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Structural and catalytic properties of the D-3-hydroxybutyrate dehydrogenase from

Pseudomonas aeruginosa

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Abstract

To put forward BDH from *Pseudomonas aeruginosa* enzymatic properties we report a two-step purification of BDH, its gene sequencing allowing the investigation of its structural properties.

Purification of BDH was achieved, using ammonium sulfate fractionation and Blue Sepharose CL-6B affinity chromatography. SDS-PAGE analysis reveals a MM of 29 kDa, whereas the native enzyme showed a MM of 120 kDa suggesting a homotetrameric structure. BDH encoding gene sequence shows a nucleotide open reading frame sequence of 771 pb encoding a 265 amino acid residues polypeptide chain.

The modelling analysis of the three dimensional structure fits with the importance of amino acids in the catalysis reaction especially a strictly conserved tetrad. Amino-acid residues in interaction with the coenzyme NAD⁺ were also identified.

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Running title: Bacterial D-3-hydroxybutyrate dehydrogenase.

Keywords

D-3-hydroxybutyrate dehydrogenase, *Pseudomonas aeruginosa*, quick purification, characterization.

Introduction

Pseudomonas aeruginosa is a gram negative bacterium and an important causative agent of nosocomial infections, particularly in intensive-care units [1] is a metabolically versatile micro-organism. While *Pseudomonas aeruginosa* is lactose negative, the NAD⁺-dependent D-3-Hydroxybutyrate

dehydrogenase (BDH: EC 1.1.1.30) plays a key role in the redox balance and in the energetic metabolism by converting 3-hydroxybutyrate to acetoacetate. Interestingly, many bacteria synthesize and accumulate poly (3-hydroxybutyrate) (PHB) as intracellular carbon storage when the availability of nitrogen, phosphorus or oxygen in the environment is restricted. In PHB-accumulating bacteria, intracellular PHB is an electron sink in some bacteria [2, 3]. The cell-accumulated PHB is degraded by PHB depolymerase to produce D-3-hydroxybutyrate. During BOH oxidation, NADH is produced as an electron carrier to provide electrons to the plasma membrane located respiratory chain. On the other hand, eukaryotic BDH is an inner mitochondrial membrane bound enzyme, associated to the NAD⁺ linked electron transport chain [4]. As a very unique property, the catalytic activity of the enzyme is lecithin-dependent [5]. In contrast to mammalian, bacterium BDH is a cytosolic enzyme, where its activity is independent of the membrane phospholipids.

In order to characterize BDH from *P. aeruginosa*, we purified this enzyme by establishing a new purification technique. Indeed, previous methods used to purify bacterial BDH were time consuming, i.e. from *Rhodopseudomonas spheroides* [6] ; from *Paracoccus denitrificans* [7] or from *Acidovorax sp* [8]. Our method is based on two quick steps: ammonium sulfate fractionation and Blue Sepharose affinity chromatography. On the other hand, the BDH encoding gene has been cloned, sequenced and modelled. The computing analysis of the three dimensional structure fits with the importance of amino acids in the catalysis reaction especially a strictly conserved tetrad. Amino-acid residues in interaction with the coenzyme NAD⁺ were also identified.

Materials and methods

- Microorganisms and growth conditions.

Bacterium *Pseudomonas aeruginosa*, strain PAO1 (Pasteur Institute, Casablanca, Morocco) was grown aerobically at 37°C without exceeding the exponential phase in nutrient broth (Topley House, Bury, UK). The exponential phase was determined spectrophotometrically at 600 nm. The culture was inoculated with 1% (v/v) overnight preculture in the same medium.

- Crude extract preparation.

Bacterial culture (5 l) was harvested by centrifugation at 2500 g for 10 min, washed three times with 50 mM potassium phosphate buffer (pH 7.5), and suspended in the same buffer containing 2 mM EDTA and 1 mM DTT (buffer A). Cells were disrupted at 4°C by sonication (30 s, 90% output, 12x) using a Bandelin Sonopuls sonifier. Cellular debris and unbroken cells were removed by centrifugation at 2500 g for 45 min at 4°C. The supernatant obtained constituted the crude bacterial extract (soluble protein fraction).

- Two steps - BDH purification.

Ammonium sulfate fractionation(1) and Blue Sepharose CL-6B chromatography(2) .

(1) The crude extract of *P. aeruginosa* was subjected to protein precipitation in the 27–42 % saturation range of ammonium sulfate at 4°C (buffer B) from buffer A containing ammonium sulfate at 50% saturation. The final pellet was dissolved in a minimal volume of buffer A. The protein solution was dialyzed overnight against 5 l of the same buffer. (2) The dialyzed enzyme preparation was applied to a Blue Sepharose CL-6B column equilibrated with two bed volumes of buffer A at 4°C. The column was washed with three bed volumes of buffer A. Finally, the enzyme was eluted with buffer A containing the coenzyme at 0.1 mM NAD⁺ at a flow rate of 6 ml/h. Active fractions were collected and conserved in 50% (v/v) glycerol at –20°C until use. Protein content was measured according to the Bradford procedure, using bovine serum albumin (BSA) as standard [9].

- Biochemical characterization of the BDH.

BDH activity, enzymatic parameters, optimal pH and temperature dependency.

BDH activity, followed at 37°C by monitoring NADH production at 340 nm ($\epsilon = 6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) and kinetic parameters were currently obtained as in [10]. Michaelis constants (K_m), dissociation constants (K_D) and maximal velocity for the oxidation of BOH and the reduction of NAD⁺ were obtained by mathematical analysis following Cleland [11]. The effect of pH on BDH activity was studied in range from

pH 4 to pH 10 using a mixture of different buffers (Tris, Mes, Hepes, potassium phosphate and sodium acetate). Thermal activation and denaturation processes were followed as described in [10].

Non-denaturing and denaturing polyacrylamide gel electrophoresis.

To determine the native molecular weight of the purified BDH, non-denaturing polyacrylamide gel electrophoresis was carried out according to the method [12]. The separating gels (6, 8, 10 and 12 % polyacrylamide) were buffered with 1.5M Tris-HCl (pH 8.8). The running buffer was composed of 25 mM Tris and 320 mM glycine (pH 8.6). All experiments were done at 4°C. The electrophoresis running conditions, staining and destaining were as described for SDS-PAGE. The relative molecular weight of the native purified BDH was estimated using native molecular weight markers (Sigma). The apparent subunit molecular weight was determined by measuring relative mobilities in denaturation conditions using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed as described by Laemmli [13] on one-dimensional 12 % polyacrylamide slab gels containing 0.1 % SDS and by comparing with the pre-stained SDS-PAGE molecular weight standards (Sigma).

Western-blotting.

After SDS-PAGE (12%) [14] and subsequent transfer in nitrocellulose [19], the proteins (30µg) were exposed to 1/100 dilution of rabbit monospecific polyclonal anti-BDH antibody raised against purified rat liver BDH [15] and detected with the secondary antibody of anti-rabbit, IgG peroxidase conjugate (diluted to 1/2500) (Promega).

- PCR and sequencing of the BDH cDNA clone from *P. aeruginosa*.

DNA was extracted from *Pseudomonas aeruginosa* using Wizard Genomic Kit. An aliquot from the obtained DNA (1 µl) was used in a subsequent polymerase chain reaction (PCR) using 1.25 U of GoTaq DNA polymerase (Promega), 0.04 µM of forward (5'-GATGACCCTGAAAGGCAAGACC-3') and reverse primer (5'-CTACTGCGCCACCAGCC-3'). PCR conditions were 35 cycles of 92°C for 30 sec, 51°C for 30 sec and 72°C for 1 min. The primers used were obtained from the alignment between the sequences of BDH of 5 *Pseudomonas* strains (*Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Pseudomonas entomophila*,

Pseudomonas fluorescens, and *Pseudomonas putida*). The PCR product was purified using QIAEX II Kit (Qiagen) and subcloned into the pGEM-T vector system (Promega). Then the nucleotide sequence was determined after sequencing independently of the available genome sequence of *Pseudomonas aeruginosa* PAO1 on both strands using universal primers T7 and SP6 (MWG Biotech, Germany) in agreement with the NCBI accession n° AAG05391.

Results and discussion

Purification of soluble BDH from *Pseudomonas aeruginosa*.

The BDH was purified to electrophoretic homogeneity from *P. aeruginosa* extract by ammonium sulfate fractionation (27 – 42 %), followed by the Blue Sepharose CL-6B chromatography. All steps were carried out at 4°C. Table 1 summarizes a representative purification protocol. The SDS-PAGE analysis of the different fractions obtained during this purification procedure showed a progressive enrichment in 29 kDa protein (Figure 1A). Only this protein band, having the same size than the BDH subunit, was seen in the electrophoretically homogeneous final enzyme preparation (Figure 1A, line 3).

After initial immunodetection tests of BDH from *P. aeruginosa* using a polyclonal rabbit antibody raised against rat liver BDH [20], we have produced specific rabbit polyclonal antibodies using purified BDH from *P. aeruginosa* as immunogen [10]. These antibodies selectively recognize a single immunoreactive band in both crude extracts and purified preparations. Figure 1B, shows that the relative molecular mass of the detected protein (29 kDa) is the expected one for the BDH monomer in denaturing conditions (Figure 1B).

Properties of the purified BDH from *P. aeruginosa*

The molecular weight obtained for the purified BDH from *P. aeruginosa* corresponds to 29 kDa (not shown), while non-denaturing PAGE showed that the native molecular weight of the *P. aeruginosa* protein was approximately 120 kDa (not shown), size in agreement with a homotetrameric structure of this BDH. The BDH kinetic parameters of the purified BDH from *P. aeruginosa* were determined. The results obtained

show a value of 15.5 U/mg for V_{\max} , 0.16 mM, 3.7 mM and 0.6 mM for $K_m\text{NAD}^+$, $K_m\text{BOH}$ and $K_D\text{NAD}^+$ respectively. Similar values have been obtained for other BDH sources. The effect of temperature on the BDH activity shows that the optimal temperature for the BDH activity was 40°C (not shown). A different optimum temperature (55°C) was found for BDH from *Acidovorax* [8]. In agreement with the soluble type of this BDH, Arrhenius-plot is linear (not shown) and doesn't show any break as it is the case for the lipid dependent and membrane bound eukaryotic BDH [10]. The optimal pH of the BDH activity is equal to 8 (not shown). Similar results were found for other bacteria; *Acidovorax*, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* [8] and for rat [10].

cDNA sequencing and protein structural modelling

In order to clone the gene encoding bacterium BDH from *P. aeruginosa*, primers were designed following the alignment of 5 BDH sequences from *Pseudomonas* strains (*Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Pseudomonas entomophila*, *Pseudomonas fluorescens*, *Pseudomonas putida*). The designed primary sequence encode N- and C-termini BDH polypeptide. PCR amplification revealed a single DNA fragment with the expected size (771 pb) (not shown). Using BioEdit program [17], the multiple alignment of *Pseudomonas aeruginosa* BDH amino-acid sequence with several other species reveals between 50 and 74% of identity between different bacterial BDH sequences. In comparison with human, the percentage of identity falls to 15.9%. Sequences analysis also shows the existence of 32 identical amino acids along the polypeptide chain in comparison between all BDH sequences (bacteria and human). The analysis of these amino acids using the data related to the crystal structure of the BDH of *Pseudomonas fragi* (the only one so far crystallized and modelised BDH [18]) indicates that these amino acids were localized next to the BDH active site (not shown) and both structures are almost identical (not shown).

In conclusion, the BDH single chain molecular weight of 29 kDa from *Pseudomonas aeruginosa* is similar from the ones obtained for BDH from *Acidovorax* (27 kDa) and *Rhodospirillum rubrum* (28 kDa) [8]. However, for eukaryotic BDH, the molecular weight was slightly higher, i.e. 31.5 kDa for eukaryotic BDH [16]. In contrast, like BDH from other bacterial source [8] the quaternary structure of BDH from

Pseudomonas aeruginosa corresponds to a tetrameric form. The large divergence between mammalian and bacterial BDH sequences is in part due to the presence of N-terminus and C-terminus extensions in the mammalian enzyme and in the other part in architectures differences of both enzymes especially since the mammalian BDH is mitochondrially membrane bound while the bacterial one is cytosoluble (highlighted in red in human BDH sequence in figure 2). Modelling analysis done using spdbv logiciel and Swiss Model Server fits with the importance of amino acids in the catalysis reaction especially the strictly conserved tetrad: Asn114, Ser142, Tyr155 and Lys159 (not shown). On the other hand, Ito [18] reported that the adenine of NAD⁺ is accommodated in the BDH hydrophobic pocket comprising Gly11, Leu64, Ala90, Ile93 and Leu113. All of these residues were found in all compared BDH sequences (Figure 2).

This work puts forward a new and two-steps simple and inexpensive procedure to quickly purify the BDH from *Pseudomonas aeruginosa* in a native and homotetrameric form and achieved between one to one and half day. The sequence of this gene encoding gene contains highly conserved tetrad of amino-acids. The exact role of this enzyme in the energetic balance of *Pseudomonas aeruginosa* remains to be understood.

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Legend of figure 1: BDH purification steps from *Pseudomonas aeruginosa*.

Proteins (40 µg) were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue (a) or subjected to Western blot (b) using the purified polyclonal anti-BDH antibodies. Lanes M, 1, 2, 3, 4, and 5 respectively represent standard proteins, crude extract, 30–50 % ammonium sulphate fraction, phenyl-Sepharose fraction, affinity chromatography fraction, and immunoaffinity chromatography eluate pool (pure protein preparation). Bound antibody was located by immunoreaction combined with peroxidase conjugated goat anti-rabbit IgG. The arrow (b) indicates the band corresponding to the BDH subunit.

Legend of figure 2: Alignment of BDH sequences

Alignment of BDH sequences from different bacterial species and from human with *Pseudomonas aeruginosa* (*P. a.*) was realized using Clustal [19]. Identical residues are highlighted in red and similar residues were shown in yellow. The high identity between bacterial sequences (from 50% to 74%) is visible with the presence of red blocks of amino acids. On the contrary, the weak identity with the human BDH sequence is located on peculiar regions corresponding to amino acids of the active site and substrate binding pocket.

Table 1 Purification steps of BDH from *Pseudomonas aeruginosa*

	Total protein (mg)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ of protein)	Total activity ($\mu\text{mol}/\text{min}$)	Purification factor (fold)	Yield (%)
Crude extract	4,600	0.22	1,012	1	100
Ammonium sulfate (27–42%)	301*	0.82	246.8	3.7	24.3
Sepharose-Blue	6	11.2	67.2	50.9	6.6

Typical experiments were reported from three independent trials

* The ammonium sulfate precipitation step allows the elimination of 94% of contaminating proteins from *P. aeruginosa* crude extracts. This value was calculated from the total protein amount subtracted from the amount of BDH (estimated from specific activities Sepharose-Blue chromatography step)

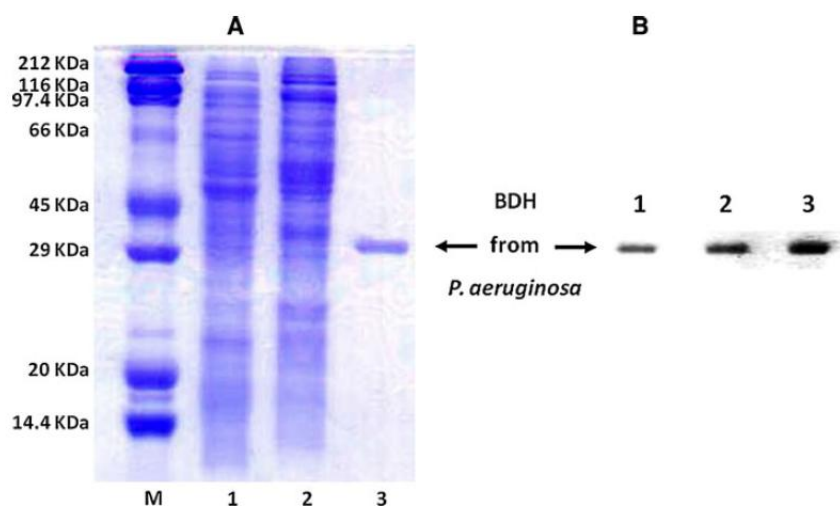


Fig. 1 BDH purification steps from *Pseudomonas aeruginosa*. Proteins were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue (a) or subjected to Western blot (b) using the purified polyclonal anti-BDH antibodies. Lanes M, 1, 2, and 3, respectively, represent standard proteins, crude extract (40 μ g), 27–42% ammonium

sulfate fraction (40 μ g), phenyl-Sepharose chromatography eluate pool (4 μ g of pure protein preparation). Bound antibody was located by immunoreaction combined with peroxidase conjugated goat anti-rabbit IgG. The arrow indicates the band corresponding to the BDH subunit

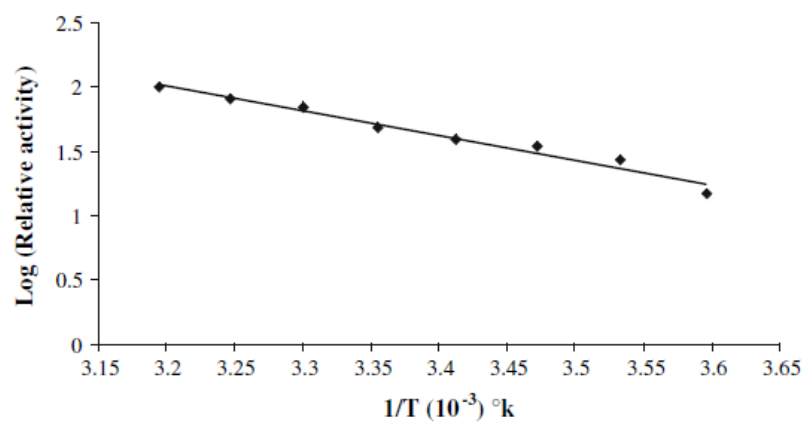


Fig. 2 Arrhenius plot of BDH. The plot was obtained by measuring the enzymatic activity using 10 μ g of the purified protein at various temperatures (from 5 to 40°C). The values represent the mean of three independent assays

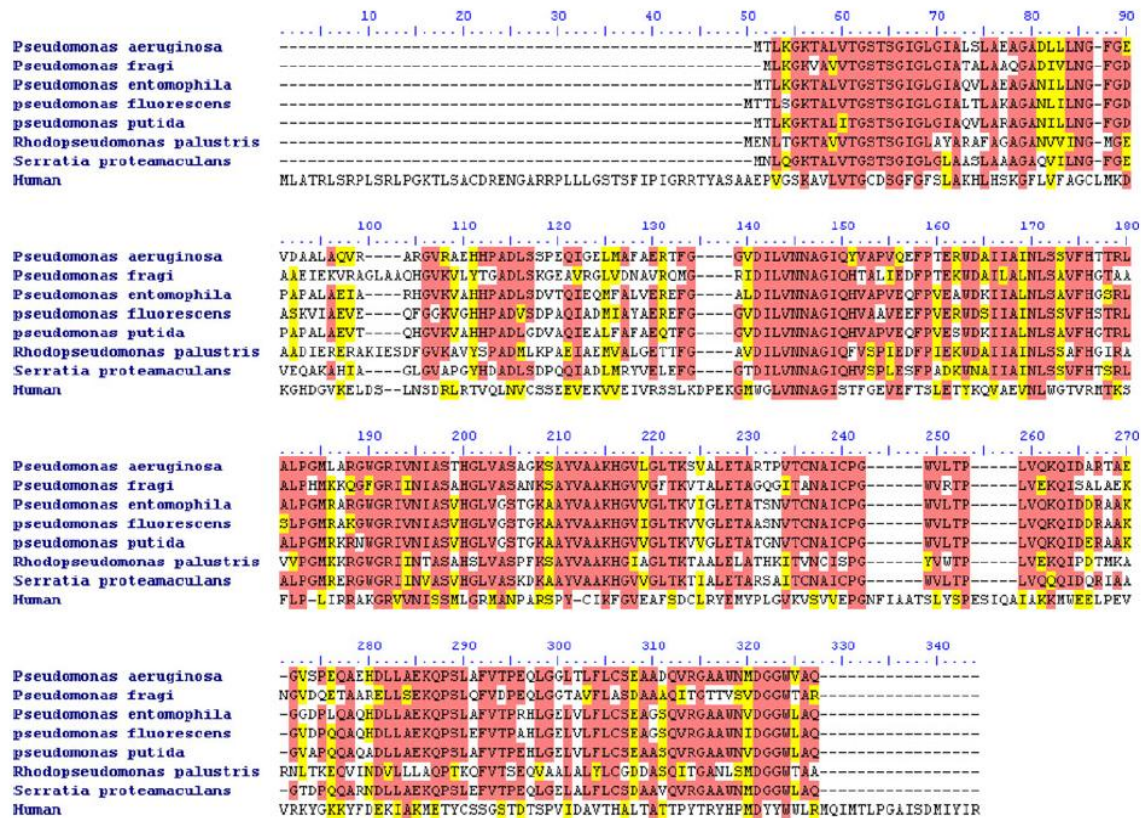


Fig. 3 Alignment of BDH sequences from different bacterial species and from human with *Pseudomonas aeruginosa* (*P. a.*) was realized using Clustal [21]. Identical residues are highlighted in red and similar residues were shown in yellow. The high identity between bacterial

sequences (from 50 to 74%) is visible with the presence of red blocks of amino acids. On the contrary, the weak identity with the human BDH sequence is located on peculiar regions corresponding to amino acids of the active site and substrate binding pocket. (Color figure online)

Fig. 4 Modeling of BDH from *Pseudomonas aeruginosa*. Stick model showing the conserved residues (identical in *blue* and similar in *yellow*) interacting with NAD^+ (in *red*). The interactions between atoms were shown by *green* lines. (Color figure online)

