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Isolation and Characterization of a Molybdenum Reducing Enzyme in *Enterobacter cloacae* Strain 48

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ABSTRAK

Enzim yang menurunkan molybdenum telah diasingkan daripada *Enterobacter cloacae* Strain 48 dengan menggunakan pemecahan amonium sulfat, kromatografi pertukaran ion DE-selulosa dan penurasan gel Sephacryl S-200. Elutan daripada penurasan gel Sephacryl S-200 yang telah dipekatkan menunjukkan kehadiran 3 subunit protein masing-masing mempunyai berat molekul 80, 90 dan 100 kDa apabila dilakukan SDS-PAGE. Pencirian aktiviti menurunkan molybdenum oleh fraksi dari elutan Sephacryl S-200 yang dipekatkan dilakukan dengan menggunakan 12-molybdofosfat (12-MoP) sebagai substrat. pH dan suhu optimum bagi tindak balas penurunan molybdenum ialah 5.0 dan 28-33°C. NADH didapati lebih baik daripada NADPH sebagai agen penurun bagi tindak balas tersebut; plot resiprokal berganda aktiviti menurunkan molybdenum melawan NADH dan NADPH masing-masing menghasilkan nilai K_m dan V_{max} 1.65 mM, 6.28 nmol molybdenum biru terhasil/min/mg dan 2.13 mM and 4.10 nmol molybdenum biru terhasil/min/mg. Plot resiprokal berganda aktiviti menurunkan molybdenum melawan 12-MoP dan 20-molybdodifosfat pula menghasilkan nilai K_m 0.3 mM dan 0.4 mM. Nilai V_{max} pula adalah sama bagi kedua-dua substrat iaitu 6 nmole molybdenum biru terhasil/min. Kaedah pengasaian aktiviti menurunkan molybdenum dengan menggunakan 12-MoP didapati lebih mudah dan cepat jika dibandingkan dengan kaedah masa kini yang menggunakan molybdat sebagai substrat.

ABSTRACT

Molybdenum reducing enzyme was isolated from *Enterobacter cloacae* Strain 48 by ammonium sulphate fractionation, DE-cellulose ion-exchange chromatography and Sephacryl S-200 gel filtration. SDS-PAGE of the concentrated Sephacryl S-200 gel filtration eluates revealed the presence of 3 protein subunits of molecular weight 80, 90 and 100 kDa. The active concentrated fraction from the Sephacryl S-200 gel filtration step was then characterized for molybdenum reducing activity with 12-molybdophosphate (12-MoP) as a substrate. The optimum pH and temperature of the reaction was 5.0 and 28-33°C, respectively. NADH was a better reducing agent in the reaction than NADPH; the double reciprocal plot of activity against NADH and NADPH revealed apparent K_m and V_{max} values of 1.65 mM, 6.28 nmole molybdenum blue produced/min/mg and 2.13 mM and 4.10 nmole molybdenum blue produced/min/mg, respectively. The double reciprocal plot of activity against 12-MoP and 20-molybdodiphosphate revealed apparent K_m

values of 0.3 mM and 0.4 mM, respectively. The apparent V_{max} values are similar for both substrates at 6 nmole molybdenum blue produced/min. The assay method for molybdenum reducing activity using 12-MoP was found to be easier and more rapid than the present method of using molybdate as a substrate.

Keywords: Molybdenum reducing enzyme, 12-molybdophosphate, *Enterobacter cloacae* Strain 48

INTRODUCTION

Several heavy metals may be detoxified in bacteria by the action of the metal reductases as shown in the reduction of Fe^{3+} in *Spirillum itersonii, Escherichia coli* and *Paracoccus denitrificans* by ferric reductases (Dailey Jr and Rascelles 1977). The ferric reductase found in *Spirillum itersonii* reduced Fe^{3+} with NADH or succinate as the electron donor and is located in the respiratory chain before cytochrome *c* while in *Azotobacter vinelandii* and *Pseudomonas aeroginosa*, the ferric reductases used NADH as an electron donor and are located in the cytoplasm (Lascelles and Burke 1978; Cox 1980; Huyer and Page 1989).

Molybdenum has been reported to be reduced to molybdenum blue by several bacteria such as *Thiobacillus ferroxidans, Escherichia coli* K12 and *Enterobacter cloacae* Strain 48 with NADH as the electron donor (Campbell *et al.* 1985; Ghani *et al.* 1993; Sugio *et al.* 1988). The molybdenum reducing enzyme in *E. cloacae* Strain 48 has been proposed to be a membrane-bound oxidoreductase, and the reduction reaction carried out anaerobically (Ariff *et al.* 1997).

The reduction of molybdenum to molybdenum blue in *E. cloacae* Strain 48 has been reported to be inhibited by high phosphate concentration (100 mM) in the culture media (6). However, the bacteria was able to reduce the metal when grown in media containing low phosphate concentration (2.9 mM). It has been suggested that high phosphate concentration prevented the formation of molybdophosphates but did not affect the reduction of molybdenum.

Molybdenum reducing activity in bacteria has been assayed using molybdate and NADH, phosphate and other salts at pH 7.0, under anaerobic conditions (Ghani *et al.* 1993). The formation of molybdophosphate complexes (Lee 1977; Sims 1961), which gives the characteristic blue colour of molybdenum blue was determined at 710 nm after the addition of a strong reducing agent such as stannous chloride (Killefer and Linz 1952). The absorption spectrum of molybdenum blue formed by the bacteria is different from that formed chemically (Glenn and Crane 1956). While most of the earlier studies used bacteria culture or crude extracts of the bacteria to demonstrate molybdenum reduction, no work has been carried out to isolate and characterize the protein that catalyses molybdenum reduction.

We report the initial isolation, partial purification and characterization of a molybdenum reducing enzyme in *E. cloacae* Strain 48 to provide evidence that molybdenum reduction in *E. cloacae* strain 48 is catalysed by an enzyme.

MATERIALS AND METHODS

Enterobacter cloacae Strain 48 was grown in 1L minimal salts media, pH 7.0, containing glucose (1.0%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.1%), $Na_2MoO_4.2H_2O$ (0.24%) and Na_2HPO_4 (1.72%) at 30°C for 24 h with gentle shaking (100 rpm), following the method of Ghani *et al.* (1993).

Isolation of Molybdenum Reducing Activity

The purification procedure for molybdenum reducing enzyme was carried out at 4°C unless stated otherwise.

Bacterial cells were harvested from 5L minimal salts media, pH 7.0 by centrifugation at 10,000 g for 20 min and the pellet obtained was rinsed three times with distilled water before being resuspended and recentrifuged. The resulting pellet was resuspended in 10 mL 50 mM Tris buffer pH 7.5 and the bacterial cells were lysed by sonication using four cycles of 1 min with 4 min cooling intervals.

The lysed fraction was then centrifuged at 10,000 g for 40 min and the resulting supernatant subjected to 0-80% ammonium sulphate fractionation. Highest molybdenum reducing activity was detected in the pellet of 40-50% ammonium sulphate fractions. The pellet obtained from the 40-50% ammonium sulphate fraction was centrifuged at 10,000 g for 40 min and resuspended in 10 mL 50 mM Tris buffer pH 7.5 containing 5 mM 2-mercaptoethanol. The suspension was then dialysed against 5L of the same buffer for 5 h.

The dialysate (3 mL) was loaded onto a DE-cellulose column (1.6 cm x 35 cm) equilibrated with 50 mM Tris buffer pH 7.5 containing 5 mM β-mercaptoethanol. Five mL fractions were collected at a flow rate of 0.5 mL per min. A linear gradient of 200 mL 0-300 mM NaCl was applied. Proteins were monitored at 280 nm and molybdenum reducing activity assayed in the collected fractions. Fractions that contained molybdenum reducing activity were pooled and concentrated to a minimal volume by ultra-filtration before being applied to a Sephacryl S-200 column (1.6 cm x 60 cm) equilibrated with the same buffer. The fraction volume and flow rate were similar to that used in the ion exchange chromatography procedure. The fractions containing high molybdenum reducing activity were pooled and concentrated by ultra-filtration.

Protein concentration was determined by the method of Bradford (1976) and SDS-PAGE carried out according to the method of Laemlli (1974).

Assay of Molybdenum Reducing Activity Using 12-MoP as the Substrate

This assay was based on the formation of MoP (as 12-MoP) from molybdate and phosphate (Campbell *et al.* 1985). The 12-MoP formed is assumed to accept the electrons from NADH in the reaction catalysed by molybdenum reducing enzyme. The electrons were then trapped in the lattice structure since MoP is known to accept electrons from reducing agents such as dithionite resulting in

the formation of molybdenum blue (Tosi *et al.* 1998). The hypothetical reaction between 12-MoP and NADH is depicted as;

$$\text{12-MoP} + \text{NADH} + H^+ \rightarrow \text{Molybdenum blue} + NAD^+$$

12-MoP was prepared as a 20mM stock solution in distilled water adjusted to pH 5.0 with HCl. To 0.8 mL of reaction mixture consisting of $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%) and NaCl (0.5%) at pH 5.0, 0.12 mL 20mM 12-MoP and 20 mL 150mM NADH were added in succession. The reaction was started by the addition of 50 mL partially purified enzyme preparation containing approximately 1 mg protein, and the absorbance monitored after every min at 865 nm.

The reducing activity was calculated based on the extinction coefficient of molybdenum blue of 16.7 $mM^{-1}cm^{-1}$ at 865 nm. One unit of molybdenum reducing activity is defined as the amount of enzyme that is required to catalyse the production of 1 nmole molybdenum blue from 12-MoP per min under the conditions specified.

Molybdenum Reducing Assay Using Molybdate as the Substrate

Molybdenum reducing activity with molybdate as the substrate was determined according to the method described by Ghani *et al.* (1993). Briefly, the reaction mixture consisted of 1 mL solution containing $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%) and NaCl (0.5%) at pH 7.0, 0.4 mL 0.5M molybdate, 0.5 mL 20mM Na_2HPO_4 and 0.1 mL 80mM NADH. To start the reaction, 20 mL of partially purified enzyme preparation containing approx. 20 mg protein was added to the reaction mixture. Nitrogen gas was pumped into the cuvette for approx. 30 s. The mixture was incubated for 1 h at 28°C after which the absorbance at 710 nm was measured. The amount of molybdenum blue was calculated from a standard curve of molybdenum reduced by stannous chloride in the presence of phosphate.

Effect of pH and Temperature on Molybdenum Reducing Enzyme

To study the effect of pH on molybdenum reducing activity, 50 uL of partially purified enzyme preparation (approx. 0.1 mg) was added to a total volume of 2.0 mL reaction mixture of various pHs containing 4.8 umol 12-MoP, 8 umol NADH and 0.4 mmol potassium antimonyl tartarate. The reaction mixture was incubated at 28°C and absorbance at 865 nm measured at 1 min intervals.

The effect of temperature on molybdenum reducing activity was tested under conditions similar to that used in the study of the effect of pH on molybdenum reducing activity. However, potassium antimonyl tartarate was added to act as a catalyst for the reduction of 12-MoP at temperatures lower than 30°C (Tosi *et al.* 1998; Clesceri *et al.* 1989). The reaction mixture was incubated at various temperatures for 20 min in a temperature-controlled water bath and the reaction started by adding 0.1 mg partially purified enzyme. The

reaction mixture was then incubated for another 10 min after which the absorbance at 865 nm was read.

Effect of NADH and NADPH on Molybdenum Reducing Activity

To 1.60 mL reaction mixture pH 5.0 containing 3 mM 12-MoP and 0.2 mM potassium antimonyl tartarate, 0.1 mL of NADH and NADPH were added. Distilled water was added to make up the total volume to 2 mL. To start the reaction, 50 uL of partially purified enzyme preparation containing approx. 0.1 mg protein, was added and after 1 min the absorbance at 865 nm was read.

Effect of 12-MoP and 20-Molybdodiphosphate on Molybdenum Reducing Activity

12-MoP($H_3Mo_{12}O_{40}P$, Mo:P ratio 12:1) and 20-molybdodiphosphate ($20MoO_4 2H_3PO_4.2H_2O$, Mo:P ratio 20:2) were prepared as 20 mM solutions in 50 mM citrate phosphate buffer pH 5.0. The cloudy solution became clear when left stirring overnight.

40 µL of 150 mM NADH was added to 1.60 mL of reaction mixture pH 5.0 containing 3 mM 12-MoP or 20-molybdodiphosphate and 0.2 mM potassium antimonyl tartarate. When 20-molybdodiphosphate was used, the potassium antimonyl tartarate was excluded. Distilled water was added so that the total volume was 2 mL. The reaction was started by the addition of 50 uL partially purified enzyme preparation containing approx. 0.1 mg protein. The absorbance at 865 nm was read after one min intervals.

RESULTS

Isolation of Molybdenum Reducing Enzyme

Molybdenum reducing enzyme in *E. cloacae* Strain 48 had been isolated by 40-50% ammonium sulphate fractionation followed by ion exchange on DE-cellulose and gel filtration on Sephacryl S-200. The results of the purification procedure are summarised in Table 1. Ammonium sulphate fraction was an excellent method for isolating the enzyme with a 6.5 fold purification while the recovery was kept high at 97% of the initial value. Ion exchange chromatography on DE-cellulose of the salt fraction yielded a single peak (*Fig. 1*). Although the purification was impressive with a high purification fold, a large reduction in activity was recorded for the ion exchange and gel filtration steps (*Fig. 2*). The final yield was 1.6% of the initial value and the final specific activity was 240 unit/mg protein realizing a purification of 40-fold over the initial value.

For both the ion exchange and gel filtration chromatographic steps, the volume of the active fractions were large and molybdenum reducing activity was low, making it necessary to concentrate the eluates. The concentrated eluates from the Sephacryl S-200 gel filtration step was subsequently used in characterization of the enzyme. SDS-PAGE of the concentrated eluates from the Sephacryl S-200 gel filtration step showed 3 protein subunits of estimated molecular weights of 80, 90 and 100 kDa (*Fig. 3*).

TABLE 1
A summary of the purification of molybdenum reducing enzyme in *Enterobacter cloacae* Strain 48

Fraction	Total Protein (mg)	Total Activity (Units)	Specific Activity (Unit/mg protein)	Fold Purification	Yield %
Crude homogenate	5000	30,000	6.0	1	100
40-50% saturated ammonium sulphate	750	29,000	39.0	6.5	97.0
DE-cellulose	40	4,500	113.0	18.8	15.0
Sephacryl S-200	2	480	240	40.0	1.6

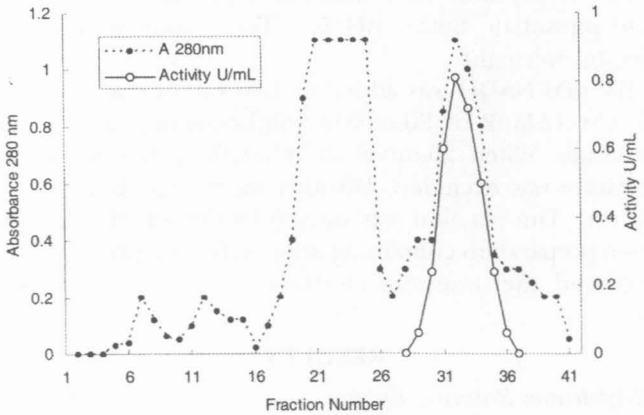

Fig. 1: DE-cellulose ion exchange chromatography profile of molydenum reducing enzyme in E. cloacae Strain 48. A 0-0.3M NaCl gradient was applied between fractions 5 - 40

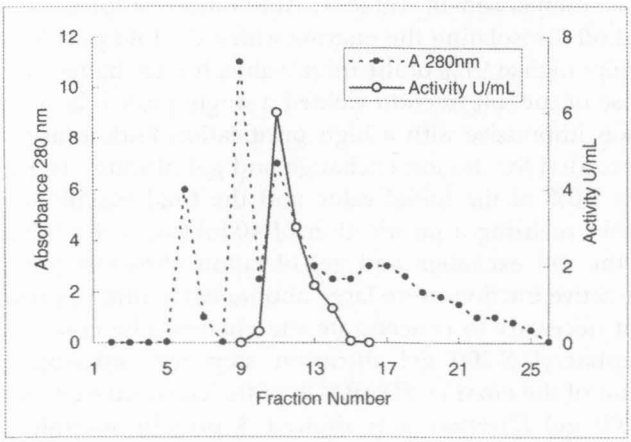

Fig. 2: Sephacryl S-200 gel filtration profile as a purification step molybdenum reducing enzyme in E. cloacae Strain 48

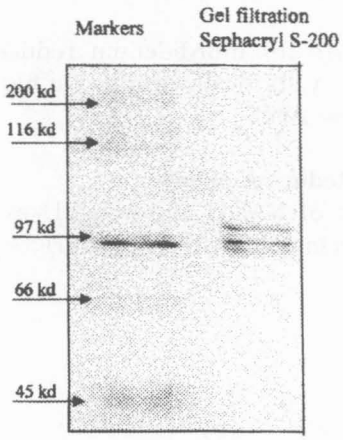

Fig. 3: SDS-PAGE of the molybdenum reducing enzyme purified in E. cloacae Strain 48. The concentrated eluate of Sephacryl S-200 gel filtration step was applied to SDS-PAGE. Staining of the electrophoresed sample with Coomassie brilliant blue yield 3 subunits of estimated molecular weight of 80, 90 and 100 kDa

Characterisation of Molybdenum Reducing Enzyme

Optimum pH

Fig. 4 shows the optimum pH for molybdenum reducing enzyme to be pH 5.0. There was almost no activity at pH 7.0 and above. The activities detected were between the range of pH 3.5 – 6.0. Molybdenum reducing activity mainly occurs in acidic pH.

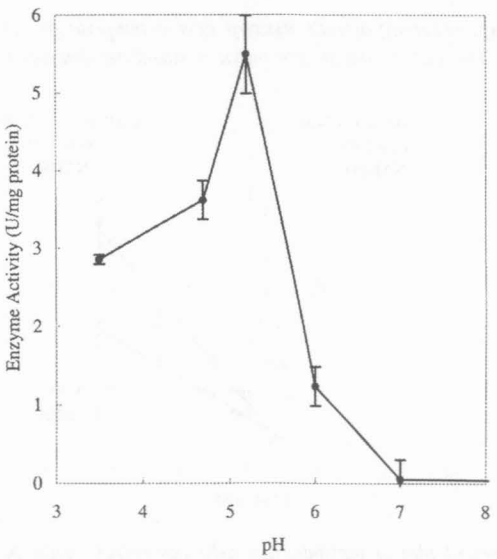

Fig. 4: Molybdenum reducing activity over pH range of 3.0 to 8.5. The values shown are mean ± standard deviation

Optimum Temperature

The optimum temperature for molybdenum reducing activity was between 28-33°C (*Fig. 5*). The activity drastically dropped at higher temperatures and no activity was detected below 20°C.

NADH and NADPH as Reducing Agents

A double reciprocal plot of NADH and NADPH as substrates revealed that NADH was a better reducing agent than NADPH (*Fig. 6*). The apparent V_{max}

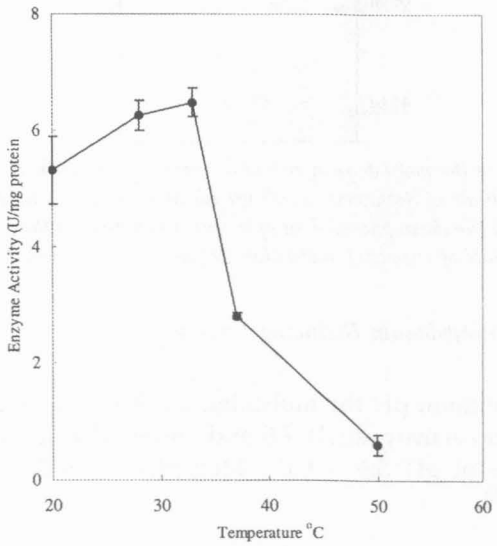

Fig. 5: Molybdenum reducing activity assayed over a temperature range of 20 – 50°C. The values shown are mean ± standard deviation

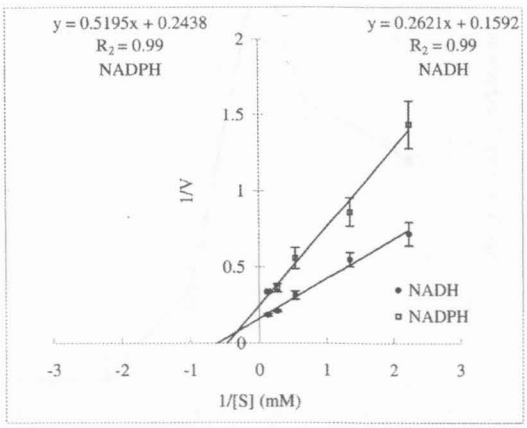

Fig. 6: Double reciprocal plot of molybdenum reducing activity with NADPH and NADH as reducing agents. The values shown are mean ± standard deviation. V, nmol molybdenum blue produced/min/mg protein under the conditions specified; (S), mM NADH or NADPH

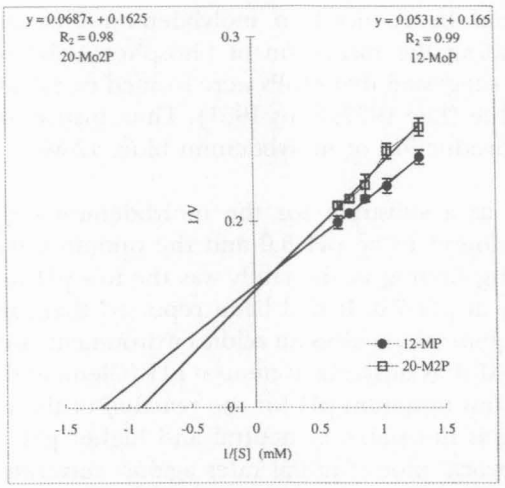

Fig 7: Double reciprocal plots of two different substrates in molybdenum reducing activity. The values shown are mean ± standard deviation. V, nmol molybdenum blue produced/ min/mg protein under the conditions specified; (S), mM 12-molybdophosphate (12-MP) or 20-molybdodiphosphate (20-M2P)

and K_m values for NADH were 6.28 nmole molybdenum blue produced/min and 1.65 mM, while for NADPH, the values were 4.10 nmole molybdenum blue produced /min and 2.13 mM, respectively.

12-MoP and 20-Molybdodiphosphate as Oxidants

A double reciprocal plot of 12-MoP and 20-molybdodiphosphate as electron acceptors for molybdenum reducing activity showed that the apparent V_{max} of 6.10 nmole molybdenum blue/min were similar for both compounds (*Fig. 7*). However, the apparent K_m values were different; 0.32 mM for 12-MoP and 0.42 mM for 20-molybdodiphosphate.

DISCUSSION

Based on the results of the isolation of molybdenum reducing activity in the present study, we propose that molybdenum reducing activity is catalysed by an enzyme comprising at least three subunits of 80, 90 and 100 kDa in size.

Further purification of the active components was not possible due to the very low yield obtained after the final Sephacryl S-200 gel filtration step. The large loss in activity during the ion exchange and gel filtration step could be linked to the properties of the enzyme. Bacterial production of molybdenum blue had been proposed to be anaerobic and NADH dependent (Campbell *et al.* 1985; Ghani *et al.* 1993; Sugio *et al.* 1988; Ariff *et al.* 1997). Possibly, the cofactors(s) that stabilized the proteins could be lost during the ion exchange step and being continuously exposed to oxygen could further contribute to the loss of activity.

Molybdate could be reduced to molybdenum blue in the presence of phosphates, indicating the formation of phosphomolybdate (Campbell *et al.* 1985). It has been suggested that MoPs were formed transiently from molybdate to molybdenum blue (Lee 1977; Sims 1961). Thus, instead of molybdate as the substrate for the production of molybdenum blue, 12-MoP may be used as an alternative.

Using 12-MoP as a substrate for the molybdenum reducing activity, the optimum pH was found to be pH 5.0 and the optimum temperature around 30°C. An interesting finding in the study was the low pH for the reaction and the lack of activity at pH 7.0. It had been reported that formation of 12-MoP and molybdates in general, requires an acidic environment (Lee 1977; Braithwaite 1981) and that 12-MoP is unstable at neutral pH (Glenn and Crane 1956). This could explain the low optimum pH for the reaction of the enzyme on 12-MoP, since the substrate is not stable at neutral and higher pH.

A double reciprocal plot of initial rates against substrate concentrations at 2.4 mM 12-MoP registered a higher apparent V_{max} for NADH than NADPH (6.28 and 4.10 nmole/min, respectively), but the apparent K_m for NADH was lower than NADPH (1.65 and 2.13 mM, respectively). It seemed that NADH is the prefered reducing agent of molybdenum reducing activity over NADPH.

At 3mM NADH, the double reciprocal plots against 12-MoP and 20-molybdodiphosphate yielded a similar apparent V_{max} value of 6.10 nmole/min but different apparent K_m values of 0.32 and 0.42 mM, respectively. It is obvious that 12-MoP is preferred over 20-molybdodiphosphate.

The concentration of 12-MoP was found to be critical for the reaction. A significant reduction in activity was observed above 3mM 12-MoP concentrations. Also, low molybdenum reducing activity was observed at low 12-MoP concentration. It seemed that molybdenum reducing activity is at an optimum between 1 - 3 mM 12-MoP. The possibility of molybdenum reducing enzyme being allosteric could not be discounted since the preparation used comprised at least 3 subunits by SDS-PAGE.

Potassium antimonyl tartarate seemed to assist in the formation of molybdenum blue from 12-MoP. It was reported that 12-MoP is unstable in very dilute solution and potassium antimonyl tartarate functioned as a stabilising agent in the phosphate determination method (Clesceri *et al.* 1989).

We have found that the assay for molybdenum reducing activity using 12-MoP as the substrate to be advantageous over the use of molybdate; it is rapid, can be completed in a few min and did not require nitrogen as the gas phase. The assay method of using molybdate as the substrate required a much longer time to complete the reaction, phosphate to be added as a reactant and nitrogen as the gas phase. It was also found that V_{max} for 12-MoP was several folds higher than molybdate, making it a more rapid assay to perform.

In conclusion, evidence has been provided to support the suggestion that molybdenum reduction in *E. cloacae* strain 48 is catalysed by an enzyme consisting of three subunits and is dependent on NADH. An assay for molybdenum reducing activity using 12-MoP as a substrate has been proposed.

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