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Purification, crystallization and X-ray crystallographic analysis of cystathionine gamma-synthase from *Corynebacterium glutamicum*

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Cystathionine gamma synthase from *Corynebacterium glutamicum* (*Cg*MetB) is a key enzyme for the production of L-methionine and it condenses O-acetyl-L-homoserine (OAHS) and cysteine to produce cystathionine. MetB is also an attractive target for the development of antimicrobial compounds because it catalyzes the first reaction of the L-methionine biosynthetic pathway. The *Cg*MetB was overexpressed and purified to homogeneity by affinity and size-exclusion chromatography. The *Cg*MetB protein was crystallized using hanging-drop vapor-diffusion method in the presence of 13% polyethylene glycol 3350 and 0.1 M Magnesium formate dihydrate at 295 K. X-ray diffraction data were collected to a maximum resolution of 1.5 Å. The crystal belonged to space group *F*222, with unit cell parameters a = 58.57 Å, b = 149.85 Å, c = 161.86, $\alpha = \beta = \gamma = 90.0°$. With one molecules per asymmetric unit, the crystal volume per unit protein mass was 2.13 $Å^3$ Da^{-1}, which correspond to a solvent content of approximately 42.27%.

INTRODUCTION

Methionine is one of the essential amino acids for human life and can be produced only in microorganisms and plants. The members of methionine biosynthetic pathway are therefore attractive targets for the design of novel antibiotics and herbicides. Methionine is also crucial for the food additives in livestock production. Due to increase in meat consumption, the global market of methionine is expected to reach USD 7.3 billion by 2022. Industrially, methionine is mainly produced by chemical synthesis. The chemical synthesis produces a mixture of D- and L-methionine (Leuchtenberger, 2008) and requires hazardous chemicals as ingredients and additional cost to separate racemic mixture. Due to the increase of demand for environment-friendly methionine production, many researchers focus on bio-based methionine production by enzymatic synthesis or by fermentation using microorganisms such as *Corynebacterium glutamicum*. Enzymatic synthesis of methionine exhibits high yields, whereas it requires expensive substrates (Chibata et al., 1957). Methionine production by fermentation utilizes microorganisms capable of producing amino acids and attempts have been made to overproduce biologically active L-methionine (Kase & Nakayama, 1975, Nakayama et al., 1978, Kumar et al., 2003, Mondal & Chatterjee, 1994).

C. glutamicum has been widely utilized for the industrial production of various amino acids (Becker et al., 2011, Georgi et al., 2005, Kase & Nakayama, 1975). In *C. glutamicum*, the methionine biosynthetic pathway is derived from homoserine. At first, homoserine is converted into O-acetyl-L-homoserine (OAHS) by homoserine O-acetyltransferase (metX). In most organisms, an acetyl group is used to activate the homoserine (Ferla & Patrick, 2014), whereas in enterobacteria and a limited amount of other organisms, succinyl group is transferred to homoserine (Rowbury & Woods, 1964, Kaplan & Flavin, 1966). In addition, plants and some bacteria utilize phosphate group. Activated homoserine can be converted into homocysteine in two different ways. The trans-sulfuration pathway via cystathionine utilizes cysteine as a sulfur donor, while direct-sulfuration pathway utilizes inorganic sulfur such as hydrogen sulfide or methanethiol. *C. glutamicum* has been reported to utilize both trans- and direct-sulfuration pathways. In trans-sulfuration pathway, cystathionine gamma-synthase (metB) catalyzes the condensation reaction of OAHS with cysteine and subsequent hydrolysis of cystathionine by cystathionine beta-lyase (metC) yields homocysteine. In direct-sulfuration pathway, OAHS reacts with free hydrogen sulfide and homocysteine is directly produced. The final step is S-methylation of homocysteine, which is catalyzed by homocysteine S-methyltransferase (metE/H).

Cystathionine gamma-synthase from *C. glutamicum* (*Cg*MetB) accomplishes the γ-replacement reaction of OAHS with cysteine, leading to cystathionine (Figure 1). This protein is an attractive target for the development of novel antimicrobial compounds because it catalyzes the first reaction in the trans-sulfuration pathway, which is unique to bacteria. MetB utilizes PLP as a cofactor and belongs to the γ-family of PLP-dependent enzymes.

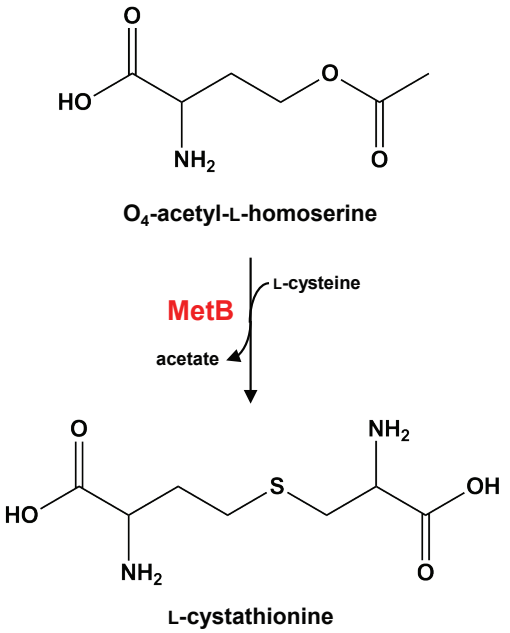

FIGURE 1 | Purification of *Cg*MetB. Enzyme reaction of *Cg*MetB.

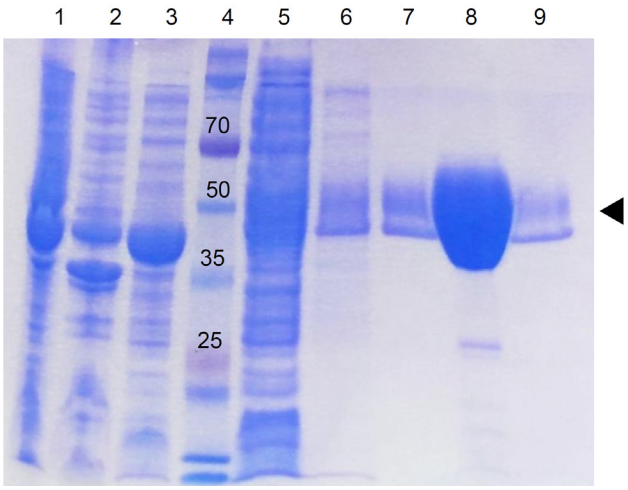

FIGURE 2 | Purification of *Cg*MetB. SDS-PAGE of purification of recombinant *Cg*MetB protein. Lane 4 shows molecular-weight markers (labelled in kDa). Land 1-3, 5-9 show the purification procedure of *Cg*MetB using Ni-NTA chromatography. Lane 1, whole cell extract; lanes 2 and 3, pellet and supernatant fraction after centrifugation of whole cell extract, respectively; lane 5, flowthrough from Ni-NTA column; lane 6 and 7, wash with 0 and 10 mM imidazole, respectively; lane 8 and 9, elution with 300 mM imidazole. Purified *Cg*MetB is indicated with an arrow.

Despite the importance of *C. glutamicum* as a producer of L-methionine, structural and biochemical studies had not been reported on MetB prior to this study. In this study, we describe the cloning, expression, purification, crystallization and X-ray crystallographic analysis of the *Cg*MetB protein.

RESULTS AND DISCUSSION

*Cg*MetB was purified to apparent homogeneity by metal-affinity chromatography followed by size-exclusion chromatography. The elution volume of the major peak corresponds to a tetrameric form, with an apparent molecular weight of ~ 160 kDa from a gel-filtration column (data not shown) calibrated with standard molecular-weight proteins. SDS-PAGE of the purified enzyme showed a single band with a monomeric molecular weight of 41.7 kDa (Figure 2).

The *Cg*MetB protein was crystallized in 13% polyethylene glycol 3350 and 0.1 M magnesium formate dihydrate (Figure 3) The crystals were transferred to cryoprotectant solution composed of the corresponding conditions described above and 30% (v/v) glycerol, fished out with a loop larger than the crystals, and flash-frozen by immersion in liquid nitrogen. X-ray diffraction data were collected to a resolution of 1.5 Å on beamline7A at the Pohang Accelerator Laboratory (PAL, Pohang, Korea), using a Quantum 270 CCD detector (ADSC, USA) (Figure 4). The *Cg*MetB crystals belonged to the space group *F*222 with unit cell parameters a = 58.57 Å, b = 149.85 Å, c = 161.86, $\alpha = \beta = \gamma = 90.0°$. Assuming one molecule of *Cg*MetB (41.7kDa) per asymmetric unit, the crystal volume per unit of protein mass was 2.13 Å3 Da-1, meaning that the solvent content was approximately 42.27% (Matthews, 1968).

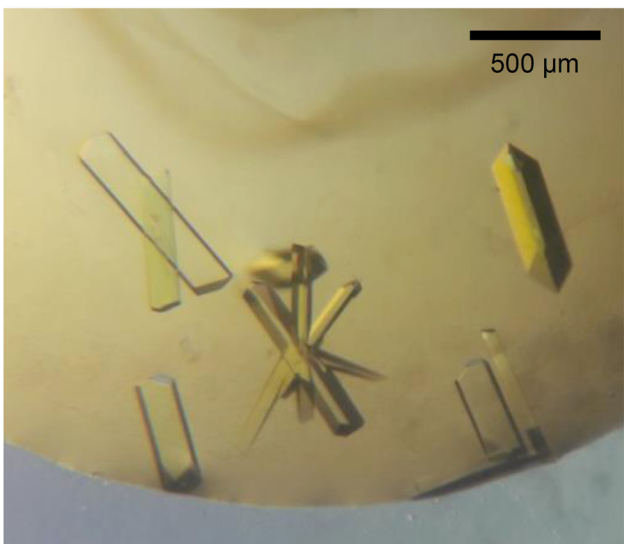

FIGURE 3 | Crystals of *Cg*MetB. Crystals of the best quality were produced from the condition 13% polyethylene glycol 3350 and 0.1 M Magnesium formate dehydrate and grew to maximum dimensions of 0.2 x 0.05 x 0.5 mm within 2 days.

The structure of *Cg*MetB was determined by molecular replacement with the CCP4 version of MOLREP (Vagin & Teplyakov, 2009) using the structure of MetB from *Mycobacterium ulcerans* (PDB code 3QHX) as a search model (Clifton et al., 2011). *Cg*MetB has 62% amino acid sequence identity to *Mu*MetB. Model building was performed manually using the program WinCoot (Emsley & Cowtan, 2004), and

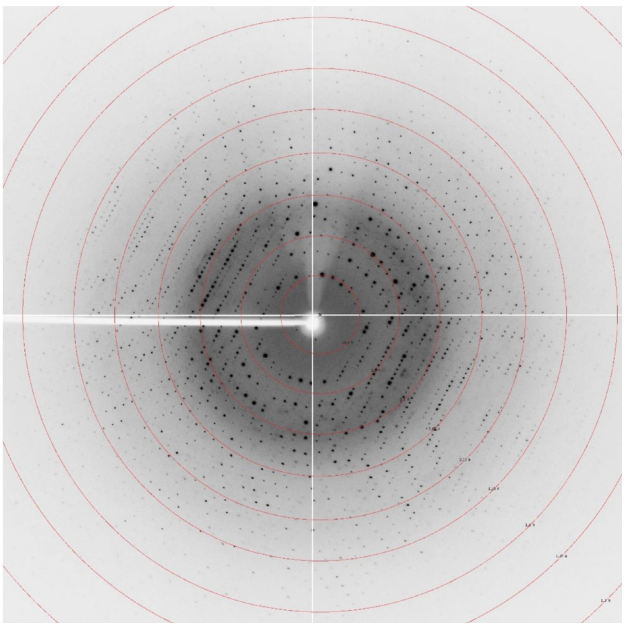

FIGURE 4 | Figure 4. Diffraction pattern of the *Cg*MetB crystal. The crystal diffracted to a maximum resolution of 1.5 Å.

refinement was performed with CCP4 refmac5 (Murshudov et al., 1997). The initial electron density map, which was good quality with backbone atoms well defined by electron density, allowed us to build a three-dimensional *Cg*MetB structure. Crystallographic model building and refinement of the structure to 1.5 Å resolution are in progress.

METHODS

Production of *Cg*MetB

The forward and reverse primers were designed as 5'-GCGCGCATATGTCTTTTGACCCAAACACCCAG-3' and 5'-GCGCGGCGGCCGCAAGGTTATTGAGGGCCTGCTC-3' to introduced NdeI and NotI restriction sites, respectively (underlined). The *Cg*MetB gene was amplified by polymerase chain reaction (PCR) using genomic DNA from *C. glutamicum* strain ATCC 13032 as a template. The PCR product was then subcloned into pET30a (Life Science Research) with a six-His tag at the C-terminus. The resulting expression vector pET30a:*CgMetB* was transformed into the *E. coli* strain BL21(DE3)-T1^R, which was grown in 1 L of LB medium containing kanamycin at 37°C. At an OD600 of 0.7, *Cg*MetB protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture medium was maintained for a further 21 h at 18°C. The culture was then harvested by centrifugation at 4,000 × *g* for 15 min at 4°C. The cell pellet was resuspended in buffer A (40 mM Tris-HCl, pH 8.0) and then disrupted by ultrasonication. The cell debris was removed by centrifugation at 13,500 × *g* for 30 min and the lysate was applied to an Ni-NTA agarose column (Qiagen). After washing with buffer A containing 10 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Finally, the trace amount of contaminants was removed by size-exclusion chromatography by using a Superdex 200 prep-grade column (320 mL, GE Healthcare) equilibrated with buffer A. All purification experiments were performed at 4°C and SDS-polyacrylamide gel electrophoresis analysis of the purified proteins shows a single polypeptide of 41.7 kDa corresponding to the estimated molecular weight of the *Cg*MetB monomer. The purified protein was concentrated to 70 mg/mL in 40 mM Tris-HCl, pH 8.0. Macromolecule-production information is given in Table 1.

TABLE 1 | Macromolecule-production information

Source organism	*Corynebacterium glutamicum* ATCC 13032
DNA source	Chromosomal DNA
Forward primer	GCGCGCATATGTCTTTTGACCCAAACACCCAG
Reverse primer	GCGCGGCGGCCGCAAGGTTATTGAGGGCCTGCTC
Cloning vector	pET-30a
Expression vector	pET-30a
Expression host	*E. coli* BL21 (DE3)-T1^R
Complete amino-acid sequence of the construct produced	MSFDPNTQGFSTASIHAGYEPDDYYGSINTPIYASTTFAQ-NAPNELRKGYEYTRVGNPTIVALEQTVAALEGAKYGRAFS-SGMAATDILFRIILKPGDHIVLGNDAYGGTYRLIDTVFTA-WGVEYTVVDTSVVEEVKAAIKDNTKLIWVETPTNPALGIT-DIEAVAKLTEGTNAKLVVDNTFASPYLQQPLKLGAHAVLH-STTKYIGGHSDVVGGLVVTNDQEMDEELLFMQGGIGPIPS-VFDAYLTARGLKTLAVRMDRHCDNAEKIAEFLDSRPEVST-VLYPGLKNHPGHEVAAKQMKRFGGMISVRFAGGEEAAKKF-CTSTKLICLAESLGGVESLLEHPATMTHQ AAGSQLEVPR-DLVRISIGIEDIEDLLADVEQALNNLAAALEHHHHHH

The underlined characters in the primers indicate restriction-enzyme sites.

TABLE 2 | Crystallization

Method	Hanging-drop vapor diffusion
Plate type	SPL plate (24-well)
Temperature (K)	293
Protein concentration (mg ml^{-1})	70
Buffer composition of protein solution	40 mM Tris-HCl pH 8.0
Composition of reservoir solution	13% polyethylene glycol 3350, 0.1 M Magnesium formate dihydrate
Volume and ratio of drop	2 µl; 1:1 ratio of protein and reservoir solutions
Volume of reservoir (ml)	0.5

TABLE 3 | Data collection and processing

Diffraction source	Beamline 7A, PAL
Wavelength (Å)	0.97934
Temperature (K)	100
Detector	Quantum 270 CCD
Crystal-to-detector distance (mm)	180
Rotation range per image (°)	0.2
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	*F*222
a, b, c (Å)	58.57, 149.85, 161.86
α, β, γ (°)	90.0, 90.0, 90.0
Resolution range (Å)	50.00-1.51 (1.54-1.51)[a]
Total No. of reflections	52555
Completeness (%)	99.3 (98.6)[a]
R_{sym} or R_{merge} (%)	7.8 (30.5)[a]
$I / \sigma I$	42.7 (16.0)[a]
Redundancy	6.6 (5.0)[a]

[a]The numbers in parentheses are statistics from the highest resolution shell.

Crystallization of *Cg*MetB

Crystallization of the purified *Cg*MetB protein was initially performed with commercially available sparse-matrix screens from Rigaku and Molecular Dimensions by using the hanging-drop vapor-diffusion method at 20°C. Each experiment consisted of mixing 1.0 µL protein solution (70 mg/ml in 40 mM Tris-HCl, pH 8.0) with 1.0 µL reservoir solution and then equilibrating this against 500 mL reservoir solution. *Cg*MetB crystals were observed from several crystallization screening conditions. After several rounds of crystal improvement, the best quality crystal appeared in 13% polyethylene glycol 3350 and 0.1 M Magnesium formate dihydrate. Crystallization information is summarized in Table 2.

X-ray diffraction analysis of *Cg*MetB

Diffraction data were collected from *Cg*MetB crystals on 7A beamline of the Pohang Accelerator Laboratory (PAL, Pohang, Korea), using a Quantum 270 CCD detector (ADSC, USA). The *Cg*MetB crystals diffracted to a resolution of 1.5 Å. The data were indexed, integrated, and scaled together using the HKL-2000 software package (Otwinowski et al., 1997). The data statistics are summarized in Table 3.

Results and discussion

CgMetB was purified to apparent homogeneity by metal-affinity chromatography followed by size-exclusion chromatography. The elution volume of the major peak corresponds to a tetrameric form, with an

apparent molecular weight of ~ 160 kDa from a gel-filtration column (data not shown) calibrated with standard molecular-weight proteins. SDS-PAGE of the purified enzyme showed a single band with a monomeric molecular weight of 41.7 kDa (Figure 2).

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Competing interests

The authors have declared that no competing interests exist.

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