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Identification of the catalytic subunit of acetohydroxyacid synthase in *Haemophilus influenzae* and its potent inhibitors

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Abstract

Acetohydroxyacid synthase (AHAS; EC 2.2.1.6) is a thiamin diphosphate- (ThDP)- and FAD-dependent enzyme that catalyzes the first common step in the biosynthetic pathway of the branched-amino acids (BCAAs) leucine, isoleucine, and valine. The gene from *Haemophilus influenzae* that encodes the AHAS catalytic subunit was cloned, overexpressed in *Escherichia coli* BL21(DE3), and purified to homogeneity. The purified *H. influenzae* AHAS catalytic subunit (Hin-AHAS) appeared as a single band on SDS–PAGE gel, with a molecular mass of approximately 63 kDa. The enzyme catalyzes the condensation of two molecules of pyruvate to form acetolactate, with a K_m of 9.2 mM and the specific activity of 1.5 μmol/min/mg. The cofactor activation constant ($K_c = 13.5$ μM) and the dissociation constant ($K_d = 3.3$ μM) of ThDP were also determined by enzymatic assay and tryptophan fluorescence quenching studies, respectively. We screened a chemical library to discover new inhibitors of the Hin AHAS catalytic subunit. Through which, AVS-2087 ($IC_{50} = 0.53$ μM), KSW30191 ($IC_{50} = 1.42$ μM), and KHG20612 ($IC_{50} = 4.91$ μM) displayed potent inhibition as compare to sulfometuron methyl ($IC_{50} = 276.31$ μM).

Keywords: *Haemophilus influenzae*; Acetohydroxyacid synthase; Catalytic subunit; Purification; Characterization; Inhibitor screening

The branched-chain amino acids (BCAAs)[2] such as leucine, isoleucine, and valine belong to a group called the essential amino acids and are synthesized by many microbes and plants [1]. Several kinds of enzymes are involved in BCAA biosynthesis. Acetohydroxyacid synthase (AHAS; EC 2.2.1.6) is one of the enzymes that catalyze the first step of BCAA biosynthesis. AHAS catalyzes the condensation of two molecules of pyruvate to form acetolactate in the biosynthesis of valine and leucine. AHAS also can condense pyruvate and 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate in the biosynthesis of isoleucine. AHAS consists of two subunits, the catalytic subunit and the regulatory subunit [1]. The AHAS enzymatic activity is produced from the catalytic subunit, while the regulatory subunit affects feedback regulation and activation of the catalytic subunit. AHAS requires three cofactors for activity: thiamin diphosphate (ThDP), a divalent metal ion (usually Mg^{2+}), and flavin adenine dinucleotide (FAD). The first two cofactors are typical for ThDP-dependent enzymes during catalysis [2]. The requirement for FAD is unusual because the catalytic mechanism of AHAS does not involve the redox reaction that is required for the private oxidase (POX)-like ThDP-dependent enzyme [2]. Recently, however, Tittmann et al. reported that FAD is reduced in the side reaction catalyzed by AHAS II in *Escherichia coli* [3].

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[2] *Abbreviations used:* BCAAs, branched-chain amino acids; AHAS, acetohydroxyacid synthase; ThDP, thiamin diphosphate; FAD, flavin adenine dinucleotide; POX, private oxidase.

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Plants and many microorganisms have the capacity to synthesize the BCAAs needed for their survival from inorganic precursors. Animals must obtain these compounds from their diet because they lack the full BCAA biosynthetic machinery. Therefore, AHAS is an attractive target enzyme for the development of herbicides and antimicrobial drugs. Herbicides that inhibit plant AHAS have been well characterized over the past ten years. The three major classes of these herbicides are sulfonylureas, imidazolinones, and triazolopyrimidines [4,5]. However, little interest has been shown for the development of AHAS inhibitors as antimicrobial drugs because of the assumption that bacterial pathogens would be able to overcome the effects of AHAS inhibitors by the intake of BCAAs from their host cell. This supposition, however, may be incorrect due to the fact that the BCAAs auxotrophic strains of mycobacterium fail to proliferate in their host cell [6]. These observations suggest that AHAS could be a potential target of new anti-TB drugs.

In order to develop potential microbial AHAS drug targets, we have cloned, expressed, purified, and characterized the catalytic subunit of AHAS from *Haemophilus influenzae* (Hin-AHAS) and then identified Hin-AHAS inhibitors through a chemical library screen.

Materials and methods

Reagents

The genomic DNA of *H. influenzae* was obtained from the Korea Institute of Science and Technology (Seoul, Korea). Herbicides used in this experiment were obtained from the Korea Research Institute of Chemical Technology (Daejeon, Korea). The chemical library was obtained from the Korea Chemical Bank (Daejeon, Korea). Sodium pyruvate, FAD, ThDP (named as cocarboxylase), creatine, and α-naphtol were purchased from Sigma (St. Louis, USA). Bacto-tryptone, yeast extract and bacto-agar were obtained from Difco Laboratories (Sparks, USA). Restriction endonuclease and other molecular biology enzymes were purchased from New England BioLabs (Beverly, USA). All other materials were of analytical grade.

Gene cloning and plasmid construction

The DNA fragment encoding the open reading frames of the catalytic subunit of acetohydroxyacid synthase (Swiss-Prot P45261) from *H. influenzae* was amplified by PCR using the gene specific primers 5′-CAT GCC ATG GGC AAG AAG TTA TCT GGC GCA GAG-3′and 5′-CCG CTC GAG GTT TGT CTC CTC TTG AGG TTT-3′. The underlined sequences indicate the location of NcoI and XhoI sites, respectively. The amplified DNA was inserted into the NcoI/XhoI sites of pET28a(+) (Novagen, Madison, USA) to produce fusion proteins with C-terminal hexa-histidine tags.

Expression and purification of recombinant Hin-AHAS

Escherichia coli BL21(DE3) cells harboring the Hin-AHAS expression plasmid were grown in 1L Luria-Bertani (LB) medium containing 100 μg/mL kanamycin at 37 °C until the A_{600nm} reached 0.7–0.8. Cells were induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further grown at 18 °C overnight. The induced cells were harvested by centrifugation and resuspended in 50 mL buffer-A (20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 20 mM imidazole) containing 1 mM PMSF, 0.5% (v/v) Triton X-100, 0.5 mg/mL lysozyme, and EDTA-free protease inhibitor cocktail (Roche, Indianapolis, USA). Cells were incubated at room temperature for 30 min, cooled on an ice-bath, and then continuously sonicated to disrupt the cell wall and nucleic acids. The crude cell extract was centrifuged at 23,000*g* at 4 °C for 30 min and the supernatant was loaded onto a Ni^{2+}-charged Chelating Sepharose column (V_c (column volume) = 8 mL; Amersham, Piscataway, USA) and run on an ACTA-Purifier FPLC (Amersham, Piscataway, USA) pre-equilibrated with buffer-A at 4 °C. All subsequent steps were performed at 4 °C. The column was washed thoroughly with the same buffer (without 40 mM imidazole) until the A_{280nm} reached baseline. The bound proteins were eluted by applying a 60 mL linear gradient of 40–500 mM imidazole in buffer-A. Protein fractions were concentrated and desalted against buffer-B (20 mM Tris–HCl, pH 8.0, 5 mM EDTA, and 5 mM β-mercaptoethanol) using a HiTrap Desalting column (Amersham, Piscataway, USA). The desalted protein was applied to a Mono-Q column (V_c = 1 mL; Amersham, Piscataway, USA), pre-equilibrated with buffer-B. After washing of the column with buffer-B, the bound protein was eluted with 60 mL of a linear salt gradient ranging from 0 to 0.5 M NaCl in buffer-B. Protein fractions were concentrated and applied to a Superdex 200 prep grade column (2.6 × 35 cm; Amersham, Piscataway, USA) that was pre-equilibrated with buffer-C (20 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 5 mM EDTA, and 5 mM β-mercaptoethanol) and fractions were collected at a 1 mL/min flow rate. Fractions containing AHAS were concentrated and stored at −80 °C in a 10% (v/v) glycerol concentration. The protein concentration was determined by Bradford assay following the manufacturer's instruction for the Bradford reagent (Bio-Rad, Hercules, USA).

Molecular weight of recombinant Hin-AHAS

The molecular weight of Hin-AHAS was determined by size exclusion chromatography using a Superdex 200 10/300 GL (V_c = 24 mL; Amersham, Piscataway, USA) column that was equilibrated with 20 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 5 mM EDTA, and 5 mM β-mercaptoethanol at a flow rate of 0.5 mL/min at 4 °C. Sample containing about 2 mg/mL of purified Hin-AHAS was loaded on a 100 μL sample loop. Protein elution was monitored by absorbance at 280 nm using the ACTA purifier FPLC. The column was calibrated using bovine serum albumin (66 kDa), alcohol dehydrogease (150 kDa), β-amylase (200 kDa) as marker proteins, and blue dextran (2000 kDa). The preparation of calibration curve and the estimation of molecular weight of AHAS were determined by following the manufacturer's instruction for the Gel filtration calibration kits (Amersham, Piscataway, USA).

Enzyme assay

AHAS activity was measured as previously described with a discontinuous colorimetric assay [7]. In the standard assay conditions, the reaction mixture contained 100 mM potassium phosphate, pH 7.5, 100 mM pyruvate, 10 mM $MgCl_2$, 1 mM ThDP, and 50 μM FAD. The enzyme and reaction mixture were each pre-incubated at 37 °C for 15 min. The reaction was initiated by adding the enzyme (1 μg) to reaction mixture (final volume, 200 μL) at 37 °C. The reaction was stopped after 1 h by adding 30 μL of 8 M H_2SO_4 and was further incubated at 65 °C for 15 min to decarboxylate acetolactate. Then 100 μL of reaction product was mixed with 90 μL of 0.5% (w/v) creatine and 90 μL of 5 % (w/v) α-naphtol (in 2.5 M NaOH, freshly prepared) one-at a time and incubated at 65 °C for 15 min. The acetoin formed (red-colored complex, $\varepsilon_{525nm} = 20{,}000\ M^{-1}cm^{-1}$) was measured at 525 nm using a Lambda25 UV–vis Spectrometer (Perkin-Elmer, Wellesley, USA). One unit (U) of activity was defined as that produces 1 μmol of acetolactate per minute under standard conditions.

ThDP binding analysis

The ThDP dissociation constant for Hin-AHAS was measured by monitoring the quenching of tryptophan fluorescence [8]. Purified Hin-