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The Catalytic Property of 3-Hydroxyisobutyrate Dehydrogenase from *Bacillus cereus* on 3-Hydroxypropionate

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Abstract The *MmsB* gene product from *Bacillus cereus* ATCC14579 exhibits 3-hydroxypropionate dehydrogenase activity. It encodes the 32-kDa enzyme protein composed of 292 amino acids. Recombinant 3-hydroxyisobutyrate dehydrogenase (3-HIBADH) was purified 100-fold from cell extract by ammonium sulfate fractionation and column chromatography. The enzyme catalyzed oxidation of 3-hydroxypropionate (3-HP) between pH7.0 and 10.0 with optimal activity between 8.8 and 9.0. A Km of 16.8 mM for 3-HP was calculated from a Lineweaver–Burk plot. The semialdehyde as products has been proven by spectrophotometric determination. The dehydrogenase apparently has no metal ion requirement. Kinetic determinations established that 3-HIBADH was more active with $NADP^+$ than NAD^+, which did not show similarity with previously reported 3-HIBADH except that from *Thermus thermophilus.*

Keywords 3-Hydroxyisobutyrate dehydrogenase · 3-Hydroxypropionate · *Bacillus cereus*

Introduction

3-Hydroxyisobutyrate dehydrogenase (3-HIBADH, EC1.1.1.31) is a key enzyme for the metabolism of valine and some keto-bodies. It exists widely in bacteria, yeasts, and mammalian tissues and can catalyze reversible conversion of 3-hydroxyisobutyrate to methylmalonate semialdehyde [1, 2]. 3-HIBADH has been purified into homogeneity and well studied from many organs such as cultured neural cells [3], *Thermus thermophilus* HB8 [4], *Pseudomonas putida* E23 [5], *Escherichia coli*, *Haemophilus influenzae* [6], and rabbit liver [7].

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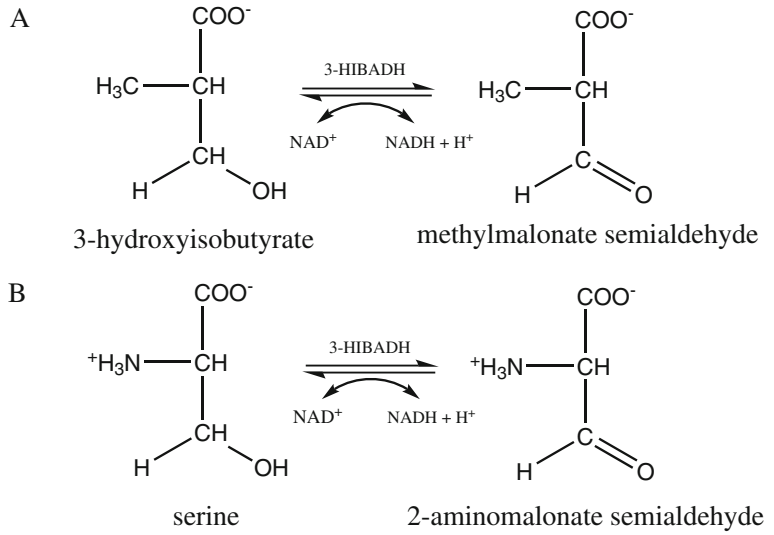

Fig. 1 **a**, **b** Reactions catalyzed by 3-hydroxyisobutyrate dehydrogenase (3-HIBADH)

A wide substrate specificity of the 3-HIBADH was found because it could catalyze not only the oxidation of 3-hydroxyisobutyrate but also L-serine, D-threonine, and other 3-hydroxyacid derivates [8] (Fig. 1). 3-HIBADH was previously classified into short-chain dehydrogenase superfamily due to its broad catalytic spectrum. Substrates of short-chain dehydrogenases are wide ranging including alcohols, sugars, steroids, aromatic compounds, and xenobiotics [9]. Although amino acid sequence alignment revealed homology with the short-chain alcohol dehydrogenases (ADH) to some extent [10], 3-HIBADH was inactive with a serial of short-chain alcohol-containing carbon backbones of four or five carbons in length [11] and it had the characteristic amino acid sequence clearly indicating a common structural framework [12]. Its substrate specificity and particular sequence features differing from ADH made it suitable for the independent 3-hydroxyacid dehydrogenases superfamily, sharing more resembling active sites and crystal structures [13]; hence, 3-HIBADH may have the similar function to 3-hydroxypropionate dehydrogenase in vivo and be the key enzyme in an autotrophic CO_2 fixation pathway, the 3-hydroxypropionate cycle [14].

3-HP has the potential to be an important biomaterial precursor as the isomer of lactate for the synthesis of other commercially valuable chemicals. There is a significant market opportunity for the development of bio-based products from 3-HP. The current challenges include the development of a low-cost fermentation and a family of catalysts for the conversion of 3-HP to other compounds [15–17]. Biosynthesis of 3-HP by 3-HIBADH induced reduction is a promising reaction from industrial viewpoints owing to its wide substrate specificity and insensitivity to long-chain alcohol.

For the first time, the cloning and overexpression of 3-hydroxyisobutyrate dehydrogenase gene from *Bacillus cereus* has been demonstrated. We report here its kinetic property for the conversion of 3-HP on the basis of the measurement of kinetic parameters. Experimental results suggest that 3-HIBADH from *B. cereus* may play a role in biosynthesis of 3-HP as a biological source.

Materials and Methods

Strains, Vectors, and Chemicals

Strain *B. cereus* ATCC 14579 from China General Microorganism Conserve Center (CGMCC) was used to get the genome and to clone *MmsB* gene. *E. coli* DH5a (Novagen) served as host for pGEM-T (Promega) and pET22b vectors (Novagen). *E. coli* BL21 was used for protein expression. pGB80 is a pGEM-T construct containing the coding region for *MmsB* in a 1-kb fragment of *B. cereus* DNA; pEB81 is a pET22b construct containing the coding region for *MmsB* in a 0.9-kb *Nde*I–*Nco*I fragment of *B. cereus* DNA oriented with a lac operator. 3-HP was purchased from Tokyo Chemical Industry. Other chemicals or agents were purchased from BBI Canada if not specified.

Culture Conditions and Media

B. cereus was cultivated in beef extract medium at 30°C. *E. coli* DH5a cells as host for pGEM-T and pET22b were cultivated in low-salt LB medium containing 1% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast extract, and 0.5% (*w*/*v*) NaCl at 37°C. *E. coli* BL21 used as the host for expression of 3-HIBADH was grown at 37°C in autoinduced LB medium containing low-salt LB medium plus 0.2% lactose and 0.02% glucose.

Construction of Recombinant Vectors

B. cereus was cultured in a centrifugal tube as the source of genome. Transformants were grown at 37°C for 12 h in 50-ml low-salt LB medium on a reciprocal shaker.

Sense and antisense primers whose sequences were 5′-GGGGATGAGAAAATGGAACA TAAAAC-3′ and 5′- CATGCCATGGTTACCCCCTTATATATTTT-3′ were designed according to the DNA sequence of 3-HIBADH in NCBI Entrez Gene (Gene ID: 1206387). PCR production contained the open reading frame of *MmsB*, an *Nde*I restriction site at 5′-end and an *Nco*I restriction site at 3′-end. After purification with agarose gel purification kit, the amplified DNA fragments were ligated into the *Nde*I–*Nco*I site of pET22b vector. The pEB81 recombinant vector was introduced into *E. coli* BL21 to overexpress 3-HIBADH protein.

Protein Overexpression and Purification

The recombined *E. coli* BL21 was grown at 37°C in the autoinduced LB medium for 12 h and then centrifuged. After majority of the cells precipitated, supernatant was removed and an ultrasonic disintegration was implemented in order to get the cell extract.

All the following steps were carried out at 4°C. Ammonium sulfate was added into the cell extract in order to obtain the protein component at the 45–85% concentration of ammonium sulfate. The precipitation was resolved in buffer A (1.5 M $(NH_4)_2SO_4$, 50 mM Tris–HCl, pH8.5, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT)) and applied to a column of Phenyl-Sepharose. The sample was eluted by a linear $(NH_4)_2SO_4$ gradient from 1.5 to 0 M in buffer A and 3-HIBADH activity was obtained between 1.2 and 1.3 M. After being dialyzed for 24 h in a 10-kDa cut dialysis bag, the sample was eluted through an anion exchange column of Q-Sepharose Fast Flow in buffer B (50 mM Tris–HCl pH7.5, 2 mM EDTA, 2 mM DTT) by a linear NaCl gradient from 0 to

1 M in buffer B. 3-HIBADH activity was obtained between 0.45 and 0.55 M. The purified enzyme sample was ultrafiltrated twice against buffer C (50 mM Tris–HCl, pH8.5, 2 mM EDTA, 2 mM DTT) and finally stored in buffer C plus 50% glycerol at −20°C.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was performed on standard size 10% slab gels for the determination of apparent subunit molecular weight. 3-HIBADH and Unstained Protein Marker (Fermentas) were dissolved in 12.5 mM Tris–HCl (pH6.8), 0.46% SDS, 5% (*w*/*v*) DTT, and 10% glycerol and boiled 5 min. Protein was visualized by Coomassie blue staining.

Enzyme Assay

The enzyme was assayed at 37°C in a volume of 200 µl containing 50 mM Tris–HCl pH8.5 and production of NADH and NADPH was measured at 340 nm in a standard 96 dual-beam spectrophotometer. Protein concentrations were determined using the Bradford protein assay with bovine serum albumin used as standard [18].

Spectrophotometric Determination with Thiobarbituric Acid

The sample was added with an equal volume of ice-cold trichloroacetic acid solution (10% trichloroacetic acid) and incubated on ice for 15 min. Then, it was centrifuged in order to remove the precipitate. Three times volume of thiobarbituric acid (TBA) solution (1.25% thiobarbituric acid (*w*/*v*), 50% ethanol (*v*/*v*)) and three times volume of acetate were successively added to the supernatant. The mixture was heated in boiling water bath for 3 min. The absorbance of cooled mixture was measured in a standard UV-Vis spectrophotometer. Another sample without 3-HIBADH was processed simultaneously as the blank control.

Results and Discussion

Overexpression and Purification of the 3-HIBADH

The SDS-PAGE shows that the target protein was overexpressed from the *E. coli* BL21 and accounts for 30% of total protein. 3-HIBADH protein was purified 96-fold at 4°C. Sequence deducted from its coded DNA is shown in Fig. 2. Recovery was 29.2% and final specific enzyme activity was 8.7 µM/min per milligram with 3-HP as substrate. Table 1 indicates that the ammonium sulfate precipitation did not increase the specific activity

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  1 MEHKTLSIGFIGIGVMGKSMVYHLMQDGHKVYVYNRTKAKTDSLVQDGAN
 51 WCNTPKELVKQVDIVMTMVGYPHDVEEVYFGIEGIIEHAKEGTIAIDFTT
101 STPTLAKRINEVAKSKNIYTLDAPVSGGDVGAKEAKLAIMVGGEKEIYDR
151 CLPLLEKLGTNIQLQGPAGSGQHTKMCNQIAIASNMIGVCEAVAYAKKAG
201 LNPDKVLESISTGAAGSWSLSNLAPRMLKGDFEPGFYVKHFMKDMKIALE
251 EAEKLQLPVPGLSLAKELYEELIKDGEENSGTQVLYKKYIRG
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Fig. 2 The sequence of 3-HIBADH protein from *B. cereus* ATCC14579

Table 1 Purification of 3-hydroxyisobutyrate dehydrogenase from *E. coli.*

Purification stage	Total protein (mg)	Specific activity (μM/min per milligram)	Total activity (μM/min)	Yield
Homogenate	4,600	0.091	418	100%
Ammonium sulfate fractionation	2,100	0.108	226	54.1%
Phenyl-Sepharose	52.0	2.75	142	34.2%
Q-Sepharose	12.8	8.77	122	29.2%

significantly and hydrophobic chromatography was the main purification step producing more than 60% of total enzyme activity (Fig. 3).

Spectrophotometric Determination for Aldehyde with Thiobarbituric Acid

It was found that the condensation product could be formed between TBA and aldehyde at a high concentration of acetate. The product usually has a peak absorbance at 450 nm approximately and this simple specific reaction could be applied to measure aldehyde in aqueous solution [19]. To corroborate this aldehyde, the reaction was studied in the presence of 3-HIBADH and blank control simultaneously. A peak absorbance observed at 453 nm suggested that the aldehyde was formed as a product during incubation.

pH Optimum and Metal Ion Effects

Oxidation of 3-HP by 3-HIBADH occurred between pH7 and 10 with optimal activity between pH8.8 and 9.0 (Fig. 4).

The following inorganic salts at 5 and 200 μM did not affect 3-HIBADH activity: $MnSO_4$, $CuSO_4$, $CaCl_2$, $MgSO_4$, and $FeSO_4$. Preincubation for 3 min with ethylene glycol tetraacetic acid or EDTA (all at 5 and 200 μM) did not affect 3-HIBADH activity as well; therefore, no evidence was found for a metal ion requirement in agreement with the previous conclusion. The only exception is $ZnCl_2$ which inhabited approximately 60% of 3-HIBADH activity at 200 μM but showed no effect at 5 μM. Although it was reported that

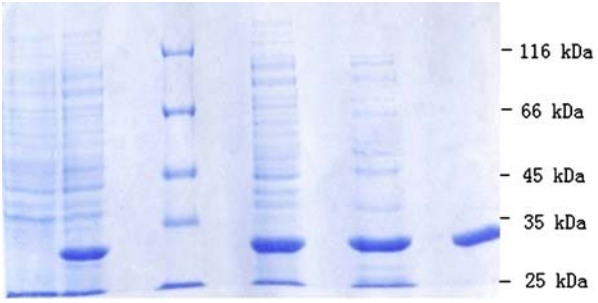

Fig. 3 Purification of 3-HIBADH expressed in *E. coli* BL21 (DE3) by column chromatography. SDS polyacrylamide gel was stained with Coomassie Brilliant Blue (G-250). *Lane 1*: total cell lysate before induction. *Lane 2*: total cell lysate after induction. *Lane 3*: high-molecular-weight protein marker. *Lane 4*: soluble proteins at 45–85% $(NH_4)_2SO_4$. *Lane 5*: resoluble proteins after phenyl-Sepharose column. *Lane 6*: purified protein through Q-Sepharose Fast Flow column at 32 kDa

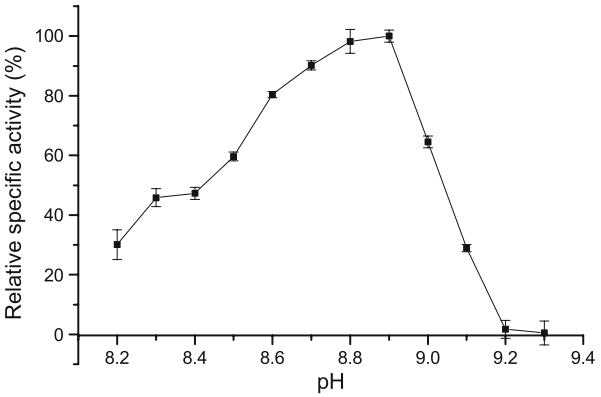

Fig. 4 Effects of pH on the enzymatic activity of 3-HIBADH. Enzyme activity was measured by a colorimetric assay using 3-HP as substrate. Each test was carried out twice in the 50 mM Tris–HCl buffer at 37°C. The *rectangles* indicate the average values

certain metal ion such as Ca^{2+} can completely inactivate 3-HIBADH [20], lack of cation active effects was found in 3-HIBADH from *B. cereus*. The inhibition of Zn^{2+} at 200 μM is quite distinct compared to ADH, as Zn^{2+} cation is active for ADH [21].

Substrate Specificity and Reaction Mechanism Analysis

Although NAD^+ can be a coenzyme during the reaction, 3-HIBADH was more active with $NADP^+$ rather than NAD^+ considering that Km_{NAD+} was 2.4 mM while Km_{NADP+} was 0.25 mM. The lower Km for $NADP^+$ indicates that the enzyme greatly favors the $NADP^+$ as cofactor rather than NAD^+. It is quite distinct from the 3-HIBADH in mammals but the same as *T. thermophilus* [7]. In mammals, the last conserved glycine residue in cofactor-binding domain is usually followed by a stretch of six hydrophobic residues which play a role in cofactor specificity for the 3-HIBADH from mammals [11], so it is a possible reason to the salient specificity for $NADP^+$ that the cofactor-binding

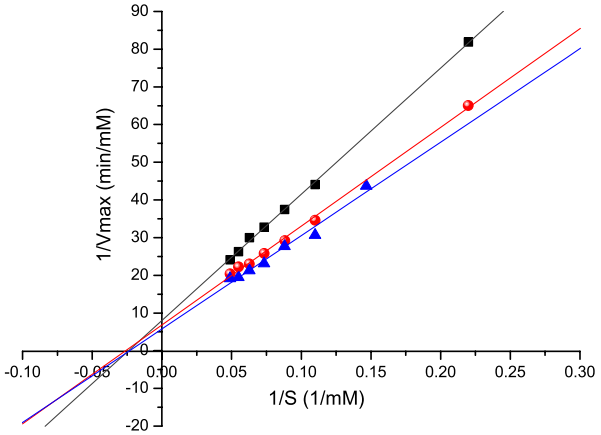

Fig. 5 Effect of varying subsaturating concentrations of both 3-HP and NAD^+ on 3-HIBADH activity. NAD^+ concentrations were, respectively, 6.25 mM (*square*), 9.375 mM (*circle*), and 12.5 mM (*triangle*)

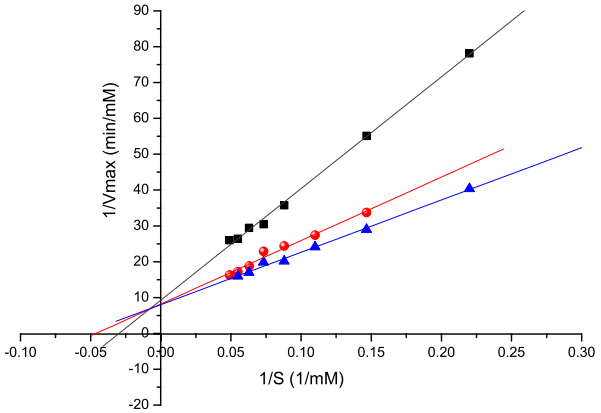

Fig. 6 Effect of varying subsaturating concentrations of both 3-HP and $NADP^+$ on 3-HIBADH activity. $NADP^+$ concentrations were, respectively, 6.25 mM (*square*), 9.375 mM (*circle*), and 12.5 mM (*triangle*)

sequence of 3-HIBADH from *B. cereus* is followed by histidine and lysine residues instead of hydrophobic residues.

Figures 5 and 6 indicate that the Vmax was 0.70 mM/min per milligram. As expected, the following substances cannot react with 3-HIBADH at concentration of 0.1 mM but they did not inhibit the activity either: 1-propanol, lactate potassium, malate potassium, malonate potassium, acetaldehyde, ethanol, and glycerin. These results also proved that the 3-HIBADH was not contaminated by other short-chain ADH. No further effort was done for the measuring the kinetic property of 3-hydroxyisobutyrate as the substrate, as this essay was focused on the reaction between 3-HP and 3-HIBADH. Apparently, Table 2 shows a much lower affiliation and a higher Km (16.8 mM) for 3-HP compared to 3-hydroxyisobutyrate whose Km is approximately 20-fold lower than 3-HP. This is partially contributed to the decreasing polarity of the substituent on the 2-carbon.

Den et al. [22] found some evidences that 3-HP could be oxidized to malonic semialdehyde in microorganism and mammals, but the metabolic importance of these reactions differing from previous short-chain alcohol dehydrogenation pathway was not completely clear. There were no definite DNA or protein sequences published for

Table 2 Kinetic constants of 3-hydroxykobutyrate dehydrogenase to 3-HP.

Constant	Value
Km	
NAD^+	2.4 mM
$NADP^+$	0.25 mM
3-HP	16.8 mM
Vmax	
3-HP	0.70 mM/min per milligram
Kcat	
3-HP	0.21 S^{-1}
Kcat/Km	
3-HP	0.0125 $mM^{-1}s^{-1}$

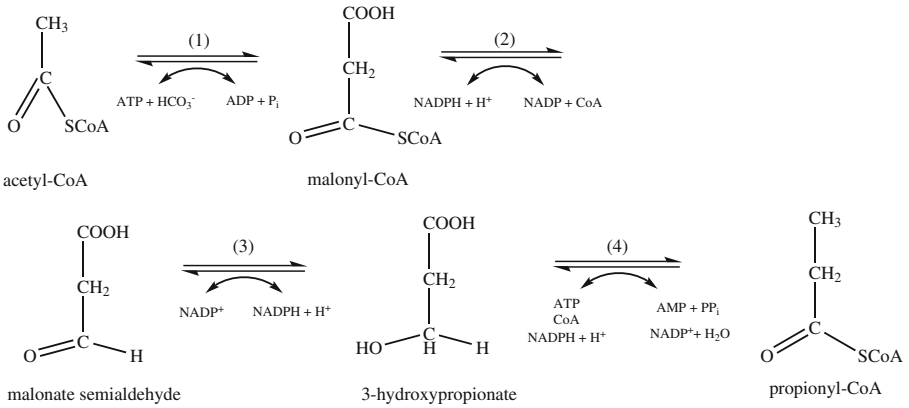

Fig. 7 A part of 3-hydroxypropionate cycle found in *Archaeal Metallosphaera*, in which step 3 indicates the conversion between 3-HP and malonate semialdehyde. Enzymes activities: *1*, acetyl-CoA carboxylase; *2*, malonyl-CoA reductase; *3*, unknown enzyme; *4*, 3-hydroxypropionyl-CoA dehydratase, acryloyl-CoA reductase, and propionyl-CoA carboxylase

3-hydroxypropionate dehydrogenase so far, so it has not come to consistency on whether 3-hydroxypropionate dehydrogenase exists in vivo. After a novel autotrophic CO_2 fixation pathway was found in *Chloroflexus aurantiacus* [23] and *Archaeal Metallosphaera* [24] (Fig. 7), more and more attention was paid to the enzymes in this metabolic cycle. But neither the enzyme catalyzing the conversion of malonic semialdehyde to 3-HP nor the key conversion step of the pathway was entirely clear [25]. Obviously, the enzyme involved in this step has the 3-hydroxypropionate dehydrogenase activity which 3-HIBADH partially revealed in this study.

Reaction Steady-State Kinetic Analysis

Reciprocal plots of activity versus varied 3-HP concentration gave intersecting lines with varied concentrations of NAD^+ and $NADP^+$ (Figs. 5 and 6). Considering that the lines with

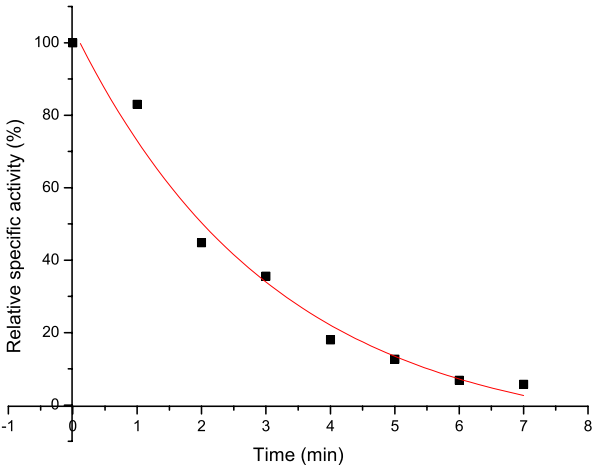

Fig. 8 Thermal stability of 3-HIBADH of *B. cereus*. *X-axis* indicates the incubation time at 55°C and pH8.8

different concentrations of coenzyme meet above the *X*-axis, the binding between coenzyme and 3-HIBADH decreased the apparent Km of substrate. These data obviously indicate a sequential reaction mechanism. The kinetic constants for the enzyme are summarized in Table 2.

Temperature Optimum and Thermal Stability

Oxidation of 3-HP by 3-HIBADH occurred between 35 and 45 °C where the enzyme could show more than 90% of its optimized activity and the maximum activity can be gained at 37 °C. This is also the optimized growing temperature of *B. cereus*. The thermal stability was acceptable. The enzyme activity was not decreased obviously during preincubation at 45 °C within 30 min, but preincubation at 55 °C in 3 min could significantly denaturate and deactivate the enzyme (Fig. 8).

Conclusion

In this study, 3-HIBADH was purified 100-fold to SDS-PAGE homogeneity and kinetic parameters were measured to determine its catalytic reaction on 3-HP. This is the first preparation of the enzyme from *B. cereus* that was demonstrated to be homogenous and determined for its kinetic property.

An oxidative function of 3-HIBADH was studied by overexpressing *MmsB* gene in *E. coli*. Oxidation of 3-HP occurred during mixing between purified enzyme and substrate. The spectrophotometric determination with TBA indicates that 3-HIBADH from *B. cereus* acts on 3-HP and has the similar function to 3-hydroxypropionate dehydrogenase in vivo. This result suggests as well that the substrate of 3-HIBADH did not limit to 3-hydroxyisobutyrate, L-serine, D-threonine, and other three-carbon backbone compound attached to a nonpolar substituent such as a methyl group.

However, there were no definite DNA or protein sequences published for 3-hydroxypropionate dehydrogenase so far; the conversion between malonate semialdehyde and 3-HP involving in putative 3-HP cycle still remains unclear [23, 24, 26]. The present study establishes that 3-HIBADH could be the key enzyme in the 3-HP cycle found in *Archaea* and *Lactobacillus*.

As ideal biomaterial precursors, 3-HP and its derivants are currently synthesized in less eco-friendly organic solution [27, 28], so the biotransformation from acetyl-CoA could be a more effective path to product 3-HP. Therefore, it may be essential for a successful transformation procedure to study the catalytic properties of 3-HIBADH.

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