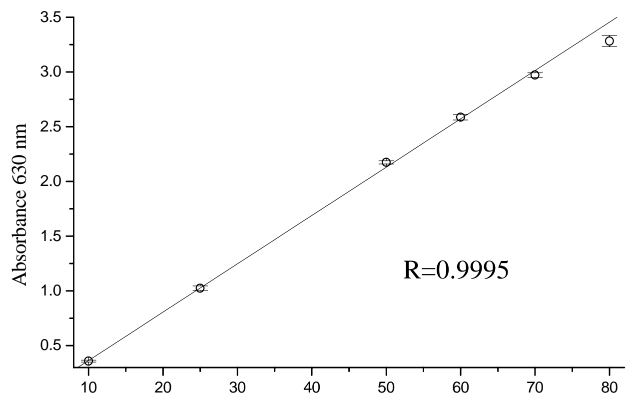
Set 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	10	10	10	10	25	25	25	25	50	50	50	50
B	60	60	60	60	70	70	70	70	80	80	80	80
C												
D												
E												
F												
G												
H												



Set 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	10	25	50	60	70	80	10	25	50	60	70	80
D	10	25	50	60	70	80	10	25	50	60	70	80
E												
F												
G												
H												



Set 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	10	25	50	60	70	80						
B												
C	10	25	50	60	70	80						
D												
E	10	25	50	60	70	80						
F												
G	10	25	50	60	70	80						
H												

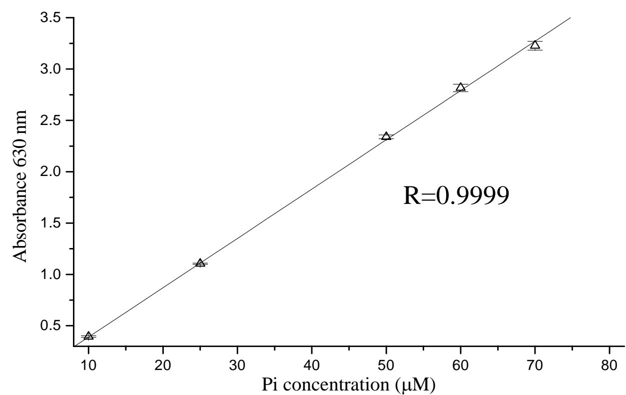


Figure 13. Graphical representation of variation between colorimetric assay replicates arranged in three ways on microtitre plate

Table 5. Statistical evaluation of three sets of four replicates analysed by the colorimetric assay of inorganic phosphate

μM Pi std.	10	25	50	60	70	80
Set 1						
Average	0.381	1.089	2.280	2.746	3.139	3.425
Standard deviation	0.008	0.002	0.010	0.017	0.019	0.018
Coeff. Variation	2.1	0.2	0.4	0.6	0.6	0.5
Set 2						
Average	0.358	1.025	2.174	2.587	2.972	3.283
Standard deviation	0.008	0.019	0.014	0.026	0.021	0.051
Coeff. Variation	2.2	1.9	0.6	1.0	0.7	1.6
Set 3						
Average	0.395	1.104	2.339	2.816	3.228	*
Standard deviation	0.007	0.006	0.019	0.036	0.043	
Coeff. Variation	1.8	0.5	0.8	1.3	1.3	

* out of range

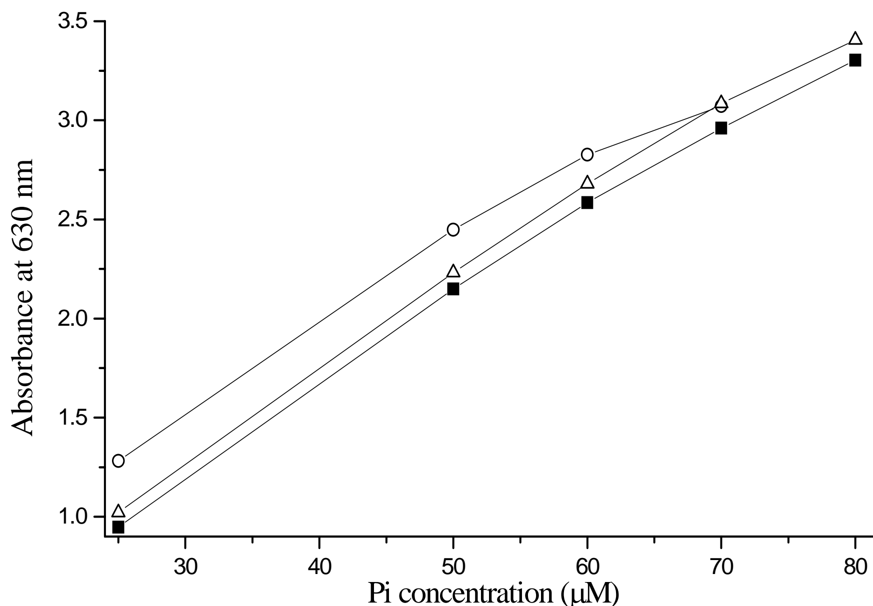


Figure 14. Effect of sample matrix on colorimetric reaction in two "problem" matrices. Milli-Q water (#), ultrafiltered farm dam extract (Δ), Diluted algal extract (∀)

3.2 Protein phosphatase 1 enzyme assay

In the early stages of this project, the phosphatase assay employed was that of Wheldrake et al. (1996), summarised in Figure 3 of Section 1.3 and described in more detail in Section 2.2. The source of PP1 enzyme used in their studies was a preparation of partially purified PP1 extracted from rabbit muscle. They did not determine the activity of their enzyme, therefore the amount of enzyme used in their assay is unknown. The first problem with the assay was that it did not work when the assay conditions were employed in our laboratory using PP1 enzyme purchased from Calbiochem. Some of the problems encountered included:

- Recombinant enzyme from Calbiochem had to be purchased as these laboratories do not have facilities to extract and purify enzyme from animals. This enzyme has different properties to rabbit muscle PP1 in that it requires the presence of Mn^{++} for full expression of its activity (Zhang et al. 1992).
- The final assay concentration of some of the buffer components, particularly dithiothreitol (DTT), was much too low, ie, 0.06 mM. The Calbiochem enzyme is an expensive component of the assay (40U for $420.00), and the buffer conditions are therefore important in optimising enzyme activity to minimise the amount of enzyme required.
- When substrate concentration was varied as part of this study, it was found that higher concentrations of phosvitin inhibited the enzyme reaction.
- There was no preincubation of PP1 with mLR and verbal discussion with one of the analysts could not resolve whether the components were added to the assay in any particular order (A. Bilney, Flinders University, personal communication).

In order to resolve these problems a thorough investigation of the enzyme assay was undertaken. After about four months work an assay that resulted in a good absorbance range in which to produce a microcystin inhibition curve was developed. This section details some of the experiments carried out with the assay developed by Wheldrake et al. (1996) before a new buffer system and conditions were adopted which greatly improved the activity of Calbiochem PP1 towards phosvitin.

3.2.1 Recovery of inorganic phosphate from PP1 assay

One of the first experiments carried out involved determining whether Pi released from the enzyme reaction was surviving through to the colorimetric part of the assay. A range of Pi standards in Milli-Q water was substituted for water samples in the assay and the recovery of Pi from the assay was determined. This was done in the presence of phosvitin, and with phosvitin solution substituted with buffer. Figure 15 graphs the recovery of spiked Pi into the assay with and without phosvitin present compared with the theoretical recovery. The theoretical recovery is the maximum amount of Pi that can be detected based on the dilution of the Pi standard with the other assay components. This experiment was conducted several times with different ranges of Pi spike solutions and different concentrations of buffer components as the assay was being optimised, therefore only the most recent data which was carried out with the modified Calbiochem buffer and a good range of spike standards (0, 10, 25, 50, 60, 70, 80, 90, 100, 200, 300, 400 µM Pi) was included.

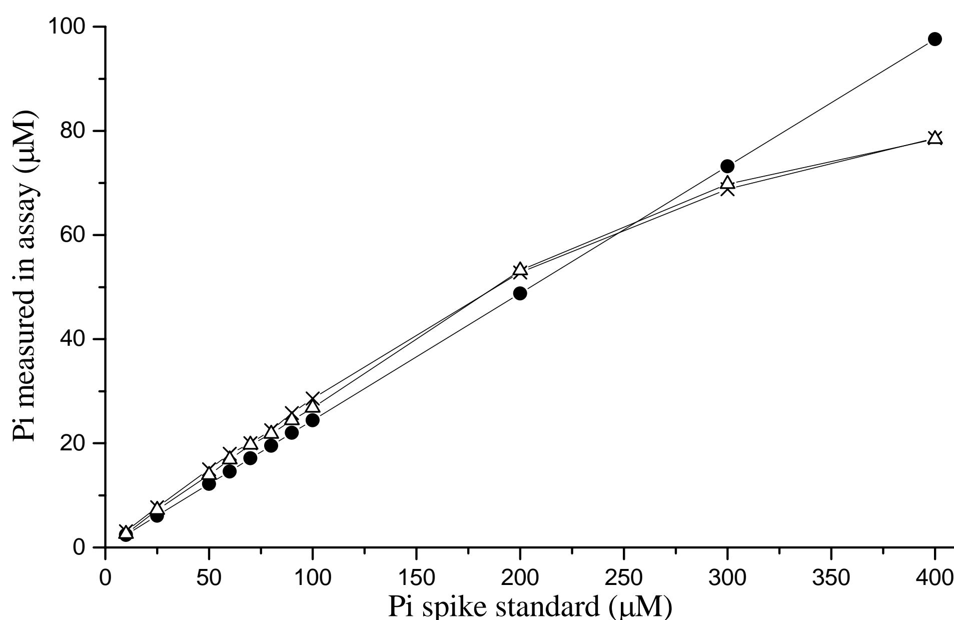


Figure 15. Recovery of Pi spiked into PP1 phosphatase assay in the presence and absence of phosvitin substrate. No phosvitin in the assay (∆), phosvitin present (x), theoretical recovery (!)

The spike experiments were reproducible irrespective of variations in the buffer components. The results indicate that any Pi released from the substrate will be detected by the colorimetric stage of the assay and is not bound or precipitated with the phosphorylated protein substrate phosvitin or any of the other buffer components. Generally, the absence of phosvitin gave recoveries similar or slightly higher than expected. In the presence of phosvitin, the recoveries were always a little higher than expected even though appropriate blanks were included which should have accounted for the presence of Pi in the phosvitin substrate itself. The graph becomes non-linear at 300 μM Pi as the absorbance values approach the limit of the linear range of the instrument. The linear regression (R) over the 10-200 μM Pi range for the data when phosvitin was present is 0.9987. When phosvitin was absent in the spiked assay R=0.9997.

3.2.2 Protein phosphatase assay developed by Wheldrake et al. (1996) in the presence and absence of manganese ion

Having determined that Pi release from the enzyme reaction is detected by the colorimetric stage of the assay, the next series of experiments involved trying to get Calbiochem PP1 to exhibit some activity to phosvitin. Figure 16 revises the enzyme assay developed by Wheldrake et al. (1996) and describes the assay conditions used to study the effect of manganese and temperature on PP1 dephosphorylation of phosvitin.

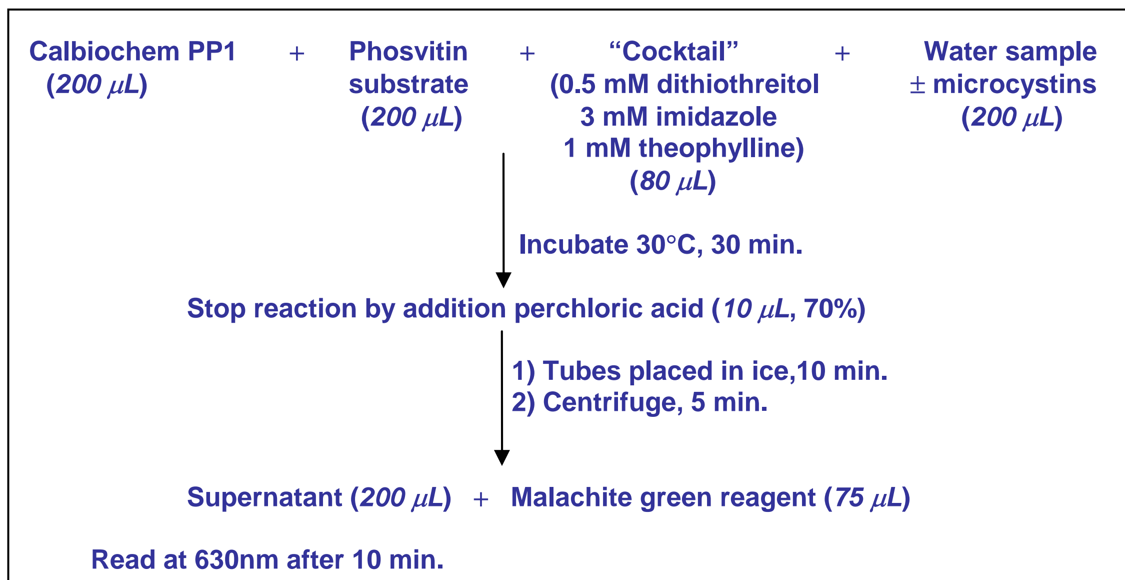


Figure 16. Revised protein phosphatase inhibition assay developed by Wheldrake et al. (1996)

As mentioned before, the assay did not work at first with the conditions used by Wheldrake et al. (1996), even when the incubation time was increased to over 3 hours. This is illustrated in Figure 17 by the curve depicted with (Θ) symbol. There was no measurable enzyme activity over this time and the curve was indistinguishable from data representing assays to which no enzyme had been added, ie, the blank (x).

Zhang et al. (1992) were the first workers to successfully express rabbit muscle PP1 in the vector *E. coli.* They found that the recombinant enzyme was slightly less sensitive to inhibitors such as okadaic acid and mLR, compared with authentic rabbit muscle PP1. The major difference between the two enzymes was the recombinant enzyme had a requirement for Mn^{++} for full expression of its activity. These authors determined about 0.06-0.2 mM Mn^{++} to be the optimum concentration for maximum activity of the enzyme. Figure 17 shows the effect of the presence of 0.2 mM $MnCl_2$ in the assay (#). The absence of Mn^{++} in the assay even when PP1 was present (Θ) had the same effect as having no enzyme present. The presence of enzyme activity in the assay was encouraging but after 3.1 hours incubation the ΔOD (OD minus blank) was still only 0.2 absorbance units. This incubation time extended the length of the assay considerably and made it difficult to achieve quantitative results in a

working day. Some of the assay conditions were therefore examined to make it faster and cheaper to perform. The concentration of Mn^{++} was optimised later and is discussed in Section 3.3.4.

It should be noted that the rate of the enzyme reaction in Figures 16 and 17 is not consistent. Variation between buffers stored in the fridge for different periods of time was noticed during the course of this work. This will be discussed further in Section 3.3.3. Also, the buffer components were concentrated and the final assay volume decreased during the early part of this work to increase the relative concentration of PP1 in the assay, however, it would be too confusing to detail all these minor changes. It should therefore be noted that the rate of reaction may not appear to be consistent between some of the data obtained in the optimisation of the enzyme dephosphorylation of phosvitin.

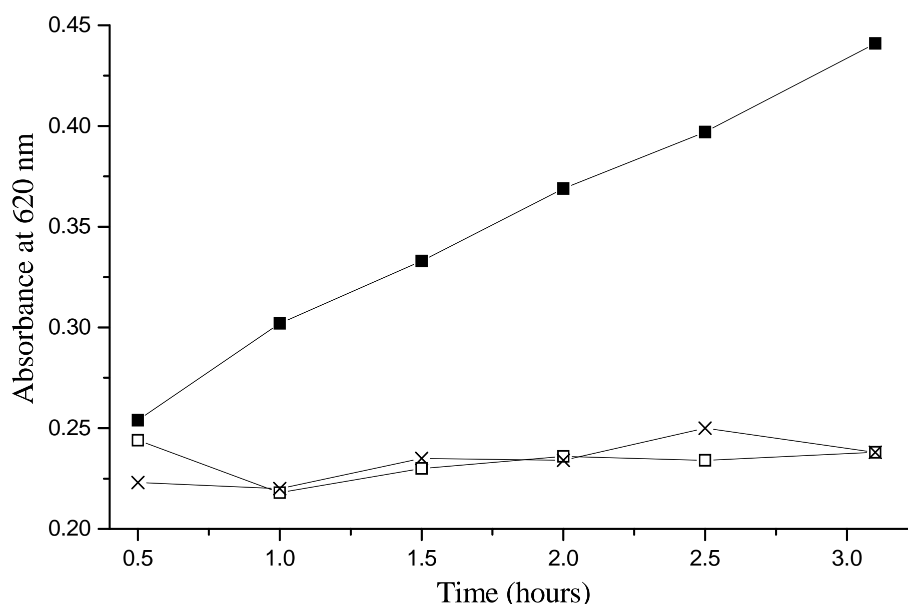


Figure 17. Protein phosphatase inhibition assay developed by Wheldrake et al. (1996) with and without manganese ion. Manganese added to assay (#), manganese absent (Θ), and PP1 enzyme absent (x)

3.2.3 Influence of temperature on protein phosphatase assay

The next parameter examined was an increase in the incubation temperature of the assay to determine if this would increase the rate of reaction so that it proceeded to completion more quickly and produced a higher absorbance change. A small ΔOD would not provide a suitable range within which to produce an accurate microcystin assay curve. Small changes in the absorbance of assay samples could result in large errors in microcystin quantitation. It was reported that using the conditions of Wheldrake et al. (1996) a ΔOD of about 0.4 or 0.5 absorbance units after an incubation time of 30 minutes was obtained (A. Bilney, Flinders University, personal communication). The activity of PP1 enzyme at 30°C and 37°C with Mn^{++} included in the assay was compared and the results are presented in Figure 18.

The enzyme activity at 37°C (!) was actually less than at 30°C (σ) and the hydrolysis of phosvitin in the blank assay was greater at 37°C (∀) compared with 30°C (Δ). Like many other phosphorylated substrates, phosvitin slowly hydrolyses and must be stored frozen. It is therefore not surprising that the blank assay at 37°C produced slightly higher absorbance readings due to spontaneous hydrolysis than the assay at 30°C. Therefore, there was no advantage in varying the temperature of the assay. It should be noted that the assay was set up in a test tube and aliquots taken out at time intervals to measure enzyme activity therefore the last points on the graph, ie, 3 hours, which were taken from the assay tube when the volume was becoming quite low, may not be very accurate.. The ΔOD at 30°C at 2 hours was 0.161 and ΔOD at 37°C at 2 hours was 0.096.

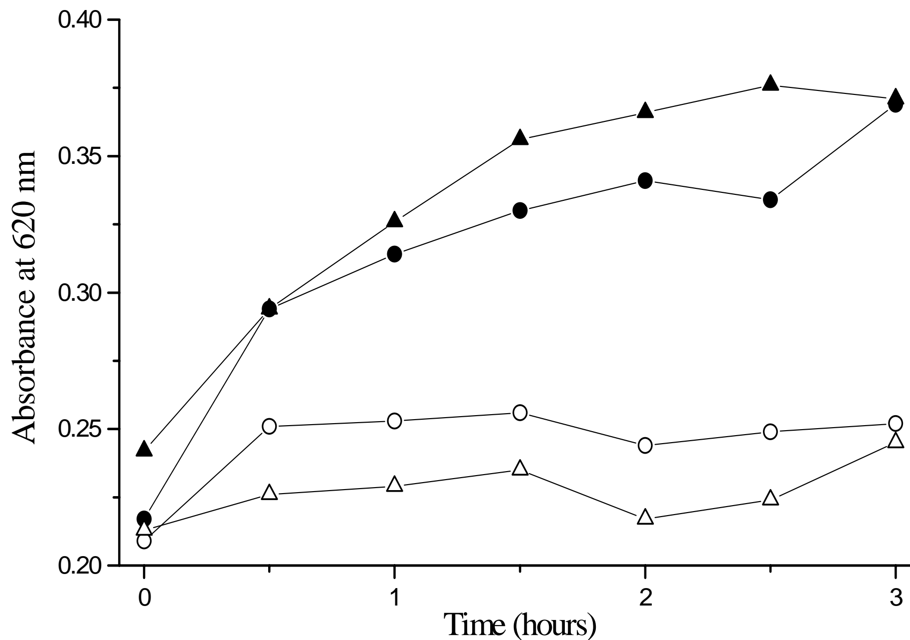


Figure 18. Activity of Calbiochem PP1 enzyme in buffer conditions of Wheldrake et al. (1996) with Mn^{++} included in the assay, at 30°C (PP1 present (σ) and PP1 absent (Δ)) and 37°C (PP1 present (!) and PP1 absent (∀))

3.2.4 Comparison of Calbiochem buffer with buffer system of Wheldrake et al. (1996)

It was next decided to try the assay buffer recommended by Calbiochem for their recombinant PP1 enzyme in order to improve enzyme activity and obtain a larger absorbance change. The details of this buffer are discussed in Section 3.3.6. Phosvitin at 5 mg/mL in this buffer was compared with the buffer currently used. It was concluded from Figure 19 that the Calbiochem buffer improved the activity of PP1 and produced more consistent results. This may be due to the fact that this buffer contains bovine serum albumin (BSA) which assists in keeping buffer components such as proteins and enzymes in solution.

It was at this stage of the project that a wide scatter of the data was apparent in assays with both buffers. The magnitude of this error varied between experiments and a slight cloudiness was noticed in some of the wells about 30 minutes after the malachite green reagent was added to the assay supernatant. A visible precipitate was apparent in these wells after the plate was stored in the refrigerator overnight. Freshly prepared buffers and phosvitin substrate did not resolve the problem. As mentioned in Section 3.2.2, there were many small changes made to the buffers in the assay which are too numerous to detail. In an attempt to increase the concentration of the enzyme in the assay, the buffer volumes were decreased as the buffer components were concentrated. This also had the effect of concentrating the substrate and is the most likely explanation for the problem becoming apparent at this stage of the experimental work. This will be discussed in more detail in the next section.

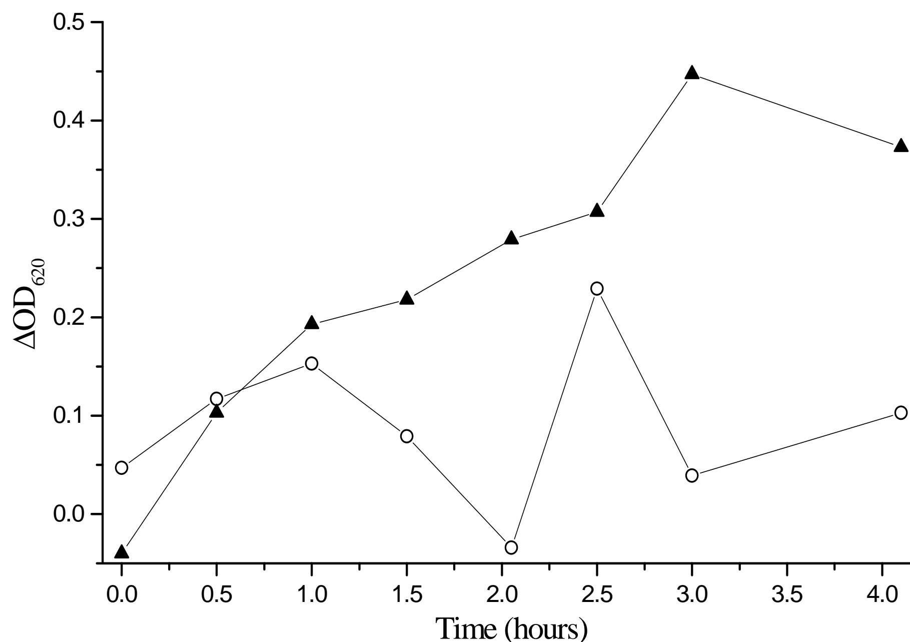


Figure 19. Comparison of recommended Calbiochem assay buffer with current buffer system and phosvitin at 5 mg/mL. Calbiochem buffer (σ) and buffer of Wheldrake et al. (1996) plus Mn^{++} (∀)

3.2.5 Examination of a precipitate problem at the colorimetric stage of the assay

Using freshly prepared buffers and phosvitin solution another assay was conducted to examine more closely the conditions causing the precipitation in the wells during the colorimetric determination of released Pi from the enzyme reaction. Figure 20 shows the PP1 activity in an assay comparing phosvitin in the Calbiochem buffer at concentrations of 1, 5, and 10 mg/mL and at 5 mg/mL in the current modified assay buffer (Section 2.2), omitting the 200 μL of Milli-Q water to represent the sample to be assayed. This had the effect of concentrating the enzyme and the other components of the assay. The concentration of the PP1 enzyme solution was 2 units/mL.

As in the previous section, some of the data did not follow a hyperbolic curve. The assay at 1 mg/mL phosvitin in the Calbiochem buffer was the most promising result achieved so far and this buffer was eventually adopted. It resulted in about 3 times the ΔOD achieved with the modified Wheldrake assay after an incubation time of 3 hours. The low absorbance values for 5 and 10 mg/mL phosvitin in Calbiochem buffer, and some of the irregular data points appeared to be caused by precipitation in the wells which caused large errors in absorbance readings. It seems unlikely that this precipitate was caused by traces of substrate which were not precipitated by the perchloric acid, since the precipitate was not evident at time 0 hours. The precipitate was worse at the higher concentrations of substrate, ie, 5 and 10 mg/mL. Nevertheless, fresh perchloric acid was purchased and the amount used was increased to ensure precipitation of the protein and enzyme and hence stop the reaction.

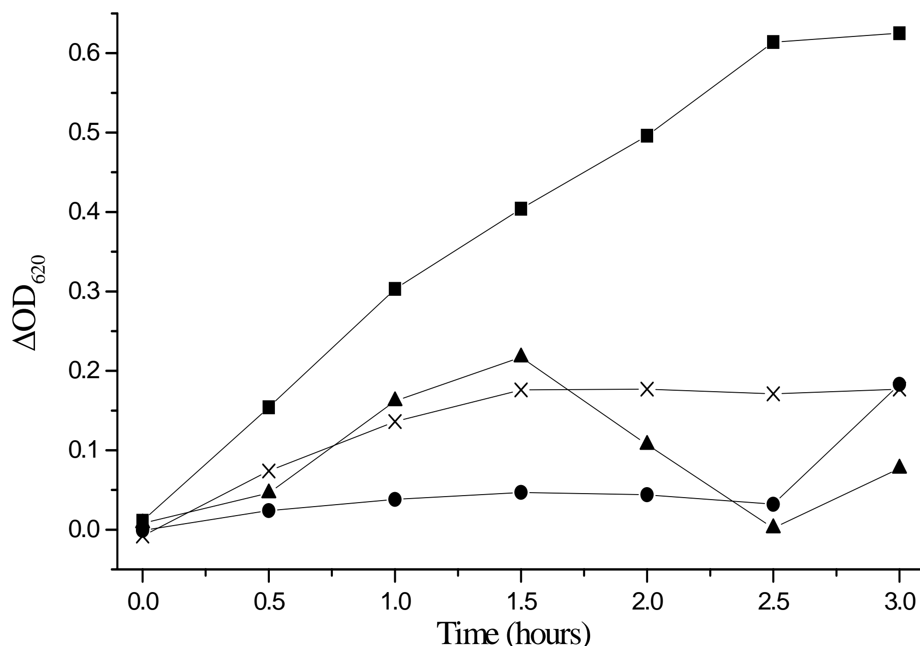


Figure 20. Comparison of Calbiochem buffer with 1, 5, and 10 mg/mL phosvitin and modified buffer of Wheldrake et al. (1996). Calbiochem with 1 mg/mL (#), 5 mg/mL (!) and 10 mg/mL (σ) phosvitin; modified buffer with 5 mg/mL phosvitin (x)

Table 6 details the degree of precipitation in the wells 30 minutes after the assay. This was estimated qualitatively on the basis of no precipitate visible, just perceptible, medium and heavy precipitate. Even though most of the wells at the time of reading had no visible precipitate this rough guide agreed reasonably well with the data in Figure 20.

The main points from this data can be summarised:

- A concentration of 1 mg/mL phosvitin in the Calbiochem buffer produced the lowest blank (data not shown) and the best enzyme activity of the conditions studied. This assay was largely unaffected by the precipitate problems.
- The higher concentrations of substrate resulted in precipitation in the wells at the colorimetric assay stage whether enzyme was present or not. Therefore it was concluded that the presence of high concentrations of phosvitin or some component in the phosvitin solution was contributing to the precipitate. It could not have been phosvitin itself as the perchloric acid should have precipitated out all the protein in the assay tubes, and the precipitate was generally not evident at time 0 hours.
- The assays with enzyme present (+E) contained more precipitate than the blanks (-E). The enzyme is precipitated by the perchloric acid and the blanks still had buffer added in place of buffer+enzyme, so it is not understood why generally these assays contained more precipitate.
- The fact that the modified buffer of Wheldrake et al. (1996) did not produce the same degree of precipitation as the Calbiochem buffer may be explained by the fact that many of the components in this buffer are more dilute than in the Calbiochem buffer.
- The heavy precipitate in the Calbiochem buffer with 10 mg/mL phosvitin accounts for the shape of this data curve in the latter stages of the assay when the absorbance readings decrease presumably due to removal of colour with the precipitate. It is not understood why the precipitate does not increase the reading. These results are the opposite of what is expected. It was difficult to say at this stage whether the graphs at 5 and 10 mg/mL substrate indicate substrate inhibition. The effect of substrate concentration on the enzyme assay will be considered further in Section 3.3.1.

The next aspect of this assay studied was the interaction of phosvitin with each component in the buffer and will be discussed in Section 3.2.6.

Table 6. Visual assessment of the degree of precipitation in the microtitre wells during the colorimetric stage of a comparison of Calbiochem buffer with 1, 5, and 10 mg/mL phosvitin and the latest modified buffer with 5 mg/mL phosvitin

	Calbiochem 1 mg/mL		Calbiochem 5 mg/mL		Calbiochem 10 mg/mL		Modified buffer 5 mg/mL	
Time (hr)	**-E**	**+E**	**-E**	**+E**	**-E**	**+E**	**-E**	**+E**
0					+	+		
0.5		+ (?)	+	++	++	+++		++
1			+	++	++	+++		++
1.5			+	+	++	+++	+	++
2			++	++	+++	+++		
2.5			++	++	+++	+++	+	++
3			++	++(+)	+++	+++	+	++

blank no precipitate visible
+ precipitate just perceptible
++ medium precipitate
+++ heavy precipitate

3.2.6 Resolution of precipitate problem between phosvitin and dithiothreitol

At about the same time the problem of precipitation in the wells during the colorimetric assay was noticed, another problem was encountered when phosvitin was made up in the Calbiochem buffer and the solution stored at -20° C. A green precipitate formed upon freezing and would not redissolve when the solution was thawed. At this stage this precipitate was considered a separate problem from the one described in Section 3.2.5, even though phosvitin was a common factor. By making up a series of buffers each missing one ingredient, the presence of dithiothreitol (DTT) in the buffer was determined to be the cause. A solution of 20 mg/mL phosvitin was made up in 50 mM Tris-HCl, 0.1 mM EDTA, pH 7.1 and to 1 mL of this solution was added 1 mL of each buffer minus a component. The results are detailed in Table 7.

Table 7. Identification of the buffer component interacting with phosvitin or an impurity of phosvitin to produce a precipitate

Phosvitin 20mg/mL	Phosvitin 20mg/mL	Phosvitin 20mg/mL	Phosvitin 20mg/mL
Tris/EDTA	Tris/EDTA	Tris/EDTA	Tris/EDTA
+ DTT	+ DTT	+ DTT	+ BSA
+ $MnCl_2$	+ $MnCl_2$	+ BSA	+ $MnCl_2$
+ BSA	- BSA	- $MnCl_2$	- DTT
Lighter green ppt.	Heavy green ppt.	Lighter green ppt.	No ppt.

The precipitate was not as heavy when BSA was also present in the buffer. Metal analysis of the precipitate by ICP spectroscopy suggested it was probably not phosvitin as a large amount of phosphorus would have been present in the precipitate. As phosvitin is not a pure protein but a protein

fraction of egg yolk, it was therefore concluded that the precipitate may be caused by some other component in the phosvitin.

Two things were changed to try and resolve the problem. The first was to make up the phosvitin in Calbiochem buffer minus dithiothreitol. The second was to decrease the final assay concentration of DTT from 5 mM to 1 mM.

3.3 Optimisation of Calbiochem buffer for PP1 dephosphorylation of phosvitin

The action suggested in Section 3.2.6 resolved the occurrence of the precipitate in the phosvitin stock solution and the precipitate in the colorimetric stage of the assay. The graph of enzyme activity with 1 mg/mL phosvitin in Calbiochem buffer shown in Figure 20 suggested the composition of this buffer was close to a system that could be used successfully with phosvitin as a substrate. The next series of experiments optimised the concentration of substrate, DTT, $MnCl_2$ and pH of the buffer to maximise enzyme activity and make the assay cheaper. These modifications allowed much smaller quantities of PP1 enzyme to be used at a cost of $0.5-1 per assay. Table 8 details the buffer components and their role in the assay.

Table 8. Application of buffer components of final assay

REAGENT	APPLICATION
Tris-HCl (2-amino-2-hydroxymethyl-1,3-propanediol HCl)	Buffering agent in incubation mixtures, pKa value: 8.1 at 25°C, pH range 7.0-9.0 Excellent biochemical buffer that maintains solubility of Ca and Mn salts.
EDTA (ethylene diaminetetra-acetic acid)	Complexing or chelating agent for divalent cations, can be used to block Ca^{++} dependent protein kinases and phosphatases (eg, 2B)
DTT (1,4-dithiothreitol)	Protective reagent for sulphydryl groups, maintains monothiols in the reduced state; also reduces disulphides. Its antioxidant activity stabilises enzymes containing sulphydryl groups, keeping the enzyme in a reduced state. May also restore enzyme activity lost by oxidation of sulphydryl group. As this compound has little odour and little tendency to be oxidised directly by air, it is superior to other thiols like β-mercaptoethanol as a protective agent.
$MnCl_2$	Zhang et al. (1992) report that recombinant PP1 enzyme requires the presence of Mn^{++} for full expression of its activity, unlike the enzyme purified from rabbit skeletal muscle.
BSA (bovine serum albumin)	Stabilises proteins and enzymes against proteolysis and denaturation, especially useful if enzyme is present in low concentration. Can minimise loss of enzyme on glass surfaces. Stops protein-protein interactions with the enzyme; seems to make enzyme preparations work better. Can also act as a carrier protein to ensure precipitation with acid works.

3.3.1 Optimisation of substrate concentration for maximum PP1 activity

Following the results obtained in Section 3.2.5 the effect of varying the substrate concentration was examined because Geladopoulos et al. (1996) also observed substrate inhibition of a protein phosphatase isolated from rat brain by substrate concentrations higher than 0.5 mg/mL in their assay. 0.25, 1 and 2.5 mg/mL phosvitin were compared to determine whether the optimum substrate concentration was significantly different from 1 mg/mL. Experiments described in Section 3.2.5

suggested 5 and 10 mg/mL substrate may inhibit activity of recombinant PP1. Figure 21 illustrates the comparison of the three substrate concentrations.

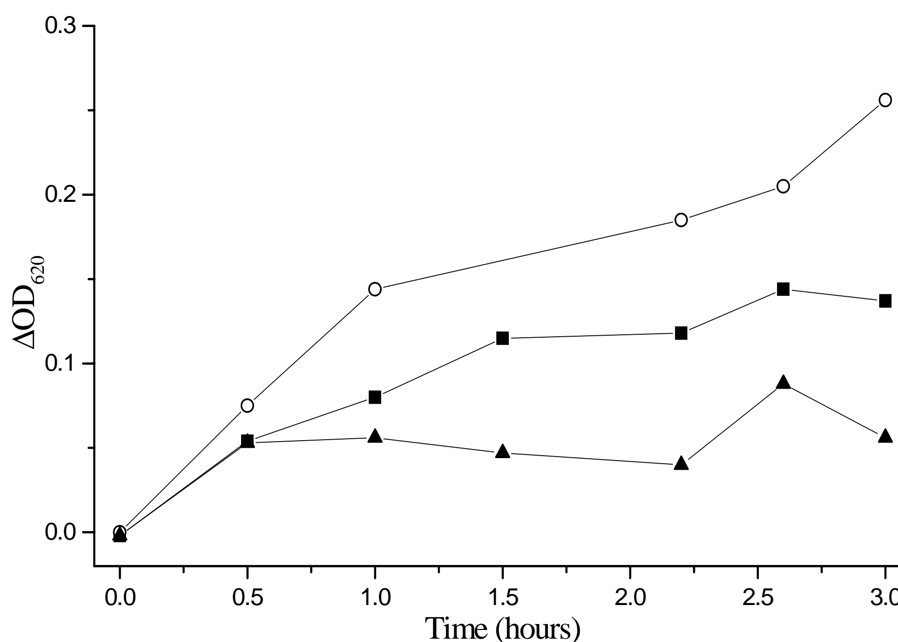


Figure 21. Optimisation of substrate concentration on PP1 activity towards phosvitin at 0.25 mg/mL (#), 1 mg/mL (∀) and 2.5 mg/mL (σ)

The data demonstrate that non-optimal levels of phosvitin significantly decreased the activity of PP1 enzyme. Both 0.25 and 2.5 mg/mL phosvitin showed poor enzyme activity compared with 1 mg/mL. 2.5 mg/mL in particular, produced much higher absorbances for the blank assays (data not presented). It was therefore concluded that 1 mg/mL is the optimum concentration for this assay. It should be noted that the last two data points were taken when the volume in the assay tubes was getting very low and may therefore be inaccurate. The maximum absorbance achieved at 1 mg/mL is also considerably lower than that obtained in previous experiments with this substrate concentration (see Figure 20). A variation in absorbance or enzyme activity between assays had been noticed for some time. Not all data sets are directly comparable because the quantity of enzyme was varied at times to save on the cost of the analysis. However, it did appear that new batches of enzyme produced more activity. This will be discussed in more detail in the next section.

3.3.2 Effect of DTT concentration on PP1 activity

The effect of varying the concentration of dithiothreitol (DTT) on the activity of the enzyme was examined by comparing five concentrations of DTT, expressed as final assay concentration: 0.1, 0.5, 1, 2 and 3 mM. All buffers were freshly prepared. 10 units/mL PP1 stock was used so that small differences in activity would be observed. The results are presented in Figure 22. The inset represents the absorbance after 2.3 hours versus DTT concentration.

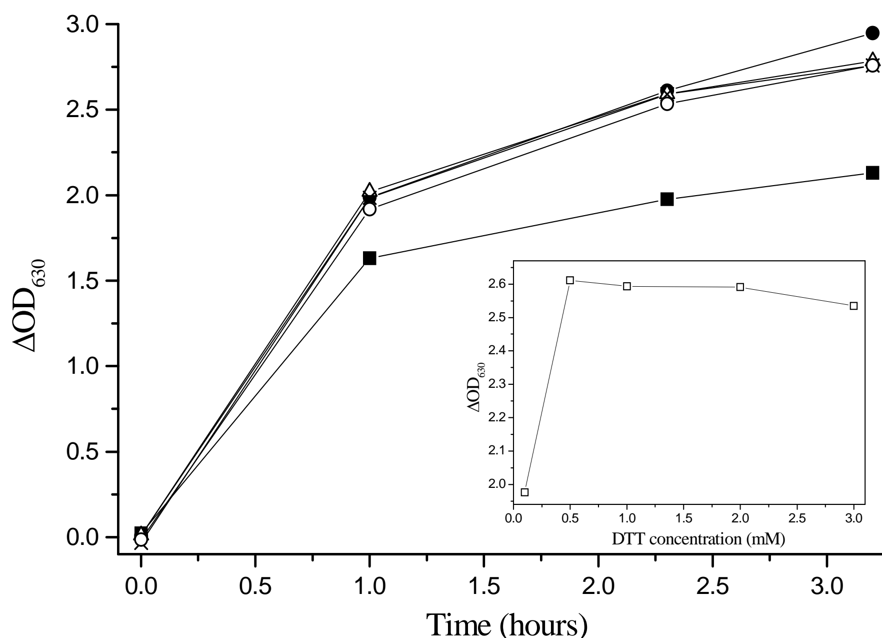


Figure 22. Effect of varying DTT concentration on the activity of PP1 towards phosvitin (0.1 mM (#), 0.5 mM (!), 1 mM (Δ), 2 mM (x) and 3 mM (∀) DTT)

The absorbances obtained were higher than expected even though a new batch of enzyme was used for this assay. Later work indicated that components such as dithiothreitol which are volatile must be added fresh to the buffer just before the assay in order to maximise enzyme activity. Other laboratories make up stock solutions of DTT and store them at -20°C (A. Bass, AWT EnSight, personal communication). The inset illustrates that levels of 0.5-3 mM DTT all produce similar levels of enzyme activity. There is very little difference between these concentrations at 1 and 2.3 hours. 0.1 mM DTT is clearly below the concentration for maximum response. The choice of 1 mM DTT is therefore an appropriate level for this component. This provides an excess of DTT and degradation of DTT may account for some of the variability in absorbance observed at times with older buffers when the DTT may have decreased to below optimum concentration. The level of 5 mM DTT originally proposed in the Calbiochem buffer provides a large excess of this component and is probably unnecessary. Given the problems of precipitation discussed in Sections 3.2.5 and 3.2.6, it was decided that 1mM DTT provides an excess of this component and avoids the precipitation problems encountered earlier.

3.3.3 Comparison of PP1 activity in old and freshly prepared buffer

DTT is a necessary component in enzyme assays in that it stabilises the enzyme against oxidation (Table 8, Section 3.3). The activity of PP1 enzyme was compared in two buffers, one which had been freshly prepared and one about a month old. The older buffer had been stored in the fridge at 4°C and had only been removed from the fridge for very short periods of time. Figure 23 illustrates the difference in activity of PP1 with phosvitin in these two buffers. The fresh buffer was clearly superior to the older one, and this was probably due to the DTT in the older buffer oxidising and not being available to keep the enzyme in a reduced state during the assay. This could have been checked by adding DTT to the old buffer and determining whether enzyme activity increased. As an added precaution, both BSA and DTT were prepared as concentrated stock solutions which were stored in the freezer at -20°C. These reagents were added to the buffer on the morning of the assay and all solutions were kept on ice until the assay was started.

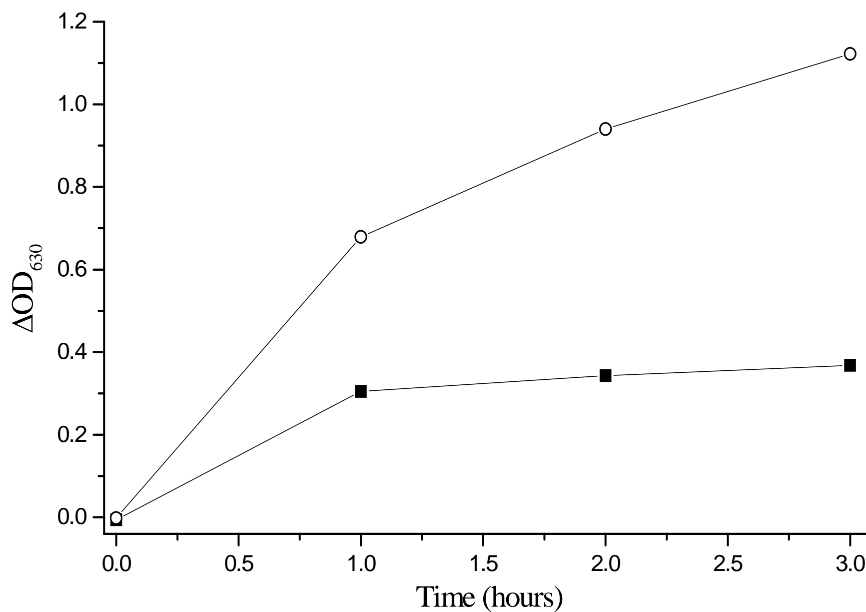


Figure 23. Comparison of PP1 activity towards phosvitin in a freshly prepared buffer (∀) and an older buffer (#)

3.3.4 Effect of manganese ion concentration on PP1 activity

As discussed in Section 3.2.2 recombinant PP1 requires the presence of manganese ion (Mn^{++}) for full expression of its activity (Zhang et al. 1992). Initially 0.2 mM Mn^{++} was selected for the final assay concentration based on the results of their work in which they determined a range of 0.06-0.2 mM Mn^{++} to be optimum for PP1. They found higher concentrations between 0.2 and 1 mM Mn^{++} inhibited the activity of the enzyme.

The concentration of manganese ion (Mn^{++}) was varied in the buffer and the activity of PP1 measured at a range of levels (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 mM Mn^{++}). The graphed data presented in Figure 24 is similar in shape to that obtained by Zhang et al. (1992) although the concentration of Mn^{++} required for optimum enzyme activity differs. They measured enzyme activity with respect to ^{32}P-phosphorylase a and *p*-nitrophenyl phosphate however, only the data for ^{32}P-phosphorylase a was presented. Based on the data graphed in Figure 24, a concentration of 0.5 mM Mn^{++} in the final assay was chosen as the optimum level for maximum activity of Calbiochem PP1 towards phosvitin. Concentrations of Mn^{++} over 1 mM began to inhibit enzyme activity. A final assay concentration of 4 mM was also tried but it was found to precipitate the BSA in the enzyme buffer. The inhibitory effect of higher concentrations of Mn^{++} may be due to undesirable complexing of the metal ion with phosvitin. Taborsky (1974) mentions that phosphoproteins are good metal-complexing agents and proteins such as phosvitin can be precipitated as a Mg^{++} complex. It is also possible that metal ions have a similar effect on enzyme proteins at higher concentrations.

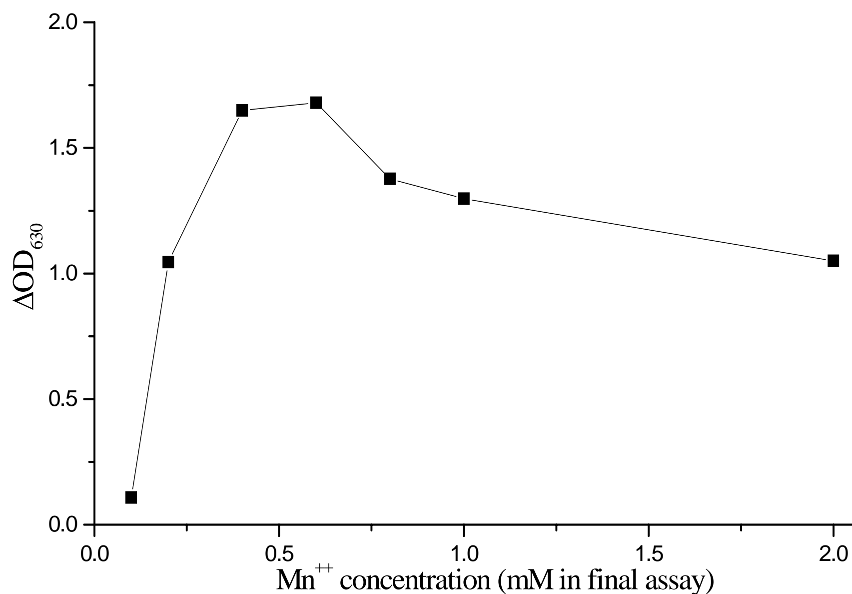


Figure 24. Activity of PP1 towards phosvitin at various levels of Mn^{++}

3.3.5 Effect of buffer pH on PP1 assay

To complete the work on optimisation of the buffer and reaction conditions of PP1 dephosphorylation of phosvitin, the pH of the buffers was varied to determine what effect this parameter had on PP1 activity. The enzyme and substrate buffers were made up at the following pH: 7.1, 7.5, 8.0, 8.5 and 9.0. BSA and DTT were added from stock solutions prior to the assay. Normally Mn^{++} is added to the Tris/EDTA buffer however, it started to precipitate out at the higher pH values so for the purpose of this study it was added into the assay mixture just before the reaction was started. The reaction mixture also contained 100 μL Milli-Q water instead of environmental sample or toxin standard so dilution would hopefully prevent precipitation of manganese. The experimental protocol is detailed in Figure 26.

The enzyme activity at these pH values is graphed in Figure 25. The maximum PP1 activity achieved was at pH 7.5. The activity at pHs 7.1, 8.0 and 8.5 were reasonably similar however, the blank values (assays without enzyme) increased as pH increased (data not shown). Under alkaline conditions, phosphoproteins such as phosvitin lose protein bound phosphate by a β-elimination hydrolysis (Taborsky 1974).

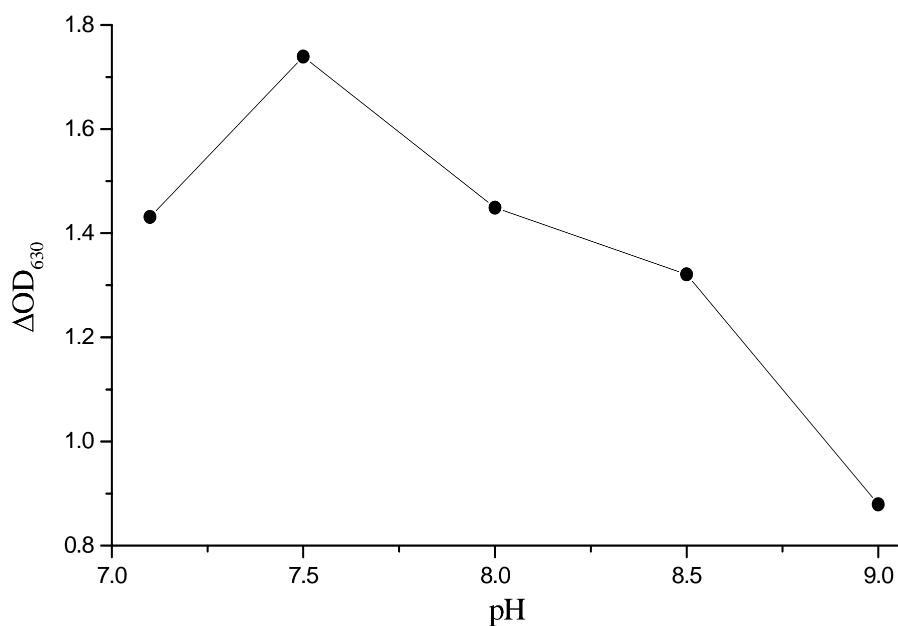
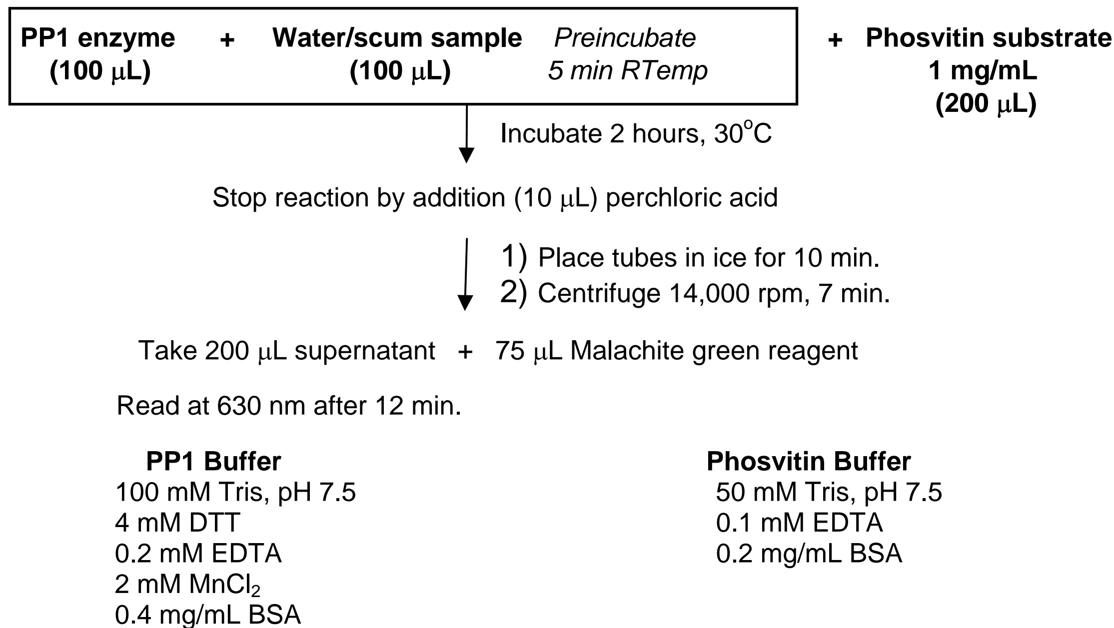


Figure 25. Enzyme activity of PP1 towards phosvitin versus pH of assay

3.3.6 Protocol of fully optimised PP1 assay for dephosphorylation of phosvitin

The final protocol for the phosphatase assay after the modifications described in Sections 3.2 and 3.3 were incorporated as outlined in Figure 26.

The final assay concentration of the buffer components is as follows.
50 mM Tris, pH 7.5
1 mM DTT
0.1 mM EDTA.
0.5 mM $MnCl_2$
0.2 mg/mL BSA



BSA and DTT are added just prior to the assay.

Principle of the assay: Protein phosphatases can use phosvitin, a highly phosphorylated protein as a substrate to produce free phosphate which can then be detected colorimetrically. If microcystin is present in the sample to be analysed, it will inhibit PP1, thus reducing the degree of hydrolysis of the substrate, ie, less phosphate is produced. The amount of phosphate produced is therefore inversely proportional to the concentration of microcystin present. From a standard curve of microcystin concentration versus absorbance, the quantity of microcystin in samples of water or scum can be determined.

Figure 26. New PP1 assay using modified Calbiochem buffer

The above concentrations can be compared with the original buffer recommended by Calbiochem for their recombinant PP1 and the final assay concentration of the buffer reagents in the assay of Wheldrake et al. (1996).

Calbiochem buffer	Wheldrake et al. buffer
50 mM Tris-HCl	29 mM Tris-HCl
5 mM DTT	0.06 mM DTT
0.1 mM EDTA	0.06 mM EGTA
0.2 mM $MnCl_2$	0.35 mM imidazole
0.2 mg/mL BSA	0.12 mM theophylline
pH 7.0	3% glycerol
	pH ~ 7.3

Table 8 (Section 3.3) details the purpose of each reagent in the optimised buffer. EDTA and EGTA are both chelating agents. EGTA is more selective for Ca^{++} (Merck Index) and was chosen for its potential to inhibit Ca-dependent phosphatases that may be present in the phosvitin (J. F. Wheldrake, Flinders University, personal communication). As EDTA can chelate Mg^{++} and Ca^{++} and the buffer recommended by Calbiochem includes this reagent, EDTA was chosen for the phosvitin assay also.

50 mM Tris was selected as the standard buffer concentration as other assays also use this level (An and Carmichael 1994, MacKintosh 1993, Ward et al. 1997).

The concentration of DTT in the Calbiochem buffer is well in excess of what the enzyme will require. After considering the data presented in Section 3.3.2, the concentration of 1mM chosen is still in excess and avoids the problems with precipitation described in Sections 3.2.5 and 3.2.6. The lower

level of 0.06 mM in the assay developed by Wheldrake et al. (1996) may partly explain the low enzyme activity in this buffer. Some assays incorporate β-mercaptoethanol as an antioxidant (MacKintosh 1993, Ward et al. 1997) although An and Carmichael (1994) use DTT. It was decided to continue using DTT once the precipitation problems had been resolved as this reagent is a solid which is not as volatile and irritating to the eyes and skin as β-mercaptoethanol.

The concentration of Mn^{++} was optimised for this assay and the requirement for this metal ion with recombinant PP1 has been well documented (An and Carmichael 1994, Zhang et al. 1992). BSA was included in the assay to stabilise the enzyme against proteolysis and denaturation. Many other published protein phosphatase assays include this reagent (An and Carmichael 1994, MacKintosh 1993, Sim and Mudge 1993, Ward et al. 1997 and Zhang et al. 1992).

Imidazole was originally included in the assay of Wheldrake et al. (1996) to extend the buffering range. Tris alone was not considered suitable for pHs below 7.5 (J. F. Wheldrake, Flinders University, personal communication). Tris buffers made up from combinations of Trizma HCl and Trizma base are recommended for the pH range 7.0 to 9.1 (Sigma Technical Bulletin No. 106B, Sigma-Aldrich). As the optimum pH for the assay was determined as 7.5, it was decided not to complicate things by adding another variable. Glycerol is added to enzyme solutions as an anti-freeze and to maintain osmotic balance (A. Humpage, University of Adelaide, personal communication). The enzyme solution sent from Calbiochem contains 50% glycerol and freeze/thaw cycles are to be avoided. Given that the assay was conducted at uniform temperature and the assay buffer recommended by Calbiochem does not contain glycerol, it was not considered necessary for this assay. Theophylline is a reagent similar to caffeine and is used to maintain glycogen phosphorylase a in solution (A. Sim, University of Newcastle, personal communication). The effect of glycerol, theophylline and imidazole on the assay was not considered due to lack of time. Once the Calbiochem buffer was optimised to detect low levels of enzyme activity, the inhibition of PP1 by microcystins and nodularin was examined. These results are discussed in Section 3.4.

Figure 27 shows the progress of the enzyme reaction at two levels of PP1. The change in absorbance is an indication of utilisation of substrate or measurement of product, i.e., increase in released Pi. As the reaction proceeds and substrate is consumed, the slope of the curve decreases. However, the fact that the curve for the lower quantity of enzyme levelled out so soon means that the enzyme may have denatured. An incubation time of two hours was selected for the inhibition assay to maximise the absorbance, but still remain in the section of the curve where the reaction proceeded rapidly. This is essentially a trade-off between time, sensitivity and reproducibility.

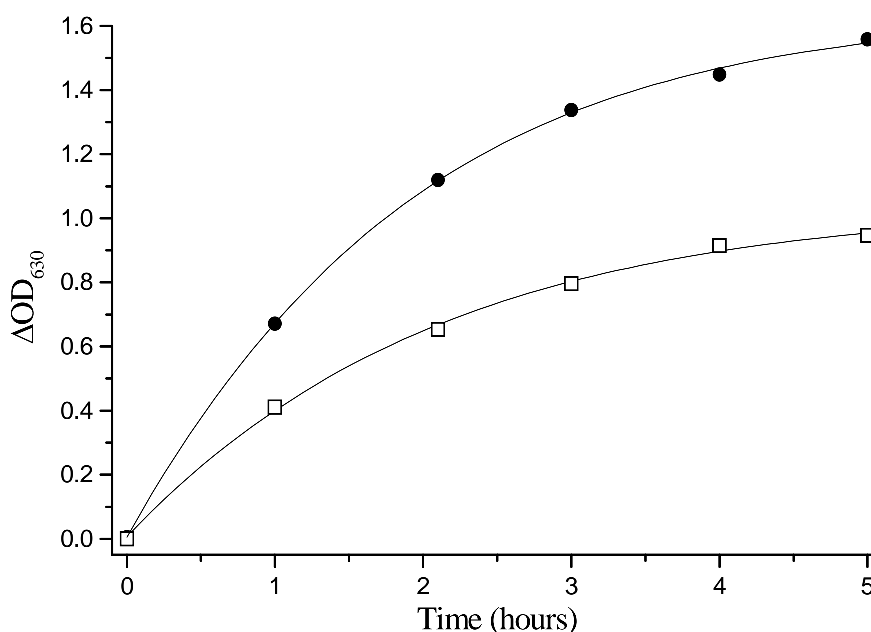


Figure 27. Kinetic assay of PP1 dephosphorylation of phosvitin. Product formation as a function of time at two levels of PP1 (0.5 unit/mL PP1 stock (Θ) and 1.0 unit/mL PP1 stock(!))

3.4 Inhibition of PP1 by hepatotoxins

As discussed in Section 3.3.6 hepatotoxin concentration is measured by the degree of inhibition of PP1 enzyme. A series of solutions containing toxin, eg, microcystin, are preincubated with PP1 enzyme at room temperature for 5 minutes. The time necessary for this incubation is not discussed in detail in many publications and Wheldrake et al. (1996) did not mention it at all, so the conditions of An and Carmichael (1994) were adopted. A plot of inhibitor concentration versus degree of inhibition of enzyme produced an inhibition curve with sigmoidal shape which can provide information that can be used to compare different assays. Section 3.4.1 discusses microcystin inhibition curves in more detail. Table 9 is a summary of IC_{50} values for protein phosphatase 1 and 2A with several substrates reported in a range of publications. These values can be used to compare sensitivity of the different assays.

Table 9. Summary of IC_{50} values for hepatotoxins towards PP1 and PP2A with a range of substrates

Reference	Substrate	IC_{50} PP1 (nM)*	IC_{50} PP2A (nM)*
An and Carmichael (1994)	*p*NPP	mLR 0.3 [DMAdda^5] mLR 1.5 [Dha^7] mLR 5 [DMAdda^3] Nod 5	-
Ash et al. (1995a)	*p*NPP	0.5	18
Ash et al. (1995b)	^{32}P-phosphorylase a	0.5	0.006
AWT Sydney (A. Bass, personal communication)	*p*NPP	-	0.04
Chaivimol et al. (1993)	Phosphorylase a	Mouse liver homogenate mLR 2.4 mRR 2.8 Nod 2.3	
Craig et al. (1993)	^{32}P-phosphorylase a	mLR 0.15 motuporin <0.1 7 new hydrophobic microcystins 0.06-0.4	
Eriksson et al. (1990)	^{32}P-myosin light chain	mLR 6 mRR 3 Nod 3	2 1 1
Heresztyn and Nicholson (this work)	Phosvitin	mLR 0.2 ng/mL mYR 0.5 ″ mRR 0.2 ″ Nod 0.2 ″	
Honkanen et al. (1990)	^{32}P-phosphorylase a ^{32}P-histone	1.7	0.04
Honkanen et al. (1994)	Phosphohistone Phosphorylase a	mLR 2.03 mLA 2.01 Nod 2.37 mLR 1.9 mLA 2.3 Nod 1.9	0.04 0.04 0.03 0.05 0.05 0.03
Lambert et al. (1994)	^{32}P-phosphorylase a	3-5 ng/mL	-
MacKintosh et al. (1990)	^{32}P-glycogen phosphorylase	0.13	>4 μM
MacKintosh (1993)	^{32}P-glycogen phosphorylase	0.1	0.1
Mez et al. (1996)	^{33}P-phosphorylase a	Rape seed protein phosphatases IC_{50} mLR = 0.25	
Nishiwaki-Matsushima et al. (1991)	^{32}P-histone H1		Mouse brain PP2A mLR 0.28 mRR 0.78 [6(Z)-Adda] mLR 80 [6(Z)-Adda] mRR 80

Table 9 continued

Reference	Substrate	IC_{50} PP1 (nM)*	IC_{50} PP2A (nM)*
Nishiwaki-Matsushima et al. (1992)		mLA 0.44 mLR 0.9	mLA 0.32 mLR 0.38
Sim and Mudge (1993)	^{32}P-glycogen phosphorylase a	Crude chicken brain extracts IC_{50} mLR = 0.33	
Suganuma et al. (1992)	^{32}P-phosphorylase a	0.1	0.1
Sugiyama et al (1996)	luciferin phosphate *p*NPP	mLR 1 (PP1γ) mLR 1	
Taylor et al. (1996)	^{32}P-phosphorylase a		mLR 0.2 synthetic peptides 1-10 mM
Ward et al. (1997)	*p*NPP	38 ng/mL	-
Wheldrake et al. (1996)	Phosvitin	Rabbit muscle extract mLR 0.7-2.6	
Yoshizawa et al. (1990)	^{32}P-histone H1	Mouse liver extract mLR 1.6 mYR 1.4 mRR 3.4 Nod 0.7	

* literature values are in nM hepatotoxin unless indicated otherwise

NB It is assumed these IC_{50} values refer to the concentration of microcystin causing 50% inhibition of PP1 in the assay, and not in the stock solution.

The data in Table 9 indicate that hepatotoxins can be detected at high levels of sensitivity by protein phosphatase inhibition assays using a variety of substrates. The work of Honkanen et al. (1994) and Ash et al. (1995) suggests PP2A may be 10-100x more sensitive to inhibition by microcystin than PP1. Takai and Mieskes (1991) found PP2A also showed a much higher level of activity towards *p*NPP and phosphorylated myosin light-chain than PP1. This was also the case for the inhibitor okadaic acid and has been used as one of the biochemical criteria to distinguish PP1 and PP2A (Table 1). PP2A has not yet been successfully cloned into *E. coli* and has only recently been commercially available (Promega Corporation) which may explain why few people have used it. Wayne Carmichael (Wright State University, personal communication) prefers to use recombinant PP1 as he has found a large degree of variability in the sensitivity of PP2A (personal communication). AWT in Sydney claim a very low IC_{50} with PP2A purchased from Promega, however large variations in enzyme sensitivity have been reported (J. Cao, AWT EnSight, personal communication).

Work from Carol MacKintosh's laboratory over a number of years suggests large variations in IC_{50}s for PP2A (Ash et al. 1995, MacKintosh 1993, MacKintosh et al. 1990) and may reflect improved techniques for isolating and purifying the enzymes or new buffer conditions. Ash et al. (1995) suggest enzymes from different sources will have different sensitivity to toxins. They found rabbit skeletal PP1 was not active towards *p*NPP unlike recombinant PP1, and suggested this was probably due to the enzymes being folded differently.

The *p*NPP assay recently modified in Geoff Codd's laboratory (Ward et al. 1997) still has a fairly high IC_{50} however some of the variations in IC_{50} values quoted in Table 9 may be due to the way in which they have been calculated. This is discussed further in Section 3.4.1.

There does not appear to be any great advantage in using a radiolabelled assay for greater sensitivity. In fact some of the assays using ^{32}P-glycogen phosphorylase a (Chaivimol et al. 1993, Honkanen et al. 1994, and Lambert et al. 1994) appear to be less sensitive than the *p*NPP assay.

3.4.1 Microcystin-LR inhibition of PP1 activity towards phosvitin

A standard curve of log_{10} microcystin concentration (ng/mL) is plotted against % of PP1 activity, where PP1 activity is defined as the degree of colour production at the various concentrations of microcystin divided by the degree of colour production when no microcystin is present (control). The IC_{50} is defined as the concentration of microcystin that causes 50% inhibition of PP1 enzyme. Blank absorbances (assay containing sample plus substrate, minus enzyme) are subtracted from sample

assays. In the graph shown in Figure 28, a stock solution of PP1 at 1 unit/mL was used in the assay. The IC_{50} is 1.7 ng/mL and the working range of the assay (ie, that area of the graph which is linear) is between 0.7 and 3 ng/mL mLR. Therefore the limit of detection of this particular assay was 0.7 ng/mL. These concentrations of microcystin quoted refer to the amount of toxin in a sample and not in the assay itself. The IC_{50} values can vary depending on the quantity of enzyme used (Section 3.4.2) and small differences were also noted between assays carried out on different days with the same quantity of enzyme. Other laboratories have also noted fluctuations in the position of the inhibition curve (J. Cao, AWT EnSight, personal communication).

It should be noted that the IC_{50} value quoted by An and Carmichael (1994) refers to the concentration of hepatotoxin in the assay well, since the toxin standards and samples for assay are diluted by the enzyme and substrate (W. Carmichael, Wright State University, personal communication). In the assay protocol described in this report the solution to be analysed for toxin is diluted 1:4 by the other components in the assay. Therefore the IC_{50} and limit of detection (LOD) for the assay in Figure 28 was 0.4 and 0.2 ng/mL respectively. It is assumed values quoted in Table 9 are the IC_{50}s in the assay itself. However, most literature references do not make it clear whether they are quoting microcystin concentration in the assay or the sample solution.

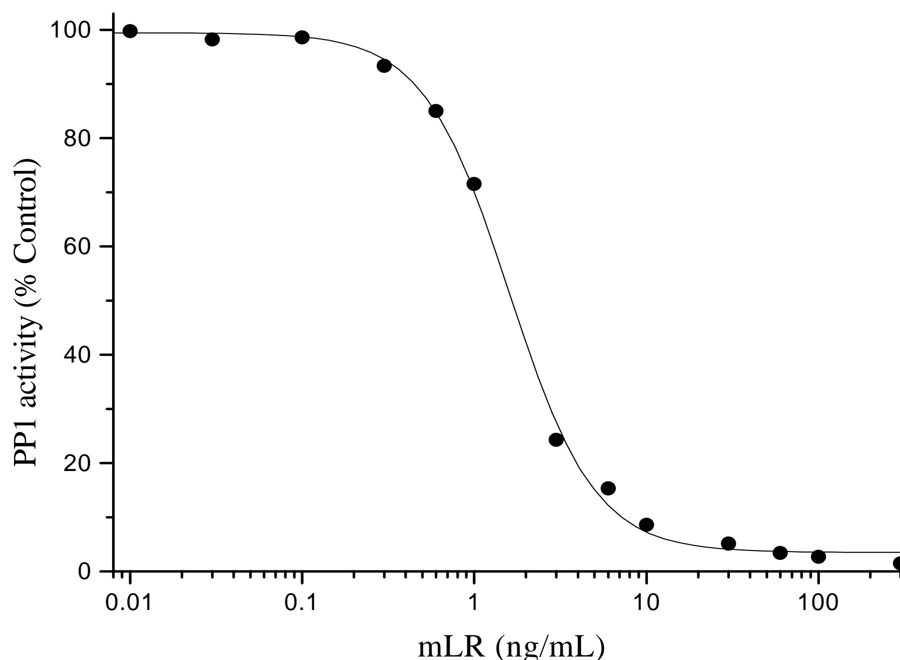


Figure 28. Inhibition of PP 1 activity by mLR (ng/mL)

3.4.2 Effect of different levels of PP1 on mLR inhibition curve

The effect of varying concentrations of PP1 on the inhibition curve was examined with a fairly broad range of enzyme solutions: 0.5, 1, 2 and 5 units/mL stock. The results are presented in Figure 29 and are graphed as absorbance at 630 nm versus log mLR concentration (a) and PP1 activity versus log mLR concentration (b) for the same assay. As expected, graph (a) shows that higher levels of enzyme produce a higher concentration of released Pi from the substrate which is reflected in higher absorbance values. When the data are graphed as PP1 activity (b), the curves are more directly comparable because they are normalised. The reason some of the values are greater than 100%, particularly at 5 units/mL PP1 is that the absorbance at very low levels of mLR was actually a little higher than in the absence of toxin. This seems to happen in some assays and not others and will be discussed further in the replicate study (Section 3.4.3).

It is apparent from Figure 29 that even at low concentrations of enzyme, a relatively flat absorbance curve still results in a symmetrical sigmoidal plot of enzyme activity versus inhibitor concentration. As

the concentration of enzyme is increased the curves progressively shift to the right because more toxin is needed to react with the enzyme. This is characteristic of inhibitors with a high affinity for the enzyme where the inhibitor and enzyme are present in similar quantities (Dixon and Webb, 1964). This translates into a higher IC_{50} value and therefore a less sensitive assay. A second feature of these graphs is that lower PP1 levels result in a flatter inhibition curve which actually extends the quantitative range of the assay as well as producing a lower IC_{50} or more sensitive assay. This is consistent with results of other groups who describe the true IC_{50} of an assay as being that level at which the curve no longer shifts to the left with decreasing quantities of enzyme (An and Carmichael 1994, Honkanen et al. 1990, and MacKintosh et al. 1990). Honkanen et al. (1990) found that inhibition of PP1 and PP2A occurred at concentrations slightly lower than the molar concentration of mLR in the assay.

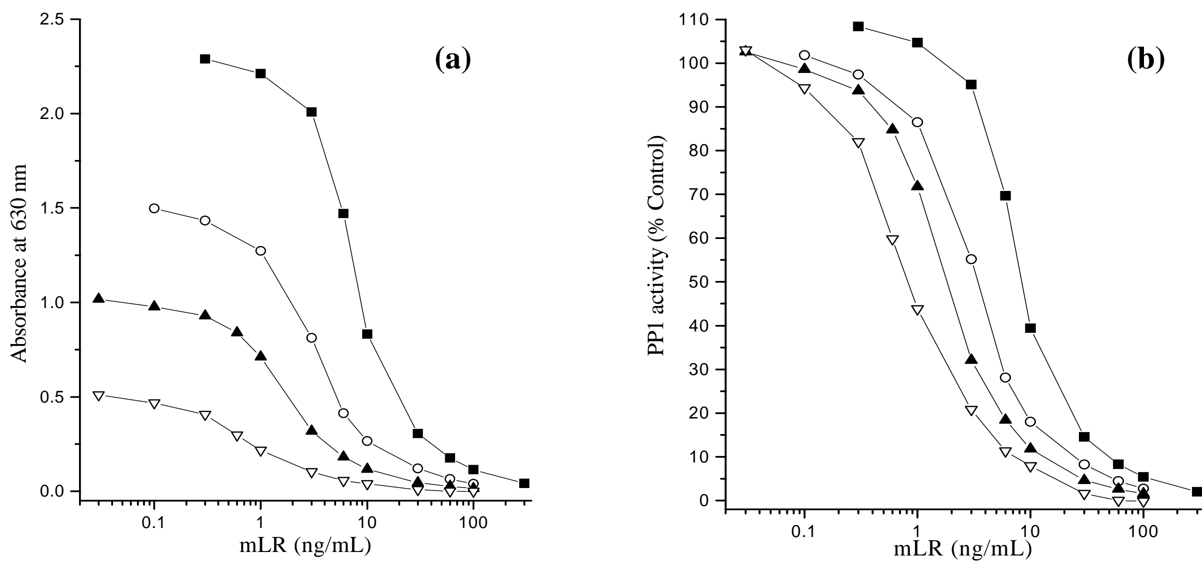


Figure 29. Comparison of PP1 inhibition by mLR at several concentrations of PP1 expressed as units/mL stock solution with phosvitin as substrate: 0.5 unit/mL (∇), 1 unit/mL (σ), 2 units/mL (∀) and 5 units/mL (#)

Dixon and Webb (1964) also discuss the effect of pH on inhibitors of enzymes. They suggest the reaction of inhibitors with enzymes can be affected by pH which can alter the ionic form of the enzyme and hence its rate of reaction. Also, the ionic form of the inhibitor may be dependent on pH. This had not been considered in this body of work, although other groups have chosen similar pHs for the enzyme-microcystin reaction. An and Carmichael (1994) chose pH 7.4, and Ash et al. (1995) used a buffer at pH 7.5 for their enzymes.

3.4.3 Replicate studies on mLR inhibition of PP1

Microcystin-LR inhibition of PP1 was studied at four enzyme concentrations (0.1, 0.25, 0.5 and 1.0 unit/mL PP1 solution) in replicates of four to determine the degree of error in the assay particularly at low PP1 levels. The mLR concentrations chosen were: 0, 0.01, 0.03, 0.1, 0.6, 1, 2, 3, 6, 10, 30, 60, 100 and 300 ng/mL. Figure 30 shows the data of the mean of four replicates graphed as absorbance versus mLR concentration (a) and as % activity of the Control (b).

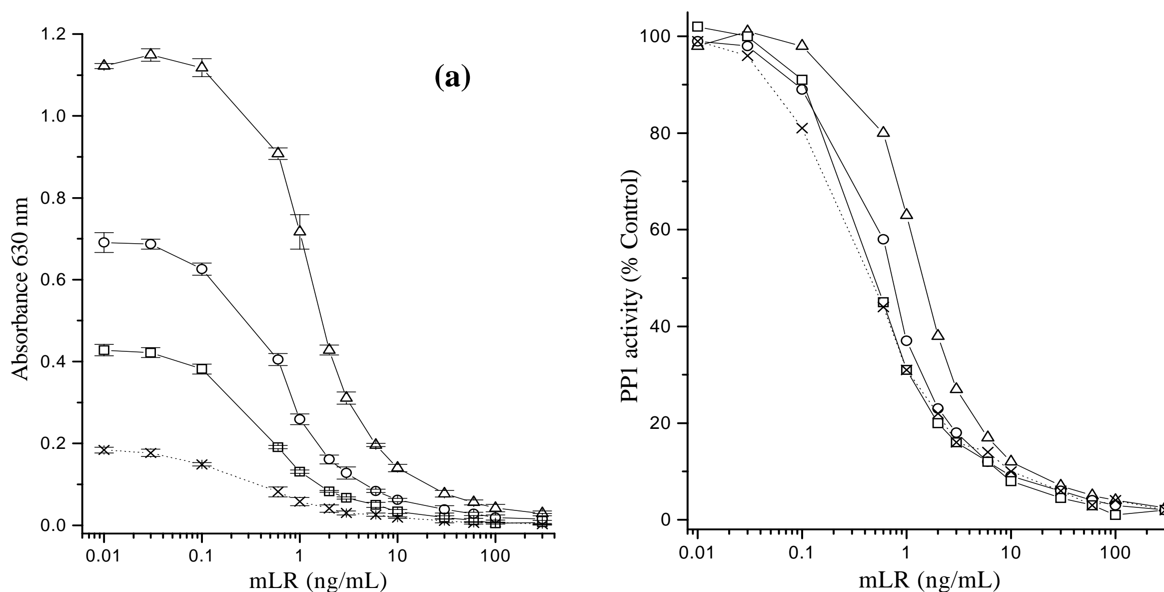


Figure 30. Microcystin-LR inhibition of PP1 (average of four replicates) at four concentrations of enzyme. Absorbance at 630 nm versus mLR concentration (a) and PP1 activity versus mLR concentration (b). PP1 solution at 0.1 unit/mL (x), 0.25 unit/mL (Θ), 0.5 unit/mL (∀) and 1.0 unit/mL (Δ)

The graphs are similar to those discussed in Section 3.4.2 in that lower concentrations of enzyme result in sigmoidal inhibition curves with lower IC_{50}s. However, this set of data includes two lower concentrations of PP1 (0.1 and 0.25 unit/mL PP1 stock solution). Graph (b) illustrates that the IC_{50} at these two levels of PP1 are essentially the same and are very close to the IC_{50} at 0.5 unit/mL PP1. Table 10 details the IC_{50}s and working range of the assay at each of these PP1 concentrations. The true IC_{50} of this assay is about 0.5 ng/mL mLR and the limit of detection (LOD) at the lower levels of PP1 is around 0.1 ng/mL mLR. As the inhibition curves can shift a little from day to day, the IC_{50} and LOD values for the assay need to be quoted from the data generated on the day the assay is conducted. It is also apparent from the data in Table 10 that the working range of the assay is wider at the lower levels of PP1 due to the flatter sigmoid curve.

Table 10. IC_{50}s and quantitation range of mLR inhibition assay at four PP1 concentrations (average of four replicates)

PP1 solution (unit/mL)	IC_{50} (ng/mL mLR in toxin soln.)	Quantitation range of assay (ng/mL)
0.1	0.45	0.1 - 2
0.25	0.5	0.15 - 1.8
0.5	0.7	0.2 - 2.2
1.0	1.4	0.6 - 4

Table 11 details statistical analysis of the four replicates at each concentration of PP1. The shaded areas represent the linear part of the inhibition curve used for quantitation. The % error with 0.1 unit/mL PP1 is clearly much higher than at the other enzyme concentrations. At 0.25 unit/mL PP1 the coefficient of variation is over 5% for almost all the data in the linear range of the curve. This is not surprising given that the absorbance range for the two lowest PP1 concentrations are 0.187 and 0.423 respectively. Small changes in absorbance due to experimental error are therefore translated into quite large percentage error in quantitation of mLR. The choice of concentration of enzyme to be used in the assay is therefore a trade-off between sensitivity and precision. 0.5 unit/mL PP1 was chosen as

the preferred enzyme concentration that would produce a standard curve with a sufficiently low IC_{50} and an acceptable percentage error.

3.4.4 Influence of methanol on PP1 inhibition of mLR

As stated earlier, high levels of inorganic phosphate (Pi) have the potential to interfere with the assay and produce high background readings in the colorimetric stage of the assay. Appendix 1 contains 5 yearly averages of some relevant parameters collated by the Australian Water Quality Centre on raw waters supplying the Metropolitan Adelaide water supply, and data collated from South Australian sewage treatment plants on effluent waters.

Samples from wastewater lagoons and concentrated algal extracts are possibly the only samples likely to contain levels of Pi that need to be removed (Appendix 1). These types of samples also need to be cleaned up and concentrated by C18 Sep-Pak extraction for HPLC analysis (Section 2.5). This procedure will serve the dual purpose of preparing the samples for HPLC analysis and removing interfering Pi for the phosphatase assay.

Samples put through the C18 cleanup at AWQC are in a final solution of 50% methanol. However, it was not known what effect this level of methanol would have on this protein phosphatase inhibition assay. Ward et al. (1997) extracted microcystins from cyanobacteria using varying concentrations of methanol (0-100% in 10% increments) and examined the efficiency of extraction of four microcystins (microcystin-LR, LY, LW, LF). They also determined the effect of methanol concentration on PP1 activity towards *p*NPP and found that methanol concentrations between 10 and 80% (v/v) did not significantly reduce the activity of PP1 to their substrate compared with controls of enzyme buffer. A loss of enzyme activity occurred at 80, 90 and 100% methanol.

The inhibition of PP1 using phosvitin substrate in the presence of mLR standards made up in Milli-Q water, 20% and 50% methanol was compared. The data are presented in Figure 31.

Table 11. Statistical analysis of replicate mLR inhibition assays of PP1; average of four replicates expressed as percent inhibition of Control for each mLR concentration (ng/mL)

		mLR (ng/mL)												
	Control	0.01	0.03	0.1	0.6	1	2	3	6	10	30	60	100	300
PP1 (0.1 unit/mL stock)														
Ave.	0.187	98.5	94.6	79.7	43.8	31.2	22.1	16.2	13.8	10.3	5.9	3.4	3.5	1.6
S.D.		2.8	4.1	2.0	6.8	5.8	4.2	2.3	2.1	1.3	2.4	1.6	0.7	1.2
CoV		2.8	4.3	2.5	15.5	18.6	19.0	14.2	15.2	12.6	40.7	47.1	20.0	75.0
PP1 (0.25 unit/mL stock)														
Ave.	0.423	101.4	100.0	90.5	45.2	31.0	19.7	15.8	11.8	8.1	4.4	2.7	1.2	1.6
S.D.		3.3	3.9	5.1	2.3	1.6	0.7	0.8	1.3	1.3	0.9	1.2	0.5	1.2
CoV		3.3	3.9	5.6	5.1	5.2	3.6	5.1	11.0	16.0	20.5	44.4	41.7	75.0
PP1 (0.5 unit/mL stock)														
Ave.	0.686	100.9	100.2	91.3	59.0	37.7	23.5	18.7	12.2	9.1	5.7	4.0	2.7	2.2
S.D.		4.3	3.6	3.3	1.2	0.8	1.4	1.6	0.4	0.6	1.2	0.5	0.8	2.0
CoV		4.3	3.6	3.6	2.0	2.1	6.0	8.6	3.3	6.6	21.1	12.5	29.6	90.9
PP1 (1.0 unit/mL stock)														
Ave.	1.146	97.9	100.2	97.6	79.2	62.6	37.4	27.2	17.1	12.3	6.8	4.9	3.7	2.5
S.D.		0.6	1.9	2.7	0.8	3.9	1.1	1.3	0.4	0.9	0.7	0.6	0.8	0.5
CoV		0.6	1.9	2.8	1.0	6.2	2.9	4.8	2.3	7.3	10.3	12.2	21.6	20.0

S.D. = standard deviation
CoV = Coefficient of Variation
Control column is the absorbance at 630 nm in absence of mLR

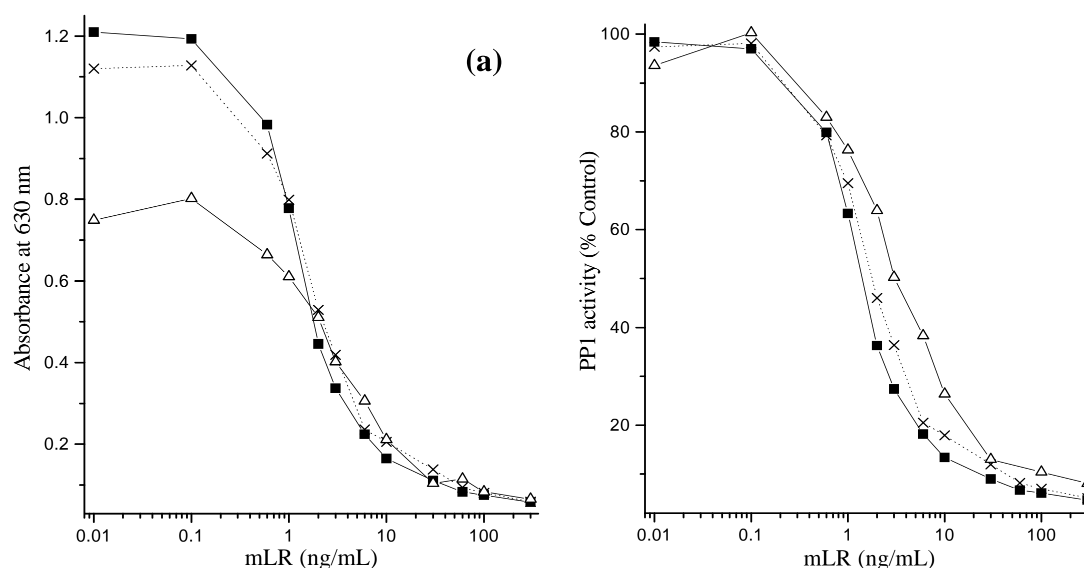


Figure 31. Comparison of Milli-Q water, 20% and 50% methanol as solvent for mLR; effect on PP1 activity. mLR in Milli-Q water (#), in 20% methanol (x) and 50% methanol (Δ)

Graph (a) initially suggests that the presence of methanol results in less Pi release from the dephosphorylation of phosvitin by PP1. However, data in Section 3.1.4 demonstrated that methanol affected the colorimetric measurement of Pi. These results therefore suggest that PP1 activity towards its substrate may be unaffected by the presence of up to 50% methanol in the mLR solutions which agrees with the results of Ward et al. (1997). However, methanol affects the colorimetric assay resulting in lower than expected absorbances of the malachite green-phosphomolybdate complex. Graph (b) indicates that a standard curve of toxin in 50% methanol may not be as sensitive as using a 20% methanol matrix as the curve has shifted to the right, decreasing its IC_{50}. It was therefore decided to make up samples cleaned up by the C18 procedure in 20% methanol to maximise the sensitivity and minimise the percentage error of the assay.

3.4.5 PP1 inhibition curves for nodularin, mYR, mRR

A series of solutions of nodularin, mLR, mRR and mYR were made in the range 0.01 to 300 ng/mL in Milli-Q water to compare the inhibition of PP1 activity by all four toxins. The concentration of PP1 chosen was 0.5 unit/mL as discussed in Section 3.4.3. The data is presented in Figure 32. The inhibition of PP1 by all four toxins is very similar and is reflected in the IC_{50}s. mYR may be a slightly weaker inhibitor (IC_{50}= 1.9 ng/mL) however, as many purchased microcystin standards can vary in purity from batch to batch, the accuracy of these IC_{50}s cannot be assumed. The IC_{50} for mLR (0.8 ng/mL) agrees well with the value obtained in the replicate study. Other research groups report similar IC_{50}s for microcystins (refer to Table 9) particularly Honkanen et al. (1994), Chaivimol et al. (1993) and Eriksson et al. (1990).

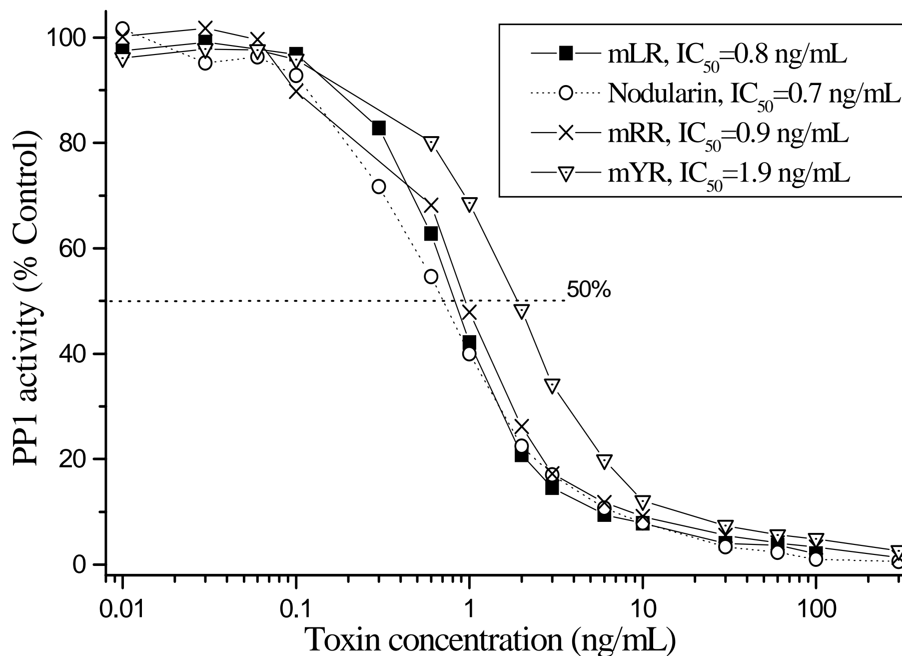


Figure 32. Protein phosphatase inhibition curves for mLR (#), nodularin (∀), mRR (x) and mYR (∇); PP1 activity versus toxin concentration (ng/mL)

3.4.6 Effect of matrix on mLR inhibition of PP1 and the implications for quantitation of toxin

Some inconsistencies in toxin quantitation were noticed when algal scum samples were analysed by the protein phosphatase method. Similar dilutions which gave responses on the linear part of the standard curve did not give the same result when the dilution factor had been factored into the quantitative value. Therefore the effect of various matrices described in Section 2.7 on mLR quantitation was examined.

The five matrices chosen were:

- humic acid solution, 9 mg/mL DOC (*humic acid*)
- diluted algal extract, (1/100) (*algal extract*)
- a raw water taken from Hindmarsh River (*raw water*)
- an ultrafiltered farm dam sample (*UF dam*)
- Milli-Q water as a control (*MQ*)

These matrices were spiked with mLR at the following concentrations: 0.1, 0.2, 0.3, 0.6, 1, 1.5, 2, 3 and 10 ng/mL. An unspiked matrix sample was also included as a blank. The resulting absorbances at 630 nm were compared with those obtained for an mLR inhibition curve from 0.01 to 300 ng/mL mLR. Table 12 contains the absorbance at 630 nm with blanks subtracted for each spiked matrix at the concentration of mLR chosen. The assay in the absence of mLR was the control and was expected to exhibit maximum PP1 activity.

Table 12. Comparison of the effect of matrix on the inhibition of PP1 by mLR; absorbance at 630 nm at various concentrations of mLR

mLR (ng/mL)	Std. curve	*Raw water*	*UF dam*	*Algal extract*	*Humic acid*	*MQ**
0 (Control)	0.991	0.858	0.510	0.473	0.950	0.991
0.01	0.971					
0.03	0.969					
0.1	0.935	0.808	0.483	0.437	0.902	0.946
0.2		0.782	0.443	0.414	0.821	0.927
0.3	0.801	0.751	0.421	0.395	0.769	0.872
0.6	0.582	0.629	0.306	0.320	0.689	0.802
1	0.417	0.487	0.170	0.238	0.511	0.549
1.5		0.333	0.096	0.208	0.311	0.492
2	0.199	0.258	0.065	0.159	0.245	0.279
3	0.146	0.181	0.052	0.147	0.169	0.258
6	0.091					
10	0.074	0.057	0.024	0.090	0.068	0.064
30	0.046					
60	0.038					
100	0.020					
300	0.007					

* MQ water was a control for the experiment on this particular day; it should give the same result as the standard curve.

Figure 33 graphs this data as absorbance versus mLR concentration. As the blanks have been subtracted, the deviation in absorbance value in each matrix is due to one of two possibilities. Either the matrix is suppressing enzyme dephosphorylation of phosvitin and hence the level of Pi released or, the matrix affects the colorimetric assay giving the impression enzyme activity has been decreased and less Pi has been liberated from the substrate. The colorimetric assay was found to be affected by methanol (Sections 3.1.4 and 3.4.4). The diluted algal extract and the ultrafiltered farm dam sample had the biggest effect on PP1 activity. The raw water from Hindmarsh River also produced a decrease in absorbance which was more noticeable at lower concentrations of toxin. It was interesting to note that the humic acid solution did not affect the assay suggesting that DOC is not a major interference in the assay.

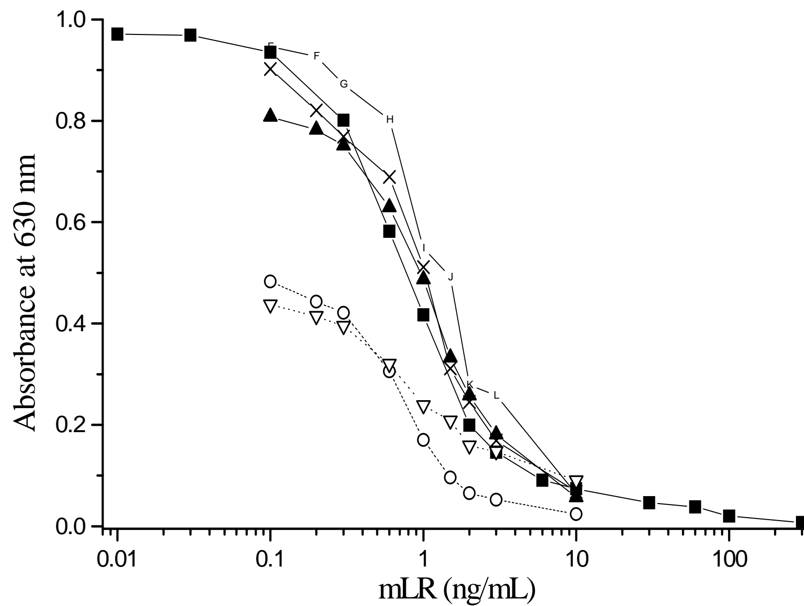


Figure 33. Comparison of different matrices on mLR inhibition of PP1, absorbance at 630 nm. (Standard inhibition curve (#), raw water (σ), UF farm dam (∀), algal extract (∇), humic acid (x), MQ water(E-M))

The data was also expressed as enzyme activity or % control in two ways. In graph (a) of Figure 34, PP1 activity in each matrix has been calculated relative to the control in each matrix. This has the effect of negating the influence matrix has on the assay, and demonstrates that a sigmoidal inhibition curve with similar IC_{50} to the standard curve in Milli-Q water is produced. In graph (b) PP1 activity has been calculated relative to the control for the standard curve (0.991). This represents how toxin levels in environmental samples are quantified and is designed to demonstrate the deviation from accurate quantification that a particular sample matrix may cause. As expected the curve is similar to that in Figure 33. The horizontal lines indicate the linear region of the standard curve between 20 and 80% of PP1 activity which is used to quantify environmental samples. The algal extract and UF dam show large inaccuracies where Milli-Q water is used as the control.

Table 13 shows the percentage of control values for each spiked matrix calculated by expressing the absorbance for each sample as a percentage of the control, 0.991 absorbance units (Table 12). The values in the second column are the toxin concentration in each sample read from the standard curve (Figure 34, graph b). This is how “unknown” samples are quantified. These values can be compared with the actual spike concentration in the first column. In the case of the algal extract and UF dam sample, the calculated values of mLR exhibit a large deviation from the spike concentrations. The data suggests the unspiked UF dam and algal extract samples contain 0.75 and 0.84 ng/mL microcystin respectively. This is unlikely as the UF dam sample was ultrafiltered through a 10,000 MW cutoff filter and the retained pigments made to original volume. Any microcystin present would have gone through the filter. The algal extract did not contain microcystins by HPLC and it is “unlikely” the 1/100 dilution did. The microcystin concentration in the 0.1 and 0.2 ng/mL spikes in these two matrices are also overestimated. It is therefore apparent that some matrices can cause false positive results and overestimation of toxin levels.

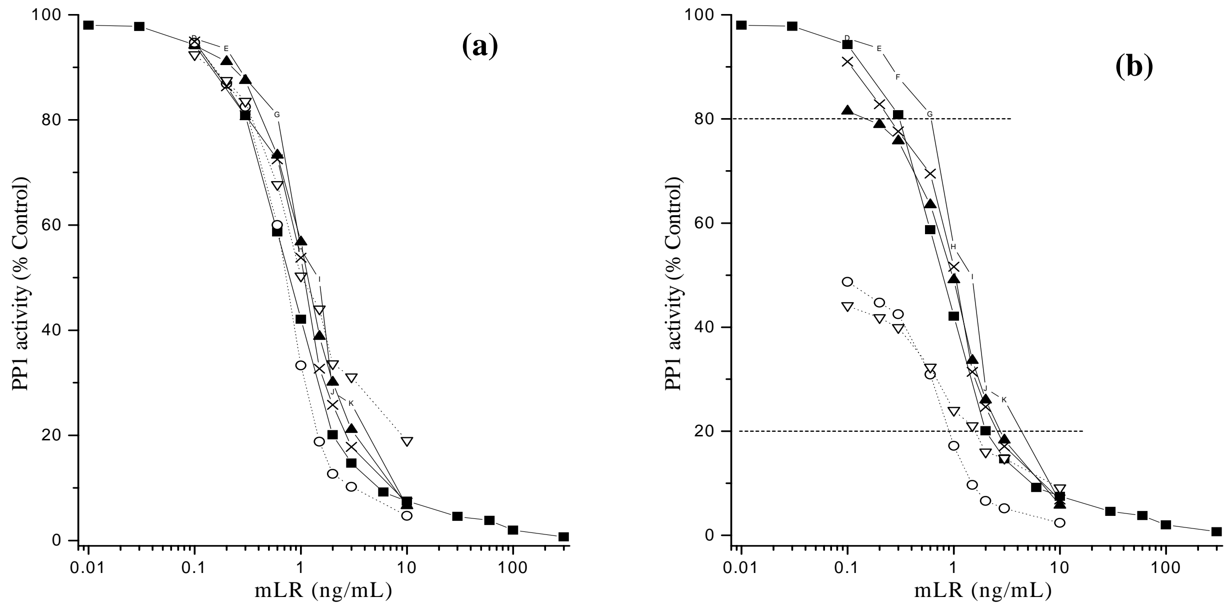


Figure 34. Comparison of different matrices on mLR inhibition of PP1, data expressed as PP1 activity relative to Control. (Standard inhibition curve (#), raw water (σ), UF farm dam (∀), algal extract (∇), humic acid (x), MQ water(D-L). Graph (a): PP1 activity as % Control from each matrix, Graph (b): PP1 activity as % Control from standard curve)

Table 13. Quantitation of mLR in spiked matrices relative to standard inhibition curve

mLR (ng/mL)	*Raw water*		*UF dam*		*Algal extract*		*Humic acid*		*MQ*	
	% C	mLR	% C	mLR	% C	mLR	% C	mLR	% C	mLR
0	86.6	-	51.5	0.75	47.7	0.84	95.9	-		
0.1	81.5	0.28	48.7	0.82	44.1	0.94	91	-	95.5	-
0.2	78.9	0.32	44.7	0.92	41.8	1	82.8	-	93.5	-
0.3	75.8	0.35	42.5	1	39.9	1.07	77.6	0.34	88	0.16
0.6	63.5	0.51	30.9	1.42	32.3	1.38	69.5	0.43	80.9	0.29
1	49.1	0.81	17.2	-	24	1.78	51.6	0.74	55.4	0.67
1.5	33.6	1.33	9.7	-	21	1.97	31.4	1.4	49.6	0.79
2	26	1.68	6.6	-	16	-	24.7	1.76	28.2	1.57
3	18.3	-	5.2	-	14.8	-	17.1	-	26	1.65
10	5.8	-	2.4	-	9.1	-	6.9	-	6.5	-

% C = % of control for standard curve (0.991)
mLR = mLR concentration in ng/mL

Dilution of spiked matrix, effect on mLR inhibition of PP1

The same algal extract described above was successively diluted to see whether this brought the curve back in line with the standard curve. The following dilutions were used: 1/50, 1/100, 1/1000, 1/10,000 and 1/100,000. The dilutions were assayed in the absence of enzyme to account for absorbance due to trace Pi (before enzyme assay) and background absorbance of the low dilutions. Each dilution was spiked at 0.3, 0.6, 1 and 2 ng/mL mLR and the absorbance minus blank plotted vs. mLR concentration. As expected, the curves approached the standard curve as the matrix was diluted. Therefore, any matrix effects are likely to occur only with relatively undiluted samples.

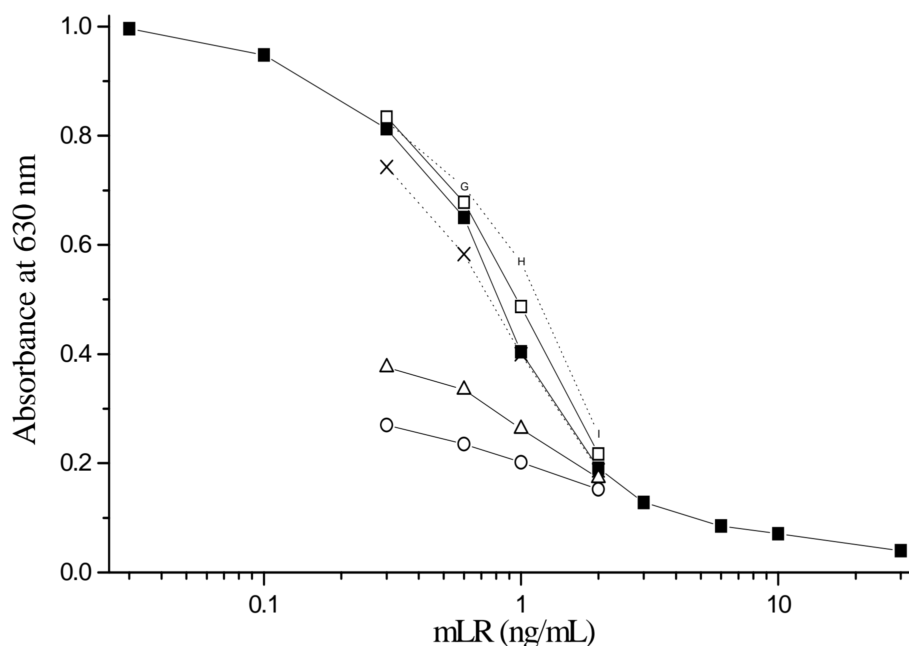


Figure 35. Effect of diluting an algal matrix on the mLR inhibition of PP1 enzyme. Standard inhibition curve (#), algal extract, 1/50 diln. (∀), 1/100 diln. (∆), 1/1000 diln. (x), 1/10,000 diln. (F-I), 1/100,000 diln. (Θ)

3.4.7 Quantitation of microcystins by HPLC and phosphatase inhibition assay

To date, only a few samples have been analysed by both HPLC and phosphatase inhibition assay. Table 14 summarises data for two scum samples which have been analysed by both techniques. The major component in the farm dam was mLR and the agreement between the two methods is good. The sample from Warren Reservoir was interesting in that it contained a large number of peaks which did not correspond to any of the more common microcystins for which we have standards. The concentration of microcystins detected by the phosphatase assay is considerably lower than that calculated by HPLC. This may be due to the presence of microcystins which produce a characteristic UV absorption spectrum, but are not toxic. The two values in the PPase column were the levels of toxin detected with two different concentrations of PP1. It is not uncommon for bioassays to produce this variation in quantitation between assays (G. Jones, CSIRO, personal communication).

The limit of detection (LOD) for mLR with the HPLC system used was 50 ng/mL. The IC_{50} and LOD for the phosphatase assay at each PP1 level were:

At 0.5 unit/mL PP1, IC_{50} = 0.8 ng/mL mLR — Working range = 0.3-3.2 ng/mL, therefore the LOD = 0.3 ng/mL mLR

At 1 unit/mL PP1, IC_{50} = 1.8 ng/mL mLR — Working range = 0.7-5.6 ng/mL, LOD = 0.7 ng/mL mLR.

Table 14. Quantitation of hepatotoxin by HPLC with diode array detection and protein phosphatase inhibition assay

Sample	HPLC comments	HPLC (μg/mL)	PPase (μg/mL)*
Farm dam	4 peaks (mLR major peak)	72	85, 65
Warren	15 peaks (mLR, RR, YR, LA not present)	76	22, 34

* PPase assay conducted at two levels of PP1 enzyme

4. SUMMARY AND CONCLUSIONS

A rapid and sensitive phosphatase inhibition assay is a useful alternative to the method of HPLC currently being used to determine hepatotoxins. This assay provides a direct indication of toxicity of the sample. An and Carmichael (1994) have found the PP1 inhibition assay using *p*NPP as substrate to be suitable for detecting toxins such as [DM $Adda^5$] mLR and [DM $Adda^3$] nodularin which are toxic to mice but not detected by ELISA. The PP1 assay will not detect esters of the D-glutamate group on the toxins such as [D-Glu-$OCH_3{}^6$] mLR which are non-toxic to mice and are detected by ELISA and HPLC. In our study reported here, we investigated a phosphatase inhibition assay using commercially available PP1 and phosvitin as substrate. Phosvitin was chosen on the basis that sensitivity might be improved relative to *p*NPP and the assay might be less sensitive to interferences. The principal findings were:

- A colorimetric assay for inorganic phosphate (Pi) conducted in microtitre plates was evaluated and found to be a quick and accurate method for detection and quantitation of Pi. The detection limit (the amount of phosphate required to cause a 0.1 absorbance unit change) was 2 μM or 0.2 μg/mL orthophosphate ∀ 2%.
- The buffer system and conditions for protein phosphatase dephosphorylation of phosvitin developed by Wheldrake et al. (1996) were modified to enable the assay to work with commercially purchased enzyme. The buffer recommended by Calbiochem for use with recombinant PP1 enzyme was modified and optimised to produce good PP1 activity towards phosvitin. The most important parameters changed were: proportion and volume of assay components, and manganese ion, dithiothreitol and phosvitin concentrations in the assay.
- The PP1 phosphatase inhibition assay using phosvitin as a substrate was evaluated extensively and found to be an accurate and sensitive assay for microcystin. Concentrations of mLR in samples as low as 0.2 ng/mL with a CV of less than 5% could be determined. The standard inhibition curves for mRR, mYR and nodularin were very similar to that of mLR. The effect of enzyme concentration on the sensitivity of the assay was also determined. The influence of endogenous phosphatases has not been fully investigated, however, these effects should be compensated for in the blanks carried through the assay.
- The phosphatase inhibition assay can be used with neat or diluted samples (water solvent) or samples that have been put through the C18 cleanup procedure for HPLC and contain a proportion of methanol (20% recommended maximum).
- The effect of matrix on the assay was examined and it was found that samples that had contained high levels of algae interfered with the inhibition assay. This resulted in false positives and overestimation of toxin concentration in these matrices if they were undiluted, ie, this simulated samples containing low levels of toxin. If the matrix were diluted, the interference in the assay was greatly decreased. The compounds interfering with the assay may be algal pigments or some other high molecular weight component of algal cells. Natural organic matter in the water sample does not interfere with the assay. The analyst needs to be aware of this possible interference whether phosvitin or *p*NPP is used as the substrate, since the *p*NPP assay may also exhibit problems with matrix interferences (W. Carmichael, Wright State University, personal communication).
- The literature was reviewed for IC_{50} values obtained with other assay methods using a variety of substrates. The inhibition assay using phosvitin as a substrate was found to be of comparable sensitivity to other assays using PP1, even radiolabelled assays. Some laboratories have found PP2A to be even more sensitive to inhibition by microcystins than PP1, but the need for magnesium by this enzyme presents problems for an assay using phosvitin as substrate. The *p*NPP assay may therefore have some advantages over the phosvitin assay, in that it is of comparable sensitivity but requires one less step.
- Several samples have been analysed for microcystins by the inhibition assay. The results for two samples compared very well with those obtained by HPLC. These techniques have different advantages. The phosphatase inhibition assay provides a measure of toxicity of the sample whereas HPLC detects all hepatotoxins present regardless of whether they are toxic or not. The inhibition assay is also about 100 times more sensitive than HPLC and does not require concentration and cleanup procedures.

5. RECOMMENDATIONS

- As the phosphatase inhibition assay using *p*NPP as substrate may have some advantages over the phosvitin assay in that it is of comparable sensitivity but requires one less step, it should be pursued as the method of choice. However, it is important to determine whether the matrix effects observed with phosvitin also apply to *p*NPP. Unfortunately literature reports where *p*NPP is used as substrate do not discuss this aspect of the assay.
- The greater sensitivity of phosphatase inhibition assays means that even lower detection limits can be achieved with C18 concentration and cleanup procedures. With a limit of detection (LOD) of about 0.5 μg/L mLR for the phosphatase assay, levels of 0.5 ng/L could potentially be detected in waters (AWQC LOD for mLR is 50 ng/L using HPLC). The ability to detect microcystin levels in water at 100 times lower than is currently achievable by HPLC justifies further investigation.

6. ACKNOWLEDGMENTS

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7. APPENDICES

APPENDIX A: Phosphate concentrations and other parameters in metropolitan raw waters - 5 yearly averages (SA Water)

Reservoir	pH	Conductivity (μS/cm)	TDS (mg/L)	Colour (HU)	Turbidity (NTU)	Phosphate-P (soluble) (mg/L)	DOC (mg/L)
Barossa	7.7	579	299	42	1.9	0.005	9.7
Little Para	8.0	796	416	23	4.6	0.009	6.0
Hope Valley	8.0	663	345	31	3.4	0.011	6.8
Happy Valley	7.9	676	338	43	5.5	0.021	7.2
Myponga	7.6	549	280	80	3.7	0.016	10.1

APPENDIX B: Phosphate levels in waste waters from South Australian Wastewater Treatment Plants - 5 yearly averages (SA Water)

Location	pH	TDS (mg/L)	Total Phosphate as P (mg/L)
Bolivar WWTP, no.1 weir effluent	7.2	1300	11.6
Christies Beach final effluent A/C	7.3	1100	10.9
Glenelg effluent A/C composite	7.6	1100	7.6
Pt. Adelaide effluent	7.1	5200	5.3
Bird-in-Hand final effluent Lagoon 10			17.6
” Lagoon 4	7.8	920	25.2
” effluent into creek	8.2	1100	13.1
Millicent STW sewage	7.7	870	4.4
” Lagoon 1	8.1	800	4.7
” Lagoon 2	8.3	790	2.2
” Lagoon 3	8.9	730	1.5

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- South East Water Ltd
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- Sydney Water Corporation
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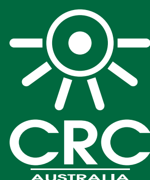

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