

A rapid method for the purification of methanol dehydrogenase from *Methylobacterium extorquens*

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Abstract

Methanol dehydrogenase (MDH) is a water soluble quinoprotein that catalyzes the oxidation of methanol as an important carbon source in methylotrophic bacteria. A rapid method for the purification of MDH from *Methylobacterium extorquens* AM1 was developed using a single cation exchange chromatographic step, followed by ultrafiltration for final purification, enzyme concentration, and buffer exchange. MDH was obtained in an excellent overall yield with a final enzyme purity of greater than 97%. Storage at -80°C in 20 mM phosphate buffer, pH 7.0, showed only a negligible loss of enzyme activity after six months.

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Methanol dehydrogenase (MDH,¹ EC 1.1.99.8) is a water soluble quinoprotein that is involved in the metabolism of small molecules such as methanol and methane in methylotrophic bacteria [1,2]. This process is recognized as a key step in providing energy for the growth and development of methylotrophs [2].

MDH has been isolated and purified from several different strains of microorganisms including *Methylobacterium extorquens* AM1 [3], *Methylosinus trichosporium* OB3b [4], *Hyphomicrobium X* [5], *Methylosinus* sp. WI 14 [6], and *Methylophaga* sp. strain 1 [7]. X-ray crystallographic studies on MDH from *M. extorquens* [1,8], *Methylophilus* W3A1 [9], and *Paracoccus dentrificans* [10] demonstrated that MDH exists in an $\alpha_2\beta_2$ tetramer.

The larger α -subunit is 66 kDa and the β -subunit is only 8.5 kDa [1]. Within each α -subunit a molecule of the noncovalently bound redox coenzyme pyrroloquinoline quinone (PQQ, 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) and a Ca^{2+} ion are found in the active site and are both key to the mechanism of action of MDH. The role of the β -subunit is unclear [1].

The cofactor PQQ undergoes an efficient pH-dependent two-electron, two-proton reversible electron-transfer reaction at relatively low potentials, which differentiates it from other redox cofactors such as flavins and nicotinamides [11–13]. PQQ has also been shown to catalyze non-enzymatic reactions at moderate pH and temperature [14–16]. The unique redox cycling properties of PQQ and the enzyme specific reactions of PQQ-dependent dehydrogenases are therefore ideal for use as bioelectrocatalysts in highly selective and sensitive electrochemical-based sensor devices. Furthermore, the PQQ-based enzymatic reactions are oxygen independent and thus can be coupled to electrode systems with a wide range of artificial electron acceptors [17]. Several

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¹ Abbreviations used: MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; ATCC, American Type Culture Collection; BSA, bovin serum albumin; PMS, phenazine methosulfate; DCIP, 6-dichloroindophenolate; Mes, 4-morpholine ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

applications include the immobilization of free PQQ in a polypyrrole membrane for the selective detection of thiols [18,19], the development of PQQ-based enzyme sensors for alcohols, glucose, and other carbohydrates [20,21] and for the fabrication of biofuel cells [21–23].

In prior separation and purification procedures for MDH, either an anion exchange chromatographic or an ammonium sulfate fractionation step, followed by many subsequent steps were employed to achieve adequately purified enzyme [3–7]. Although these procedures produced purified MDH they are labor intensive, time consuming, and result in relatively low overall yields of the enzyme. In this paper, the rapid purification of MDH from *M. extorquens* AM1 was achieved using a single cation exchange chromatographic step, followed by ultrafiltration. The details of this approach and an examination of the optimal conditions for long-term storage are described.

Materials and methods

Materials

The microorganism, *M. extorquens* AM1 (ATCC14718), was purchased from the American Type Culture Collection. Separation experiments were performed using SP Sepharose HP resin (Amersham Biosciences) and Amicon Centriplus YM-50 centrifugal filter devices (Fisher). Pre-stained protein molecular weight marker with a range of 6–175 kDa was obtained from New England Biolabs. Bradford reagent, bovine serum albumin (BSA), phenazine methosulfate (PMeS), sodium 2,6-dichloroindophenolate hydrate (DCIP), and 4-morpholine ethanesulfonic acid (Mes) were purchased from Sigma. All other reagents were analytical reagent grade and used as received. Solutions were prepared with water distilled and deionized to a resistivity of at least 17.5 MΩcm by a Barnstead B-pure water purification system (Dubuque, IA).

Cell growth

Methylobacterium extorquens AM1 was grown aerobically at 30 °C in a rotatory shaker according to the method of Day and Anthony [3]. The bacteria were harvested at the end of an exponential growth period of 2–3 days by centrifugation at 10,000 rpm for 15 min in a Beckman J2-HS centrifuge. The cell paste was stored at –20 °C without further treatment for subsequent enzyme purification.

Enzyme isolation and purification

Cell paste (20 g) was suspended in 80 mL H₂O and placed in an ice bath to slowly thaw while stirring. The cells were then broken by ultrasonication (Heat Systems Ultrasonics, Model W-380) with 20 repetitive cycles of

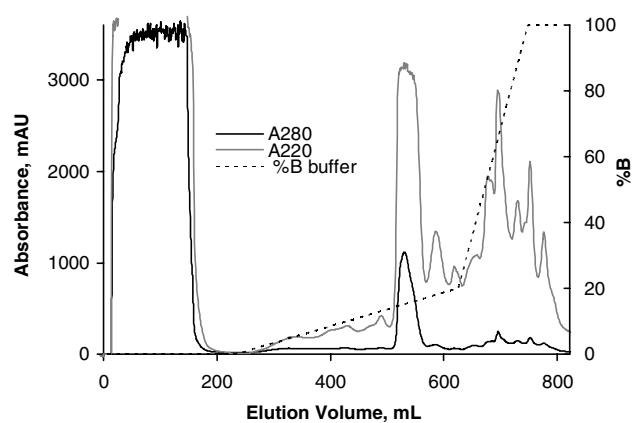


Fig. 1. Chromatogram of the crude cell extract on a SP Sepharose HP cation exchange column. The column was preconditioned and washed with buffer A, Mes 25 mM, pH 5.5. The enzyme was eluted by a programmed gradient (-----) of buffer B, Mes 25 mM, pH 5.5, with NaCl 250 mM. The flow rate was 3 mL/min.

ultrasonication for 30 s followed by cooling for 30 s in an ice bath. Cell debris was removed by centrifugation at 12,000 rpm for 30 min at 4 °C. The resulting supernatant was mixed with 100 mM Mes, pH 5.5 (v/v = 3:1) at 4 °C to a final buffer concentration of 25 mM.

Enzyme purification from the crude cell extract was conducted on an ÄKTA Explorer 100 (Pharmacia) chromatographic system at 4 °C. Crude extract was first filtered through a 0.8 mm cellulose acetate disposable syringe filter and then applied onto a SP Sepharose HP Model XK16 column (bed volume = 20 mL), which was preconditioned with 25 mM Mes, pH 5.5. The extract was first washed with 25 mM Mes, pH 5.5, to remove unbound proteins and then eluted with a programmed gradient of 0–100% of 25 mM Mes, pH 5.5, containing 250 mM NaCl (Fig. 1). Active enzyme fractions were combined and concentrated by ultrafiltration with Amicon Centriplus YM-50 filters. For long-term storage, the buffer medium was exchanged to 20 mM phosphate, pH 7.0, by two ultrafiltration cycles. The final MDH solution (16.6 mg/mL) in 20 mM phosphate, pH 7.0, was stored at –80 °C.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of MDH

Gel electrophoresis was performed using a Bio-Rad Mini Protein II apparatus. MDH solutions with protein concentrations of 0.5–1.0 mg/mL were treated with 4× SDS sample buffer (Novagen) containing dithiothreitol (DTT) at 85 °C for 2 min to denature the protein. Solutions were then applied to a pre-cast polyacrylamide 10–20% Tris–glycine gel (Bio-Rad) and separated at a constant voltage of 150 V with a running buffer of 25 mM Tris, 190 mM glycine, and 0.1% (w/v) SDS, pH 8.3. Protein bands were visualized by Coomassie blue staining for 1 h followed by overnight destaining. The enzyme purity was

estimated by digitizing the SDS gel image with UN-SCAN digital software (Silk Scientific).

Enzyme assay

MDH activity was determined spectrophotometrically with PMeS and DCIP as electron acceptors at room temperature [3]. The assay mixture contained 100 mM Tris-HCl, pH 9.0, 15 mM NH₄Cl, 10 mM CH₃OH, 50 μM DCIP, 0.33 mM PMeS, and a fixed volume (1–20 μL) of enzyme solution that contained approximately 50 mU of MDH. The reaction was initiated by the addition of PMeS, and the MDH activity was determined by measuring the decrease of DCIP absorbance at 600 nm ($\epsilon = 1.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) within 10–30 s. The assay mixture without enzyme served as the control. One unit of enzyme activity was defined as the amount that catalyzed the reduction of 1 μmol DCIP per minute. Protein concentrations were determined by the Bradford method with BSA as the standard [24].

Results and discussion

Purification of MDH

MDH from *M. extorquens* AM1 has a basic *pI* value of 8.8 and therefore will be positively charged in low pH solutions [3]. For this reason, cation exchange chromatography was chosen as the first step for enzyme purification. Preliminary experiments were conducted in a batch adsorption mode to evaluate several possible column conditions for the initial purification of MDH. The conditions tested included 25 mM Mes, pH 5.5, 25 mM phthalic acid, pH 5.5, 25 mM Mes, pH 6.2, 25 mM phosphate, pH 6.5, and 25 mM phosphate, pH 7.2. Between pH 6.0 and 7.5 MDH binding to SP Sepharose HP was not strong enough to achieve complete adsorption. At pH 5.5 complete adsorption of MDH by SP Sepharose HP was observed when 25 mM Mes ($I=2 \text{ mM}$) buffer was used, while no adsorption occurred for 25 mM phthalic acid ($I=50 \text{ mM}$) due to the higher ionic strength. These studies identified the optimal binding conditions for MDH and suggested the use of a salt gradient for protein elution.

Twenty-five millimolar Mes, pH 5.5, was used as the initial column conditions for loading the crude MDH extract on the SP Sepharose HP column. The crude extract was then washed with 25 mM Mes, pH 5.5, until the absorbance at 220 and 280 nm returned to baseline, which signified removal of unbound protein material and other impurities (Fig. 1). MDH was then eluted by slowing increasing the ionic strength of the buffer by introducing a gradient of 25 mM Mes, pH 5.5, with 250 mM NaCl. MDH was eluted when the NaCl concentration reached 35–45 mM. Once MDH was eluted, the

NaCl gradient was rapidly increased to 100% 25 mM Mes, pH 5.5, 250 mM NaCl to remove tightly bound proteins from the column. A final column wash with two column volumes of 1 M NaOH removed a large red band of unwanted cell extract material.

The enzyme fractions were pooled and then analyzed by SDS-PAGE (Fig. 2). A dramatic improvement in the purity of the isolated enzyme mixture resulted after the single chromatographic purification step. Comparison of lanes 2 and 3 in Fig. 2 demonstrates this improvement, and shows the major remaining bands are at the expected positions of the α -subunit (~62 kDa) and the β -subunit (~7.5 kDa) of MDH. The pooled enzyme also showed very faint impurity bands at 17 and 35 kDa. Further purification and concentration was accomplished by two ultrafiltration cycles through a 50 kDa cutoff membrane. As a result, the impurity bands were minimized (Fig. 2, lane 4). Purification of smaller quantities of cell extract or additional ultrafiltration cycles resulted in undetectable impurity bands in SDS-PAGE experiments as compared to Fig. 2, lane 4. The specific activity of MDH increased by a factor of 20 (from 0.25 to 5.0 U/mg) from the crude extract to the pooled enzyme and then from 5.0 to 5.4 U/mg after the ultrafiltration step. Densitometry scanning of the SDS-PAGE gel showed that the purity of MDH was higher than 97%. The total enzyme activity after cation exchange chromatography was 139% relative to the crude extract. In addition, the overall yield after ultrafiltration was determined to be 130%, which is much higher than previously reported yields for MDH, i.e., typically less than 60% [3–7]. This increase in the total enzyme activity relative to the crude extract is presumably due to the reduced accuracy of the enzyme assay at low enzyme purity and the possible presence of interfering or inhibitory compounds in the

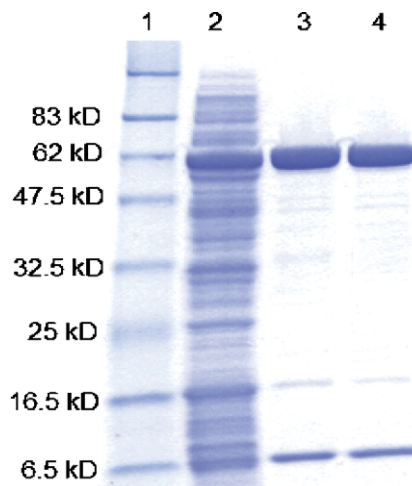


Fig. 2. SDS-PAGE of the crude cell extract and purified MDH. Lane 1, molecular weight marker; lane 2, crude cell extract; lane 3, MDH pooled fractions after cation exchange chromatography; lane 4, MDH after two ultrafiltration cycles.

Table 1
Purification of MDH from *Methylobacterium extorquens* AM1

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Yield (%)	Adjusted yield ^a (%)
Crude extract	896	224	0.25	—	100	100
SP Sepharose HP	63	312	5.0	20	139	71
YM-50 ultrafiltration	54	291	5.4	22	130	67

^a Calculation based on digitized SDS–PAGE bands for MDH at a 9% expression level relative to the total soluble protein in the crude extract.

crude extract. Further examination of the SDS–PAGE gel by densitometry provided an estimate of the MDH expression levels at about 9% of the total soluble proteins in the crude extract (Fig. 2). Based on the specific activity of the purified enzyme this translates into an expected total of 436U in the cell extract, and an adjusted overall yield on purification of nearly 70%. The purification results are summarized in Table 1.

Storage stability of MDH

When MDH was stored at 4 °C approximately 80% of the enzyme activity was lost after one week in 20 mM phosphate, pH 7.0. The enzyme stability was then moni-

tored as a function of several additives to identify improved conditions for long-term storage. The addition of Ca²⁺ and DTT yielded little stabilization for MDH. EDTA and NaN₃ were also used and provided a slightly greater benefit, but more than 60% of the enzyme activity was still lost within one month at 4 °C (Fig. 3A). Increasing the ionic strength of the enzyme solution with KCl concentrations varying from 0.05 to 0.5 M or storing the enzyme at pH values ranging from 5 to 7 did not result in a significant increase in enzyme stability. At –20 °C approximately 56% of the enzyme activity was retained after 4 weeks if no additive was present (Fig. 3B). Sucrose (25%) and glycerol (20%) further enhanced the MDH stability, but glycerol was also found to be a substrate, although a poor one, for MDH. At –80 °C, minimal loss in enzyme activity was observed over a six-month period in the absence of an additive when stored in a 20 mM phosphate, pH 7.0, buffer solution. Virtually all of the activity was retained within one month at –80 °C in 20 mM phosphate, pH 7.0, within 6 h in an ice bath in 20 mM phosphate, pH 7.0, and within 2 h at room temperature in 100 mM Tris buffer, pH 9.

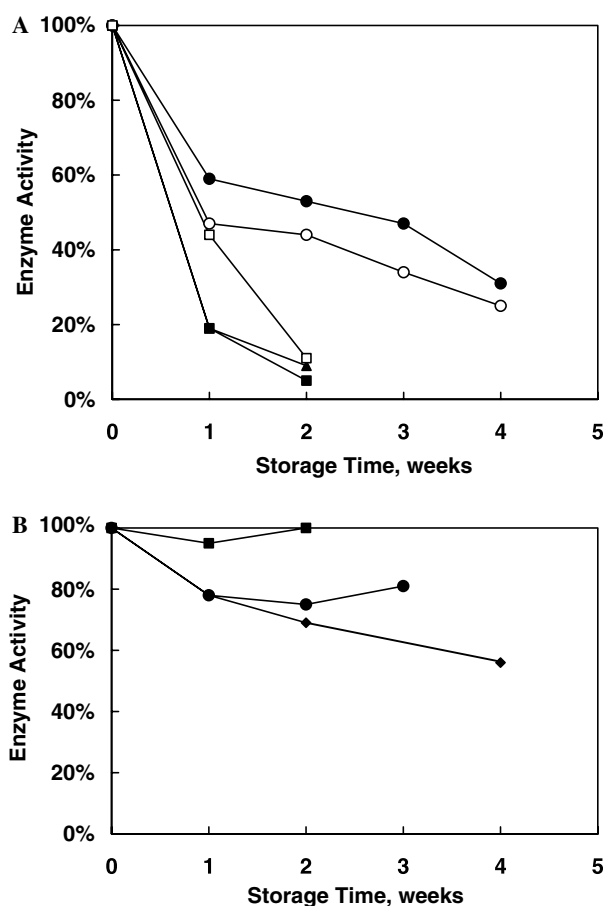


Fig. 3. The effect of different additives on the enzyme activity of MDH as a function of time. (A) Storage at 4 °C: (■) no additive; (▲) 0.5 mM CaCl₂; (□) 0.5 mM DTT; (○) 0.5 mM EDTA; (●) 0.5% NaN₃; (B) storage at –20 °C: (◆) no additive (■) 20% glycerol; (●) 25% sucrose.

Summary

MDH was purified from *M. extorquens* AM1 by a single cation exchange chromatographic step followed by ultrafiltration. This straightforward procedure resulted in high yields of enzyme with purities greater than 97%. The optimum storage conditions were determined to be at –80 °C in 20 mM phosphate, pH 7.0, with no detectable loss in enzyme activity within one month and minimal loss after six-months.

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