Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 46 (2006) 316–320
Protein Expression & Purification
www.elsevier.com/locate/yprep

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

Qinfeng Liu, Jon R. Kirchhoff *, Christopher R. Faehnle, Ronald E. Viola, Richard A. Hudson

Department of Chemistry, The University of Toledo, Toledo, OH 43606, USA

Received 1 July 2005
Available online 11 August 2005

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.

Keywords: Methanol dehydrogenase; Purification; *Methylobacterium extorquens* AM1; Cation exchange chromatography

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], *Methylophilius* 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-1*H*-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

* Corresponding author. Fax: +1 419 530 4033.
E-mail address: jkirchh@uoft02.utoledo.edu (J.R. Kirchhoff).

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

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2005.07.014

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.

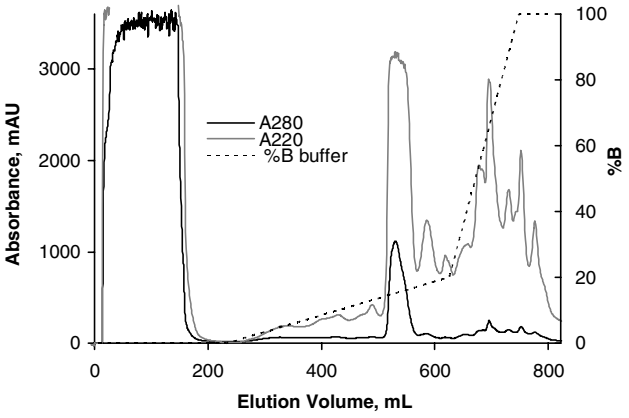

Fig. 1. Chromatogram of the crude cell extract on a SP Sepharose HP cation exchange column. The column was preconditioned and washed with buffer A, Mes 25 mM, pH 5.5. The enzyme was eluted by a programmed gradient (---------) of buffer B, Mes 25 mM, pH 5.5, with NaCl 250 mM. The flow rate was 3 mL/min.

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). Prestained 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 H_2O 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 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