Purification and Characterization of a Methanol Dehydrogenase Derived from *Methylomicrobium* sp. HG-1 Cultivated Using a Compulsory Circulation Diffusion System

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Abstract Methanotrophs are microorganisms that possess the unique ability to utilize methane as their sole source of carbon and energy. A novel culture system, known as the compulsory circulation diffusion system, was developed for rapid growth of methanotrophic bacteria. Methanol dehydrogenase (MDH, EC 1.1.99.8) from *Methylomicrobium* sp. HG-1, which belongs to the type I group of methanotrophic bacteria, can catalyze the oxidation of methanol directly into formaldehyde. This enzyme was purified 8-fold to electrophoretic homogeneity by means of a 4 step procedure and was found in the soluble fraction. The relative molecular weight of the native enzyme was estimated by gel filtration to be 120 kDa. The enzyme consisted of two identical dimers which, in turn, consisted of large and small subunits in an $\alpha_2\beta_2$ conformation. The isoelectric point was 5.4. The enzymatic activity of purified MDH was optimum at pH 9.0 and 60°C, and remained stable at that temperature for 20 min. MDH was able to oxidize primary alcohols from methanol to octanol and formaldehyde.

Keywords: methanol dehydrogenase, *Methylomicrobium* sp. HG-1, methanotrophic bacteria

INTRODUCTION

Methane plays an important role in global warming, and its atmospheric concentration has been increasing over many decades [1,2]. Methane-oxidizing bacteria (methanotrophs) are widespread in nature and are considered important regulators of atmospheric methane fluxes in nature [3,4]. Methanotrophs possess the unique ability to utilize methane as their sole source of carbon and energy. These aerobic bacteria convert methane to methanol in the presence of methane monooxygenase in the first step of their metabolic pathway. The current classification system separates all known methanotrophs into 3 groups –types I, II, and X– on the basis of multiple criteria, including cell morphology, the arrangement of intracytoplasmic membranes, the pathway for formaldehyde assimilation, the guanine plus cytosine content of the DNA, and major cellular fatty acid profiles [2].

Methanol dehydrogenase (MDH) is an enzyme responsible for the oxidation of methanol to formaldehyde, which is assimilated into the cellular biomass or oxidized further to CO_2 to provide the reducing power needed for biosynthesis. *In vitro* MDH is coupled to the electron transport chain at the level of cytochrome *c* [5-9]. When prepared aerobically, this coupling is irreversibly destroyed. As a result, MDH requires ammonium salt to be activated (this can be replaced by methylamine, but not by di- or trimethylamine) [10]. Most of the MDH enzymes that have been described, especially those derived from facultative or obligate methylotrophic bacteria, are very similar in their molecular and biochemical properties, with exceptions in those derived from several gram-positive and thermotolerant bacteria [11].

The oxidation of methanol is usually catalyzed by a MDH that was originally described in *Pseudomonas* M27 [12]. This enzyme is found in large amounts especially in organisms that grow on one-carbon compounds (*e.g.*, methane and methanol) and is known for its relatively broad spectrum of substrate specificity [13]. MDH has pyrroloquinoline quinone as a prosthetic group and cytochrome c_L as its primary electron acceptor.

MDH has been characterized in several methanotrophs [14,15]. *Methylomicrobium* sp. HG-1 is a type I novel obligate methane-oxidizing bacterium that was isolated from the effluent of manure by this lab. In this study, we describe a novel culture system for the rapid growth of methanotrophic bacteria and purification as well as some of the biochemical and molecular properties of MDH derived from *Methylomicrobium* sp. HG-1.

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MATERIALS AND METHODS

Microorganism, Medium, and Cultivation

Methylomicrobium sp. HG-1 was isolated from the effluent of manure distributed at Goksung, Jeon-Nam, South Korea. The modified nitrate-mineral-salt (NMS) medium [13,16] with 1 μM copper ions was used for cultivation. Cells were cultured in a 500 mL Erlenmeyer flask, containing 50 mL of the medium and covered with a gas tight seal. The head space in the flask was displaced with a methane mixture (methane:air = 1:1, v/v), that was used as the sole source of carbon and energy for cell growth. Cultivation was carried out in the 30°C incubator. The pre-cultured cells were inoculated into a 5 L Erlenmeyer flask containing 3 L of the medium, and gas mixture contained in a separated reservoir was supplied to the flask (Fig. 1A). As soon as a fresh methane mixture in a gas bag was injected into the gas reservoir, it was transferred to the flask through an air filter using an air bubbler. The mixture dispersed evenly in the NMS medium by continuous stirring and was consumed by the bacteria. Part of methane mixture was fed back to the gas reservoir and recirculated into the flask until the fresh gas mixture in the gas bag was consumed completely. This compulsory gas circulation system, which was developed in this laboratory, was very effective in cultivating methanotrophic bacteria.

Enzyme Purification

All purification procedures were carried out at 4°C. Cells were harvested when the optical density of the culture reached 1.0 at 600 nm. Harvested cells were washed with 25 mM Tris-HCl buffer (pH 8.0, standard buffer) and disrupted by 2 passes through a French Pressure Cell (SLM Instruments, USA). Cellular debris was removed by centrifugation at 15,000 × g for 15 min and, when necessary, the membrane fraction was removed by further centrifugation at 100,000 × g for 60 min [17,18].

The soluble fraction was concentrated with Centricon (Millipore, Billerica, Mass, USA) and applied to a POROS 20 HQ column equilibrated with standard buffer. After elution with a standard buffer, bound MDH was released along a linear gradient of 0 to 1 M NaCl dissolved in standard buffer. Active fractions were pooled, concentrated, and applied to a FPLC Superose 12 HR 10/30 column that had been equilibrated with 20 mM Tris-HCl buffer containing 0.15 M NaCl. The purity of the MDH was determined by 15% SDS-PAGE.

Enzyme Assay

The protein concentration was determined using the Bradford method [19], with bovine serum albumin (BSA) used as a standard. MDH activity was assayed photometrically at 30°C using a two-dye linked assay system, phenazine ethosulfate (PES) was used as an artificial electron acceptor, and 2,6-dichlorophenol indophenol (DCPIP) as a terminal acceptor. The reaction mixture (3
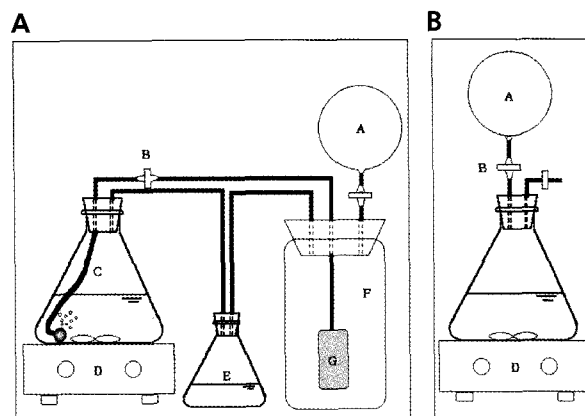

Fig. 1. Schematic diagram of cultivation system for methanotrophic bacteria. (A) Compulsory gas circulation diffusion system, which was developed in this lab. (B) Simple diffusion system. A, gas bag; B, 0.2 μm pore size air filter; C, bioreactor; D, stirrer; E, water trap; F, gas reservoir; G, air bubbler.

mL) contained 100 mM Tris-HCl buffer (pH 9.0), 13.4 mM methanol, 1.1 mM PES, 0.04 mM DCPIP, 1 mM KCN and 15 mM NH_4Cl. Reactions were initiated by adding MDH, and the reduction of DCPIP was measured by the decrease in absorbance at 600 nm [20]. One unit was defined as the amount of enzyme needed to catalyze the reduction of DCPIP at a rate of 1 mol/ min.

Analysis of MDH

The molecular weight of the native MDH was determined by gel filtration on a Sephacryl S200 column using the following standard proteins: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). The molecular weight of the subunit was determined by SDS-PAGE using 15% polyacrylamide gels, as described by Laemmli [21].

Native 8% polyacrylamide gel was used to determine the activity staining of purified MDH. After electrophoresis, the gel was stained with an active staining solution that contained 100 mM Tris-HCl buffer (pH 9.0), 13.4 mM methanol, 1.1 mM PES, 0.04 mM nitro blue tetrazolium, 1 mM KCN and 15 mM NH_4Cl.

The isoelectric point for MDH derived from *Methylomicrobium* sp. HG-1 was determined by native isoelectric focusing [22]. The MDH was visualized by active staining.

RESULTS AND DISCUSSION

Effect of a Novel Culture System on Cell Growth

The novel culture system developed in this laboratory is summarized in Fig. 1A. As described in the Materials and Methods section of this article, a methane mixture that was kept in a separate reservoir was supplied com-
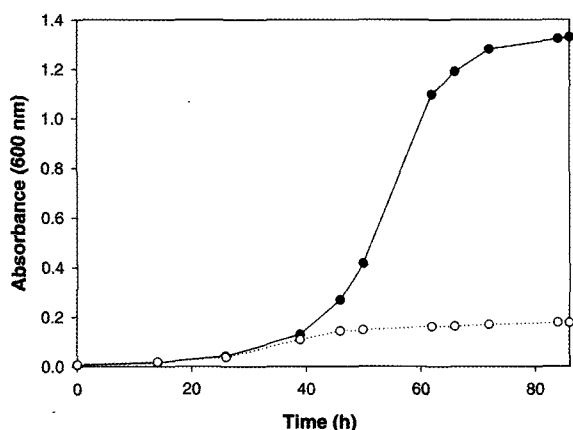
Fig. 2. Comparison of specific growth rates for each culture system. Symbols: ●, compulsory gas circulation diffusion system; ○, simple diffusion system.

pulsorily to the flask using an air bubbler. Because methane solubility in water is poor, the concentration of methane in the medium acts as a limiting factor for cell growth. Compared with the conventional simple diffusion culture system (Fig. 1B), a compulsory circulation diffusion system is better able to increase methane solubility. We found that cells growing in the compulsory circulation diffusion system demonstrated higher optical density than those growing in the simple diffusion culture system (Fig. 2). The growth rate (μ) and doubling time (D_T) of cells in the simple diffusion culture system were 0.067 h^{-1} and 14.9 h, respectively, compared with 0.127 h^{-1} and 7.9 h, respectively, for cells in the compulsory circulation diffusion system. This suggests that our compulsory circulation diffusion system is very effective in cultivating bacteria growing on methane.

Purification of MDH

After *Methylomicrobium* sp. HG-1 cells were disrupted, more than 95% of MDH activity was found exclusively in the soluble fraction and only a trace of MDH was detected in the membrane fraction. This observation differed from that seen with MDH derived from the type I methanotrophic bacterium, *Methylocystis* sp. GB25 [14]. It has been known that after cell disruption, MDH derived from type II methanotrophs appears mostly in the soluble fraction [15,23], however, in the type I and type

X methanotroph, MDH activity was found both in the soluble and particulate fractions in nearly equal amounts [24]. Although *Methylomicrobium* sp. HG-1 is a type I obligate methanotroph, MDH was found only in its soluble fraction. It has been suggested that the MDH in methylotrophs is a peripheral membrane protein localized to the periplasmic face of the membrane [25-28]. Methanotrophs are distinguished from other methylotrophs by the presence of an intracytoplasmic membrane (ICM). MDH has been found in both the soluble and particulate fractions of the methanotroph [24,29]. The formation of an ICM in a methanotroph is dependent on its growth conditions [30-33]. It has been suggested that the ICM is continuous with the cytoplasmic membrane [34-36] and that this continuity further implies continuity between the periplasm and intra-ICM space.

Copper is known to be important in the formation of ICMs. High copper concentrations are essential for the formation of extensive ICMs, and copper is thought to play an active role at both the catalytic site and along the electron transport chain [37,38]. *Methylomicrobium* sp. HG-1 also required copper for growth. As the copper concentration increased, the formation of ICM in cell increased remarkably. When the cells were grown on a medium containing 1 μM copper, 99% of the MDH activity was found in the soluble fraction. By comparison, when the cells were grown in a medium containing 50 μM copper, 10% of the MDH activity was detected in the membrane fraction. This indicates that the distribution of MDH is dependent on the formation of ICM. MDH derived from *Methylomicrobium* sp. HG-1 is detected only in the soluble fraction; authentic MDH is bound loosely to the cytoplasmic membrane and ICM, but it was released into the soluble fraction when the cells were forcefully disrupted through French pressure. Moreover, the cells needed for MDH purification were cultivated in the 1 μM copper containing medium, because the cells grown in the presence of a high copper concentration were not easily disrupted (data not shown).

The steps involved in the purification of MDH are summarized in Table 1. MDH derived from *Methylomicrobium* sp. HG-1 was purified about 8-fold through a 4 step process, with a yield of 17.6% and specific activity of 4.21 units/mg protein. An electrophoretically homogeneous preparation was obtained during the final purification on a Superose 12 HR 10/30 column. Purified MDH, dissolved in 50 mM potassium phosphate buffer (pH 7.0), lost 20% of its activity during 24 h of exposure to air at 4°C.

Table 1. Purification of MDH from *Methylomicrobium* sp. HG-1

	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Cell free extracts	205.8	109.6	0.53	1.0	100.0
Soluble fraction	143.1	97.6	0.66	1.2	89.0
POROS 20 HQ	21.4	64.5	3.01	5.6	58.8
Sephacryl S200	4.6	19.3	4.21	7.9	17.6

[a] One unit is the amount of enzyme that catalyzes the reduction of 1 μmol DCPIP per minute. Enzyme activity represents the mean of 3 independent experiments.
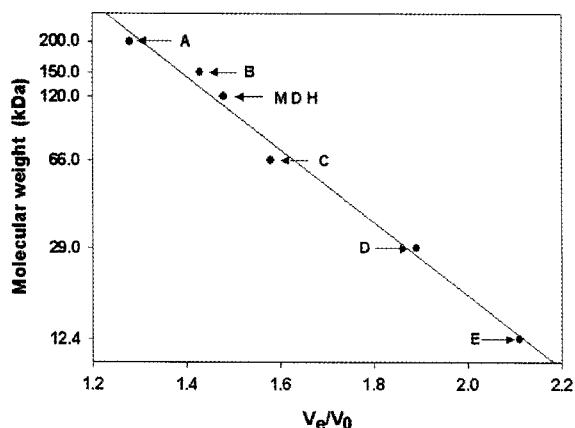
Fig. 3. Determination of the molecular mass of MDH by gel filtration on a Sephacryl S200 column using the following standard proteins: β-amylase (200 kDa); B, alcohol dehydrogenase (150 kDa); C, bovine serum albumin (66 kDa); D, carbonic anhydrase (29 kDa); E, cytochrome c (12.4 kDa).

Molecular Mass and Isoelectric Point

The molecular mass of the MDH derived from *Methylomicrobium* sp. HG-1 was estimated to be 120 kDa (Fig. 3). This enzyme consists of two identical subunits with a molecular weight of 62 kDa each (Fig. 4A). It has been suggested that the MDH derived from methylotroph has an $\alpha_2\beta_2$ conformation, which indicates that they consist of two large (60 kDa) and two small (10 kDa) subunits [39]. However several MDHs derived from methanotrophs, such as *Methylosinus trichosporium* OB3b [40], *Methylocystis* sp. GB25 [14], *Methylomonas methanica* [41], and *Methylosinus sporium* [23], consisted of only two identical large subunits, although the molecular masses are slightly different. An analysis of methanol oxidation genes (*mxaFJGI* genes) in the *Methylomonas* sp. strain A4, a type I marine methanotroph, showed that its MDH has an $\alpha_2\beta_2$ conformation and that it consists of two different subunits with a molecular weight of 59 kDa (*mxaF*) and 12 kDa (*mxaI*) [42]. Similarly, a genetic analysis of the MDH derived from our isolate showed that it also has an $\alpha_2\beta_2$ conformation and consists of two different subunits with molecular weights of 62 and 10 kDa (GenBank No. DQ463162). Consequently, it is assumed that the MDHs of methanotroph have an $\alpha_2\beta_2$ conformation, although the small subunit was not detected by SDS-PAGE or gel chromatography. The results of activity staining of purified MDH are shown in Fig. 4B. The isoelectric point was 5.4.

Substrate Specificity

Purified MDH showed broad substrate specificity with primary alcohols and aldehydes (Table 2). Similar to the MDH derived from *Methylocystis* sp. GB25, the activity of MDH of *Methylomicrobium* sp. HG-1 decreased as the number of carbons in the alcohol increased; however, it showed relatively high activity with hexanol and hep-

Fig. 4. (A) SDS-PAGE of MDH derived from *Methylomicrobium* sp. HG-1. Lane 1, molecular size marker; lane 2, cell free extract; lane 3, soluble fraction; lane 4, POROS 20 HQ column; lane 5, MDH purified through the Sephacryl S200 column. (B) Activity staining of MDH.

Table 2. Substrate specificity of MDH derived from *Methylomicrobium* sp. HG-1

Substrate	Relative activity[a] (%)	Km (mM)
Methanol	100.00	0.81
Ethanol	85.13	5.73
Propanol	59.93	184.0
Butanol	49.13	186.0
Pentanol	67.40	172.0
Hexanol	94.10	162.0
Heptanol	101.88	80.78
Octanol	16.33	ND[b]
Formaldehyde	73.24	ND
Acetaldehyde	7.06	ND

[a] Activity in the presence of methanol was assumed to be 100%.
[b] Not determined.

tanol among the alcohols tested in this study. Unfortunately it was very difficult to obtain a real value for MDH activity with primary alcohols with longer carbon chains, because those alcohols could not be solubilized sufficiently in the enzyme reaction mixture before the assay was carried out. Sometimes a period of vigorous shaking is required to ensure complete solubilization. Similar to other MDHs, the MDH of *Methylomicrobium* sp. HG-1 showed high activity with formaldehyde, but low affinity with acetaldehyde [14].
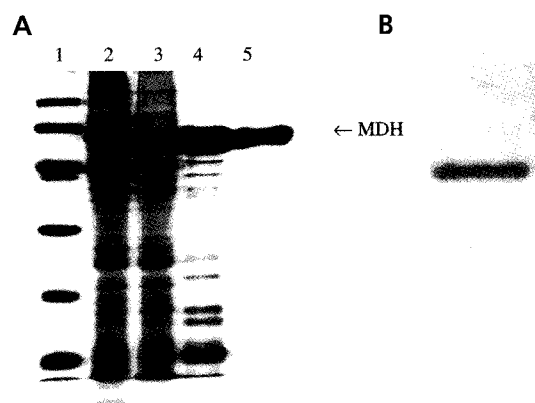
Effect of Temperature and pH

The optimum temperature was determined in a 100 mM Tris-HCl buffer. MDH activity was highest at 60°C. After a 60 min incubation period at a temperature below 60°C, more than 70% of enzyme activity remained. The

Table 3. Effect of divalent cations on MDH activity

	Concentration (mM)	Relative activity (%)
Control		100.00
Zn^{2+}	1.0	77.16
Mg^{2+}	1.0	90.16
Ca^{2+}	1.0	81.89
Fe^{2+}	0.1	93.59
Co^{2+}	0.1	92.50
Cu^{2+}	0.1	80.16
Mn^{2+}	0.1	0.00
EDTA	0.1	110.59

effect of pH on MDH activity was tested in the range of pH 7.0 to 10. The optimum pH was found to be 9.0.

Effect of Divalent Cations

The effect of divalent cations on MDH activity is shown in Table 3. As indicated, 1 mM of Zn^{2+}, Mg^{2+}, and Ca^{2+} did not affect MDH activity significantly, whereas the same concentrations of Cu^{2+}, Co^{2+}, Fe^{2+}, and Mn^{2+} inhibited completely. The inhibitory effects of these cations at a lower concentration are shown in Table 3. MDH is not inhibited by metal chelating agents such as EDTA. It is well known that most MDH activity, while strongly inhibited by EDTA *in vivo*, is not inhibited by EDTA when phenazine methosulfate is used for electron transfer [10]. This may be due to the functional coupling of MDH with the electron transport chain at the level of cytochrome *c in vivo* [14].

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