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Short Communication

# Enzymatic reduction of levulinic acid by engineering the substrate specificity of 3-hydroxybutyrate dehydrogenase

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## HIGHLIGHTS

- A novel activity toward levulinic acid was generated by engineering of 3HBDH.
- Levulinic acid was converted to 4-hydroxyvaleric acid by the engineered 3HBDH.
- Engineering strategy was proposed and validated by molecular docking simulation.
- Approximately 60% conversion of levulinic acid was achieved by the engineered enzyme.

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## ABSTRACT

Enzymatic reduction of levulinic acid (LA) was performed for the synthesis of 4-hydroxyvaleric acid (4HV) – a monomer of bio-polyester and a precursor of bio-fuels – using 3-hydroxybutyrate dehydrogenase (3HBDH) from *Alcaligenes faecalis*. Due to the catalytic inactivity of the wild-type enzyme toward LA, engineering of the substrate specificity of the enzyme was performed. A rational design approach with molecular docking simulation was applied, and a double mutant, His144Leu/Trp187Phe, which has catalytic activity ($k_{cat}/K_m$ = 578.0 $min^{-1}$ $M^{-1}$) toward LA was generated. Approximately 57% conversion of LA to 4HV was achieved with this double mutant in 24 h, while no conversion was achieved with the wild-type enzyme.



## 1. Introduction

Levulinic acid (LA) is a chemical building block that can be obtained from cellulosic biomass through a relatively simple chemical treatment with acid catalysts (Girisuta et al., 2008; Rackemann & Doherty, 2011). Many reports have described the conversion of LA to its derivatives such as liquid fuels and commercially valuable chemicals (Bond et al., 2010; Bozell et al., 2000; Bozell and Petersen, 2010). However, most of the methods are focused on the use of chemical processes that require harsh conditions with high temperature and pressure and/or the use of expensive metal catalysts (Chen et al., 2011; Corma Canos et al., 2007; Deng et al., 2009; Rackemann & Doherty, 2011).

Several studies on the conversion of LA with mild conditions – by bio-based methods – have also been reported. These studies used cell-based production systems with LA as a component of the growth medium for the production of 3-hydroxyvalerate (3HV), 4-hydroxyvalerate (4HV) (Martin and Prather, 2009) and γ-valerolactone (4VL) (Martin et al., 2010). The hydroxyvalerates can be used as components of biodegradable polyesters (polyhydroxyalkanoates, PHAs) with interesting physical properties (Gorenflo et al., 2001; Hazer and Steinbüchel, 2007), and 4VL, which can be obtained by the lactonization of 4HV, has been reported as a versatile compound that can be used as fuels, solvents and precursors of carbon-based chemicals (Corma Canos et al., 2007).

In this study, the reduction of LA using an isolated enzyme – not a living cell system – was attempted for the production of 4HV. Because no enzyme that has LA as its native or major substrate has been reported, engineering of the substrate specificity of an enzyme is required. 3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) from *Alcaligenes faecalis* was selected as a target enzyme

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for the engineering of substrate specificity. Reduction of LA with this enzyme has not been reported, but the structural similarity between LA and acetoacetate (the native substrate of 3HBDH) – the former is a linear γ-keto acid with 5 carbon chains, and the latter is a linear β-keto acid with 4 carbon chains – encouraged us to apply the enzyme to the bio-reduction of LA.

Using rational design with mutational studies, the substrate specificity of 3HBDH was engineered, and the catalytic characteristics of the engineered enzymes were analyzed.

## 2. Methods

### 2.1. Materials

The 3HBDH gene was commercially synthesized by Bioneer (Daejeon, Korea). Restriction enzymes (NdeI, XhoI) and DNA ligase were purchased from Enzynomics (Daejeon, Korea). Competent *Escherichia coli* Top 10 and *E. coli* BL21 (DE3) were purchased from Invitrogen (Carlsbad, CA, USA) and Novagen (Madison, WI, USA), respectively. Levulinic acid, lithium acetoacetate, NADH and ammonium formate were purchased from Sigma (St. Louis, MO, USA). As a substrate for the enzyme assay, a solution of the salt of levulinic acid (sodium levulinate) was prepared (Vasavada et al., 2003).

### 2.2. Site-directed mutagenesis, expression and purification of enzymes

The 3HBDH gene was cloned into the pET-22b(+) vector (Novagen, USA) to produce a recombinant plasmid for expression of the protein with a C-terminal 6-histidine tag. The mutants were constructed by a modified QuickChange™ site-directed mutagenesis protocol (Zheng et al., 2004). The plasmids of the wild-type and mutants were transformed into competent *E. coli* BL21 (DE3) for protein expression. The cells were induced by adding 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and cultured at 20 °C for 24 h to express 3HBDH. The wild-type and mutant enzymes were purified using Ni-NTA agarose column and the purity was estimated by 12% SDS–PAGE. The enzyme concentration was determined by Bradford assay (Bradford, 1976).

### 2.3. Enzyme assay and kinetic analysis

To assay enzyme activity, reduction of acetoacetate and LA was monitored by measuring the oxidation rate of NADH with a UV-spectrophotometer (Varian, USA) at 340 nm. For the selection of mutants that have catalytic activity toward LA, the activity was measured in 50 mM sodium phosphate (pH 6.0) supplemented with 2.7 μM 3HBDH, 0.20 mM NADH and 0.60 mM LA at 30 °C. For the kinetic analysis of 3HBDH, the $K_m$ and $k_{cat}$ values for each substrate were determined at the optimum pH in 50 mM sodium phosphate buffer (pH 6.0) containing 0.25 mM NADH at 30 °C.

### 2.4. Enzyme reaction and HPLC analysis

The enzyme reaction was initiated by the addition of 12 μM 3HBDH (final concentration) to 50 mM sodium phosphate buffer (pH 6.0) containing 30 mM NADH and 12 mM LA at 30 °C. Reaction samples were acquired at fixed time intervals and analyzed by HPLC with a modified method described in Martin and Prather (2009), and Valentin et al. (1992). The HPLC samples were analyzed on a Finnigan Surveyor Plus HPLC System (Thermo Scientific) equipped with an Agilent ZORBAX SB-Aq reverse phase column (4.6 × 250 mm, 5 μm).

### 2.5. Molecular docking simulation

The molecular docking simulation was conducted by partial protein modeling using the SYBYL-X package (Tripos, ver. 1.2). The crystal structure of 3HBDH (pdb code: 3VDR) was used to determine the best docking position of LA at the binding pocket. The Surflex dockings of the substrate were performed using the Run-Multiple ligand option of Surflex-Dock, and $\Delta G_b$ (free energy of binding) values were calculated.

## 3. Results and discussion

### 3.1. Rational design and mutational study of 3HBDH

Because the wild-type enzyme did not exhibit any catalytic activity toward LA (Table 2), engineering of substrate specificity of 3HBDH was conducted for the enzymatic reduction of LA. To engineer the substrate specificity, the binding features of wild-type 3HBDH with native substrate (acetoacetate) were analyzed, and the strategies for the mutations were suggested as described in Table 1. Following the strategies, the 6 single mutants (Gln94Asn, His144Leu, Lys152Ala, Gln196Asn, Trp187Phe and Trp257Phe) were selected for the engineering of the substrate specificity of 3HBDH (Table 1).

*In vitro* activity assays of the selected 6 single mutants with LA were performed to select the mutants that have catalytic activity toward LA, and the His144Leu mutant was found to be the only mutant that exhibit catalytic activity toward LA (Table 2).

According to the reaction mechanism of 3HBDH from *Alcaligenes faecalis*, the distance between the C4 atom of NADH and the C3 atom of acetoacetate ($d_{1,std}$ = 3.8 Å), and the distance between the oxygen atom of Tyr155 and the oxygen atom of the ketone group of acetoacetate ($d_{2,std}$ = 3.1 Å) were considered to be important for the catalysis (Hoque et al., 2008, 2009). In this regard, it was assumed that the distance between the C4 atom of NADH and the C4 atom of LA ($d_1$), and the distance between the oxygen atom of Tyr155 and the oxygen atom of the ketone group of LA ($d_2$) should be within the comparable value of $d_{1,std}$ and $d_{2,std}$, respectively, for the reduction of LA by the engineered 3HBDH. This assumption was applied to the validation of the 3HBDH mutants with molecular docking simulation.

The molecular docking simulations for the 6 "single" mutants were performed, and the $d_1$, $d_2$ and the $\Delta G_b$ values were obtained from each run – a negatively larger value of $\Delta G_b$ implies a greater binding affinity between enzyme and substrate (Table 2). Of the docking results of the 6 single mutants, only one mutant – His144Leu – displayed suitable distance values of $d_1$(3.4 Å) and $d_2$(3.0 Å) with a favorable value of $\Delta G_b$ (−9.1 kcal/mol). Based on this result, the His144Leu mutant was found to have an activity toward LA and was selected as an initial mutant for further improvement of the activity of the mutant.

### 3.2. Additional mutation studies for the selection of improved mutant

In addition to the His144Leu mutant, the 15 additional double mutants were prepared with the combination of the 6 single mutations ($_6C_2$), and tested with *in vitro* activity assay (data not shown). Only one double mutant (His144Leu/Trp187Phe) exhibited catalytic activity toward LA (Table 2), and showed approximately 11-fold higher activity compared with the single mutant His144Leu (Table 2). From this result, His144Leu/Trp187Phe was selected as the best double mutant for the enzymatic reduction of LA.