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# An Improved Enzyme Assay for Molybdenum-Reducing Activity in Bacteria

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**Abstract** Molybdenum-reducing activity in the heterotrophic bacteria is a phenomenon that has been reported for more than 100 years. In the presence of molybdenum in the growth media, bacterial colonies turn to blue. The enzyme(s) responsible for the reduction of molybdenum to molybdenum blue in these bacteria has never been purified. In our quest to purify the molybdenum-reducing enzyme, we have devised a better substrate for the enzyme activity using laboratory-prepared phosphomolybdate instead of the commercial 12-phosphomolybdate we developed previously. Using laboratory-prepared phosphomolybdate, the highest activity is given by 10:4-phosphomolybdate. The apparent Michaelis constant, $K_m$ for the laboratory-prepared 10:4-phosphomolybdate is 2.56±0.25 mM (arbitrary concentration), whereas the apparent $V_{max}$ is 99.4±2.85 nmol Mo-blue $min^{-1}$ $mg^{-1}$ protein. The apparent Michaelis constant or $K_m$ for NADH as the electron donor is 1.38±0.09 mM, whereas the apparent $V_{max}$ is 102.6±1.73 nmol Mo-blue $min^{-1}$ $mg^{-1}$ protein. The apparent $K_m$ and $V_{max}$ for another electron donor, NADPH, is 1.43±0.10 mM and 57.16±1.01 nmol Mo-blue $min^{-1}$ $mg^{-1}$ protein, respectively, using the same batch of molybdenum-reducing enzyme. The apparent $V_{max}$ obtained for NADH and 10:4-phosphomolybdate is approximately 13 times better than 12-phoshomolybdate using the same batch of enzyme, and hence, the laboratory-prepared phosphomolybdate is a much better substrate than 12-phoshomolybdate. In addition, 10:4-phosphomolybdate can be routinely prepared from phosphate and molybdate, two common chemicals in the laboratory.

**Keywords** Assay · Mo-reducing enzyme · Molybdate · Phosphomolybdate · Molybdenum blue

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## Introduction

The pollution of the heavy metal molybdenum has been recorded globally. For example, Japan has recorded evidence of molybdenum pollution in the sea in the Tokyo Bay where molybdenum level reaches hundreds of parts per million [1], and in Tyrol, Austria, molybdenum pollution has contaminated large pasture areas, reaching as high as 200 ppm causing scouring in ruminants. It is in this area that the first documented case of bioremediation of molybdenum was carried out using a combination of microbes and plants [2]. In Malaysia, molybdenum in the form of molybdenite is mined as a by-product of copper mining, and there have been reports of several cases of pollution caused by accidental leakage of pipe-carrying metal system and leaching of the metals from the mining site causing contamination of 2,000 acres of paddy field and the Ranau River [3]. Thus, there is a great need for isolating molybdenum-reducing bacteria as a tool for bioremediation. Understanding the mechanism of reduction would help in improving the bioremediation potential of the molybdenum-reducing bacteria.

Molybdate reduction to molybdenum blue by microbes is an old phenomenon. According to Levine [4], the phenomenon was first reported in *Escherichia coli* [5]. Since then, reports on molybdate reduction by other bacterium trickle in ([6–9]). After a silence of nearly 13 years, microbial molybdate reduction resurfaced again in a report on its reduction by *E. coli* K12 [10]. Its reduction by another chemolitotrophic bacterium; *Thiobacillus ferreoxidans* was reported, and the sulfur/ferric ion oxidoreductase (SFORase) was identified as the enzyme responsible for the reduction [11]. Further studies, however, showed that molybdate reduction in *T. ferreoxidans* is chemically mediated by ferrous iron added into the media [12]. The *T. ferreoxidans* media is very acidic, and the presence of molybdate and phosphate would result in the formation of phosphomolybdate. Ferrous iron is known as a common reducing agent of phosphomolybdate, and its presence would convert the latter to molybdenum blue [13, 14]. In 1993, another heterotrophic bacterium, *Enterobacter cloacae* strain 48 (EC 48), was found to be able to reduce molybdate to molybdenum blue [15].

The mechanism of chemical reduction of molybdate to molybdenum blue is well known. At neutral pH, molybdenum exists as molybdate ions ($MoO_4^{2-}$). Under acidic conditions, molybdate would be converted to polymolybdates (polyions). In the presence of anions such as phosphate, arsenate, sulfate, or silicate, the heteropolymolybdates would form instead. A heteropolymolybdate such as phosphomolybdate is easily reduced by a variety of reducing agents, such as ferrous ions, stannous ions, and dithionite. The mechanism of heteropolymolybdate reduction to molybdenum blue or Mo-blue has been extensively studied. According to the electron spin resonance work, dithionite, a reducing agent, donates two electrons to a heteropolymolybdate, $PMo_{12}O_{40}^{3-}$ (12-molybdophosphate) converting it to molybdenum blue. The introduced electrons are uniformly dispersed over the whole polymetallate sphere by a thermally activated hopping process. The electrons in the two-electron reduced forms were shown by $^{17}O$ nuclear magnetic resonance spectroscopy to be very mobile, thus averaging the valence of all 12 molybdenum atoms [16]. This explains the mixed valence (between 5+ and 6+) properties of the molybdenum in molybdenum blue [13, 14].

It was also shown that the reduction of molybdate to molybdenum blue in EC 48 is predominantly enzymatic using the modified method of Munch and Ottow [17]. We enclosed EC 48 in dialysis tubing and immersed the tubing in molybdenum media. Because molybdenum blue is colloidal, it would not pass through the dialysis tubing. We found that the reduced product, molybdenum blue, is found only in the dialysis tubing. We suggest

that this indicates that the reduction requires the presence of cells and hence is not chemically mediated [18].

The molybdenum-reducing enzyme from the heterotrophic bacterium has never been purified to homogeneity. An attempt to purify this enzyme beyond ammonium sulfate fractionation proved unsuccessful [19]. The original assay employed molybdate as the electron acceptor substrate. We observed that the molybdenum blue produced both from whole cells and enzymatic reduction closely resembles to that of reduced phosphomolybdate. Using this information, we suggest that the formation of phosphomolybdate is an important event during microbial molybdate reduction and proceed to develop a new enzyme assay based on 12-phosphomolybdate (12-phoshomolybdate) as the electron acceptor and found the activity increased several folds. In addition, the assay can be carried out in an open-cuvette manner, whereas the original assay requires complete anaerobicity. Using this assay allowed us to partially purify the molybdenum reducing activity using ion exchange and gel filtration chromatography [20]. In a previous publication on the development of assays for aldehyde oxidase and xanthine oxidase using phosphomolybdate and silicomolybdate as substrates, an improvement of the existing was found accidentally. The authors discovered that laboratory-prepared phosphomolybdate gave much higher activity than 12-phosphomolybdate as the electron acceptor substrate [21]. In this work, we described an improved molybdenum-reducing enzyme assay using a similar approach. We found this new substrate very convenient to prepare, as molybdate and phosphate are common chemicals in the laboratory.

## Materials and Methods

### Chemicals

All chemicals are of analytical grade. Buffers were prepared at the appropriate temperatures according to their final use by mixing the appropriate basic and dibasic salts [22].

### Source of Bacteria and Preparation of Enzyme

*Enterobacter cloacae* Strain 48 was originally isolated from Chengkau, Malaysia and was grown on agar plate and in 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%), $Na_2MoO_4.2H_2O$ (0.242%), and $Na_2HPO_4$ (0.05%) [15]. Although the high phosphate inhibits molybdate reduction to molybdenum blue, the cells contain active enzymes [15]. Growth on low phosphate resulted in a blue sticky culture that complicated the preparation of crude enzyme and enzyme assay. For large-scale growth, EC 48 was grown in 5 l of high phosphate media in separate large conical flasks with a total of 5 l capacity at 30°C for 48 h on an orbital shaker (100 rpm, Kubota). The following experiment was carried out at 4°C unless stated otherwise. Cells were harvested through centrifugation 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 pH 7.5 (Tris buffer prepared at 4°C) containing 0.5 mM dithiothreitol or 2-mercaptoethanol and 0.1 mM phenyl-methyl-sulfonyl-chloride (PMSF) as an anti-protease. Cells suspension was sonicated for 1 min on an ice bath with 4 min cooling with a Branson Sonifier 450 (50% power output at 50% duty cycle) 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. The supernatant was subjected to ultracentrifugation at 105,000×*g* for 1 h, and the supernatant fraction or cytosolic fraction was collected. Protein was assayed using the Coomassie dye-binding method [23] using crystalline bovine serum albumin as the standard.

## 12-Phosphomolybdate as a Substrate for Molybdenum-Reducing Enzyme

Into 1 ml of reaction mixture containing 3 mM of the electron acceptor substrates 12-phoshomolybdate in 50 mM citrate phosphate buffer pH 5.0 at room temperature, 100 μl of NADH (30 mM stock) is added to a final concentration of 3 mM. Fifty microliters of enzyme fraction (1 mg protein) was added to start the reaction. The absorbance increase in 1 min was read at the wavelength of 865 nm [20]. This solution is stable at this pH for several months. When precipitate is seen, the solution is discarded.

## Laboratory-Prepared Phosphomolybdate as a Substrate for Molybdenum-Reducing Enzyme

Ten to one phosphomolybdate or 10:1 and other ratios of phosphomolybdate was prepared arbitrarily as a 20 mM in 50 mM citrate-phosphate buffer pH 5.0 by mixing 200 mM molybdate ($Na_2MoO_4.2H_2O$) with 20 mM phosphate ($Na_2HPO_4.2H_2O$). For 10:2 phosphomolybdate, the ratio is 200 mM molybdate to 40 mM phosphate in 50 mM citrate-phosphate buffer pH 5.0. The procedure for preparation of 10:3, 10:4, and 10:5 phosphomolybdate is the same. To increase the concentration of a particular ratio such as 10:1 from 20 mM arbitrary concentration to 60 mM, we simply increased the concentration of both molybdate and phosphate ratio from 200:20 to 600:120 mM. Final pH adjustment of phosphomolybdate solution in citrate phosphate buffer to pH 5.0 was done using HCl and NaOH. This solution is stable at this pH for several months. When precipitate is seen, the solution is discarded.

Into 1 ml of reaction mixture containing the laboratory-prepared electron acceptor substrates in 50 mM citrate-phosphate buffer pH 5.0 at room temperature, 100 μl of NADH (30 mM stock) is added to a final concentration of 3 mM. Fifty microliters of partially purified Mo-reducing activity fraction (1 mg protein) was added to start the reaction. The absorbance increase in 1 min was read at the wavelength of 865 nm [20]. The resultant molybdenum blue was scanned (Cintra UVPC) between the wavelength of 400 and 980 nm. The kinetic parameters were determined using standard Michaelis–Menten approach utilizing Graph Pad Prism Version 4.00 for Windows (Graphpad Software, San Diego, CA, USA).

## Enzyme Activity

One unit of Mo-reducing activity is defined as that amount of enzyme that produce 1 nmol molybdenum blue (in terms of equivalent reduced 12-phoshomolybdate) per minute at room temperature. There is almost no lag period observed during molybdenum blue production. This increase of absorbance was linear up to 10 min of incubation. The extinction coefficient or molar absorptivity at 865 nm for molybdenum blue using 12-phoshomolybdate as a standard is 16.7 $mM^{-1}$ $cm^{-1}$ and at 710 nm is 11.7 $mM.^{-1}cm^{-1}$ (absorption at 710 nm is 30% less than at 865 nm) [24]. An increase in OD 865 nm of 1.00 unit absorbance per minute per milligram protein would yield 60 nmol of 12-phoshomolybdate or 60 units of enzyme activity in a 1-ml assay mixture.

## Results and Discussions

### Absorption Spectrum

The absorption spectrum of molybdenum blue from the enzymatic reduction of phosphomolybdate using laboratory-prepared phosphomolybdate as a substrate in comparison with the molybdenum blue spectrum from cellular reduced molybdate is shown in Fig. 1. Both spectra have a maximum peak at 860–870 nm and a shoulder at 700 nm. This spectrum is similar to the molybdenum blue produced by ascorbic-acid-reduced phosphomolybdate. Previously, we discussed on the similarity of the molybdenum blue from bacterial reduction to that of the molybdenum blue produced by the phosphate determination method. We suggest that this similarity provide corroborative evidence on the formation of molybdophosphate during the reduction of molybdate to molybdenum blue [24]. This similarity suggests that phosphomolybdate is likely the intermediate formed from the acidification of molybdate in the low phosphate media. It is the phosphomolybdate that is reduced by molybdenum-reducing enzyme into molybdenum blue and not molybdate. The molybdenum-reducing enzyme probably catalyses a similar mechanism to the two-electron transfer of dithionite to phosphomolybdate as mentioned before.

### Kinetic Studies

The results show that, of the entire phosphomolybdate ratios tested, namely from 10:1 (molybdate to phosphate ratio) to 10:5, the highest activity is given by 10:4-phosphomolybdate (Fig. 2). The electron acceptor substrate, 10:4-phosphomolybdate, and the electron donors NADH and NADPH follow a classical Michaelis–Menten rectangular hyperbolic curve when its initial velocity was plotted against its concentration (Figs. 3 and 4). The correlation coefficient of 0.99 and 0.99 for NADH and NADPH, respectively, suggests good correlation between theoretical and experimental data. The $K_m$ and $V_{max}$ were determined using a non-linear regression analysis software, GraphPad Prism version 4.00 for Windows, (GraphPad Software). The apparent Michaelis constant, $K_m$ for the laboratory-prepared 10:4-phosphomolybdate (±SD, $n$=3) is 2.56±0.25 mM (arbitrary concentration), whereas the apparent $V_{max}$ is 99.4±2.85 nmol Mo-blue $min^{-1}$ $mg^{-1}$ protein. The apparent

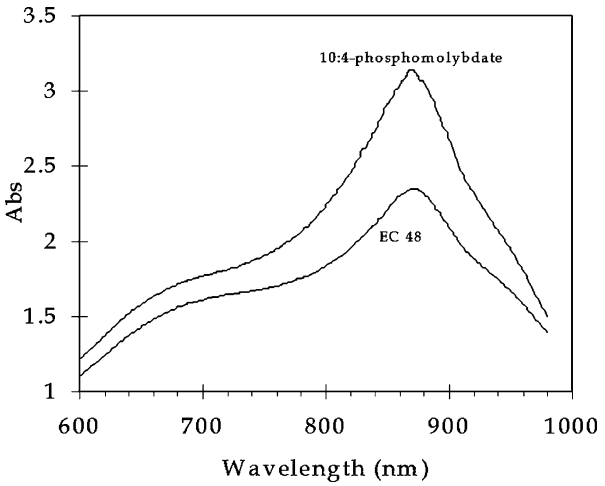


**Fig. 1** Scanning spectra of the molybdenum blue from enzymatic reduction of 10:4-phosphomolybdate and from EC 48 cellular reduction of molybdate

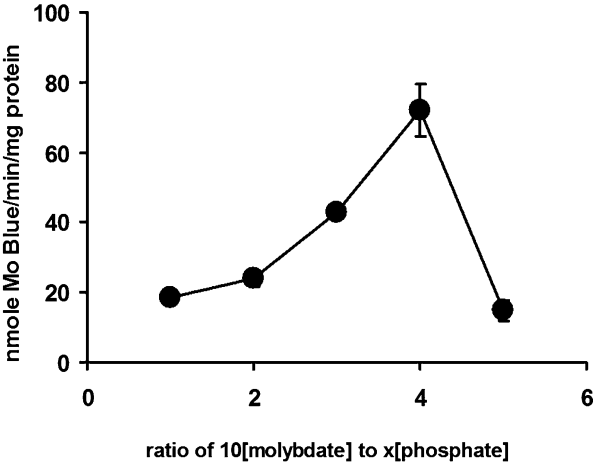


**Fig. 2** Activity of molybdenum-reducing enzyme at various ratios of phosphomolybdate to phosphate. The *error bars* represent mean±standard deviation for three replicates

Michaelis constant or $K_m$ for NADH as the electron donor is 1.38±0.09 mM, whereas the apparent $V_{max}$ is 102.6±1.73 nmol Mo-blue $min^{-1}$ $mg^{-1}$ protein. The apparent $K_m$ for another electron donor, NADPH, at 1.43±0.10 mM is slightly lower than the previously reported value using the 12-phosphomolybdate at 2.2 mM, whereas the apparent $V_{max}$ is nearly halved to that of NADH at 57.16±1.01 nmol Mo-blue $min^{-1}$ $mg^{-1}$ protein using the same batch of molybdenum-reducing enzyme. The apparent $V_{max}$ (measured as specific activity) obtained for NADH and 10:4-phosphomolybdate is approximately 13 times better than 12-phoshomolybdate using the same batch of enzyme, the latter having an apparent $V_{max}$ at 6.06 nmol Mo-blue $min^{-1}$ $mg^{-1}$ protein [20] showing that the laboratory-prepared phosphomolybdate is a much better substrate than 12-phoshomolybdate. In a similar work, the assay of xanthine and aldehyde oxidase using laboratory-prepared heteropolymolybdate (silicomolybdate) was found to give considerably higher $V_{max}$ than commercial silicomolybdate [21]. However, the authors offer no explanation as to the reason for the increase in activity. Literature search also offers no plausible explanation for the increase in activity of laboratory-prepared phosphomolybdate over commercial phosphomolybdate, and hence, this phenomenon remains to be studied further. Literature search also shows that the

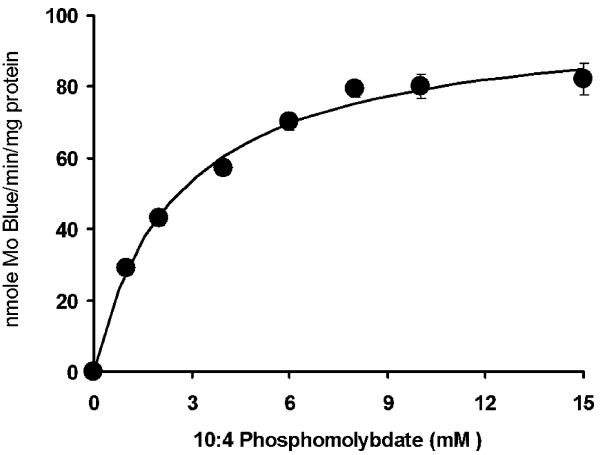


**Fig. 3** Michaelis–Menten plot of initial velocity vs substrate (laboratory-prepared 10:4-phosphomolybdate). The concentration of the $e^-$ donor, NADH, was at saturation level at all points. The *error bars* represent mean±standard deviation of three replicates

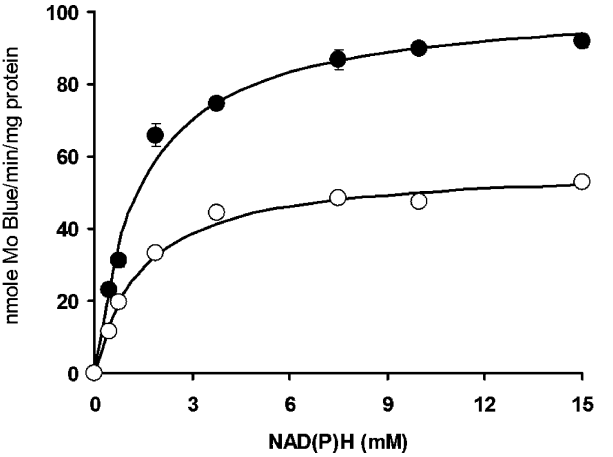


**Fig. 4** Michaelis–Menten plot of initial velocity vs the electron donor substrates NADH (*filled circle*) and NADPH (*open circle*). The concentration of the electron acceptor, 10:4-phosphomolybdate, was at saturation level at all points. The *error bars* represent mean±standard deviation of three replicates

use of phosphomolybdate as an enzyme substrate is rare, and hence, cross-reference is limited. AOD and XOD remains the few enzymes assayed using phosphomolybdate as a substrate. The possible reason why phosphomolybdate is not preferred as a substrate is its notoriety as a sensitive test for the presence of reducing agents ([13, 14, 25]) and it is difficult to distinguish between chemical and enzymatic reduction.

Although the apparent Michaelis constant for 10:4-phosphomolybdate at 2.56 mM is much higher than 12-phoshomolybdate at 0.3 mM [20], the actual composition (phosphomolybdate species) of 10:4-phosphomolybdate is unknown and prevents direct comparison. Using 10:4-phosphomolybdate as a standard electron substrate for Mo-reducing enzyme assay offer several advantages over 12-phoshomolybdate. For instance, the $V_{max}$ obtained is considerably higher, approximately 13 times better than 12-phoshomolybdate with a $V_{max}$ at 8.15±1.29 nmol Mo-blue$^{-1}$ min$^{-1}$ mg$^{-1}$ protein using the same batch of enzyme. In addition, 10:4-phosphomolybdate can be routinely prepared from phosphate and molybdate, two common chemicals in the laboratory.

## Conclusions

In summary, we have devised an open-cuvette alternative substrate for Mo-reducing activity using laboratory-prepared phosphomolybdate. This substrate is approximately 13 times better than the commercial phosphomolybdate 12-phoshomolybdate in terms of $V_{max}$. The absorption spectrum for the reduced form of 10:4-phosphomolybdate (molybdenum blue) is similar to that of 12-phoshomolybdate validating the use of the laboratory-prepared phosphomolybdate as an electron acceptor substrate for Mo-reducing activity. We suggest 10:4-phosphomolybdate to be an alternative substrate for Mo-reducing activity, as it is easily prepared from common chemicals. The principle similarity of the Mo-reducing enzyme assay to that of XOD and AOD assays in relation to phosphomolybdate (another XOD and AOD substrate) is that all of the assays above use phosphomolybdate as the final electron with the reduced product becoming molybdenum blue. The principle difference is that, in the XOD and AOD assays, phosphomolybdate is an artificial substrate, whereas in the Mo-reducing enzyme assay, it is hypothesized as an actual physiological substrate formed from the conversion of molybdate under acidic fermenting conditions.

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