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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. 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, 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. 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. 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 Na₂HPO₄·12H₂O, 4.6 g/l KH₂PO₄, 2.0 g/l NH₄Cl, 1.0 g/l MgSO₄·7H₂O, 0.2 g/l FeCl₃·6H₂O, and 84 μM CaCl₂·2H₂O) 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 (Δbdh1) and bdh2 mutant (Δbdh2) of *R. picketti*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'-GG-TGGCCTGGTTGACGGACGCCTC-3' for *bdh1* and 5'-ACATGAGCAAGGCATCGGA-3' and 5'-GTTCTTCTGCCGCATGCCG-3' for *bdh2*.

*R. picketti*T1 wild type, Δbdh1, and Δbdh2 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 Δbdh1 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 resuspended 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% [v/v] glycerol, and 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’-ATATATACATATGCTAAAGGAAAGTCC-3’ and 5’-GG-TGGCCTGGTTGACGGACGCCTC-3’ for *bdh1* and 5’-ACATGAGCAAGGCATCGGA-3’ and 5’-GTTCTTCTGCCGCATGCCG-3’ for *bdh2*.

The 4.2-kbp *Pst*I fragment,
including bdh3, was isolated and inserted into the cloning vector pUC19 digested with PstI. The resultant plasmid (pTB342) was digested with BamHI and PstI, yielding pTB319 carrying a 1.9-kbp BamHI–PstI 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'-CCGGATCCCATATGACTACCACCCCTCTGCCGCGGTCCAGCCGGCTC-3' and 5'-CCGGATCTTACTGCAGCCGCCGTC-3'. The PCR products were inserted into the expression vector pET23b to yield pETT13. E. coli BLR (DE3)/pLysS (Novagen) harboring pET713 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.

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, Δbdh1, and Δbdh2 were grown on MM with PHB, and BDH1 and BDH2 were separated 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) (BDH1RpT1) and peak (II) (BDH2RpT1). Δbdh1 had peak (II), but did not peak (I), whereas Δbdh2 had the two peaks like in the wild type.

To identify the BDH of peak (II) in Δbdh2, 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 BDH1RpT1 (31 kDa) or BDH2RpT1 (31 kDa) (data not shown). The BDH partially purified had 630 units mg⁻¹ of specific activity, which was higher than BDH1RpT1 (180 units mg⁻¹) and BDH2RpT1 (85 units mg⁻¹) (Table 1). The N-terminal amino acid sequence of the BDH besides BDH1RpT1 in Δbdh2 was determined chemically to be MTTTPSAAPLAGKTALVTGSTGILGIK, whose sequence differed from BDH1RpT1 and BDH2RpT1.

**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 BamHI-digest; at 1.5 kbp in the PstI-digest), a weak signal band at 4.2 kbp in PstI-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 PstI 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 (GxxGxG) and putative active-site residues (Ser139, Tyr152, and Lys156 with the numbering of alcohol dehydrogenase from *Drosophila melanogaster* [AF175211]) conserved in many dehydrogenases.

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.
BDH3 was purified from 37 g in wet weight of bdh2 mutant cells.

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.

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.

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 detect 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).

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.

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

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Table 1. Partial purification of BDH3 from bdh2 mutant

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<th>Total protein (mg)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield (%)</th>
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<tr>
<td>Q Sepharose phenyl-650-M</td>
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<tr>
<td>red- Sepharose</td>
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*BDH3 was purified from 37 g in wet weight of bdh2 mutant cells.

† 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.

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Table 2. Purification of the gene product of bdh3 expressed in E.coli

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<th>Step</th>
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<th>Total protein (mg)</th>
<th>Specific activity (U mg⁻¹)</th>
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<td>crude extract</td>
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<td>100</td>
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<tr>
<td>DEAE 650-M red- Sepharose</td>
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<td>390</td>
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*The enzyme was purified with 2.0 g in wet weight of E. coli cells.
roles in utilization of 3HB in R. pickettii T1.

References