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PURIFICATION AND CHARACTERIZATION OF RECOMBINANT ACETOHYDROXYACID SYNTHASE FROM *Haemophilus influenzae*

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ABSTRACT: Acetohydroxyacid synthase (AHAS) presents only in plants and microorganisms. The enzyme catalyzes the first common step in the biosynthesis of branch chain amino acids (BCAAs), including isoleucine, leucine and valine. AHAS is also a potential target for controlling *Haemophilus influenzae*. In this study, the recombinant catalytic subunit of AHAS from *H. influenza* (Hin-AHAS) was expressed in *Escherichia coli*. The purified Hin-AHAS protein exhibited a molecular weight of approximately 63 kDa on SDS-PAGE gel. The apparent V_{max} and K_m values of the purified Hin-AHAS were determined to be 0.236 U/mg protein and 2.503 mM pyruvate, respectively. Two inhibitors of plant AHAS, namely ethoxysulfuron (ETS) and pyrazosulfuron ethyl, were shown to inhibit Hin-AHAS in a non-competitive manner with the IC_{50} values of 90.14 µM and 376.6 µM, respectively. This result showed that the purified enzyme can be used for screening of inhibitors against Hin-AHAS.

Keywords: Haemophilus influenzae, acetohydroxyacid synthase, enzymatic activity, inhibitor, purification.

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INTRODUCTION

Haemophilus influenzae is a Gram-negative, coccobacillary, facultatively anaerobic bacterium which causes a variety of infections in both children and adults, ranging from respiratory tract infection to invasive diseases including meningitis, bacteraemia, epiglottitis, cellulitis and septic arthritis [8]. Among 3 main types: non-encapsulated strains, encapsulated type b strains, and encapsulated non-type b strains (types a and c-f), type b strains commonly known as Hib, are responsible for causing childhood pneumonia (infection in the lungs), meningitis, and bacteremia [12]. A wide variety of antimicrobial drugs has been developed to treat Hib diseases, in which, ampicillin was considered as an effective antibiotic for treatment of *H. influenzae* infection diseases until 1974 [11], however, an increasing number of cases of *H. influenzae* has been recorded that resistant to various antibiotics [7].

Acetohydroxyacid synthase (AHAS) is a transferase acting on aldehyde or ketone residues [5]. As a transketolase, it has both catabolic and anabolic forms that act on a ketone (pyruvate) and can go back and forth in the metabolic chain. AHAS consists of two subunits, in which, the large one gives rise to the enzymatic activity of AHAS, while a small regulatory subunit plays an important role in the feedback regulation and the activation of the catalytic. Moreover, there are three cofactors needed for the enzymatic activity of AHAS, namely thiamine diphosphate (ThDP), divalent metal ion (usually Mg^{2+}), and flavin adenine dinucleotide (FAD) [5]. The enzyme catalyzes the first step in the biosynthesis of branch chain amino acids (BCAAs), including isoleucine, leucine and valine in microorganisms and plants which needed for the survival of living organisms as well as their development [5]. Hence, AHAS is an attractive target for scientists to develop new herbicides for

controlling weeds as well as antimicrobial drugs for controlling disease-causing bacteria.

Regardless of appealing characteristics of AHAS and its inhibitors, the development of AHAS inhibitors as antimicrobial drugs have been limited and received little interest due to the supposition that bacterial pathogens would be capable to overcome the impacts of AHAS inhibitors by taking up of BCAAs from their host cells. This assumption, however, may be inaccurate because of the fact that the BCAAs auxotrophic strains of mycobacterium fail to multiply in their host cells [6]. Indeed, the AHAS mutant of *Burkholderia pseudomallei* had shown that the AHAS of pathogenic microorganisms could be a potential target for antimicrobial drugs [1]. Hence, the main objectives of this study are to express, purify and characterize the catalytic subunit of AHAS from *Haemophilus influenzae* (Hin-AHAS) and evaluate the inhibition kinetics of several AHAS inhibitors.

MATERIALS AND METHODS

The AHAS catalytic subunit coding gene of *H. influenzae* was inserted into a pET28a vector containing kanamycin-resistant gene. The vector was a gift from Hanyang University, Korea. Pyruvate, FAD, ThDP, creatine and α-naphthol, isopropyl-β-D-1-thiogalactopyranoside (IPTG) and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (USA). Luria-Bertani (LB) medium was purchased from SERVA Electrophoresis GmbH (Germany). The inhibitors pyrazosulfuron ethyl and ethoxysulfuron are available in commercial herbicides namely Pyrasus 10WP (Nicotex, Vietnam) and Sunrice 15WG (Bayer, Germany), respectively.

Protein expression

A single colony of *Escherichia coli* BL21 (DE3) cells harboring AHAS-coding gene was cultivated in 250 mL of LB medium containing 50 µg/ml of kanamycin at 37°C and 250 rpm in a shaking incubator until the optimal density at A_{600} reached 0.8-1.0. Protein expression was induced by the supplement of 0.5 mM IPTG. The cells were further grown to allow protein expression at 30°C for approximately 5 hours, then recovered by centrifuging at 5,000 rpm at 4°C for 5 minutes.

Protein purification

The recombinant protein with C-terminal fused to a hexahistidine tag was purified by affinity chromatography using nickel-charged sepharose resin (Qiagen). Three kinds of buffer, namely lysis, wash and elution buffers, were titrated at pH 7.4. The lysis buffer contained 50 mM of NaH_2PO_4, 300 mM of NaCl and 10 mM of imidazole, while washing and elution buffers contained the same concentrations of NaH_2PO_4 and NaCl, and their concentrations of imidazole were 20 and 250 mM, respectively.

The cells were dissolved in 5 ml lysis buffer supplemented 0.15 mM of PMSF, freezed and thawed for several times, then sonicated intermittently on ice to release protein. Crude cell extract solution was centrifuged at 12,000 g at 4°C for 10 minutes, then the supernatant was harvested and loaded onto a Ni^{2+}-charged chelating sepharose column for affinity chromatography. Washing buffer was applied 2 times and the target protein was eluted with elution buffer. The protein concentration was determined by measuring A_{280} using a Nano Drop 2000 UV-Vis Spectrophotometer, then stored at -80°C in 10% (v/v) glycerol. The purity of the desired protein was determined on 10% SDS-polyacrylamide gel electrophoresis.

Determination of the enzymatic activity of *H. influenzae* AHAS

The enzymatic activity of AHAS was determined by a discontinuous colorimetric assay as described previously [15]. Enzyme of 0.75 µg was added to a total of 200 µl mixture of 100 mM potassium phosphate buffer, pH 7.4, 10 mM $MgCl_2$, 1 mM ThDP and 50 µM FAD and a series of pyruvate concentration from 0 mM to 128 mM which was already pre-incubated at 37°C for 10 minutes. The reaction was allowed to take place at 37°C for exactly 1 hour. The reaction was terminated by adding 30 µl of 6N H_2SO_4 and further incubating at 65°C for 15 minutes to convert decarboxylate acetolactate into acetoin. 200µL of 0.5% (w/v) creatine and then 200 µl of 5% (w/v) α-naphthol (in 2.5M NaOH, freshly prepared) were added

into each 200 µl of reaction mixture to produce color of the product acetoin and incubated at 65°C for 15 minutes. The acetoin (red-colored complex, ε_{525nm} = 20,000 M^{-1} cm^{-1}) was measured at 525 nm using a UV-Vis Spectrometer. One unit (U) of activity was defined as the amount of enzyme which produces 1 µmol of acetolactate per minute under the assay conditions described above.

Data analysis

The experimental data were analyzed by the GraphPad Prism program, version 6.0. The Michaelis-Menten equation (equation 1) was fitted to substrate and cofactor saturation curves, where v and S represent the initial velocity and substrate concentration, respectively. The 50% inhibition concentration (IC_{50}) was analyzed by fitting to (equation 2), in which V_0 is the reaction rate without inhibitor, V_f is the rate at maximal inhibition and [I] is an inhibitor concentration. Equation 1, 2 and the equations used to determine the enzyme activity and specific activity (equation 3 and equation 4, respectively) are described below.

$$V = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

$$V = \frac{(V_0 - V_f) \times IC_{50}}{IC_{50} + [I]} + V_f \quad (2)$$

$$Activity = \frac{\Delta OD \times Volume}{\varepsilon \times time} \quad (3)$$

$$Specific\ Activity = \frac{Activity}{Amount\ of\ protein} \quad (4)$$

In which, $\Delta OD = ODx - OD_0$; "OD_x" and "OD_0" are the optical density reading of reaction solution at x mM and 0 mM of substrate concentration. Time = 60 min; ε = 20 µmol/mL; Volume = 0.63 mL; Amount of protein = 0.00075 g.

RESULTS AND DISCUSSION

Protein purification

The recombinant catalytic subunit of *H. influenzae* AHAS was expressed in *E. coli* BL21 (DE3) as a fusion protein with a hexahistidine tag in the C-terminal. As visualized on SDS-PAGE gel, Hin-AHAS protein was highly expressed in the cells (fig. 1, lane L) and that all the Hin-AHAS were bound on the column strongly without being washed out (fig. 1, lanes L, FT, W1 and W2). The molecular weight of the purified fusion protein was approximately 63 kDa (fig. 1, lane E2). Through SDS-PAGE analysis, only one clear band of AHAS was obtained (fig. 1, Lane E2), while most of other non-specific proteins were washed away. Some fainted non-specific bands were presented as a result of overloading protein samples in SDS-PAGE.

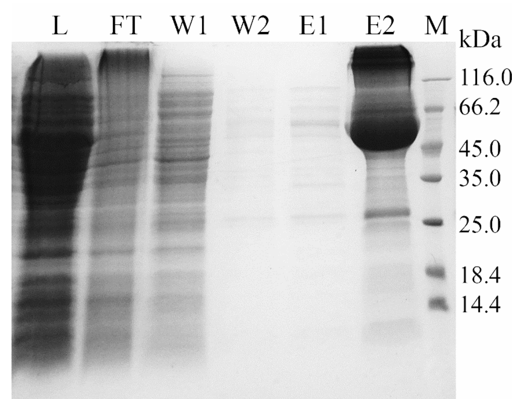

Figure 1. SDS-PAGE analysis of the purification of Hin-AHAS. L: Load; FT: Flow-through; W1: Wash 1; W2: Wash 2; E1: Elution 1; E2: Elution 2 (containing desired protein); M: protein marker.

The enzymatic activity of Hin-AHAS

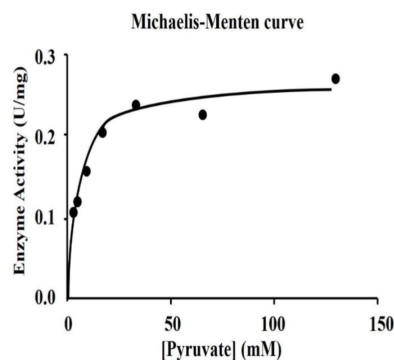

Figure 2. Pyruvate saturation curve of the AHAS enzymatic reaction

Kinetic parameters of Hin-AHAS were measured by fitting the data to equation 1, resulting in the pyruvate saturation curve of the AHAS enzymatic reaction (fig. 2). The V_{max} and K_m of Hin-AHAS were calculated by GraphPad Prism to be 0.236 U/mg protein and 2.503 mM, respectively.

Determination of inhibition kinetics

The inhibition mechanisms of ethoxysulfuron (ETS) and pyrazosulfuron ethyl (PSE) were determined by a discontinuous colorimetric assay with fix concentration of inhibitors (100 µmol) under different concentrations of the pyruvate substrate ranging from 0 to 128 mM. The Lineweaver-Burk plot of Hin-AHAS in the absence and presence of 100 µmol of ETS and PSE is shown on fig. 3, suggesting the inhibition mechanism to be non-competitive.

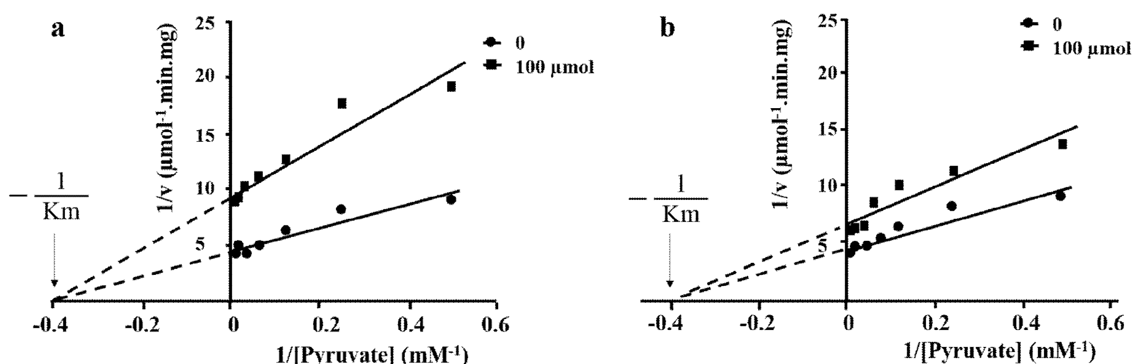

Figure 3. Kinetics of Hin-AHAS inhibition. **a**: Extended Lineweaver-Burk plot of Hin-AHAS in the absence and presence of 100 µmol of ETS and **b**: Extended Lineweaver-Burk plot of Hi-AHAS in the absence and presence of 100 µmol of PSE.

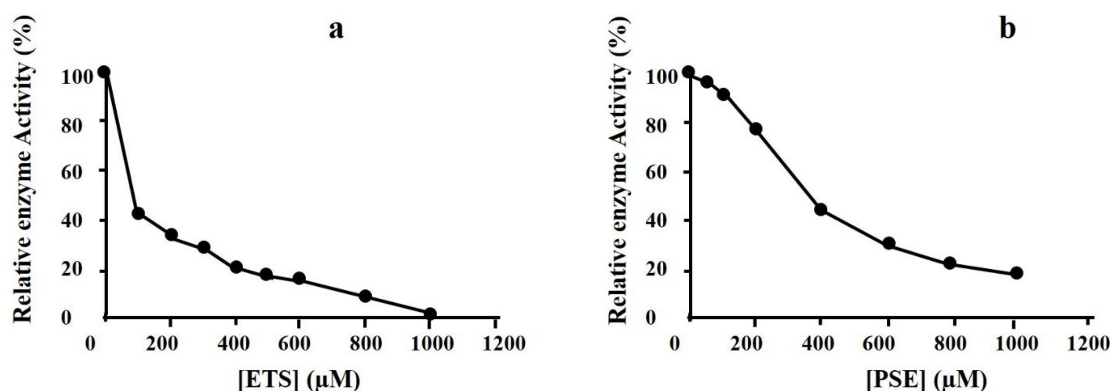

Figure 4. The relative activity of Hin-AHAS as a function of the concentration of (**a**) ETS and (**b**) PSE

In the presence of 100 µmol of ETS, K_m remained the same; however, ETS reduced specific activity from 0.1176 to 0.0518 U/mg protein. Regarding inhibition of PSE, it also experienced similar results. In particular, although K_m was not altered by the addition of 100 µmol of PSE, there was a significant decline in specific activity, approximately from 0.1722 to 0.0714 U/mg protein. Thus, both ETS and PSE are non-competitive inhibitors of Hin-AHAS. In non-competitive inhibition, the binding of the inhibitor to the enzyme decreases the rate of the reaction to form the enzyme-

product complex but does not have an effect on the binding of substrate.

To determine the apparent inhibition constants of the 2 inhibitors, activities of the enzyme in the presence of various concentrations of the inhibitors were measured and fitted into equation 2. As shown in fig. 4, the IC50 values of ETS and PSE were found to be 90.14 and 376.6 μM, respectively.

ETS was shown to be a 4-fold more potent inhibitor than PSE. Moreover, in the presence of 1000 μM of ETS, activity of Hin-AHAS was completely inhibited (fig. 4A), however, the same concentration of PSE can only inhibit up to 80% of the enzyme activity.

The Hin-AHAS was highly soluble and the affinity-purified protein has similar molecular weight as that reported previously [4, 9]. Via gel filtration, the catalytic subunit of *E. coli* AHAS has been observed as a monomer while previous studies revealed that the *E. coli* AHAS II catalytic subunit exists predominantly as a dimer [5].

The specific activity of Hin-AHAS (0.236 U/mg) is somewhat similar to those of other purified catalytic subunits from other bacteria: 0.12 U/mg for *E. coli* AHAS I [11], 0.37 U/mg for *E. coli* AHAS III [10], and 0.117 U/mg for *Shigella sonnei* [9] but lower than *Mycobacterium tuberculosis* (2.8 U/mg) [2]. Regarding the K_m values for pyruvate, it can be seen that K_m values are varied between different microorganisms (table 1). Among species, while *E. coli* AHAS I had very high K_m, indicating the weak affinity to bind to the substrate, K_m values of *E. coli* AHAS II or *M. tuberculosis* AHAS were quite low, indicating the strong affinity to bind to the substrate. Thus, by comparing the kinetic parameters for the catalytic subunit of AHAS of various bacteria, the purified Hin-AHAS obtained in this study can be considered as having good catalytic efficiency and good affinity to pyruvate.

Table 1. Comparison of kinetic parameters for the catalytic subunit of Hin-AHAS and other bacterial AHASs

Bacteria	K_m (pyruvate) (mM)	Reference
E. coli I	25	[14]
E. coli II	5.0±0.5	[14]
E. coli III	86±14	[13]
M. tuberculosis	2.76±0.12	[13]
S. sonnei	8.01	[9]

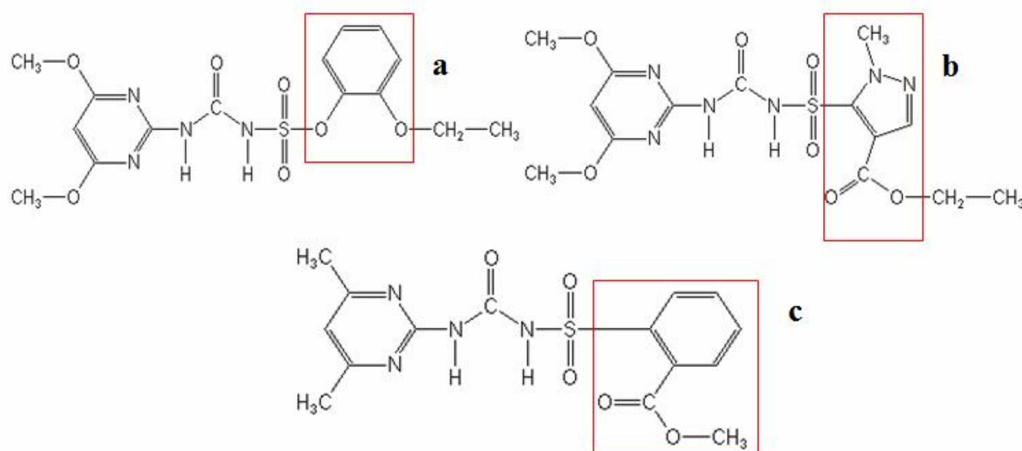

Figure 5. Chemical structure of a: ETS;
b: PSE and c: Sulfometuron methyl (SMM)

Sulfonylurea, imidazolinone and triazolopyrimidine derivatives are three main types AHAS inhibitors [7]. In this study, ETS and PSE belong to sulfonylureas family. Both inhibitors were identified as non-competitive inhibitors for the catalytic subunit of Hin-AHAS similar to the inhibition of *Arabidopsis* and barley AHASs by chlorsulfuron and *E.coli* AHAS I, II, III by sulfometuron methyl and chlorsulfuron [3].

In terms of half maximal inhibitory concentrations (IC_{50}), both ETS and PSE showed medium to weak inhibition capacity for the catalytic subunit of Hin-AHAS [15]. ETS was approximately 4-time more potent than PSE probably due to a minor difference in the chemical structure as highlighted in the red boxes (fig. 5). SMM, a sulfonylurea (fig. 5c), was also considered as a weak inhibitor with an IC_{50} value 276.31 μM, a 3-fold less potent and 1.4-fold more potent in comparing to ETS and PSE, respectively [4].

CONCLUSION

In this study, AHAS catalytic subunit was characterized for its kinetic parameters. Two inhibitors belonging to sulfonylurea family were evaluated for their inhibition capacity. This result can be applied to demonstrate promising structural template for the development of novel AHAS inhibitors against *H. influenzae* strains. AHAS is still attractive target to identify potent inhibitors to defense against various infectious diseases, especially when antimicrobial drug resistances have been increasing considerably. Assessing and analyzing chemical structure of various kinds of potent inhibitors also give raise to useful information to develop not only novel and effective herbicides but also antimicrobial agents.

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TINH SẠCH VÀ NGHIÊN CỨU TÍNH CHẤT CỦA ENZYME TÁI TỔ HỢP ACETOHYDROXYACID SYNTHASE TỪ *Heamophillus influazae*

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TÓM TẮT

Acetohydroxyacid synthase (AHAS) là enzyme chỉ xuất hiện ở thực vật và vi khuẩn. Enzyme xúc tác cho phản ứng đầu tiên của quá trình sinh tổng hợp amino acid có mạch nhánh (BCAAs), bao gồm isoleucine, leucine và valine. AHAS cũng là mục tiêu tiềm năng để kiểm soát vi khuẩn *Haemophilus influenzae*. Trong nghiên cứu này, tiểu đơn vị xúc tác của enzyme tái tổ hợp AHAS từ *H. influenza* (Hin-AHAS) được biểu hiện trong vi khuẩn in *Escherichia coli*. Protein Hin-AHAS tinh sạch có trọng lượng 63 kDa được thể hiện trên gel SDS-PAGE. Giá trị V_{max} và K_m tương ứng của enzyme Hin-AHAS được xác định là 0,236 U/mg protein và 2,503 mM pyruvat. Hai chất kìm hãm của AHAS là ethoxysulfuron (ETS) và pyrazosulfuron ethyl (PSE) được sử dụng trong các thí nghiệm, cho thấy chức năng kìm hãm không cạnh tranh với Hin-AHAS với giá trị IC_{50} tương ứng là 90,14 μM và 376,6 μM. Kết quả nghiên cứu này cho thấy, enzyme tinh sạch có thể được sử dụng để sàng lọc các chất kìm hãm kháng Hin-AHAS.

Từ khóa: Haemophillus influenzae, acetohydroxyacid synthase, hoạt tính enzyme, chất ức chế, tinh sạch.

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