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Characterization of Third 3-Hydroxybutyrate Dehydrogenase in *Ralstonia pickettii* T1

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Abstract: We previously reported that *Ralstonia pickettii* T1, a bacterium growing on extracellular poly-3-hydroxybutyrate (PHB), have two 3-hydroxybutyrate dehydrogenases (BDH1 and BDH2). By analysis of knockout mutants of *bdh1* or *bdh2* using anion-exchange column chromatography, it was shown that a novel BDH besides BDH1 and BDH2 was present in *R. pickettii* T1. The third BDH (BDH3) was partially purified by column chromatography, and the enzyme had the N-terminal amino acid sequence different from those of BDH1 and BDH2. In Southern blotting with *bdh2* as a probe, *bdh3* was detected and cloned, and the purified gene product of *bdh3* expressed in *Escherichia coli* showed higher specific activity than those of BDH1 and BDH2.

Keywords: 3-hydroxybutyrate dehydrogenase, poly-3-hydroxybutyrate, *Ralstonia pickettii* T1

Introduction

Poly-3-hydroxybutyrate (PHB) is a natural biodegradable polymer that is biosynthesized and accumulated as an internal reserve of carbon and energy in many microorganisms [1-6]. In bacteria, it has been believed that 3-hydroxybutyrate dehydrogenase (BDH; EC 1.1.1.30), which catalyzes the oxidation of 3HB to acetoacetate or the reverse reaction, is involved in the metabolism of 3-hydroxybutyrate (3HB) as degradation products of intracellular or extracellular PHB. However, most studies on bacterial BDHs have reported on their biochemical properties [7-17], but not on the physiological property.

In a PHB-accumulating bacterium *Shinorhizobium meliloti*, it was reported recently that the expression of the transcriptional fusion gene *bdh-lacZ* was associated with the growth phase, when the expression level increased from the lag to log phase and leveled off at the stationary phase [18]. In addition, a bacterium growing on extracellular PHB, *R. pickettii* T1, had a substantial BDH activity in growth on various carbon sources, such as nutrient broth, succinate, and citrate as well as 3HB and PHB as carbon sources [19]. In summary, it is not necessarily true that BDH works only to utilize 3HB.

In this study, we report the characteristics of a novel BDH (BDH3) isolated from *bdh2* mutant of *R. pickettii* T1.

Materials and Methods

Strains, plasmids, and cultivation conditions

Ralstonia pickettii T1 (BBCM/LMG 18351) was precultured in nutrient broth (NB) (Difco Laboratories, Sparks, Md.), and the cultures were inoculated into a minimal medium (MM; 11.6 g/l $Na_2HPO_4 \cdot 12H_2O$, 4.6 g/l KH_2PO_4, 2.0 g/l NH_4Cl, 1.0 g/l $MgSO_4 \cdot 7H_2O$, 0.2 g/l $FeCl_3 \cdot 6H_2O$, and 84 μM $CaCl_2 \cdot 2H_2O$) [20] with a carbon source and ampicillin (Ap; 50 μg ml^{-1}) which was incubated at 30°C for 24 h. bdh mutants of R. pickettii T1 were cultured in NB or MM with a carbon source plus Ap (50 μg ml^{-1}) and chloramphenicol (Cm; 34 μg ml^{-1}). Escherichia coli strains were cultured

at 37°C overnight in Luria-Bertani (LB) medium with Ap (50 μg ml^{-1}), Cm (34 μg ml^{-1}), and/or tetracycline (Tc; 10 μg ml^{-1}) when necessary. pUC19 (Takara, Kyoto, Japan), pET23b (Novagen, Madison, Wis.), and pZJD2 [21] were used for cloning, expression, and construction of knockout mutants, respectively.

Enzymatic assay, protein assay, electrophoresis, and immunoblotting

Enzyme activity of BDH3 was measured in the presence of 0.5 mM NAD^+ and 3 mM D(–)-3HB in 10 mM Tris-HCl (pH 8.0). Protein concentration was determined with bovine serum albumin (BSA) as a standard [22]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [23], and immunoblotting was carried out with a transfer buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, and 20% [v/v] methanol) using a semi-blotter (Bio-Rad; Hercules, CA, USA) according to the method of Towbin [24].

Analysis of *bdh1* mutant and *bdh2* mutant

To construct *bdh1* mutant (Δ*bdh1*) and *bdh2* mutant (Δ*bdh2*) of *R. pickettii* T1, suicide vectors (pZJDT11 and pJZDT12) were prepared. The *bdh* mutants were constructed by homologous recombination according to the methods of Simon et al [25]. The internal sequence of *bdh1* or *bdh2* was amplified from template pTB118 (pUC19 carrying a 1.8-kb *Bam*HI fragment containing *bdh1*) and pTB215 (pUC19 carrying a 1.5-kb *Pst*I fragment containing *bdh2*), and the PCR fragments were inserted into pZJD2 to yield pZJDT11 and pJZDT12. The primers used were 5'-ATGCAGCTCAAAGGAAAGTCC-3' and 5'-GGTGGCCTGGTTGACGGACGCCTC-3' for *bdh1* and 5'-ACATGAGCAAGGCATCGGA-3' and 5'-GTTCTTCTGCCGCATGCCG-3' for *bdh2*.

R. pickettii T1 wild type, Δ*bdh1*, and Δ*bdh2* were precultured in NB overnight, and then inoculated into 500 ml of MM with 0.15% (w/v) PHB as a sole carbon source. The cells were cultured at 30°C for 24 h, except that Δ*bdh1* needed 48 h to obtain the minimum necessary cell weight, and harvested by centrifugation. The pellet was resuspended with 5 volumes of buffer A (20 mM Tris-HCl [pH 8.0] and 20% glycerol), and sonicated. The cell extracts were applied to a Q Sepharose Fast Flow (FF) column (5 × 3 cm; GE Healthcare, Buckinghamshire, UK) equilibrated with buffer A. After a wash with buffer A, BDHs were eluted and separated with a linear NaCl gradient (0–0.2 M, 500 ml) in buffer A. Active fractions were collected, and the total activity of BDH(s) of each peak was determined.

Purification of BDH3 from *bdh2* mutant

Δbdh2 was cultured at 30°C for 24 h in 5 liters of MM with 0.15% (w/v) PHB, and the cells were harvested by centrifugation. The pellet was re-suspended with 5 volumes of buffer A, and sonicated. The cell extracts were centrifuged at 15,000 × g for 40 min at 4°C, and the supernatant was applied to a Q Sepharose FF column (5 × 5 cm; GE Healthcare) equilibrated with buffer A. After a wash with buffer A, BDHs were eluted with a linear NaCl gradient (0–0.2 M, 500 ml) in buffer A. Active fractions (180 ml) were mixed with 20 ml of 1 M Tris-HCl (pH 8.0) and 53 g of ammonium sulfate, and applied to a TOYOPEARL phenyl-650M (1.5 × 6 cm; Tosoh, Tokyo, Japan) equilibrated with buffer B (0.1 M Tris-HCl [pH 8.0], 20% glycerol, and 2 M ammonium sulfate). After a wash with buffer B, the enzyme was eluted with a linear ammonium sulfate gradient (2–0 M, 200 ml) in buffer B. Active fractions were dialyzed against buffer C (20 mM phosphate [pH 7.0] and 20% glycerol) for 4.5 h, and applied to a red-Sepharose CL-4B column (1.5 × 7 cm) [17] equilibrated with buffer C. After a wash with buffer C, the enzyme was eluted with 20 mM phosphate buffer (pH 7.0) with 20% [v/v] glycerol, 1 mM 3HB, and 0.5 mM NAD^+. After dialysis against buffer C, the enzyme was stored at –20°C.

Cloning of *bdh3* and purification of gene product of *bdh3* expressed in *E. coli*

To clone *bdh3*, Southern hybridization was performed with a [α-^{32}P]-labeled *bdh2* as a probe according to standard techniques [26]. The *bdh2* fragment (798-bp) was amplified from pETT12 as a template with primers: 5'-ATATATACATATGCTTAAAGGCAAGACGG-3' and 5'-TCGGATCCTATTGCGCGAACCAGCC-3'. A 4.2-kbp *Pst*I fragment,

including *bdh3*, was isolated and inserted into the cloning vector pUC19 digested with *Pst*I. The resultant plasmid (pTB342) was digested with *Bam*HI and *Pst*I, yielding pTB319 carrying a 1.9-kbp *Bam*HI-*Pst*I fragment, including *bdh3*. The nucleotide sequence of *bdh3* and the adjacent regions was determined, and analyzed with the GENETYX–WIN/ATSQ (version 5.1; Software Development, Tokyo, Japan).

To overexpress *bdh3* in *E. coli*, *bdh3* was amplified from pTB319 as a template with primers: 5'-CCGGATCCCATATGACTACCACCC-CCTCTGC CGCC-3' and 5'-CCGGATCCTTACTG-CGCGGTCCAGCCGCCGTC-3'. The PCR products were inserted into the expression vector pET23b to yield pETT13. *E. coli* BLR (DE3)/pLysS (Novagen) harboring pETT13 was cultivated at 37°C to reach the optical density at 600 nm of 0.3 to 0.5. The expression of *bdh3* was induced with the addition of isopropyl-β-D-thiogalactopyranoside (final 0.1 mM), and cultured at 18°C overnight. The pellet was harvested by centrifugation, and resuspended with 5 volumes of buffer A, and then sonicated. The supernatant was applied to a TOYOPEARL DEAE-650M [2.5 × 3.5 cm; Tosoh] and a red-Sepharose CL-4B [5 × 8 cm]), and the gene product was purified according to the procedures described previously [19].

The nucleotide and amino acid sequence data reported in this paper have been submitted to the EMBL/GenBank/DDBJ nucleotide sequence databases under accession number AB330992 for *bdh3* of *R. pickettii* T1.

Results

Isolation of BDH3 from *bdh2* mutant and characterization of BDH3

R. pickettii T1 wild type, Δ*bdh1*, and Δ*bdh2* were grown on MM with PHB, and BDH1 and BDH2 were speparated with anion-exchange column chromatography (data not shown).

In the wild type, two peaks of BDH activity appeared at approx. 0.1 M and 0.15 M of a linear NaCl gradient (0 to 0.2 M). The two peaks were termed peak (I) ($BDH1_{RpT1}$) and peak (II) ($BDH2_{RpT1}$). Δ*bdh1* had peak (II), but did not peak (I), whereas Δ*bdh2* had the two peaks like in the wild type.

To identify the BDH of peak (II) in Δ*bdh2*, the enzyme was partially purified. The protein showed a subunit molecular mass of approx. 28 kDa on SDS-PAGE, which differed from that of either $BDH1_{RpT1}$ (31 kDa) or $BDH2_{RpT1}$ (31 kDa) (data not shown). The BDH partially purified had 630 units mg^{-1} of specific activity, which was higher than $BDH1_{RpT1}$ (180 units mg^{-1}) and $BDH2_{RpT1}$ (85 units mg^{-1}) (Table 1) [19]. The N-terminal amino acid sequence of the BDH besides $BDH1_{RpT1}$ in Δ*bdh2* was determined chemically to be MTTTPSAAPLA-GKTALVTGSTSGIGLGIAK, whose sequence differed from $BDH1_{RpT1}$ and $BDH2_{RpT1}$ [19].

Cloning and genetic properties of *bdh3*

To clone the gene of a novel BDH (BDH3), Southern blotting was carried out with the DNA fragments of *bdh2* as a probe (Fig. 1). In addition to the strong signal bands of *bdh2* (at 3.8 kbp in the *Bam*HI-digest; at 1.5 kbp in the *Pst*I-digest), a weak signal band at 4.2 kbp in *Pst*I-digested was observed. The DNA fragment of 4.2 kbp was inserted into pUC19, and cloned by colony hybridization with *bdh2* as a probe.

The cloned *Pst*I fragment (4,217 bp) contained an open reading frame (ORF) of 795 bp (72% G+C content) that coded for 264 amino acid residues whose predicted molecular weight was 26,910. The amino acid sequence deduced from the ORF had a putative N-terminal coenzyme-binding motif (GxxxGxG) and putative active-site residues (Ser_{139}, Tyr_{152}, and Lys_{156} with the numbering of alcohol dehydrogenase from *Drosophila melanogaster* [AF175211]) conserved in many dehydrogenases [27].

The amino acid sequence of BDH3 had 39% identity with BDH1 (AB239333), 66% identity with BDH2 (AB239334), and 50–70% identity with BDHs from other bacteria. BDH3 showed greater similarity with BDH2, but less similarity with BDH1.

Purification of the gene product of *bdh3* from *E. coli*

bdh3 was expressed in *E. coli*, and the gene product ($BDH3_{Eco}$) was purified by two steps of column chromatography. The specific activity of

Table 1. Partial purification of BDH3 from *bdh2* mutant[a]

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)
crude extract [b]	770	680	1.1	100
Q Sepharose	630	160	3.9	100
phenyl-650-M	620	49	13	98
red-Sepharose	490	0.78	630	78

[a] BDH3 was purified from 37 g in wet weight of *bdh2* mutant cells.
[b] Cell extract contained BDH1 (130 units), which was separated from BDH3 by a linear NaCl gradient (0–0.2 M) in a Q Sepharose FF column.

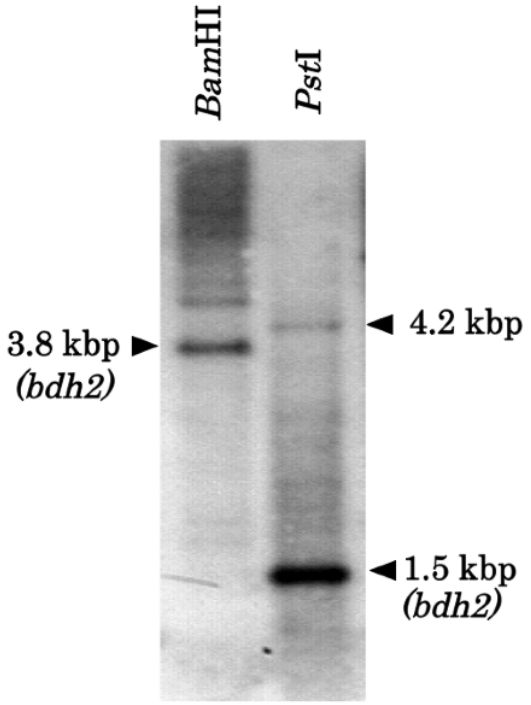

Fig. 1. Southern blotting to clone *bdh3*. Chromosomal DNA of *R. pickettii* T1 digested with *Bam*HI- or *Pst*I was blotted on the nylon membrane, and hybridized with *bdh2* fragment as a probe. Arrows indicate the positions of *bdh2* and *bdh3*.

the purified BDH3$_{Eco}$ (1,200 units mg^{-1}) was higher than those of BDH1$_{Eco}$ (450 units mg^{-1}) and BDH2$_{Eco}$ (360 units mg^{-1}) purified from *E. coli* (Table 2) [19]. BDH3$_{Eco}$ showed a subunit molecular mass of approx. 28 kDa on SDS-PAGE (data not shown), corresponding to that of BDH3$_{RpT1}$. The N-terminal amino acid sequence of BDH3$_{Eco}$ corresponded to that of BDH3$_{RpT1}$.

Table 2. Purification of the gene product of *bdh3* expressed in *E.coli*[a]

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)
crude extract	34000	1200	28	100
DEAE 650-M	37000	94	390	110
red-Sepharose	37000	30	1200	110

[a] The enzyme was purified with 2.0 g in wet weight of *E. coli* cells.

Discussion

We recently described that *R. pickettii* T1, a bacterium growing on an extracellular PHB, has two BDHs (BDH1 and BDH2) with different biochemical and physiological properties [19].

In analysis of mutants lacking *bdh1* or *bdh2*, it was indicated that *R. pickettii* T1 has the third BDH (BDH3) different from BDH1 and BDH2. We could not detected the presence of BDH3 until the mutant lacking BDH2 was examined with column chromatography, because BDH3 was always accompanied by BDH2 in the process of purifying BDH2 so far. It was interesting that BDH3 had 2 to 3-fold greater specific activity than BDH1 and BDH2 (Table 1 and 2) [19]. Possibly, BDH3 is better than BDH1 and BDH2 in utilization of 3HB.

The cloning of *bdh3* was achieved with difficulty, that is, *bdh3* was detected with very weak signal in Southern blotting with *bdh2* as a probe regardless of greater homology between *bdh2* and *bdh3* (Fig. 1). Perhaps, *R. pickettii* T1 contains three BDHs or more. It has not ever been reported that multiple BDHs were identified and characterized biochemically in a bacterium, although it was reported that some *Rhizobium* strains produce multiple forms of BDH in electrophoresis [28].

In this study, it was demonstrated that *R. pickettii* T1 contains three BDHs (BDH1, BDH2, and BDH3), and a novel BDH (BDH3) differs greatly from BDH2 in specific activity despite the similarity in the amino acid sequence. Probably, these three BDHs play different physiological

roles in utilization of 3HB in *R. pickettii* T1.

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