Development of an inhibitive enzyme assay for copper

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Abstract: In this work the development of an inhibitive assay for copper using the molybdenum-reducing enzyme assay is presented. The enzyme is assayed using 12-molybdophosphoric acid at pH 5.0 as an electron acceptor substrate and NADH as the electron donor substrate. The enzyme converts the yellowish solution into a deep blue solution. The assay is based on the ability of copper to inhibit the molybdenum-reducing enzyme from the molybdate-reducing Serratia sp. Strain DRY5. Other heavy metals tested did not inhibit the enzyme at 10 mg l\(^{-1}\). The best model with high regression coefficient to measure copper inhibition is one-phase binding. The calculated IC\(_{50}\) (concentration causing 50% inhibition) is 0.099 mg l\(^{-1}\) and the regression coefficient is 0.98. The comparative LC\(_{50}\), EC\(_{50}\) and IC\(_{50}\) data for copper in different toxicity tests show that the IC\(_{50}\) value for copper in this study is lower than those for immobilized urease, bromelain, Rainbow trout, R. meliloti, Baker’s yeast dehydrogenase activity, Spirillum volutans, P. fluorescens, Aeromonas hydrophilia and synthetic activated sludge assays. However, the IC\(_{50}\) value is higher than those for Ulva pertusa, and papain assays, but within the reported range for Daphnia magna and Microtox™ assays.

Key words: Inhibitive enzyme assay, Copper, Mo-reducing enzyme

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Introduction

Nowadays, heavy metal pollution has become a global concern. The early detection of heavy metal ions, especially bioavailable metal ions, in the environment is very important to safeguard human health. Bioassay and inhibitive enzyme assays are excellent detection method for bioavailable ions as they are inhibited only by the bioavailable form (Selifonova et al., 1993) whereas instruments, such as atomic absorption and emission spectrophotometry, usually do not discriminate between toxic and non-toxic forms of metal ions. Numerous enzymes have been used for the inhibitive determination of heavy metal traces, such as peroxidase, xanthine oxidase, invertase, glucose oxidase, urease and the proteases papain and bromelain (Jung et al., 1995; Shukor et al., 2006, 2008). Most of these enzymes are cheap, do not require costly instruments and are amenable to field testing.

It is very difficult to design an assay that is sensitive to a particular heavy metal. Although it is advantageous to detect a spectrum of heavy metals, the ability to detect a particular heavy metal at a very sensitive level is equally advantageous. Hence, an antibody-based system for detecting specific metal ions, such as mercury, has been developed (Mehrabian et al., 1998). Unfortunately, the assay is costly and the sensitivity is lower than the detection level required by many monitoring bodies.

In Malaysia, states with extensive industrial development have high levels of heavy metal contamination. One of the most commonly reported heavy metal contaminants in Malaysian waters is copper. In fact, 50% of sampling sites contain copper at levels that exceed the interim standards (DOE, 2002). The limit established by the Malaysian Department of Environment is 0.05 ppm (DOE, 2002). Copper, when present at elevated levels, is toxic to living organisms and plants (Lim et al., 2006; Zengin and Kirbag, 2007; Singh et al., 2007). The ubiquitous presence of copper in Malaysian waters is due to the fact that it is often added to feedstock for pigs at several ppm to control parasites (Foulkes et al., 2006), and commercial pig production in Malaysia is among the highest in South East Asia (Sommor et al., 2005). Consequently, a rapid and easy test for the presence of copper in waters and rivers in Malaysia must be developed. In this work we report on the development of a novel copper assay using the molybdenum-reducing enzyme from Serratia marcescens Strain DRY5. This assay is sensitive to copper at the sub mg l\(^{-1}\) level. The assay is suitable for use as a routine biomonitoring method to detect copper in the environment.

Materials and Methods

Preparation of reagents: Pesticides with chemical purities of >99%, (Ehrenstorfer, Augsburg, Germany and Pestanal®, Riedel de Häën, Germany) such as metolachlor, glyphosate, diazinon, endosulfan sulphate, coumaphos, imidacloprid and dicamba were prepared by dissolving the pesticides in the appropriate solvents or used directly from the liquid solutions. The final concentration of all these pesticides in the reaction mixture was 4 mg l\(^{-1}\). The xenobiotics tested are as follows; acetonitrile (Merck), ethylene glycol (Merck), ethyl acetate (Merck), ethanol (BDH), isopropanol (BDH), methanol (BDH), triethanolamine, polyethylene glycol (PEG) 400, 600 and 1000 (Sigma), diethylamine (Sigma), acrylamide (Sigma), Nonidet-P40 (Sigma), Triton-X-100 (Sigma) and SDS (Sigma). These xenobiotics were prepared as 2% (v/v) solution in deionized water and added.

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into the reaction mixture to a final concentration of 0.4% (v/v). The concentration of pesticides and xenobiotics chosen in this study is generally much higher than normally found in natural water and also limited to the solubility of pesticide and xenobiotics in water.

Isolation of molybdate reducing bacterium: Soil samples, each measuring approximately 10 g were taken randomly to a depth of 5 cm from the topsoil using sterile spatula and stored in sterile screw-capped polycarbonate tubes. The soil samples were taken from an abandoned metal recycling ground near the King Edward VII(2nd) Primary School in the city of Taiping, Perak, Malaysia. The samples were immediately placed on ice until returned to University for further examination. Five grams of a well-mixed soil sample were suspended in 45 ml of 0.9% saline solution. A suitable serial dilution aliquot (0.1 ml) of soil suspension was spread plated onto an agar of low phosphate (2.9 mM phosphate) media (pH 7.0) containing glucose (1%), (NH₄)₂SO₄ (0.3%), MgSO₄·7H₂O (0.05%), NaCl (0.5%), yeast extract (0.05%), Na₂MoO₄·2H₂O (0.242%) and Na₂HPO₄ (0.05%). Glucose was autoclaved separately (Ghani et al., 1993). Growth in liquid media uses the same media as in the solid media above. Molybdenum blue is produced in this media but not at high phosphate media (100 mM phosphate). The only difference between the high and low phosphate media is the phosphate concentration. Several white and blue colonies appeared after overnight incubation at room temperature. Blue colonies signify molybdenum-reducing bacteria. One single blue colony was inoculated into 50 ml of low phosphate media and incubated at 30°C for 24 hr. The production of molybdenum blue from the media was measured at 865 nm.

Identification at species level was performed by using Biolog GN micro-plate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and molecular phylogenetics studies. Each Biolog plate contains 95 different carbon sources in addition to a tetrazolium dye. The utilization of a carbon source by this bacterium results in the reduction of the dye and formation of a purple color that can be quantified and monitored over time. The carbon utilization profile fingerprint produced is unique to a particular species of bacterium and the identity of the bacterium can be ascertained by matching the fingerprint with the database in the system.

A pure culture of a bacterium was grown on a Biolog Universal Growth agar plate. The bacterium was swabbed from the surface of the agar plate, and suspended to a specified density in GN Inoculating Fluid. A hundred fifty µl of a bacterial suspension was pipetted into each well of the micro-plate. The micro-plate was inoculated into one liter of Growth in liquid media uses the same media as in the solid media above. Molybdenum blue is produced in this media but not at high phosphate media (100 mM phosphate). The only difference between the high and low phosphate media is the phosphate concentration. Several white and blue colonies appeared after overnight incubation at room temperature. Blue colonies signify molybdenum-reducing bacteria. One single blue colony was inoculated into 50 ml of low phosphate media and incubated at 30°C for 24 hr. The production of molybdenum blue from the media was measured at 865 nm.

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Molybdenum reducing enzyme assay: Into 800 µl of reaction mixture containing 12- phosphomolybdate (in 50 mM citrate phosphate buffer pH 5.0) at room temperature (28 to 30°C), 20 µl of NADH (150 mM stock) was added to a final concentration of 2.5 mM. Fifty microlitres of partially purified molybdenum-reducing enzyme fraction (1 mg ml⁻¹ final protein) was added to start the reaction. Distilled water or buffered heavy metals and samples were added so that the total reaction mixture was 1 ml. The absorbance increase in one minute was read at the wavelength of 865 nm.

One unit of molybdenum-reducing activity is defined as that amount of enzyme that produce 1 nmole molybdenum blue (in terms of equivalent reduced 12-phosphomolybdate standard (Shukor et al., 2000). The reference standard curve was prepared as follows:

12-phosphomolybdate (12MoO₄H₂PO₄·2H₂O, Sigma-Aldrich Chemical Co., St. Louis, USA) was prepared in distilled water as a 5 mM stock solution and adjusted to pH 5.0. Ascorbic acid was prepared fresh as a 25% (w/v) solution in distilled water and was kept at 4°C for a maximum period of one week. One hundred to six hundred microlitres from the 12-phosphomolybdate stock solutions was added to 100 ml ascorbic acid and the final volume adjusted to one ml with distilled water. After 12 hr of incubation, the absorbance was read at 865 nm wavelength. The molar extinction coefficient or molar absorptivity at 865 nm for molybdenum blue using 12-phosphomolybdate as a standard is 16.7 mM⁻¹·cm⁻¹ (Shukor et al., 2000).

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Preparation of crude enzyme: Bacteria were grown in one liter of media containing high phosphate at 30°C for 24 hr on an orbital shaker (100 rpm). Although high phosphate inhibits molybdate reduction to molybdenum blue, the cells contain active enzymes (Ghani et al., 1993). Growth on low phosphate resulted in a blue sticky culture that complicated the preparation of crude enzyme and enzyme assay. The following experiment was carried out at 4°C unless stated otherwise. Cells were harvested through centrifugation.
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at 10,000 g for 10 min. Cells were washed at least once with distilled water, resuspended and recentrifuged. The pellet was reconstituted with 10 ml of 50 mM Tris buffer (prepared at 4°C, pH 7.5). Cells were sonicated for 1 min on an ice bath with 4 min cooling until a total sonication time of at least 20 min was achieved. The sonicated fraction was centrifuged at 10,000 g for 20 min and the supernatant consisting of the crude enzyme fraction was taken. Freeze-dried preparation of the crude enzyme is stable for one year when stored at -20°C.

Preparation of heavy metals and interference solutions:
Heavy metals and metals were prepared from analytical grade commercial salts such as chromium (vi) (K₂Cr₂O₇, BDH), selenium (vi) (Na₂SeO₄, BDH), nickel (ii) (NiCl₂, Ajax Chemicals), zinc (ii) (ZnSO₄, anhydrous J.T. Baker), tungsten (vi) (Na₂WO₄·2H₂O, BDH), manganese (ii) (MnSO₄·H₂O, BDH), borate (iii) (H₃BO₃·H₂O, anhydrous BDH), cobalt (ii) (CoCl₂·6H₂O, J.T. Baker), aluminium (iii) (Al₂(SO₄)₃, anhydrous BDH), cesium chloride (CsCl, BDH), lithium chloride (LiCl, BDH) and barium (BaCl₂·2H₂O, Sigma) and from atomic absorption spectrometry standard solutions from Merck such as mercury (ii), arsenic (v), cadmium (ii), lead (ii), copper (ii) and silver (ii). Heavy metals were initially diluted in 0.1 M Tris.Cl buffer pH 7.0 to the final concentration of 20 mg/l to ensure that the nitric acids from the commercial heavy metals solution are neutralized. Solvents such as ethylene glycol, DMSO, methanol, acetonitrile, ethanol and triethanolamine were taken directly from commercial reagent bottles and added into enzyme reaction mixture to a final concentration of 10% (v/v). The mixture was then added into the enzyme reaction mixture as before. The final volume of the reaction mixture was 1 ml.

Statistical analysis: Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student’s t-test or a one-way analysis of variance with post hoc analysis by

Table 1: Sensitivity of the assay to copper in comparison to EC₅₀, LC₅₀ or IC₅₀ of several assays

<table>
<thead>
<tr>
<th>Assays</th>
<th>EC₅₀, LC₅₀ or IC₅₀</th>
</tr>
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<tbody>
<tr>
<td>Immobilized urease</td>
<td>0.41±0.14</td>
</tr>
<tr>
<td>Papain</td>
<td>0.004</td>
</tr>
<tr>
<td>15 min Microtox™</td>
<td>0.076±3.8</td>
</tr>
<tr>
<td>Bromelain</td>
<td>0.163±1 to 0.3048</td>
</tr>
<tr>
<td>96 hr Daphnia magna</td>
<td>0.020±0.093</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>0.25</td>
</tr>
<tr>
<td>R. melilot</td>
<td>0.95±0.181</td>
</tr>
<tr>
<td>Baker’s yeast</td>
<td>5.6±0.3</td>
</tr>
<tr>
<td>Dehydrogenase activity (TTC or INT)</td>
<td>5.6 (INT)</td>
</tr>
<tr>
<td>Spirillum volutans</td>
<td>8.6</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>0.2-3.2</td>
</tr>
<tr>
<td>P. fluorescens (18 hr)</td>
<td>16.8</td>
</tr>
<tr>
<td>Synthetic activated sludge (180 min)</td>
<td>29</td>
</tr>
<tr>
<td>U. pertusa (5 days)</td>
<td>0.061 (0.049-0.081)（95% confidence interval）</td>
</tr>
<tr>
<td>This study</td>
<td>0.099±0.013</td>
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Fig. 1: Screening results for the inhibitory effect of heavy metals on the Mo-reducing enzyme assay. Data is mean ± standard error of the mean (n=3)
Tukey’s test (Miller and Miller, 2000). p < 0.05 was considered statistically significant.

**Results and Discussion**

**Inhibition of Mo-reducing enzyme by metals:** Molybdate reduction to molybdenum blue by microbes is an old phenomenon. According to Levine (1925), the phenomenon was first reported in *E. coli* (Capaldi and Proskauer, 1896). Since then, reports on molybdate reduction by other bacteria have trickled in (Marchal and Gerard, 1948; Jan, 1939; Woolfolk and Whiteley, 1962; Bautista and Alexander, 1972; Campbell et al., 1985; Sugio et al., 1988; Ghani et al., 1993). Using phosphomolybdate as a substrate, the Mo-reducing enzyme was partially purified and characterized (Shukor et al., 2003).

Of the 18 metals tested at a final concentration of 10 mg l\(^{-1}\), only copper showed more than 50% inhibition (Fig. 1) suggesting specificity to copper. Lead showed a slight inhibition of 20% but the
permissible limit for lead is 0.05 mg/l (DOE, 2002), far lower than the concentration used in the preliminary screening. An analysis of the variance showed that the differences in the percentages of activity for lead and copper are significant (p<0.05) compared to the control. The rest of the metal ions showed percentages of activity with no significant differences from control (p=0.05). The result shows that the assay is selective to copper and thus can be used for the biomonitoring of bioavailable copper in waste water. Since little is known about the molybdenum-reducing enzyme, the mechanism of copper inhibition must be studied further. When plotted in the form of delta change in absorbance, copper exhibited rectangular hyperbolic inhibition curves with the best model to determine the IC_{50} was one-phase binding (Fig. 2). Using the GraphPad software (GraphPad software, Inc., San Diego, CA), the calculated IC_{50} for copper was 0.099 mg l^{-1} and the regression coefficient is 0.98. Repeated measurement of the enzyme inhibition by the heavy metals suggests the assay is reproducible with CV (Coefficient of variation) of the replicated data ranging from 7 to 15%. The delta absorbance, measured at 865 nm at 1.10 between the control and the highest copper concentrations tested, allows inhibition to be seen visibly. This is an important feature for the development of color charts for semiquantitative determination of copper in fieldworks (Fig. 1). In the papain assay for copper, a linear relationship between copper concentration and absorbance was obtained (Shukor et al., 2006). In the urease assay, a linear relationship between the log of copper concentration and absorbance is seen (Jung et al., 1995) while in Ulva pertusa an approximate radioactive decay type curve is seen instead (Han and Choi, 2005) The comparative LC_{50}, EC_{50} and IC_{50} data for copper in different toxicity tests are shown in Table 1. The results show that the IC_{50} value for copper in this study is lower than those for immobilized urease, bromelain, rainbow trout, R. mellioti, Baker’s yeast dehydrogenase activity, Spirillum volutans, P. fluorescens, Aeromonas hydrophilia and synthetic activated sludge assays. However, the IC_{50} value is higher than those for Ulva pertusa and papain assays, but within the reported range for Daphnia magna and Microtox™ assays.

Since many of the reported IC_{50}, LC_{50} or EC_{50} values are compilations of repeated works by several workers and sometimes reported without confidence intervals or any measurement of uncertainty, statistical comparison between this study and published results cannot be made. The IC_{50} value of immobilized urease is used instead of free urease, since the ubiquitous presence of ammonia in environmental samples interferes with the assay and hence the need to immobilize the urease (Jung et al., 1995). The effects of solvents on the assay for copper were investigated. Of all the solvents tested, only triethanolamine significantly (p<0.05) inhibited the assay, exhibiting more than 50% inhibition at 10% (Fig. 3). However, the concentrates of interference agents used in this study are rarely found at this level (10% v/v) in the environment. The findings in this work suggest that the Mo-reducing enzyme assay system is suitable for use to detect copper in the environment. The assay has been shown to be simple and rapid with good sensitivity for the assay of copper. There are several advantages of testing the toxicity of copper using the Mo-reducing activity assay. The bacterium is easy to grow, the assay of the enzyme is rapid and its crude extract is easily prepared. The rapidity and simplicity of the system permit the development of a simple kit to monitor copper in the environmental or in other samples.

References


