Expression of Acetohydroxyacid Synthase from *Bacillus anthracis* and Its Potent Inhibitors

**Kyoung-Jae Choi,[a] Chien Ngoc Pham, Hoeil Jung, Sung-Hwan Han,
Jung-Do Choi,[†] Jinheung Kim,[‡] and Moon-Young Yoon[*]**

Department of Chemistry, Hanyang University, Seoul 133-791, Korea. [*]E-mail: myyoon@hanyang.ac.kr
[†]School of Life Sciences, Chungbuk National University, Cheongju 361-763, Korea
[‡]Department of Chemistry, Ewha Womans University, Seoul 120-750, Korea
Received April 26, 2007

Acetohydroxyacid synthase (AHAS, EC 2. 2. 1. 6) is the enzyme that catalyses the first step in the common pathway of the biosynthesis of the branched chain amino acids, valine, leucine and isoleucine. For the first time, the AHAS gene from *Bacillus anthracis* was cloned into the expression vector pET28a(+), and was expressed in the *E. coli* strain BL21(DE3). The purified enzyme was checked on 12% SDS-PAGE to be a single band with molecular weight of 65 kDa. The optimum pH and temperature for *B. anthracis* AHAS was at pH 7.5 and 37 $^{\circ}$C, respectively. Kinetic parameters of *B. anthracis* were as follows: K_m for pyruvate, $K_{0.5}$ for ThDP and Mg^{2+} was 4.8, 0.28 and 1.16 mM respectively. AHAS from *B. anthracis* showed strong resistance to three classes of herbicides, Londax (a sulfonylurea), Cadre (an imidazolinone), and TP (a triazolopyrimidine). These results indicated that these herbicides could be used in the search for new anti-bacterial drugs.

Key Words : Acetohydroxyacid synthase, Herbicides, Resistance, Kinetic parameters

Introduction

AHAS catalyses the condensation of two molecules of pyruvate to form acetolactate in the biosynthesis of valine and leucine, or the condensation of pyruvate and 2-keto-butyrate to form 2-aceto-2-hydroxybutyrate in the bio-synthesis of isoleucine (Figure 1).[1] Bacterial AHASs are composed of large (60 kDa) catalytic and small (9 to 18 kDa) regulatory subunits. Isolated catalytic subunits have lower than the holoenzymes are similar to them in their cofactor dependence and specificity towards the different substrate. The sensitivity of AHAS to feedback inhibition is completely dependent on the small subunit.[2]

AHAS is present in bacteria, fungi, algae and plants. Biochemical studies have shown that AHAS requires three cofactors: flavin adenine dinucleotide (FAD) as non-cata-lytic cofactor, ThDP (Thiamine diphosphate) and a bivalent metal ion such as Mg^{2+} or Mn^{2+}, for its activity.[3,4] The reaction, catalyzed by ThDP-dependent enzyme has involv-ed in the cleavage of a carbon-carbon bond, which is adjacent to a keto group.[5] The function of FAD is unclear. Two hypotheses were proposed for requirement of FAD for AHAS, the first suggested that FAD is required for purely structure purpose, that is, unless FAD is present the active site can not attain correct geometry for substrate binding and/or catalysis to occur.[6] The second hypothesis assumed that FAD plays a protective role in the catalytic cycle.[7]

Bacillus anthracis, a gram-positive, rod shaped bacterium which is etiological agent of anthrax, is transmitted by its highly resistant spores. Inhalation anthrax is a severe di-sease, with a fatality rate > 40%, despite antibiotic therapy.[8] With the emergence of *B. anthracis* spores as a weapon of terror, the development of new therapies is essential.[9] It is extremely necessary to create a tool that could be used in the search of new anti-bacterial drugs. To this end, in the present study we have purified the *B. anthracis* AHAS catalytic subunit and studied some physical and biochemical properties of this enzyme.

Materials and Methods

Materials. Brain Heart Infusion (BHI), Bacto-trypton, yeast and bacto-agar were purchased from Becton Dickinson and Company, Sparks (USA), Restriction enzyme, *Pfu* DNA polymerase and T4 DNA ligase were obtained from Pro-mega. Sodium pyruvate, FAD, ThDP, $MgCl_2$, IPTG (iso-propyl-β-D-thiogalactopyranoside), creatine, β-ME (β-mercaptoethanol) and α-naphtol were obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals were obtained from commercial sources and were of the highest quality available.

Gene cloning. *B. anthracis* sterne strain was transferred into BHI medium (Brain Heart Infusion medium, difco) at 30 $^{\circ}$C. Total genomic DNA of *B. anthracis* was isolated and the DNA fragment encoding the open reading frames of the catalytic subunit of AHAS (TIGR locus: NT05BA1436) from *B. anthracis* was amplified by polymerase chain reaction (PCR) using total genomic DNA as a template. In experiment, the PCR was carried out with the specific primer which has sequence: 5'-<u>GGA TCC</u> ATG TCT TCA AAA ACG GAA GAG A-3' and 3' <u>CTC GAG</u> TCA CCT TTT TTC CAC TCC CTC-5'. Underlined nucleotide sequences represented the linker of *Bam*HI and *Xho*I,
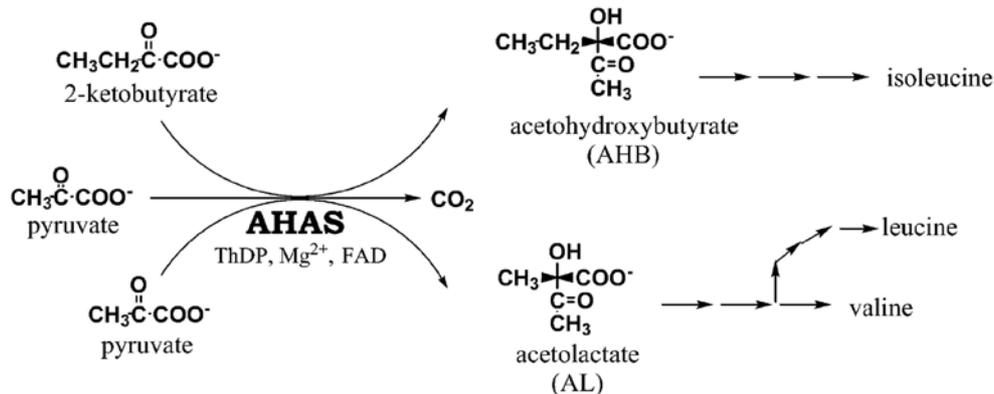
Figure 1. Pathway of branched-chain amino acid biosynthesis

respectively. The conditions during PCR reaction were: 95 °C, 10 min, 25 cycles [95 °C, 2 min; 55 °C, 2 min; 72 °C, 2 min], 72 °C, 10 min. The PCR product was gel purified, digested with *Bam*HI and *Xho*I and cloned into the expression vector pET28a(+) which was digested with the same restriction enzymes. The resulting plasmids were sequenced to verify the correct clones. The clone harboring correct AHAS sequence was used to transform it to *E. coli* BL21 (DE3) and transformants were collected on LB plate containing 100 μg/mL Kanamycin.

Expression of AHAS. The plasmid containing AHAS insert was transformed into an expression host, BL21 (DE3), which has relatively low protease activity. The inoculum was prepared by growing single colony of transformed cells at 37 °C for overnight. This overnight grown culture was transferred into 1 L Luria-Bertani (LB) culture medium containing 100 μg/mL kanamycin and incubated further at shaking condition (200 rpm). When OD_{600} of approximately 0.7-0.8 was reached, the over expression of AHAS was induced by addition of 0.4 mM IPTG and the culture was incubated at 18 °C for overnight. Cell pellets were harvested by centrifugation at 5000 rpm for 10 min at 4 °C. The cell pellets were washed once with PBS and then the intact cell pellets were stored at −80 °C till further use.

Purification of AHAS protein. All the steps were carried at 4 °C unless otherwise stated. The cell pellets were resuspended in 50 mL buffer containing 20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 10 mM imidazole, 1 mM PMSF, 0.5 mg/mL Lysozyme, 0.5% Triton X-100 and incubated at room temperature for 30-40 min and followed by lysis with ultra-sonication. Cell debris was removed by centrifugation at 14000 rpm for 40 min. The clear supernatant was filtrated through syringe filter (0.45 μm) and directly loaded on Ni^{2+}-charged Hi-Trap chelating HP column pre-equilibrated with 20 mM sodium phosphate pH 8.0, 0.5 M NaCl, 10 mM imidazole at a flow rate of 0.5 mL/min. The loaded column was washed with 100 mL of buffer containing 20 mM Sodium phosphate, pH 8.0, 0.5 M NaCl and 40 mM imidazole. The bound AHAS protein was eluted by applying a linear gradient of imidazole (20-500 mM) at a flow rate of 1 mL/min in 60 minutes. The fractions containing expressed protein were dialyzed against 0.1 M Tris-HCl,

pH 8.0 containing 10 mM EDTA and 5 mM β-ME and was concentrated by ultrafiltration (Amicon), then stored at –80 °C until used.

Enzyme assay. The activity and specificity of AHAS were measured by using the method of Singh *et al.*[3] with the following modifications. The assay solution contains final concentration of 100 mM potassium phosphate buffer (pH 7.4), 1 mM ThDP, 10 mM $MgCl_2$, 50 μM FAD and in the presence or absence of various concentrations of cofactors and/or inhibitors. The reaction mixture was pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of 0.8 μg enzyme (total volume of 200 μL). After 1 h incubation, the enzyme reaction was terminated by adding of 30 μL of 8 M-H_2SO_4 and decarboxylation of acetolactate was followed at 65 °C for 15 min. Then 100 μL of reaction product was mixed with 90 μL of 0.5% creatine (w/v) and 90 μL of 5% (w/v) α-napthol solution in 2.5 M NaOH, and incubated at 65 °C for 15 min to measure acetoin formed. The colour developed was then measured by its absorbance at 525 nm. Since acetolactate is chemically unstable, acetoin was used as the standard to determine the absorption coefficient of 20,000 M^{-1} cm^{-1} for the coloured complex produced. One unit of enzyme activity is defined as that producing 1 μmol of acetolactate/min under the above condition. Specific activity is expressed as enzyme units/mg of catalytic subunit as determined by the Bradford protein assay, unless otherwise stated.

Optimum pH and temperature determination. The determination of pH and temperature dependence activity of AHAS was carried out according to the method described in enzyme assay section. For characterization, the purified recombinant enzyme was used (see above). Partially purified *B. anthracis* AHAS was assayed over a range of pH values using three kinds of different buffer systems: MES, pH 5.0-6.0; HEPES, pH 6.0-8.0; CHES, pH 8.0-10.0. All buffers were titrated to the appropriate pH with NaOH. The pH was maintained using the buffers at 100 mM concentration and optimum pH determination was repeated several times. In order to determine the optimum temperature, the activity of the enzyme was measured in the range of 10-50 °C.

Protein concentration. Protein concentrations were determined by the Bradford method with modifications to

the protocol supplied by manufacture. BioRad reagent was diluted 1:4 in water, 995 μL of diluted dye reagent was added into tube, 5 μL protein solutions was then added into tube. The blue colour developed was measured at wavelength of 595 nm.

SDS-PAGE. SDS-PAGE was performed using the method of Leaemmli. Protein were separated on a 12% polyacrylamide gel using a Bio-Rad model 1000/500 power supply and detected by staining with 0.1% coomassie blue R-250.

Determination of kinetic parameters. All experimental data was processed using programs based on Origin Pro 6.1. The values of V_{max} and K_m for the substrate, cofactors were determined by fitting the date into equation (1)

$$v = (V_{max}S)/(K_m + S) \tag{1}$$

In this equation, v is the reaction velocity, V_{max} is the maximum velocity, [S] is substrate concentration, K_m is Michaelis Menten constant.

Results

Expression and purification. The PCR amplification of the AHAS gene from genomic DNA of *B. anthracis* was resulted in a fragment of expected size, 1.7 kb corresponding to the AHAS sequence. This fragment was cloned into pET28a(+) vector, and *E. coli* BL21 (DE3) cells were transformed with the recombinant plasmid as described in experimental procedures. The resulting construct contains additional 34 (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMG-RGS-) residues at the N-terminus of AHAS. This was resulted in an AHAS protein of 65 kDa instead of 62 kDa for the native protein (data not shown).

For purification of the soluble fraction containing a decent amount of AHAS, the recombinant protein was purified by using Ni^{2+}-charged Hi Trap chelating HP column as described in experimental procedures. As expected, most of the AHAS was bound to the resin, and a wash with 40 mM imidazole removed non-specifically bound proteins. Finally purified AHAS protein was eluted with 500 mM imidazole. The purity of the protein was estimated to be 96% homogeneity by SDS-PAGE, and protein content was determined using Bradford (data not shown).

Optimum temperature and pH of AHAS. In order to determine the optimum pH and temperature of AHAS, partially purified *B. anthracis* AHAS was assayed over a range of pH values using three different buffers. As shown in Figure 2A, the optimum pH for the activity of *B. anthracis* AHAS was 7.5. The activity of AHAS was determined in the range of 10-50 °C (Figure 2B) and the results showed that the optimum temperature of AHAS was 37 °C.

Biochemical characterization of the *B. anthracis* AHAS. Enzymatic parameters for the interaction of the enzyme with its substrate, cofactors were determined using a discontinuous colorimetric assay as described in materials and methods. The properties of *B. anthracis* AHAS were as follows: K_m for pyruvate, ThDP and Mg^{2+} were, respectively, 4.8, 0.28 and 1.16 mM, the saturation curves for substrate as well as
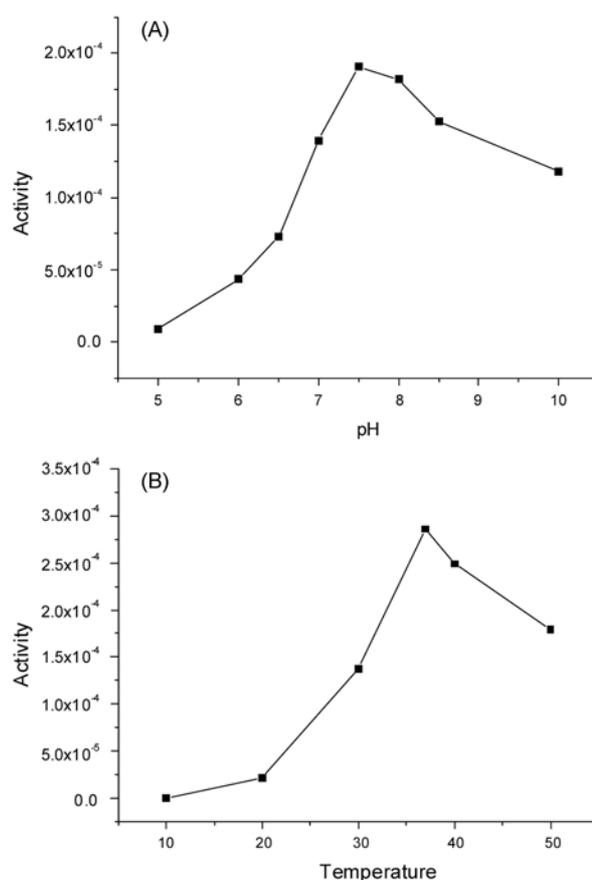

Figure 2. Optimum pH (A) and temperature (B) for *Bacillus anthracis* AHAS. AHAS was assayed as in materials and methods except that pH of the assay was varied using three kinds of different buffer system and temperature was performed in a range of values

cofactors were hyperbolic (Figure 3), isoelectric point was at 6.03. The specific activity of the catalytic subunit was found to be about 1.5 U/mg. Compared to the specific activities of AHASs of *M. tuberculosis* (2.8 U/mg)[10] and *A. thaliana* (7.88 U/mg),[11] the value of 1.5 U/mg protein reported here is not high. The values of the various kinetic parameters from other sources are summarized in the Table 1.

Inhibition of AHAS by herbicides. The sensitivity of the enzyme to herbicides was determined for three class of herbicides, Londax (a sulfonylurea), Cadre (an imidazolinone), and TP (a triazolopyrimidine). The K^{app}_i were determined by fitting the data to eq. (2)

$$v_i = v_o/(1 + [I]/K^{app}_i) \tag{2}$$

In this equation, v_i and v_o represent the rates in the presence or absence of the inhibitor, respectively, and [I] is the concentration of the inhibitor. The K^{app}_i is apparent K_i, that is the concentration of the inhibitor giving 50% inhibition under a standard assay condition and which is also known as IC_{50}. Three different classes of herbicides, Londax, Cadre, and TP are potent inhibitors of *B. anthracis* AHAS with K^{app}_i values of 22.4, 12.3, and 146.5 nM, respectively (Figure 4).

1112   *Bull. Korean Chem. Soc.* **2007**, Vol. 28, No. 7
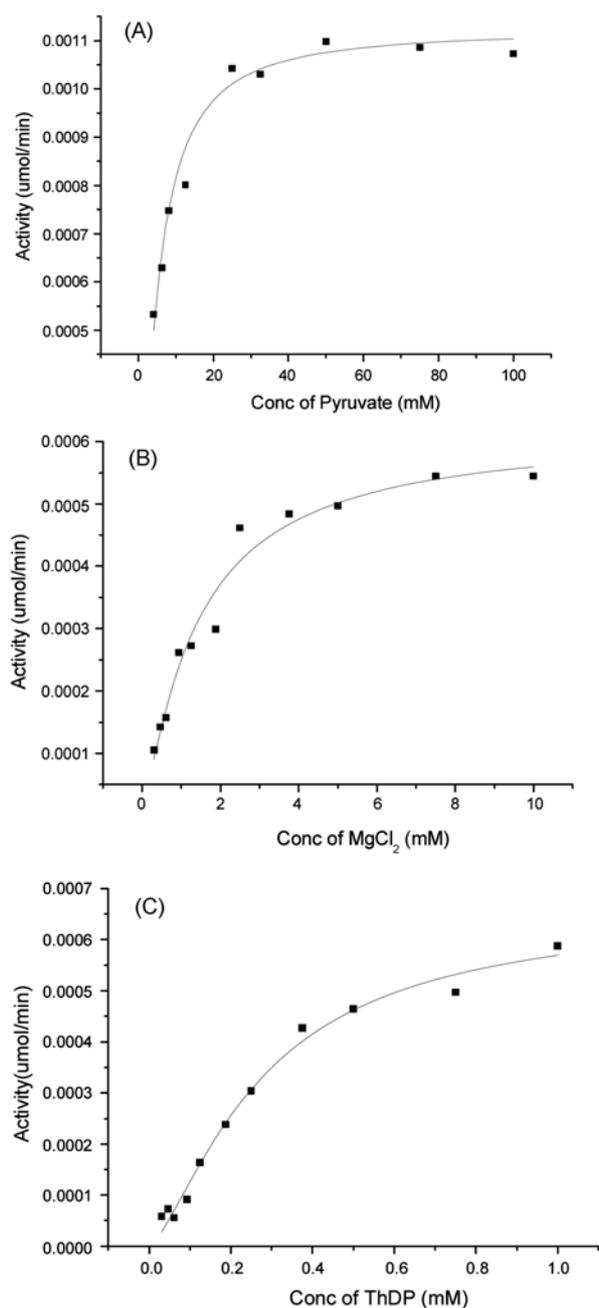
K.-J. Choi et al.

Figure 3. The saturation curve of pyruvate (A), Mg^{2+} (B) and ThDP (C) for catalytic subunit of *Bacillus anthracis*. Experiments were carried out in 100 mM potassium phosphate buffer, pH 7.5. The curves shown represent the fit of the date to the Hill equation.
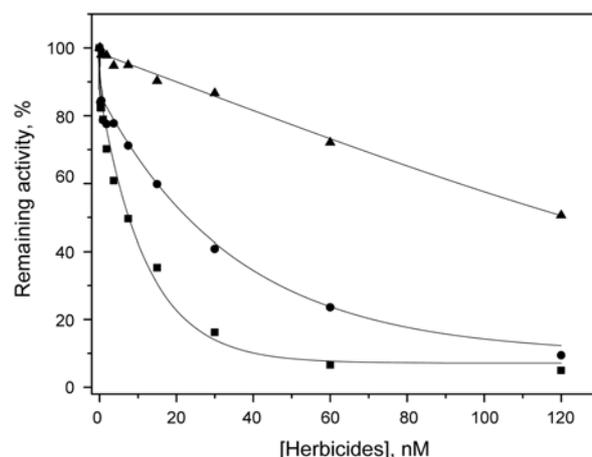

Figure 4. Inhibition of the purified AHAS by three classes of herbicides. Londax (a sulfonylurea) (●), the Cadre (an imidazolinone) (■) and the TP (a triazolopyrimidine) (▲). The activity was measured as described in Materials and methods.

Discussion

In this report, we described the isolation of *B. anthracis* AHAS gene, its heterogeneous expression, purification and characterization. The purification of AHAS for biochemical characterization required the subcloning of its gene into the expression vector pET28a(+). The AHAS from *B. anthracis* is less active than isolated from other microorganisms (Table 1); it is not clear whether this has any physiological significance. The inferred molecular mass (65 kDa) of the polypeptide encoded by *ilvB* gene was confirmed (data not shown).

Table 1 shows the biochemical properties of *B. anthracis* AHAS with that of other purified AHASs. The specific activity of this enzyme is (1.5 U/mg) similar to that of AHAS from the bacteria *M. tuberculosis* (2.8 U/mg). However, its activity is several folds lower than that of AHAS from *N. tabacum* (2.8-8.1 U/mg)[12] and *A. thaliana* (7.88 U/mg).[11] It has been suggested that the value of 1.5 U/mg protein reported here is not high.

In common, with related enzymes that catalyze the decarboxylation of pyruvate, the AHAS enzyme requires ThDP, FAD and a divalent metal ion Mg^{2+} as obligatory cofactors for its catalytic activity. Kinetic parameters for the affinities of the enzyme with its substrate and cofactors were determined and compared with those of other purified AHASs (Table 1). The K_m for pyruvate is similar to *E. coli*

Table 1. Comparison of enzymatic properties of *B. anthracis* AHAS to those of a varieties of AHASs

AHAS	Specific activity (U/mg)	K_m for pyruvate (mM)	$K_{0.5}$ for ThDP (μM)	$K_{0.5}$ for Mg^{2+} (μM)	$K_{0.5}$ for FAD (μM)
B. anthracis [a]	1.5	4.8 ± 0.45	280 ± 40	1160 ± 130	ND
M. tuberculosis [b]	2.8	2.76 ± 0.12	51.23 ± 2.3	270 ± 20.8	ND
N. tabacum [c]	2.8 – 8.1	8.1 – 12.8	80 – 120	ND	1.4 – 2.6
A. thaliana [d]	7.88	8.01 ± 0.66	25.3 ± 1.4	198 ± 19	1.46 ± 0.22

[a]Except where indicate, the enzyme assay was carried out under standard conditions as described in material and method, ND = not determined. [b]Data taken from.[10] [c]Data taken from.[11] [d]Data taken from.[12]

isozyme II AHAS (5.0 mM). The AHAS from other micro-organisms, on the other hand, showed a significant increased in affinity for ThDP with $K_{0.5}$ 2-11 fold and for Mg^{2+} with $K_{0.5}$ 4-6 fold higher than the *B. anthracis* AHAS.

It is important to determine the stability of an enzyme over the temperature and the pH range studied. The temperature dependence of the acetohydroxyacid synthase activity was bell shaped, in the range of 10-50 °C. The enzyme has low activity at low and high temperature, respectively (Figure 2B), which implied that temperature can effect the enzymatic activity in the reaction. The optimum temperature (37 °C) and pH (7.5) results for *B. anthracis* AHAS are consistent with *Escherichia coli* I AHAS (pH 7.5)[13] and permeabilized yeast cell (pH 7-8).[14]

Lately AHAS has been intensively studies,[15-17] since AHAS has been known a potential target for various classes of herbicides. Therefore, the inhibition of *B. anthracis* AHAS by the three classes of herbicides, Londax (a sulfonylurea), Cadre (an imidazolinone), and TP (a triazopyrimidine) was determined. The *B. anthracis* AHAS exhibited a large effect on the sensitivities to the three herbicides. Londax and Cadre showed strong resistant to *B. anthracis* AHAS, however TP relatively showed weak resistance.

In conclusion, we have introduced successfully AHAS gene by extension PCR, cloned into the expression vector, the base changes confirmed by DNA sequencing (data not shown) and the *B. anthracis* AHAS protein was expressed and purified. This is the first report on biochemical characterization of the *B. anthracis* AHAS protein as optimum temperature and pH respectively, enzymatic parameters for interaction of the enzyme with its substrate and cofactors. Also we have tested the possible use of herbicides as portent inhibitors of *B. anthracis* AHAS. Further research on identification of compounds from the chemical library to develop potent inhibitor of purified *B. anthracis* AHAS protein as anti-bacterial drug is underway.

References

1. Umbarger, H. E. In *Synthesis of Amino Acid and Protein*; Arbstein, H. R. V., Ed.; MTP International Review of Science: Butterworths, London, 1975; pp 1-56.
2. Duggleby, R. G.; Pang, S. S. *J. Biochem. Mol. Biol.* **2000**, *33*, 1-36.
3. Singh, B. K.; Stidham, M. A.; Shaner, D. L. *Anal. Biochem.* **1988**, *171*, 173-179.
4. Davies, M. E. *Plant Physiol.* **1963**, *39*, 53-59.
5. Khaleeli, N.; Li, R.; Townsend, C. A. *J. Am. Chem. Soc.* **1999**, *121*, 9223-9224.
6. Schloss, J. V.; Ciskanik, L. M.; Van Dyk, D. E. *Nature* **1988**, *331*, 360-362.
7. Schloss, J. V.; Aulabaugh, A.; Barak, Z.; Chipman, D. M.; Schloss, J. V., Eds.; *Biosynthesis of Branched Chain Amino Acids*; VCH press: Weinheim, Germany, 1988; pp 329-359.
8. Spencer, R. C. *Bacillus Anthracis*, *Int. Clin. Pathol.* **2003**, *56*, 182-187.
9. Jerigan, J. A.; Stephens, D. S.; Ashford, D. A. *Emerg. Infect. Dis.* **2001**, *7*, 933-944.
10. Choi, K. J.; Yu, Y. G.; Hahn, H. G.; Choi, J. D.; Yoon, M. Y. *FEBS Letter.* **2005**, *579*, 4903-4910.
11. Kil, M.; Chang, S. I. *J. Biochem. Mol. Biol.* **1998**, *31*, 287-293.
12. Poulsen, C.; Stougaard, P. *Eur. J. Biochem.* **1989**, *185*, 433-439.
13. Chang, A. K.; Duggleby, R. G. *Biochem. J.* **1997**, *327*, 161-169.
14. DeFelice, M.; Squires, C.; Levinthal, A. *Biochem. Biophys. Acta* **1978**, *241*, 9-17.
15. Karim, M.; Shim, M. Y.; Kim, J.; Choi, K. J.; Kim, J.; Choi, J. D.; Yoon, M. Y. *Bull. Kor. Chem. Soc.* **2006**, *27*, 549-555.
16. Choi, K. J.; Noh, K. M.; Choi, J. D.; Park, J.; Won, H. S.; Kim, J. R.; Yoon, M. Y. *Bull. Kor. Chem. Soc.* **2006**, *27*, 1697-1700.
17. Chang, A. K.; Duggleby, R. G. *Biochem. J.* **1997**, *327*, 161-169.