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Purification, crystallization, and X-ray crystallographic analysis of Acetolactate synthase from *Hydrogenobacter thermophilus*

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Acetolactate synthase (ALS) catalyzes the chemical reaction in plants and microorganisms that converts two pyruvate molecules to an acetolactate molecule and a carbon dioxide. To confer change on its substrate specificity for one carbon chemicals, structural study was initiated. For this, the recombinant ALS protein from *Hydrogenobacter thermophilus* (HtALS) was expressed in *Escherichia coli*, and homogeneously purified. The purified HtALS protein was crystallized from the precipitant composed of 0.3 M Ammonium sulfate, 0.1 M Sodium cacodylate (pH 6.5), and 18% (w/v) polyethylene glycol 4000. Diffraction data was collected to 2.4 Å resolution. The crystal belongs to the primitive orthorhombic P2₁2₁2₁ space group with unit-cell parameters $a = 75.24$ Å, $b = 76.62$ Å, $c = 194.69$ Å, and $\alpha = \beta = \gamma = 90^\circ$. There are two HtALS molecules in the asymmetric unit.

INTRODUCTION

One carbon chemicals, such as methane, methanol, formaldehyde, and carbon dioxide, are easily diffused into air. They are also regarded as major chemicals for evoking greenhouse effects and global warming (Nordhaus, 1991). Even though plants and some algae catalyze a reaction for the incorporation of carbon dioxide into large molecules (Merrett and Lord, 1973), an enzyme that directly introduces a covalent bond between two one carbon chemicals is seldom found in nature.

Recently, a biological process for the conversion of formaldehyde into chemicals of two or three carbons is being attempted by using non-natural enzymes. Formolase is a modified enzyme of benzaldehyde lyase (BAL), and produces glycolaldehyde and dihydroxyacetone from formaldehydes (Siegel et al., 2015). A glycolaldehyde synthase is also a modified version of benzoylformate decarboxylase (BFDC), and converts two formaldehydes into a glycolaldehyde (Lu et al., 2019). These two enzymes commonly require a cofactor of thiamine diphosphate (ThDP). Even though it is rather obscure how the way in which they aid the formation or breakdown of covalent bonds between two carbon atoms, it is generally accepted that carbanion formation of a ThDP cofactor by the glutamate residue of an enzyme initiates the chemical reaction (Breslow and McNelis, 1959), implicating that other ThDP-dependent enzymes might be developed as a biocatalyst for the conjugation of one carbon chemicals.

Acetolactate synthase (ALS), which is also known as acetohydroxy acid synthase, catalyzes the chemical reaction that converts two pyruvate molecules to an acetolactate molecule, and releases a carbon dioxide molecule as a by-product. The reaction requires three cofactors of flavin adenine dinucleotide

(FAD), ThDP, and Mg²⁺. ThDP in the ALS enzyme is activated as it is deprotonated by glutamate, which generates an anion at the C2 atom between the sulfur atom and the nitrogen atom of the thiazolium ring of ThDP. Then, this carbanion acts as a nucleophile that attacks the carbonyl carbon of substrate (Breslow and McNelis, 1959). In the ALS-catalyzed reaction, the first pyruvate is decarboxylated by this attack, and the remnant is covalently bound to ThDP. Since this adduct also possesses a carbanion, its conjugation with the second pyruvate can proceed. The last reaction step is helped by the FAD cofactor, which provides an electron to give off the final product, (2S)-2-acetolactate.

The suggested catalytic mechanism of ALS is very similar to that of BFDC itself and that of BAL, except for the last reduction by FAD. Comparison of the known structures of ALS to those of BAL and BFDC reveals the presence of close structural relationships between these enzymes, suggesting that ALS might also be developed as an enzyme for the formation of a covalent bond between two carbon atoms, like other ThDP-dependent enzymes. Therefore, the increased structural information of ALS is expected to contribute to developing a biocatalytic system for converting one carbon chemicals into more than two carbon carrying chemicals.

In this study, ALS from *Hydrogenobacter thermophilus* (HtALS) (Kawasumi et al., 1984) has been targeted for an enzyme candidate for converting one carbon chemicals. Since minute structural information at the molecular level may lessen trial-and-error for variation of the substrate specificity of an enzyme, elucidation of the three-dimensional structure of HtALS has been initiated. For this, the codon-optimized HtALS-encoding gene was cloned into a bacterial expression plasmid. After heat

treatment, the expressed recombinant protein was affinity-purified to high purity, and the purified protein was highly concentrated. The recombinant HtALS protein was successfully crystalized, and its X-ray diffraction data was collected. The electron density calculated by molecular replacement is enough to trace most residues of the molecule.

RESULTS AND DISCUSSION

The codon-optimized gene encoding the recombinant HtALS was synthesized, and subsequently cloned into the pProEx HTa

TABLE 1 | Protein production information

Source organism	<i>Hydrogenobacter thermophilus</i>
DNA source	Synthesized DNA (Cosmogenetech, Seoul, Korea)
Cloning vector	Modified pProEX HTa
Expression vector	Modified pProEX HTa
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino acid sequence of the construct produced (N–C)	<u>MSYYHHHHHH</u> HDYDIPTTENLYFQGGHMARKGADIVI ETLKEEGVEYIFGHPGGAIMVEYDLYRDGSIKHILT RHEQGAGHMAEGYAKATGKVGAMSTSGPGATNL VTAIADAYMDSVPVVFITGQVPTHLIGNDAFQEV GITRPITKHNFLVKRIEDLPMLRQAFYIAKTGRPGPV LVDIPKDITQKISDVEIPSLEEVKSSLPGYKPHTEGNP QQIKKAARLAEAKRPVLYVGGGAVQAEAKELVEL AELMKVPVTTTNNMGKGAFPEHPLALHMLGMHGT YYANMAVYNCDLLIAVGARFDDRTGKVEEFAPQA SIIHIDIDPASISKNIIVDVPVGDVWLRKLEIEIKKEG AKLLYPEERQKWLEQIEKWKLHPLTYRKSDKVIKP QYVIEEIWKVTKGDIIATGVGQHQMWAAMFYRSF PRQFINSGLGTMGFLPAGIGAKIGRPEKEVVID GDGSFMMTMQELITAVHYRVPVKAIINNGYLGMR QWQELFYEKRYAEVDLSIQPDFVKLAECGAVGFR AEKPSEVREVLEEAAMKVQDKPVLDDFHVDRREENVL PMVPAGKSYRDMILEDGKKGVEADTMYLVG

*Underlined amino acid sequence indicates an N-terminal 6xHis tagging and the TEV cleavage sequence.

FIGURE 1 | SDS-PAGE analysis of the Ni ion affinity chromatography of HtALS. 12% (w/v) SDS-PAGE of the Ni affinity chromatography. Lane 1, Cell debris; lane 2, Precipitant fraction with thermal treatment; lane 3, Supernatant; lane 4, Unbound fraction; lane M, protein size marker; lanes 5–8, Fractions eluted at 25, 50, 300, 500 mM Imidazole concentration of buffer, respectively. The expected size of the recombinant HtALS protein is indicated by the black arrow.

vector that contains an N-terminal 6xHis-tagging cleavable with a tobacco etch virus (TEV) protease (Table 1). The expressed recombinant protein was initially purified by Ni-ion affinity chromatography after heat treatment, and further with ion exchange chromatography (Figure 1). The purified HtALS protein was concentrated up to 5 mg·ml⁻¹ using Ultra centrifugal filter units (Merck Millipore) in the buffer consisting of 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl.

Initial protein crystal screening was performed at 291 K using sparse-matrix crystal screening solutions of ~1,000. Yellow-colored crystals suitable for X-ray diffraction (Figure 2) were obtained within 2 days at 291 K by mixing 0.5 µl of protein solution and 0.5 µl of the reservoir solution including 0.3 M Ammonium sulfate, 0.1 M Sodium cacodylate (pH 6.5), and 18% (w/v) polyethylene glycol 4000 (Table 2).

For X-ray diffraction experiments, the obtained crystals were shortly immersed in the precipitant solution enriched with the cryo-protecting agents of 25% (w/v) polyethylene glycol 4000. The best diffraction data were collected to a resolution of 2.39 Å (Figure 3), and integrated and scaled using HKL2000 software package (Otwinowski and Minor, 1997). The crystal belongs to the primitive orthorhombic P2₁2₁ space group with unit cell

FIGURE 2 | A representative photograph of HtALS crystals. The crystal of HtALS protein was obtained from the reservoir solution consisting of 0.3 M Ammonium sulfate, 0.1 M Sodium cacodylate (pH 6.5), and 18% (w/v) polyethylene glycol 4000. The crystal was grown at 291 K within 2 days.

TABLE 2 | Crystallization

Method	Hanging-drop vapor diffusion
Plate type	VDX™ Plate without sealant (24-well)
Temperature (K)	291
Protein concentration (mg·ml ⁻¹)	5
Buffer composition of protein solution	20 mM Tris-HCl (pH 7.5), 0.2 M NaCl
Composition of reservoir solution	0.3 M Ammonium sulfate, 0.1 M Sodium cacodylate (pH 6.5), and 18% (w/v) Polyethylene glycol 4000
Volume and ratio of drop	1 µl; 1:1 ratio of protein and reservoir solution
Volume of reservoir (µl)	200

parameters $a = 75.24 \text{ \AA}$, $b = 76.62 \text{ \AA}$, $c = 194.69 \text{ \AA}$, and $\alpha = \beta = \gamma = 90^\circ$ (Table 3). With two molecules in the asymmetric unit, the crystal volume per unit protein molecular weight corresponds with $2.19 \text{ \AA}^3 \cdot \text{Da}^{-1}$, corresponding to 44% solvent in the crystal (Matthews, 1968).

The electron density of the HtALS crystal was obtained with molecular replacement (MR) by PHENIX (Adams et al., 2010), using *A. thaliana* acetohydroxy acid synthase structure (PDB ID 3E9Y, sequence identity 49%) as the initial search model. The calculated electron density is clearly enough to trace most of the $C\alpha$ atoms of the protein. Currently, preliminary structure refinement and model building are in progress. The diffraction data statistics is summarized in Table 3. The crystal structure of HtALS is expected to provide molecular information that develops HtALS as a formaldehyde-converting enzyme.

METHODS

Cloning, expression, and purification of HtALS

The HtALS-expressing gene (ilvB) has 1,752 nucleotides that encode a polypeptide of 584 residues. The codon-optimized target gene was synthesized (Cosmogenetech, Seoul, Korea), and cloned into the pProEx HTa vector (Invitrogen) that allows the recombinant protein to be expressed with the 6xHis-TEV protease recognition site at the N-terminus (Table 1). The recombinant plasmid was transformed into *E. coli* BL21(DE3) (Thermo Fisher Scientific), and was cultivated in Luria-Bertani media that was supplemented with $50 \mu\text{g} \cdot \text{mL}^{-1}$ of ampicillin at 310 K with shaking at 200 rpm. When the optical density at 600 nm reached 0.5, the expression of the HtALS protein was induced at 291 K for 18.5 hours, after adding 0.1 mM isopropyl β -D-1-thiogalactopyranoside into the cultural media. The cell was

harvested by centrifugation at $5,000 \times g$ for 10 min at 277 K. The collected cell was resuspended with buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 1 mM β -mercaptoethanol (buffer A), and disrupted by ultrasonicator. The cell debris was removed by centrifugation at $11,000 \times g$ for 30 min at 277 K. The supernatant was heated at 353 K for 30 min in water bath. The denatured precipitation was removed by centrifugation, and only the supernatant was used for purification of the HtALS protein. The supernatant was purified initially by immobilized metal ion affinity chromatography (IMAC), using Ni-NTA resin (ELPIS, Daejeon, Korea) equilibrated with buffer A. The bound protein in the resin was eluted by a discontinuous gradient of 0–500 mM imidazole. To eliminate the 6xHis-tag, the target protein eluate was mixed with recombinant TEV protease, incubated at 291 K overnight, and dialyzed to remove the salt. After that, the TEV protease was removed by a second IMAC using Ni-NTA resin (Bio-Works) equilibrated with 20 mM Tris-HCl (pH 7.5) and 1 mM β -mercaptoethanol (buffer B). HtALS protein was further purified by anion exchange chromatography using a 5 ml HiTrap™ Q column (GE Healthcare, Uppsala, Sweden). The target protein was eluted by a linear gradient from 0–1 M NaCl in buffer B.

Crystallization

The purified HtALS was concentrated up to $5 \text{ mg} \cdot \text{mL}^{-1}$ in a buffer consisting of 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl for crystallization (Figure 1). The protein concentration of HtALS was determined considering the molar extinction coefficient of $0.932 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm. Initial crystallizing condition was obtained at 291 K using the sitting-drop vapor-diffusion method in 96-well crystallization plate (Corning, USA). A mixture of $0.3 \mu\text{l}$ of protein

solution and 0.3 μ l of protein drop was placed in each well of the plate, and was equilibrated with 55 μ l of reservoir solution at 291 K. The initial conditions were obtained from a precipitant solution No.32 of MCSG-1T (Microlytic North America Inc., Massachusetts, USA), which contains 0.2 M Ammonium sulfate, 0.1 M Sodium citrate:HCl (pH 5.6), and 25% (w/v) polyethylene glycol 4000. This condition was further optimized by changing the buffer pH, precipitant concentration, and salt concentration. The best crystals (Figure 2) were grown in 0.3 M Ammonium sulfate, 0.1 M Sodium cacodylate (pH 6.5), and 18% (w/v) polyethylene glycol 4000 using hanging-drop vapor-diffusion method at 291 K, where 0.5 μ l protein and 0.5 μ l precipitant were mixed and equilibrated with 200 μ l precipitant solution (Table 2).

X-ray diffraction data collection and processing

The obtained HtALS protein crystals were immersed shortly in the precipitant solution enriched with the cryo-protecting agents of 25% (w/v) polyethylene glycol 4000, and placed in the flowing 100 K nitrogen gas system. The X-ray diffraction data set of the HtALS protein crystal of 180 frames was collected using the Pilatus 6M detector at beamline 11C of the Pohang Light Source III (PLS III) at the Pohang Accelerator Laboratory (PAL, Pohang, Korea), with the per-frame oscillation of 1°, per-frame exposure of 1 s, and the crystal-to-detector distance of 500 mm (Figure 3). The indexing, integration, and scaling of HtALS were conducted with the HKL-2000 suite (Table 3). The experimental electron density of the HtALS crystal was determined by the MR method in the PHENIX suite, using the structure of acetohydroxy acid synthase from *A. thaliana* (PDB ID 3E9Y) as a model.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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