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Research article

The development of a specific inhibitive enzyme assay for the heavy metal lead

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ABSTRACT

The development of an inhibitive assay for lead using a molybdenum-reducing enzyme assay system is presented for the first time. The assay is based on the ability of lead to inhibit the molybdenum-reducing enzyme of a bacterium. The molybdenum-reducing enzyme was assayed using 12-molybdophosphoric acid at pH 5.0 as an electron acceptor substrate and NADH as the electron donor substrate. The enzyme in the crude extract converted the yellowish solution into a deep blue solution with a maximum peak at 865 nm and a shoulder at 710 nm. Lead exhibited a sigmoidal inhibition curve. The calculated IC_{50} using the four-parameter logistic model for lead was 2.186 mg l^{-1} and the regression coefficient was 0.998. The limit of detection (LOD) for lead was 0.021 mg l^{-1} while the limit of quantitation (LOQ) for leady was 0.237 mg l^{-1}, respectively. The comparative LC_{50}, EC_{50} and IC_{50} data for lead in different toxicity tests show that the IC_{50} value for lead was higher than *Ulva pertussae*, lower than the value for immobilised urease, *Daphnia magna*, and rainbow trout and within the range of the papain assay.

INTRODUCTION

The presence of toxic xenobiotics in water bodies such as azo dyes [30], detergents [22], acrylamide [23-24], diesel [25-26], pesticides [31] and heavy metals [5,27,28,29] is a global phenomenon that have warranted the development of technologies to detect and remediate these xenobiotics. Of all the xenobiotics, heavy metals are so prevalent in various parts of the world that many researchers have begun to embark upon various methods to monitor toxic heavy metals [8]. Heavy metals, unlike organic pollutants, cannot be detoxified via degradation and thus they persist in the ecosystem. Their toxic effects to aquatic organisms are well documented [10-12]. The infamous case of mercury poisoning in Minamata, Japan resulting in a massive outbreak of methylmercury poisoning represents a tragic example [32]. The Department of Environment, Malaysia has revealed that states with high industrial development (Pulau Pinang, Johor and Perak) have high levels of heavy metal (lead and mercury) contamination. Copper was found highest in the waters of Kedah and Langkawi with 50% of sampling sites exceeding the Interim Standards [3]. Thus, there is a need for simple and fast procedures as a preliminary screening method to determine the presence of toxic heavy metals.

Biosensors and bioindicator/bioassay provide rapid measurements for the analysis of heavy metal compounds without the disadvantage of the classical methods often used in analytical works such as atomic emission spectroscopy, inductively coupled plasma mass spectrometry, ion selective electrodes, polarography, and voltammetry. Recently, many workers have concentrated on enzymes to develop more specialize assay systems. Numerous enzymes have been used for the inhibitive determination of heavy metal traces, e.g. peroxidase, xanthine oxidase, invertase, glucose oxidase, the proteases trypsin [15], papain [18], bromelain [19] and the molybdenum-reducing enzyme [27] but the most frequently applied is urease as it is relatively cheap and easily available although it suffers from high interference [8]. During our work with molybdate-reducing bacterium *Serratia marcescens* Strain Dr.Y10, we found out that the molybdenum-reducing enzyme or activity is strongly inhibited by lead. In this work we reported the development of a novel lead assay using the molybdenum-reducing enzyme from this bacterium.

MATERIALS AND METHODOLOGY

Isolation of molybdate-reducing bacterium

Soil samples were taken 15-20 centimetres (cm) beneath the surface from the grounds of Universiti Putra Malaysia in Serdang, Malaysia in January 2005 using a sterile spatula and were placed in sterile screw-capped vials. The samples were immediately placed in a freezer and stored at -20°C until returned to the laboratory. 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%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2MoO_4.2H_2O$ (20 mM) and Na_2HPO_4 (5 mM) [20]. Glucose was autoclaved separately. 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 was the phosphate concentration. Several white and blue colonies appeared after overnight incubation at room temperature. One single colony exhibiting strong molybdate reduction was inoculated into 50 ml of low phosphate media and incubated at 10 ^oC for 24 hours. The production of molybdenum blue from the media was measured at 865 nm. The results obtained from Biolog^{TM} Identification system gave very high probability (99%) to *Serratia marcescens*. Genomic DNA extraction, PCR of the 16s rDNA and comparison of the partial sequence obtained (1279 base pairs) with the GenBank database using the Blast server at NCBI [1] showed that the sequence to be 99% similar to *Serratia marcescens*. The 16s rRNA ribosomal gene sequence for this isolate have been deposited in GenBank under the following accession number DQ226211. At this juncture, the isolate is assigned tentatively as *Serratia marcescens* strain Dr.Y10 (unpublished results).

Molybdenum-reducing Enzyme Assay

The enzyme assay used in this work was developed by Shukor *et al*. (2008c) [21] using phosphomolybdate instead of the original molybdate [4] for the 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 ^oC), 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 reductase activity is defined as that amount of enzyme that produce 1 nmole molybdenum blue (in terms of equivalent reduced 12-MP) per minute at room temperature. The specific extinction coefficient is 16.7 $mM.^{-1}.cm^{-1}$ at 865 nm [16]. An increase in OD 865 nm of 1.00 unit absorbance per minute per mg protein would yield 60 nmole of 12-MP or 60 units of enzyme activity in a 1 ml assay mixture.

Preparation of Crude Enzyme

Bacteria were grown in one liter of media containing high phosphate at 30 ^oC for 24 hours on an orbital shaker (100 rpm). Although the high phosphate inhibits molybdate reduction to molybdenum blue, the cells contain active enzymes [4]. 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 ^oC unless stated otherwise. Cells were harvested through centrifugation at 10 000 g for 10 minutes. Cells were washed at least once with distilled water, resuspended and recentrifuged. The pellet was reconstituted with 10 ml of 50 mM Tris buffer pH 7.5 (Tris buffer prepared at 4 ^oC). Cells were sonicated for 1 minute on an ice bath with 4 minutes cooling until a total sonication time of at least 20 minutes was achieved. The sonicated fraction was centrifuged at 10 000 g for 20 minutes and the supernatant consisting of the crude enzyme fraction was taken.

Preparation of heavy metals solutions

Heavy metals and metals such as chromium (vi) ($K_2Cr_2O_7$, BDH), selenium (vi) (Na_2SeO_4, BDH), nickel (ii) ($NiCl_2$, (Ajax Chemicals), zinc (ii) ($ZnSO_4$ anhydrous J.T. Baker), tungsten (vi) ($Na_2WO_4.2H_2O$, BDH), manganese (ii) ($MnSO_4.H_2O$, BDH), borate (iii) (H_3BO_3, anhydrous BDH), cobalt (ii) ($CoCl_2.6H_2O$,

J.T. Baker), aluminium (iii) ($Al_2(SO_4)_3$, anhydrous BHD) and barium ($BaCl_2.2H_2O$, Sigma) were prepared from commercial salts or 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^{-1} to ensure that the nitric acids from the commercial heavy metals solution are neutralized.

Preparation of pesticides and miscellaneous xenobiotics

Pesticides with chemical purities of >99%, (Ehrenstorfer, Augsburg, Germany and Pestanal®, Riedel de Häen, 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 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.

Enzyme inhibition studies

Suitable volumes of the heavy metals or xenobiotics of up to 50 µl were then incubated with 50 µl of enzyme for 1 hour at 4^oC. The mixture was then added into the enzyme reaction mixture as before. Traces of ferrous iron in the samples at several mg l^{-1} can reduce the phosphomolybdate reagent to molybdenum blue. Although ferrous iron in environmental samples is usually negligible due to oxidation to ferric irons, some samples which contain high amount of ferric irons do give appreciable ferrous iron especially if nitric acid is added. In order to remove this interference, samples were incubated with the phosphomolybdate reagent for one hour. If blue solution develops then the sample contains ferrous iron. The sample was extensively bubbled with air, the pH adjusted to neutral and manganese (MnO_2) was added to a final concentration of 10 mg l^{-1} to oxidise the ferrous iron to ferric iron. Samples which contain excessive ferric irons are diluted and the process repeated until no further formation of molybdenum blue was observed upon reacting with the reagent. 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 Graph Pad In Stat version 3.05. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test (Miller and Miller, 2000). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Enzyme inhibition studies

Molybdate reduction is a mysterious phenomenon since the mechanism and why bacteria reduce molybdate to molybdenum blue is unknown [2]. Bacteria that could reduce molybdate seems are not affected by molybdenum since these bacteria could tolerate high concentrations of molybdate in excess of 100 mM [2,4,20]. What is known is that an enzyme(s) is responsible for the reduction and its purification has been attempted by Ariff *et al*. (1993) [30] and Shukor *et al*. (2003) [17]. The substrate for the enzyme is NADH as the electron donor and molybdate as the electron acceptor [4]. However the assay took hours and under fully anaerobic conditions and works only for crude fraction. An attempt to purify the enzyme beyond crude fraction was not successful [16,30] and Campbell *et al*. (1985) [2] realise the similarity of the molybdenum blue spectrum with that of the phosphate determination method with a peak maxima from 820 to 870 nm and a shoulder at 700 nm. It is also known that phosphomolybdate has been used previously as an electron acceptor substrate for the enzymes xanthine and aldehyde oxidase (AOD and XOD) with molybdenum blue as a product. The spectrum for the molybdenum blue is also similar to that of the phosphate determination method [6]. With this information, we replaced molybdate with phosphomolybdate (12-phosphomolybdate) and found the assay to be approximately 200 times faster than using molybdate and can be assayed under open-cuvette conditions [17]. Later on we developed an even better enzyme assay using laboratory-prepared phosphomolybdate instead of the commercial 12-phosphomolybdate [21]. We proposed that during microbial molybdate reduction, an intermediate; phosphomolybdate, would form under acidic fermenting conditions of static growth and this intermediate is reduced by the molybdenum-reducing enzyme to molybdenum blue in a similar fashion to that of AOD and XOD. Difficulties in conducting the assay and purifying the enzyme are probably the main reasons why works in this area has not flourished. This is reflected in the literature search that showed the scarcity of information on molybdate reduction to molybdenum blue by bacterium.

Using the newly developed assay we screened for the inhibitive effects of heavy metals on the assay. Out of the 18 metals tested at the final concentration of 10 mg l^{-1}, only lead showed more than 50% inhibition (Fig. 1). Lead exhibited a sigmoidal inhibition curve (Fig. 2). The calculated IC_{50} using the GraphPad software (GraphPad software, Inc., San Diego, CA) using the four-parameter logistic model for lead was 2.186 mg l^{-1} and the regression coefficient was 0.998. The limit of detection (LOD) is the lowest concentration which can be detected with confidence (99% confidence interval) and is usually assigned as three times the standard deviation of the blank for the y-intercept. The LOD for lead was 0.021 mg l^{-1}. The limit of quantitation (LOQ) is the concentration level above which the concentration can be determined with acceptable precision (usually RSD < 10 to 25%) and accuracy (usually 80-120% recovery) and is usually assigned as ten times the standard deviation of the blank for the y-intercept. The LOQ for lead was 0.237 mg l^{-1}, respectively. The LOD value was well below the maximum permissible limit for mercury (0.05 mg l^{-1}) outlined by Malaysian Department of Environment[5].

The comparative LC_{50}, EC_{50} and IC_{50} data for lead in different toxicity tests is shown in Table 1. The results show that the IC_{50} value for lead was higher than *Ulva pertussae*, lower than the value for immobilised urease, *Daphnia magna*, and rainbow

trout and within the range of the papain assay. The IC_{50} value of immobilized urease is used instead of free urease since the ubiquitous presence of ammonia in environmental samples interferes the assay, hence the need to immobilize the urease [8]. Repeated measurement of the assay for lead suggests the assay was reproducible with CV (Coefficient of Variation) of the replicated data ranging from 3 to 7.5%.

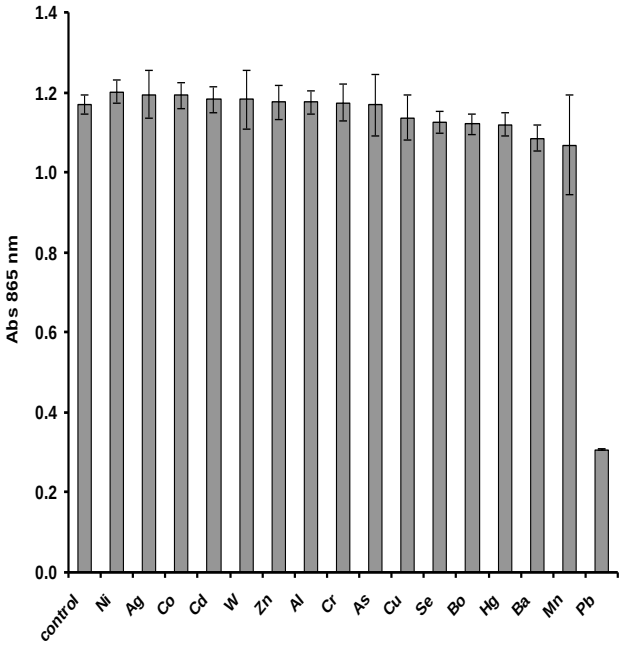

Fig. 1. Screening results for the inhibitory effect of heavy metals on the Mo-reducing enzyme assay. Data is mean± standard error (n=3).

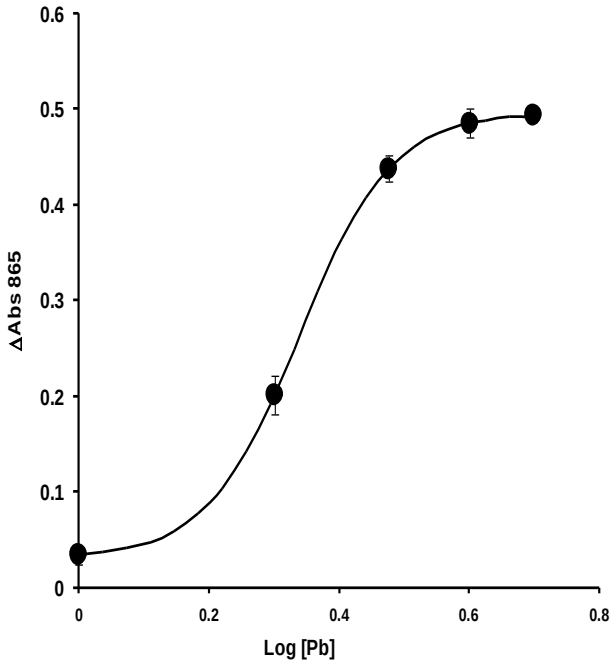

Fig. 2. Inhibition of molybdenum-reducing enzyme by lead. Absorbance of the resultant molybdenum blue from enzyme incubated with lead was subtracted from control. Data is mean± standard error (n=3).

Table 1. Sensitivity of the assay to lead in comparison to EC_{50}, LC_{50} or IC_{50} of several assays.

Assay	IC_{50} or EC_{50} for lead
Immobilized Urease[a]	>250
Papain[b]	2.16±0.53
15 min Microtox™ [a]	1.7-3.0
48 hours *Daphniaa magna* [c]	3.6
96 hours Rainbow trout [c]	8
EC_{50} *Ulva pertussae* (5 days)[d]	0.625(0.745-0.947)
This study	2.186 (2.163-2.208)

[a]Jung *et al*., 1995
[b]Shukor *et al*., 2006
[c]Rodgers *et al*., 1997
[d]Han *et al*., 2005

Interference Study

The influence of foreign species on the assay was investigated. ANOVA analysis showed that of the 13 xenobiotics tested at the final concentration of 0.4%, only SDS significantly inhibited the assay strongly ($p<0.05$). The IC_{50} for SDS was 25 mg l^{-1}. Two of the pesticides tested; metolachlor, and coumaphos at 4 mg l^{-1} showed appreciable inhibition at 13.8 and 12.3% respectively. The concentration of xenobiotics tested in this assay at 4000 mg l^{-1} is not normally found in aquatic bodies at this high level. The concentration of pesticide chosen in this work is generally much higher than normally found in natural water and also limited to the solubility of these pesticides in water and thus not a major interference factor to the lead assay. The results for the interference studies suggest that this inhibitive assay could be a specific assay for lead.

CONCLUSION

In this work a molybdenum-reducing enzyme assay system was used to detect lead. The use of this assay to detect lead is the first of its kind. The assay has been shown to be simple, reproducible and rapid with good sensitivity for the assay of lead. There are many advantages of testing toxicity of heavy metals using this assay. The bacterium is easy to grow and its crude extract is easily prepared. The assay is also not interfered by many xenobiotics with the exception of SDS. The rapidity and simplicity of the system would allow development of a simple kit to monitor lead in environmental or other samples. Currently work is under way to test the assay for environmental samples containing lead contaminant.

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