The BhbA Enzyme Assay

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[Abstract] Reductive dehalogenation has been found primarily in anaerobic communities and is originally thought to rarely occur in aerobes. A reductive dehalogenase (BhbA) was characterized from an aerobic strain of *Comamonas* sp. 7D-2, which was isolated from a bromoxynil octanoate-contaminated soil sample collected in Jiangsu, China. BhbA catalyzes the reductive dehalogenation of bromoxynil and its derivative 3,5-dibromo-4-hydroxybenzoate under aerobic conditions. BhbA is membrane-associated and found to have the key features of anaerobic respiratory reductive dehalogenases. This protocol describes the method for enzyme analysis of the aerobic reductive dehalogenase (BhbA) in the membrane fraction.

Materials and Reagents

1. *Comamonas* sp. 7D-2
2. LB medium
3. Substrate, 3,5-dibromo-4-hydroxybenzoate (DBHB) or 3-bromo-4-hydroxybenzoate (BHB) (Sigma-Aldrich)
4. Electron donor, NADPH or NADH (Sangon Biotech, catalog numbers: Y4433000-100 mg and NB0642-1 g, respectively)
5. Reaction inhibitor, sodium dithionite ($Na_2S_2O_4$, Sigma-Aldrich, catalog number: 7775-14-6)
6. Protein Quantification Kit (Sangon Biotech, catalog number: BE530-100 ml)
7. Phosphate buffered saline (PBS) (Sambrook and Russell, 2001) (see Recipes)
8. Mobile phase of HPLC (see Recipes)

Equipment

1. 7 ml centrifuge tube
2. Membrane filtration (pore size, 0.22 μm)
3. Centrifuge
4. HPLC (600 controller, Rheodyne 7725i manual injector and 2487 Dual λ Absorbance Detector) (Waters)
5. Ultrasonic instrument

6. Fast protein liquid chromatography

Procedure

1. *Comamonas* sp. 7D-2 was inoculated in 100 ml LB medium (supplemented with 0.25 mM DBHB) and cultured at 30 °C for 14 h.
2. Late exponential-phase cultures (approximately OD_{600}=1.0) of strain 7D-2 were pelleted by centrifugation at 15,000 *x g* for 5 min, washed two times with PBS (pH 7.4), resuspended in PBS (containing 2 mM dithiothreitol) and then disrupted by sonication at 4 °C. BhbA was partially purified by fast protein liquid chromatography (Amersham Biosciences) at 4 °C (Chen *et al.*, 2013; van de Pas *et al.*, 1999).
3. Protein concentrations were determined by the Bradford method (Bradford, 1976) with Protein Quantification Kit.
4. A standard enzyme assay was performed under aerobic conditions in a 7 ml centrifuge tube containing 3.0 ml PBS, 0.1 mM DBHB (BHB) and 0.2 mM NADPH (NADH). The reaction was initiated by the addition of 30 µl of enzyme preparation and incubated at 30 °C for 60 min. Negative control without added enzyme was performed.
5. The reaction was stopped by the addition of 15 µl sodium dithionite (1 M).
6. The mixture was centrifuged at 16,000 *x g* for 10 min and filtered by membrane filtration; then, the DBHB concentration was analyzed using HPLC (Chen *et al.*, 2013).
7. One unit of BhbA activity was defined as the amount of BhbA that catalyzes the reduction of 1 nmol of DBHB per minute. Specific activity was expressed in units per milligram of protein. All assays were performed independently three times, and the means and standard deviations were calculated.

Recipes

1. Phosphate buffered saline (PBS)
   8.0 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4 per liter of water
   pH 7.4
2. Mobile phase of HPLC
   Acetonitrile: water: acetic acid (50:50:0.5/v:v:v)

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