Cloning and Characterization of GST Fusion Tag Stabilized Large Subunit of *E. coli* Acetohydroxyacid Synthase I

Heng Li,^a Nan Liu,^a Wen-Ting Wang, Ji-Yu Wang, Wen-Yun Gao^{*,1}

College of Life Sciences, Northwest University, 229 North Taibai Road, Xi'an, Shaanxi 710069, P. R. China.

Running title: Stabilizing effect of the GST tag

¹ Dr. Wen-Yun Gao, Phone: +86-29-88303446 ext. 832; Fax: +86-29-88303572; E-mail: gaowenyun@nwu.edu.cn

a. Li and Liu contributed equally.

There are three acetohydroxyacid synthase (AHAS, EC 4.1.3.18) isozymes (I, II, and III) in the enterobacteria *E. coli* among which AHAS I is the most active. Its large subunit (LSU) possesses full catalytic machinery, but is unstable in the absence of the small subunit (SSU). To get applicable LSU of AHAS I, we prepared and characterized in this study the polypeptide as a His-tagged (His-LSU) and a GST (glutathione S-transferase)-tagged (GST-LSU) fusion protein, respectively. The results showed that the His-LSU is unstable, whereas the GST-LSU displays excellent stability. This phenomenon suggests that the GST polypeptide fusion tag could stabilize the target protein when compared with histidine tag. It is the first time that the stabilizing effect of the GST tag was observed. Further characterization of the GST-LSU protein indicated that it possesses the basic functions of AHAS I with a specific activity of $20.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and a K_m value for pyruvate of 0.95 mM. These observations imply that introduction of the GST fusion tag to LSU of AHAS I does not affect the function of the protein. The possible reasons that the GST fusion tag could make the LSU stable are initially discussed.

[**Keywords:** Acetohydroxyacid synthase I; *E. coli*; Large subunit; Characterization; Stability; GST fusion tag]

Acetohydroxyacid synthase (AHAS, EC 4.1.3.18), a thiamin diphosphate (ThDP) dependent enzyme that is also known as acetolactate synthase (ALS), is the first common enzyme in the biosynthetic pathway of the branched chain amino acids (1-3). This pathway is present in microorganism and in plants but is absent in animals, making it an appealing target

for the screening of new compounds to be developed as herbicides or antimicrobial drugs (1,4). Two different classes of commercial herbicides, the imidazolinones and the sulfonylureas target AHAS and they have been used for crop protection for more than 30 years thereby providing an incalculable benefit to society (5,6). The function of AHAS is to catalyze the enantiospecific condensation of two molecules of pyruvate to form (S)-2-acetolactate (the precursor of valine and leucine) or one molecule of pyruvate with a molecule of 2-ketobutyrate to generate (S)-2-aceto-2-hydroxybutyrate (the precursor of isoleucine) (Figure 1). Three cofactors are essential for the catalysis of AHAS: ThDP, divalent metal ion such as Mg^{2+} and Mn^{2+} , and flavin adenine dinucleotide (FAD). Furthermore, AHAS is also an effective catalyst of the acyloin-like reaction of pyruvate with benzaldehyde to generate the chiral-hydroxyketone (R)-phenylacetylcarbinol (R-PAC) (Figure 1) (7,8) which is the key precursor in the production of several drugs with adrenergic properties, such as L-ephedrine, pseudoephedrine, and norephedrine (9,10).

AHASs from microorganisms comprise two large (60-kDa) catalytic subunits and two small (9- to 18-kDa) regulatory subunits in equimolar amounts (1). For both bacterial and fungal enzymes, the LSU contains the complete catalytic machinery to perform the chemical reactions while the small one is necessary for stabilization of the LSU and also for valine sensitivity (11,12). It has been demonstrated that the AHAS extracted from *Neurospora crassa* is extremely labile (13-16). Similar phenomena were observed with yeast AHAS (17).

AHAS from the enterobacteria *E. coli* is the most thoroughly investigated of all the AHASs from different organisms. Three isozymes, namely AHAS I, II and III have been characterized in this enteric bacterium and up to date, the individual subunits of each isozyme

have been expressed and purified to homogeneity (18-22). It is noteworthy that AHAS I is not only the most active one of the three isozymes, but also one of the most potent enzymes catalyzing the formation of acetolactate amongst all the known AHASs of bacterial origin (1,21). The *E. coli* isozymes that, to date, have been expressed and purified suffer from the fact that they are relatively unstable (22-25). Despite the fact that the LSU of AHAS possesses full catalytic machinery, its stability, turnover numbers, or both are drastically decreased in the absence of the SSU (21,22). Thus, in order to get active AHAS, both of its subunits have to be prepared and subsequently reconstituted (18,19,21). In particular for yeast AHAS, reconstitution is challenging as the storage buffer needs to be of high ionic strength (about 1 M potassium phosphate) to maintain the activity of the complex (26).

In this paper, the *ilvB* gene of *E. coli* encoding the LSU of AHAS I was cloned respectively into the expression vectors pPROEXTM HTb (with histidine-tag) and pGEX-4T-1 (with GST-tag) and was expressed in *E. coli* BL21(DE3) strain. Two fusion proteins, namely the LSU of AHAS I with histidine-tag (His-LSU) and with GST-tag (GST-LSU) were obtained and characterized, respectively. The results showed that both of the proteins could mediate the condensation of pyruvate to form acetolactate, but the specific activity of the His-LSU was low and decreased very quickly, whereas the GST-LSU not only had specific activity comparable to ASAH I, but was also stable. Here we present the experimental details and discuss the possible reasons that the GST-LSU protein is more stable than the His-LSU protein.

Materials and methods

Reagents Ampicillin, ThDP, dithiothreitol (DTT), FAD, sodium pyruvate, amino

acids, HPLC-grade methanol were purchased from Sigma-Aldrich (Shanghai, China). DNA restriction enzymes, T4 ligase, Taq RNA polymerase, Gel DNA Extraction Kit, Plasmid DNA Preparation Kit and other molecular biology reagents were obtained from Takara Biotech. Co. (Dalian, China). The pPROEXTM HTb vector was from Life Technologies Inc. (Gaithersburg, MD, USA). pGEX-4T-1 vector was purchased from Amersham Pharmacia Biotech (USA). Bradford Protein Assay Kit was purchased from Bio-Rad Laboratories (Hercules, USA). Anti GST-tag rabbit monoclonal antibody and alkaline phosphatase conjugated goat anti-rabbit IgG were from Bio Basic Inc. (Amherst, New York). All other chemicals were of analytical grade unless stated otherwise.

Preparation of recombinant His-LSU protein. The *E. coli ilvB* gene encoding the LSU of AHAS I was amplified by PCR from the genomic DNA of *E. coli* DH5 α strain using the primers 5'-GCAGGATCCATGGCAAGTTCGGGCACA-3' and 5'-GATCTCGAGTTATTCCCCCACCATTTC-3'. The amplified gene was inserted into the pPROEXTM HTb vector *via* BamHI and XhoI restriction sites introduced by the primers (restriction sites were underlined) to yield the recombinant plasmid pPROEXTM HTb-ilvB, in which the encoded protein was endowed with an amino-terminal His₆-tag (Fig. S5). The recombinant plasmid pPROEXTM HTb-ilvB was transformed into *E. coli* BL21(DE3) strain for protein production. Protein expression was induced in LB medium containing 100 μ g/mL ampicillin by adding 1.0 mM IPTG at mid-log phase at 30 °C for 5 h after the A₆₀₀ of the bacterial culture had reached 0.5-0.6. Bacteria from a 1.0-liter culture were harvested by centrifugation at 8,000 rpm for 15 min. The precipitate (about 1.5g) was resuspended in 6.0 mL lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 5 mM

β -mercaptoethanol, and 10 mg/mL lysozyme, pH 8.0) and disintegrated by sonication. After centrifugation of the cell lysate at 11,000 rpm for 30 min (4°C), the supernatant was filtered through a 0.45 μ m membrane and the filtrate was loaded onto a Nickel-Sepharose column. The column was first washed with a wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0), then with an elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0). The protein was analyzed by 12% SDS-PAGE and stained with Coomassie Blue R. Overnight dialysis of the purified protein was carried out at 4 °C against 50 mM phosphate buffer (pH 7.5) containing 2 mM DTT and 20% glycerol. The His-LSU protein produced was aliquoted into 0.5 mL Eppendorf tubes (50 μ L each), flash-frozen, and stored at -80°C. Protein concentrations were determined by the Bradford method using BSA as a standard.

Preparation of recombinant GST-LSU protein and Western-blot clarification. The *E. coli ilvB* gene amplified above was cloned into pGEX-4T-1 vector to create a recombinant plasmid pGEX-4T-1-*ilvB* (Fig. S1). Upon expression, the GST sequence is at the N-terminus of the fusion protein (Fig. S5). The protein production was accomplished by using the similar method described for the preparation of His-LSU. The differences were that the concentration of isopropylthiogalactopyranoside (IPTG) was 0.5 mM and the time for induction was 6 h. The bacterial pellet (3-4 g per liter culture) was resuspended in 6-8 mL lysis buffer (50 mM KH₂PO₄, 300 mM NaCl, 5 mM KCl, 5 mM BME, 1 mM phenylmethanesulfonyl fluoride, and 5 mg/mL lysozyme, pH 7.5) and centrifuged at 11,000 rpm for 30 min (4 °C). The supernatant was incubated under gentle shaking with 2.5 mL Glutathione Sepharose 4B at room temperature for 0.5 h before it was slowly poured out. The residue resin was washed twice

with a wash buffer (50 mM KH_2PO_4 , 300 mM NaCl, 5 mM KCl, 5 mM BME, pH 7.5, 3 mL each time) and then eluted with 3 mL elution buffer (50mM Tris-HCl containing 15mM reduced glutathione, pH 8.0). Overnight dialysis of the purified protein was carried out at 4 °C against dialysis buffer (50 mM KH_2PO_4 , 300 mM NaCl, 5 mM KCl, 5 mM BME, pH 7.5). The GST-LSU thus obtained was analyzed by SDS-PAGE and aliquoted into 0.5 mL Eppendorf tubes, flash-frozen, and stored at -80°C. To further verify the resulting protein, Western blot was tested with anti-GST rabbit monoclonal antibody (diluted 1:1000 with 5% skimmed milk powder) and alkaline phosphatase conjugated goat anti-rabbit IgG (diluted 1:10 000 with 5% skimmed milk powder). To detect the activity of alkaline phosphatase, a solution of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt was used as a substrate.

Activity of His-LSU and GST-LSU. The activity of His-LSU and GST-LSU was determined using the colorimetric assay described previously by Singh et al. (27). The standard assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 100 mM sodium pyruvate, 10 mM MgCl_2 , 1 mM thiamine pyrophosphate, and 10 μM FAD. The enzyme and the assay mixture were respectively pre-incubated at 30 °C for 5 min before the enzyme (end conc. 0.25 $\mu\text{g}/\mu\text{L}$) was added to initialize the reaction (total volume: 200 μL). The reaction mixture was maintained at 30°C for 1 h and was then quenched by addition of 10 μL 4M H_2SO_4 . Subsequent incubation at 60°C for 15 min decarboxylates acetolactate into acetoin. The reaction mixture was treated with 100 μL creatine (1.25 mg/mL) and 100 μL 1-naphthol (12.5 mg/ml in 4 M NaOH, freshly prepared) at 60°C for 15 min and then kept at room temperature for another 15 min. The UV absorption of the color complex was measured

at 525 nm after 100-fold dilution with ddH₂O. Each determination was run at least in duplicate and the experiments were repeated three to five times.

Stability of His-LSU and GST-LSU. Purified His-LSU protein was stored at -80°C in a buffer (50 mM phosphate buffer, pH 7.5 containing 2 mM DTT and 20% glycerol or 100 mM Tris-HCl buffer, pH 7.5 containing 2 mM DTT and 20% glycerol) or in the same buffer containing extra protective agents (10 mM pyruvate, 10 mM ThPP, 1 mM FAD, 10 mM Mg²⁺). Purified GST-LSU protein was stored at -80°C in a buffer (50 mM KH₂PO₄, 300 mM NaCl, 5 mM KCl, 5 mM BME, pH 7.5 or 100 mM Tris-HCl, 5 mM BME, pH 7.5) or in the same buffer containing extra protective agents (10 mM pyruvate, 10 mM ThPP, 1 mM FAD, 10 mM Mg²⁺). Their activities were measured in a certain time interval using the photometric method described above.

Optimum pH and temperature for GST-LSU activity. The influences of the pH and the incubation temperature on the activity of GST-LSU were tested using the above photometric protocol under standard assay conditions. For measurement of pH dependence, following buffers were selected: 50 mM sodium acetate/acetic acid (pH 5.0-5.5), 50 mM sodium phosphate (pH 6.0-7.5), and 50 mM Tris-HCl buffer (pH 8.0 and 9.0). All the buffers were titrated to required pH levels with 2 M HCl or 2 M NaOH and the samples were incubated at 30°C for 1h. For determination of the effect of temperature, incubation was performed over a 20-45°C range for 1 h and the pH was maintained at 7.0.

Kinetic analysis of GST-LSU. The K_m of GST-LSU for pyruvate was measured according to the above described photometric method. Typically, assays contained 2 µg GST-LSU, pyruvate at various concentrations, and all the cofactors, FAD, ThDP and Mg²⁺ at

saturating concentrations were incubated at 30 °C for 10 min. Initial rates were measured for 2-10% formation of acetolactate. K_m was obtained by fitting the experimental data to the Michaelis-Menten equation using GraphPad Prism.

Determination of the substrate preference of GST-LSU. The substrate preference of GST-LSU were measured according to the published procedure (28). The reaction mixture (3 ml) contained 10 mM $MgCl_2$, 20 μM FAD, 10 mM pyruvate, and 2 mM ThPP in 80 mM potassium phosphate buffer (pH 7.5). 2-Ketobutyrate was added at various concentrations (1-30 mM). The reaction was initialized by addition of 0.1U GST-LSU. After incubation at 37°C for 10 min, the reaction was stopped by 0.3 ml of 4 M phosphoric acid. The products, 2-acetolactate and 2-aceto-2-hydroxybutyrate were determined simultaneously by the GC method developed by Gollop et al. (29).

GST-LSU mediated formation of R-PAC. The formation of L-PAC from condensation of pyruvate and benzaldehyde mediated by GST-LSU was carried out in 1 mL reaction mixture containing 0.1 M Hepes/KOH buffer (pH 7.0), 5 mM $MgCl_2$, 0.1 mM ThDP, 0.05 mM FAD, 0.5 mM DTT, 60 mM KCl, 5% (v/v) DMSO, 100 mM pyruvate, and 40 mM benzaldehyde. The reaction was initiated by addition of 100 μg GST-LSU and incubation was continued at 30°C for 1 h before the reaction was terminated by boiling for 2 min. After centrifuged at 6000 rpm for 3 min, the supernatant was extracted with chloroform (1 mL x 3) and the organic layers were combined and concentrated to about 0.2 mL under a gentle flow of nitrogen gas. The residue was first analyzed by LC-MS on a Shim-pack VP-ODS column (4.6 mm x 250, 5 μm , Shimadzu) using 30% aqueous acetonitrile as mobile phase at a flow rate of 0.7 mL/min, and then the product was isolated on a silica gel column developed by

petrol ether-ethyl acetate (3:1). The fractions that contained the target molecule were pooled and evaporated in vacuum. The residue thus generated (ca. 4.3 mg, 72%) was further elucidated by HRESI-MS and ¹H-NMR. HRESI-MS (negative mode) *m/z*: 149.0605 (100%) [M-H]⁻, calcd. 149.0671; ¹H-NMR (400MHz, CDCl₃) δ 2.09 (s, 3H, Me), 4.30 (broad s, 1H, OH), 5.14 (s, 1H, CH), 7.30 (m, 5H, aromatic) (7).

Results

Characterization of His-LSU protein. The LSU of AHAS I was first expressed as a histidine-tagged fusion protein. The soluble fraction was purified through His-tag affinity chromatography with a yield of 3 mg/L medium. The SDS-PAGE result indicated that the purity of the His-LSU was more than 95% and its molecular weight was found to be approximately 65 kD (Figure 2), which is similar in size to other bacterial LSU (20,21). The protein was subsequently evaluated by using the photometric method described in experimental section. The result showed that the specific activity of the protein is about 5.8 μmol min⁻¹ mg⁻¹, which is only around 10% of the maximal catalytic activity of *E. coli* AHAS I (21) and is about 3-4 times larger than that of the same protein generated by Yoon and co-workers (Table 1) (20). Further measurement showed that the His-LSU was unstable; its activity reduced quickly even when it was stored at -80°C in phosphate buffer or Tris-HCl buffer and after 7 days storage, most its activity had disappeared. It was reported that the use of extra protective agents such as fresh pyruvate, FAD, ThPP, and divalent metal ions in the storage buffer could be helpful for keeping the activity of AHAS (13). Our results showed that addition of these reagents could only slightly improve the stability of His-LSU protein. (Figure 3)

Expression, purification, and Western-blot clarification of GST-LSU. Because of the instability of His-LSU, we decided to insert the LSU gene into the pGEX-4T-1 expression vector to construct a new plasmid and to express the enzyme as a GST-fused protein. The result showed that the GST-LSU was successfully generated as a soluble protein in a quite good amount. Following affinity chromatography on a glutathione-Sepharose column produced GST-LSU in a yield of 1.2 mg/L medium. Further SDS-PAGE analysis (Figure 4A) indicated that the enzyme acquired was more than 95% pure with a molecular weight of about 90 kD, which was as expected (65 kD (LSU) + 23 kD (GST)). Western blot experiment confirmed the above result (Figure 4B).

Stability of GST-LSU. We also evaluated the stability of GST-LSU protein and to our delight, after the enzyme had been stored at -80°C in a phosphate buffer without any extra protective agents for at least 2 months, more than 90% of its activity was remained. After 4 months the protein was around 70% active. Even after 8 month storage under same condition, 40% of its activity was still present. It seems that addition of the extra protective agents to the buffers had no effect on stabilizing the protein. Furthermore, it is obvious that phosphate buffer is better than Tris-HCl buffer for both of the activity of GST-LSU protein and its stability. (Figure 5) Same observation has been reported by Yoon and coworkers (20). To the best of our knowledge, it is the first time that the LSU of *E. coli* AHAS I was expressed as a GST-tagged fusion protein. More importantly, through this way, the stability of LSU was highly promoted.

Optimum pH and temperature for GST-LSU activity. The measurements of the optimum pH and reaction temperature were carried out in a phosphate buffer. The results

showed that the optimum conditions for GST-LSU activity were pH at 7.0-7.5 (Figure 6A) and temperature at 37°C (Figure 6B), respectively, which were very similar to those for *E. coli* AHAS I (20).

The kinetic parameters of GST-LSU. The kinetic parameters of GST-LSU were determined using the photometric method. The K_m value for pyruvate was 0.95 mM (Figure S2), which was in the same level as those of the LSUs of the other bacterial AHASs (18-21). The specific activity of GST-LSU on pyruvate condensation is about 20.8 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, which is about one third of the maximal catalytic activity of *E. coli* AHAS I holoenzyme (entry 1 of Table 1), about three times larger than that of the His-LSU protein prepared in this study (entry 8), and is approximately 7 to 170-fold greater than that of the LSUs of the other bacterial AHASs (entries 3-7).

Substrate preference of GST-LSU. Barak et al. (28) has defined the product ratios (R) for the AHAS isozymes and by using the equation they deduced we calculated the R value of GST-LSU based on the experimental determination (Figure S3). The result showed that the R value was around 0.99 ± 0.05 , indicating that GST-LSU displayed little or no preference for either of its two natural substrates, which was identical with that of AHAS I of *E. coli* (28).

Formation of R-PAC from pyruvate and benzaldehyde. We also checked whether GST-LSU was able to catalyze the condensation of pyruvate and benzaldehyde to form R-PAC, which is a very important function of AHAS I. The reaction mixture contained DMSO to facilitate dissolution of benzaldehyde in aqueous buffer. The LC-MS profile showed that the main peak at about 11 min has a molecular weight of 149.0605 $[\text{M-H}]^-$ which was identical with R-PAC (Figure S4). Moreover, the little peak at about 22 min

corresponding to benzaldehyde indicated that the conversion of the starting material was almost complete. Further silica gel purification gave the target compound with a yield over 70% (un-optimized). ¹H-NMR determination confirmed that the compound acquired was R-PAC.

Discussion

AHAS is a promising target for the screening of herbicides and anti-infectious agents. Also, it is a very effective tool in stereoselective C-C bond formation in organic synthesis. Because its LSU is not stable alone, both subunits have to be synthesized and then reconstituted in vitro to obtain the active enzyme. It might be the reason that AHAS is not commercially available up to now, despite its scientific importance. Meanwhile, the unavailability of the enzyme largely hampers the screening of its inhibitors and its application in synthetic organic chemistry.

Several attempts have been carried out to prepare stable samples of AHAS (18,19,21). In these studies, both of the large and small subunits of the *E. coli* AHAS isozymes I, II, and III were expressed. Activity tests showed that neither subunit alone has significant enzymatic activity but, upon mixing the large and small subunits respectively, each isozyme could be reconstituted and displayed similar activity to its correspondent holoenzyme. Investigations performed by Yoon and co-workers centered on the preparation and characterization of AHAS large subunits from *E. coli* (AHAS I) (20), *Haemophilus influenza* (30), *Shigella sonnei* (31), and *Mycobacterium tuberculosis* (32) and the searching for their inhibitors. The data showed that the specific activities of all the four LSUs were very low (Table 1). Meanwhile, the authors did not evaluate the stability of them.

We prepared in this study the LSU of *E. coli* AHAS I as fusion proteins with two different tags, respectively. The specific activity of His-LSU is low if compared to that of the *E. coli* AHAS I holoenzyme but is in the comparable level of that of the same protein reported (Table 1) (21). Unfortunately, stability test of the His-LSU gave negative result. Sharp drop of its activity was observed even after only two days storage and after one week, it was completely inactive (Figure 3). Same phenomenon has been reported by Chipman and Silverman, respectively (21,22).

It has been reviewed that the use of GST polypeptide as a fusion tag could be advantageous in increasing the expression of target proteins and enhancing their solubility (33,34). The AHASs of higher plants usually exist in low abundance and are labile, however, several plant AHASs have been functionally expressed in *E. coli* as GST-fusion proteins recently (35-38). So we thought it might be possible to create a GST-fused LSU which is active and stable. Based on the assumption, we prepared and evaluated the GST-tagged LSU of *E. coli* AHAS I (GST-LSU). To our delight, the acquired fusion protein displayed quite high specific activity on the pyruvate condensation reaction (Table 1). More important, the determination showed that the stability of the protein is excellent. Also, the GST-LSU could mediate the reaction between pyruvate and benzaldehyde to form R-PAC with a quite good yield and clean product. Meanwhile, the pH dependence, temperature preference and the kinetic properties of GST-LSU were very similar to those of the native enzyme. These data plus the result of His-LSU protein indicated that introduction of GST label to the LSU of AHAS could stabilize the LSU protein almost without influence on its catalytic properties. The possible reason that the GST label acts as a stabilizer of LSU of *E. coli* AHAS might be

that when GST is fused into LSU, it effects to some extent like SSU through interaction with LSU and thus the LSU is stabilized. But because i) the mechanism of how the SSU stabilizes the LSU is yet unclear; ii) the sizes of GST (23kD) and SSU (9kD) are quite different and their amino acid sequences share almost no similarity, further work is still necessary to determine the exact reason that the GST tag can make the LSU of *E. coli* AHAS stable.

In this study, the GST fusion tag stabilized LSU of *E. coli* AHAS I was prepared and evaluated. The result showed that the fusion protein not only possesses all the fundamental functions of AHAS I, but is stable as well. Whether the GST-LSU protein also possesses the extra activities of AHAS I such as catalyzing the formation of peracetate and the conversion of acetolactate to peracetate and pyruvate in the presence of oxygen (39,40) is still under investigation.

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Figure legends

FIG. 1. Condensation reaction catalyzed by AHAS

FIG. 2. SDS-PAGE analysis of the expression and purification of His-LSU protein. Lane 1: protein marker; lane 2: whole cell extract of the *E. coli* BL21 (DE3) harboring pPROEXTM

HTb-*ilvB* before IPTG induction; lane 3: after IPTG induction; lane 4: the soluble fraction of lane 3; lane 5: Ni-NTA purified His-LSU protein.

FIG. 3. Stability of His-LSU protein in different buffers with and without extra protective agents. Curve 1: 50 mM phosphate buffer, pH 7.5 containing 2 mM DTT and 20% glycerol; Curve 2: 100 mM Tris-HCl buffer, pH 7.5 containing 2 mM DTT and 20% glycerol; Curve 3: 50 mM phosphate buffer containing extra protective agents; Curve 4: 100 mM Tris-HCl buffer containing extra protective agents. Extra protective agents: 10 mM pyruvate, 10 mM ThPP, 1 mM FAD, and 10 mM Mg²⁺.

FIG. 4. SDS-PAGE result of preparation of GST-LSU protein (A) and Western-blot clarification (B). A. Lane 1: whole cell extract of the *E. coli* BL21 (DE3) harboring pGEX-4T-1-*ilvB* before IPTG induction; lane 2: after IPTG induction; lane 3: the soluble fraction of lane 1; lane 4: the soluble fraction of lane 2; lane 5: glutathione sepharose purified GST-LSU protein; M: protein marker. B. Lane 1: Glutathione sepharose purified GST-LSU; M: protein marker; lane 3: whole cell extract of the *E. coli* BL21 (DE3) harboring pGEX-4T-1-*ilvB* after IPTG induction.

FIG. 5. Stability of GST-LSU protein in different buffers with and without extra protective agents. Curve 1: 50 mM phosphate buffer, pH 7.5 containing 300 mM NaCl, 5 mM KCl, 5 mM BME; Curve 2: 100 mM Tris-HCl buffer, pH 7.5 containing 5 mM BME; Curve 3: 50 mM phosphate buffer containing extra protective agents; Curve 4: 100 mM Tris-HCl buffer

containing extra protective agents. Extra protective agents: 10 mM pyruvate, 10 mM ThPP, 1 mM FAD, 2 mM mercaptoethanol, and 10 mM Mg^{2+} .

FIG. 6. Determination of optimum pH (A) and temperature (B) for GST-LSU activity.

FIG. 1.

FIG. 2.

Fig. 3

FIG. 4.

Fig. 5

FIG. 6.

Table 1. The specific activities of LSU of microbial AHASs and of microbial AHASs

Entry	Sources of AHAS	Sp. activities ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)		Fusion tag	Ref.
		LSU	Holoenzyme		
1	<i>E. coli</i> AHAS I	6-12	63.6	His	[21]
2	AHAS II	trace	24	With no tag	[18]
3	AHAS III	0.23	2.6	With no tag	[19]
4	<i>E. coli</i> K12 AHAS I	1.5	NR*	His	[20]
5	<i>H. influenza</i>	1.5	NR	His	[30]
6	<i>S. sonnei</i>	0.12	NR	His	[31]
7	<i>M. tuberculosis</i>	2.8	NR	His	[32]
8	<i>E. coli</i> AHAS I	5.8	ND**	His	This study
9	<i>E. coli</i> AHAS I	20.8	ND	GST	This study

*Not reported in the cited literature. **Not detected in this study.