JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 101, No. 6, 501–507. 2006
DOI: 10.1263/jbb.101.501

Characterization of Two 3-Hydroxybutyrate Dehydrogenases in Poly(3-Hydroxybutyrate)-Degradable Bacterium, *Ralstonia pickettii* T1

Masahiko Takanashi[1] and Terumi Saito[1,2]*

Laboratory of Molecular Microbiology, Department of Biological Science, Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan[1]
and Research Institute for Integrated Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan[2]

Received 6 February 2006/Accepted 30 March 2006

Two D-(−)-3-hydroxybutyrate (3HB) dehydrogenases, BDH1 and BDH2, were isolated and purified from a poly(3-hydroxybutyrate) (PHB)-degradable bacterium, *Ralstonia pickettii* T1. BDH1 activity increased in *R. pickettii* T1 cells grown on several organic acids as a carbon source but not on 3HB, whereas BDH2 activity markedly increased in the same cells grown on 3HB or PHB. To examine their biochemical properties, *bdh1* and *bdh2* were cloned and overexpressed in *Escherichia coli*, and their purified products were characterized. The kinetic parameters indicate that BDH1 is more suitable for converting acetoacetate to 3HB than BDH2, whereas BDH2 is more efficient for the reverse reaction than BDH1. Thus, *R. pickettii* T1 contains two BDHs with different biochemical properties and physiological roles: BDH1 for cell growth on organic acids other than 3HB and BDH2 for cell growth on 3HB or PHB.

[**Key words:** 3-hydroxybutyrate dehydrogenase, *Ralstonia pickettii* T1, poly(3-hydroxybutyrate) (PHB)]

Many bacteria synthesize and accumulate poly(3-hydroxybutyrate) (PHB) as an intracellular carbon store when the availability of nitrogen, phosphorus or oxygen in the environment is restricted. In PHB-accumulating bacteria, intracellular PHB is used not only as a reserve of carbon and energy in various bacteria but also as an electron sink in some bacteria (1, 2). PHB accumulated in the cell is degraded by intracellular PHB depolymerase. Released 3HB is converted to acetoacetate by 3HB dehydrogenase (BDH; EC 1.1.1.30), and activated to acetoacetyl-CoA by succinyl-CoA transferase (EC 2.8.3.5) or acetoacetyl-CoA synthetase (EC 6.2.1.16). The resulting acetoacetyl-CoA is cleaved into two acetyl-CoA molecules by β-ketothiolase (EC 2.3.1.9). Finally, acetyl-CoA is metabolized via the TCA cycle and glyoxylate route (3).

Many BDHs have been purified from PHB-accumulating bacteria, and their biochemical properties have been reported (4–9). In this study, we investigated the biochemical and physiological properties of two BDHs from a PHB-degradable bacterium, *Ralstonia pickettii* T1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions *R. pickettii* T1 (BBCM/LMG 18351) cells were grown aerobically on nutrient broth (NB) (Difco Laboratories, Sparks, MD, USA), and the precultured cells were transferred to a minimal salt medium (Stinson and Merrick medium [SM]) (10) with carbon sources and grown at 30°C. *Shinorhizobium meliloti* (ATCC 9930) was grown on a nutrient medium (K_2HPO_4, 0.7; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 1.0; and mannitol, 5 [in g/*l*]). *Escherichia coli* cells were grown at 37°C overnight in Luria–Bertani (LB) medium supplemented with ampicillin (Ap, 50 μg/ml), chloramphenicol (Cm, 34 μg/ml), or tetracycline (Tc, 10 μg/ml), when necessary. pUC19 (Takara, Kyoto) and pET23b (Novagen, Madison, WI, USA) were used for cloning and overexpression, respectively.

Enzyme assay and enzyme kinetics BDH activity was measured with 3HB and NAD^+ as substrates in oxidation or with acetoacetate and NADH as substrates in reduction using a molar absorption coefficient of 6.2×10^3 M^{-1} cm^{-1} at 340 nm. One unit of enzyme was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NAD^+ per minute or the oxidation of 1 μmol of NADH per minute at 30°C. BDH activity was measured at 30°C in 10 mM Tris–HCl (pH 8.0) supplemented with 3 mM D(−)-3HB and 0.5 mM NAD^+, unless otherwise indicated. The optimal pHs of BDH1 and BDH2 were assayed at 30°C with the gene products (BDHs) purified from *E. coli* in a 20 mM buffer of phosphate–NaOH (pHs 6.5–8.0), Tris–HCl (pHs 7.5–9.0), and glycine–NaOH (pHs 8.5–10.0) in the oxidation reaction and in a 20 mM buffer of citrate–NaOH (pHs 3.0–6.0) and phosphate–NaOH (pHs 6.0–8.0) in the reduction reaction. Michaelis constant (K_m) and maximal velocity (V_{max}) were determined at the optimal pH by a linear regression analysis of the data plotted according to the method of Lineweaver–Burk (11).

Purification of BDH1 and BDH2 from *R. pickettii* T1 *R. pickettii* T1 cells were precultured in NB at 30°C overnight and grown aerobically in SM with both succinate (0.4% w/v) and PHB (0.15% w/v) at 30°C for 24 h. All subsequent manipulations were

* Corresponding author. e-mail: 43saito-bio@kanagawa-u.ac.jp
phone/fax: +81-(0)463-59-8673

carried out at or below 4°C. The culture cells were harvested by centrifugation at 7000×*g* for 10 min, resuspended with 5 volumes of buffer A (20 mM Tris–HCl [pH 8.0], 20% glycerol, and 10 mM 2-mercaptoethanol [2ME]) and sonicated (50 W for 5 min, 4 times). The lysate was centrifuged at 15,000×*g* for 60 min, and the supernatant was applied to a Q Sepharose Fast Flow (FF) column (5×3 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer A. After being washed with buffer A, BDH1 and BDH2 were separated with a 500-ml linear NaCl gradient of 0 to 0.2 M in buffer A. BDH1 and BDH2 were eluted at 0.1 M and 0.15 M NaCl, respectively. Fractions containing BDH1 were concentrated with an ultrafiltration membrane (Biomax-50 filter; Millipore, Bedford, MA, USA) and dialyzed for 5 h against 10 volumes of buffer B (5 mM piperazine-*N,N*′-bis-[2-ethanesulfonic acid] (PIPES) [pH 6.6] and 20% glycerol). The enzyme was applied to a red-Sepharose CL-4B column (2.5×5 cm) equilibrated with buffer B. After being washed with buffer B, BDH1 was eluted stepwise with buffer B containing 0.2 mM NAD^+ and 0.5 mM 3HB, buffer B containing 0.5 mM NAD^+ and 1 mM 3HB, and 5 mM PIPES buffer (pH 7.0) containing 20% glycerol, 0.5 mM NAD^+, and 1 mM 3HB. The active fractions were combined, concentrated with an ultrafiltration membrane, and dialyzed for 5 h against 10 volumes of buffer C (10 mM PIPES [pH 7.5] containing 20% glycerol, 10 mM 2ME, and 1 M ammonium sulfate). The dialyzed enzyme was applied to a phenyl Sepharose high-performance (HP) column (0.7×3.5 cm; Pharmacia Biotech) equilibrated with buffer C. The column was washed with buffer C, and the enzyme was eluted with a linear ammonium sulfate gradient of 1 to 0 M in 30 column volumes of buffer C. The purified BDH1 was concentrated with an ultrafiltration membrane and dialyzed for 3 h against 10 volumes of buffer D (20 mM phosphate [pH 7.0], 50% glycerol, 1 mM dithiothreitol [DTT], and 5 mM $MgCl_2$), and stored at –20°C. BDH2 was purified similarly to BDH1, except that the enzyme was applied to a phenyl Sepharose HP column in a larger volume (1.5×8 cm).

Electrophoresis The subunit molecular masses of BDHs were determined by SDS–PAGE according to the method of Laemmli (12). The proteins were stained with Coomassie Brilliant Blue (CBB) R-250.

Cloning, expression of genes, and purification of gene products According to standard techniques (13), chromosomal DNA from strain T1 was prepared and digested with various restriction endonucleases. The DNA fragments were separated by agarose gel electrophoresis and blotted on a nylon membrane. The DNA on the membrane was hybridized with a *bdh* fragment from *S. meliloti* as a probe, which was prepared by labeling with [α-^{32}P] dCTP using a nick translation kit (Pharmacia Biotech) after the amplification of *bdh* from the chromosomal DNA of *S. meliloti* with the primers SMN (5′-ATGACCAAGACTGCGGTG) and SMC (5′-CCATCG AAACATGCGTCC) designed on the basis of a published nucleotide sequence (AF080548).

A 3.0-kbp *Pst*I fragment with *bdh1* was isolated and inserted into pUC19 digested with *Pst*I. The resultant plasmid (pTB130) was digested with *Bam*HI to yield pTB118 carrying a 1.8-kbp *Bam*HI fragment with *bdh1*. For the cloning of *bdh2*, a 3.8-kbp *Bam*HI fragment with *bdh2* was isolated and inserted into pUC19 digested with *Bam*HI. The resultant plasmid (pTB238) was digested with *Pst*I to yield pTB215 carrying a 1.5-kbp *Pst*I fragment with *bdh2*.

To overexpress *bdh* in *E. coli*, the open reading frames of *bdh1* and *bdh2* were amplified from pTB118 and pTB215 with the primers T1B1EN (5′-CC*GGATCC*CATATGCAGCTCAAAGGAAAG TCCGCCATC) and T1B1EC (5′-CC*GGATCC*TTACTGCATATA CCAGCCGTGGCTCAC) for *bdh1*, and T1B2EN (5′-ATATATA CATATGCTTAAAGGCAAGACGG) and T1B2EC (5′-TC*GGAT CC*TAATTGCGCGAACCAGCC) for *bdh2*, respectively. The resultant PCR product of *bdh1* was double-digested with *Nde*I (sites underlined) and *Bam*HI (sites in italics), and ligated into the expression vector pET23b digested with *Nde*I and *Bam*HI, yielding pETT11. pETT12 carrying *bdh2* was constructed using similar procedures.

bdh1 was expressed in *E. coli* BLR (DE3)/pLysS (Novagen) harboring pETT11. The transformed cells were grown at 37°C overnight in 2 *l* of LB supplemented with antibiotics (Ap, Cm, and Tc). When the optical density at 600 nm (OD_{600}) reached about 0.6–0.7, isopropyl-β-thiogalactopyranoside was added to the culture (50 mM), and the culture was grown at 22°C overnight. The expression of *bdh2* was achieved using similar procedures.

The gene products from *E. coli* were purified as follows: For BDH1, *E. coli* cells harboring pETT11 were harvested by centrifugation at 5000×*g* for 10 min, resuspended in 10 volumes of buffer E (10 mM Tris–HCl [pH 8.0] and 20% glycerol), and sonicated (50 W for 5 min, 4 times). The lysate was centrifuged at 15,000×*g* for 60 min, and the supernatant (50,000 units of BDH1 activity) was applied to a Q Sepharose FF column (2.5×7 cm) equilibrated with buffer E. After being washed with buffer E, the enzyme was eluted with a 150-ml linear NaCl gradient of 0 to 0.2 M in buffer E. The active fraction was dialyzed against 10 volumes of buffer F (10 mM Tris–HCl [pH 7.0] and 20% glycerol) for 5 h. The enzyme was applied to a red-Sepharose CL-4B column (5×8 cm) equilibrated with buffer F. The column was washed with buffer F, and the enzyme was eluted with 20 mM Tris–HCl buffer (pH 8.0) containing 20% glycerol, 3 mM 3HB, and 0.5 mM NAD^+. The purified BDH1 was dialyzed for 5 h against 20 volumes of 10 mM Tris–HCl buffer (pH 7.0) containing 50% glycerol, and stored at –80°C. BDH2 was purified similarly, except that the cell extract (81,000 units of BDH2 activity) obtained from 1 *l* of LB culture was applied to a Q Sepharose FF column (2.5×9 cm). The purified BDH2 was stored in 10 mM Tris–HCl buffer (pH 8.0) containing 50% glycerol at –80°C.

Other methods Protein concentration was determined according to the method of Bradford with bovine serum albumin (BSA) as the standard (14). The red-Sepharose CL-4B column was prepared by coupling Reactive Red 120 (Sigma Chemicals, St. Louis, MO, USA) with Sepharose CL-4B (Pharmacia Biotech) activated by CNBr. The native molecular masses of BDHs were determined by gel filtration using a Sephacryl S-200 HR column (0.78×30 cm; 0.15 ml/min; Pharmacia Biotech) equilibrated with

TABLE 1. Summary of purifications of BDH1 and BDH2 from *R. pickettii* T1

Step	Enzyme	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	BDH1 and BDH2	1300	700	1.9	100
Q Sepharose FF	BDH1	590	540	1.1	45
	BDH2	730	480	1.5	56
Red Sepharose CL-4B	BDH1	520	14	37	40
	BDH2	520	120	4.3	40
Phenyl Sepharose HP	BDH1	230	1.3	180	18
	BDH2	400	4.7	85	31

The enzyme was purified from 45 g (wet weight) of *R. pickettii* T1 cells.