3,6-Anhydro-L-galactonate cycloisomerase from *Vibrio* sp. strain EJY3: crystallization and X-ray crystallographic analysis

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3,6-Anhydro-L-galactonate cycloisomerase (ACI), which is found in the marine bacterium *Vibrio* sp. strain EJY3, converts 3,6-anhydro-L-galactonate into 2-keto-3-deoxygalactonate. ACI is a key enzyme in the metabolic pathway of 3,6-anhydro-L-galactose (AHG). Study of AHG metabolism is important for the efficient fermentation of agar and biofuel production, because AHG is a sugar that is non-fermentable by commercial microorganisms. The *aci* gene from *Vibrio* sp. strain EJY3 was cloned, and the recombinant protein was overexpressed and crystallized in order to determine the structure and understand the function of the protein. The crystals diffracted to 2.2 Å resolution and belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 87.9$, $c = 143.5$ Å. The Matthews coefficient was $2.3 \text{ Å}^3 \text{ Da}^{-1}$, with a solvent content of 47%.

1. Introduction

3,6-Anhydro-L-galactose (AHG) is a monomeric unit of agar polysaccharides and is a rare sugar that is only found in marine red algal biomass (Day & Yaphe, 1975; Yun, Choi *et al.*, 2015). Recently, microbial AHG metabolism has been revealed through the study of *Vibrio* sp. strain EJY3, which is capable of growth using AHG as a sole carbon source (Yun, Lee *et al.*, 2015). AHG is recruited into the central metabolic pathway through successive reactions catalyzed by two key enzymes: AHG dehydrogenase (EC 1.2.1.92; designated AHGD) and 3,6-anhydro-L-galactonate cycloisomerase (EC 5.5.1.25; designated ACI) (Yun, Lee *et al.*, 2015). AHGD transforms AHG into 3,6-anhydro-L-galactonate (AHGA), a novel metabolite that was reported for the first time in the agar metabolic pathway. The resultant AHGA is converted to 2-keto-3-deoxygalactonate (KDGal) by ACI (Fig. 1; Yun, Lee *et al.*, 2015). This two-step conversion of AHG to KDGal is a bypass route to the central metabolic pathway for metabolizing AHG as a carbon source.

The enolase superfamily has been divided into seven major subgroups on the basis of sequence, structure and molecular function: (i) mandelate racemases (MRs), (ii) muconate-lactonizing enzymes (MLEs), (iii) enolases, (iv) D-mannonate dehydratases (D-ManDs), (v) D-glucarate dehydratases (D-GlucDs), (vi) β -methylaspartate ammonia lyases (MALs) and (vii) galactarate dehydratases (GalcDs) (Babbitt *et al.*, 1996; Lukk *et al.*, 2012). The overall reaction of the enolase super-

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Table 1

Macromolecule-production information.

Target molecule	ACI
Source organism	<i>Vibrio</i> sp. strain EJY3
DNA source	<i>Vibrio</i> sp. strain EJY3
Forward primer (43-mer)	5'-GAAGGAGATATAAGGATGAAAACAACA ATCAAAGACATCAAAA-3'
Reverse primer (38-mer)	5'-ATGATGGTGATGGTGCACCTTCGTACTG AGCAATTTTGT-3'
Cloning vector	<i>E. coli</i> DH5 α
Expression vector	pJL
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MKTTIKDIKTRLFKIPLEKILSDAKHGHDH HFELITTTVTLEDGSQGTGYTYTGKGG YSIKAMLEYDIQPALIGKDATQIEEYD FMEWHIHYVGRGGISTFAMSAVDIALWD LKGKREGLPLWKAGGKNNCKAYCGGI DLQFPLEKLLNNICGYLES GFNAVKIKI GRENMQEDIDRIKAVRELIGPDITFMID ANYSITVEQAIIKLSKAVEQYDITWFEEP TLPDDYKGF AEIADNTAIPAMGENLHT IHEFGYAMDQAKLGYCQPDASNCGGITG WLKAADLITEHNIPVCTHGMQELHVSIV SAFDTGWLEVHSFPIDEYTKRPLVVENF RAVASNEPGIGVEFDWDKIAQYEVHHHH HH

family involves the Mg²⁺-assisted enolization of a carboxylate anion substrate (Gerlt *et al.*, 2005). The total number of members of the enolase superfamily is greater than 8000, but the exact molecular function of many of the members remains unknown or unclear (Gerlt *et al.*, 2012). Based on the sequence–structure–function relationship, the ACI enzyme belongs to the MR subgroup of enzymes (Yun, Lee *et al.*, 2015). However, the crystal structure of ACI, which has molecular function towards the novel substrate AHGA, is unclear. The structure is essential to study the mechanism of the ACI enzyme. In this study, we report the cloning, expression, purification, crystallization and X-ray crystallographic analysis of ACI from *Vibrio* sp. strain EJY3.

2. Materials and methods

2.1. Macromolecule production

The *aci* gene from *Vibrio* sp. strain EJY3 (gene ID 11666919), which encodes 362 amino-acid residues, was amplified by PCR from the genomic DNA of *Vibrio* sp. strain EJY3 using the forward and reverse primers shown in Table 1. The PCR products were cloned into the pJL plasmid, which is a modified pET-21a vector with a six-histidine tag at the

Table 2

Crystallization of ACI from *Vibrio* sp. strain EJY3.

Method	Sitting-drop vapour diffusion for initial screening; hanging-drop vapour diffusion for crystal optimization
Plate type	96-well Intelli-Plates for initial crystal screening; 24-well VDX plate for crystal optimization
Temperature (K)	295
Protein concentration	15 mg ml ⁻¹ for initial screening and crystal optimization
Buffer composition of protein solution	20 mM Tris–HCl pH 8.0
Composition of reservoir solution	0.2 M magnesium chloride hexahydrate, 0.1 M bis-tris pH 6.5, 25% (w/v) polyethylene glycol 3350
Volume of reservoir	70 μ l for initial screening; 500 μ l for crystal optimization

C-terminus (Lee & Kim, 2009). The cloned vector with the *aci* gene was transformed into *Escherichia coli* BL21 (DE3) cells. The cells were grown in 1 l Luria–Bertani (LB) medium (BD, USA) containing 100 μ g ml⁻¹ ampicillin at 310 K until the OD₆₀₀ reached 0.8, and protein expression was then induced by 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 289 K for 24 h in a shaking incubator (150 rev min⁻¹). The cells were harvested by centrifugation at 5000 rev min⁻¹ for 40 min at 277 K. The harvested cells were lysed by sonication in lysis buffer (20 mM Tris–HCl pH 8.0). To obtain soluble protein, the lysate was centrifuged at 18 000 rev min⁻¹ for 40 min at 277 K. The recombinant protein was purified by Ni²⁺-affinity chromatography using a 5 ml HisTrap HP column (GE Healthcare, USA). The column was equilibrated with 20 mM Tris–HCl pH 8.0. The *Vibrio* sp. strain EJY3 ACI (*Vej*ACI) protein was eluted with a linear gradient of elution buffer (20 mM Tris–HCl pH 8.0, 500 mM imidazole). The eluted protein was further purified by anion-exchange chromatography using a 5 ml HiTrap Q FF column (GE Healthcare, USA) equilibrated with 20 mM Tris–HCl pH 8.0 and eluted with a linear gradient of 0–500 mM NaCl in the same buffer.

Buffer exchange of the *Vej*ACI protein was performed using a HiPrep 26/10 desalting column (GE Healthcare, USA) pre-equilibrated with 20 mM Tris–HCl pH 8.0, and the protein was concentrated using a 30 kDa molecular-weight cutoff Centricon centrifugal filter (Millipore, USA) to 15 mg ml⁻¹ for crystallization. The concentration of the purified *Vej*ACI was determined using the Bradford assay kit (Bio-Rad, USA) and BSA was used for calibration.

Figure 1

Scheme showing the initial metabolic pathway of AHG.

2.2. Crystallization

Initial crystallization of *Vej*ACI was attempted by the sitting-drop vapour-diffusion method using Crystal Screen HT, Index HT, Natrix (Hampton Research), Wizard 1 & 2 and Wizard 3 & 4 (Rigaku Reagents) in 96-well Intelli-Plates (Hampton Research). All drops were made by mixing 0.2 μ l protein solution (15 mg ml⁻¹) with an equal volume of reservoir solution on 96-well plates and were equilibrated against 70 μ l reservoir solution at 295 K. Crystal optimization was carried out by the hanging-drop vapour-diffusion method at various pH values and precipitant concentrations. Bipyramidal crystals of *Vej*ACI were obtained in 0.2 M magnesium chloride hexahydrate, 0.1 M bis-tris pH 6.5, 25%(w/v) polyethylene glycol 3350 at 295 K after one week (Table 2). The diffraction-quality crystals grew to 0.2 $\times$ 0.15 $\times$ 0.15 mm in size.

2.3. X-ray data collection and processing

Crystals were mounted on a nylon loop (Hampton Research, USA). X-ray diffraction data were collected on beamline 7A at Pohang Accelerator Laboratory (PAL). The best diffraction data for *Vej*ACI were collected over a 360° oscillation range at a wavelength of 0.97934 Å. All diffraction images were recorded using an ADSC Quantum 270r detector at a crystal-to-detector distance of 250 mm. All data were processed using *HKL*-2000 (Otwinowski & Minor, 1997). The Matthews coefficient was calculated using the *CCP4* program suite (Winn *et al.*, 2011). The data-processing statistics are summarized in Table 3.

3. Results and discussion

The *Vibrio* sp. strain EJY3 *aci* gene was cloned with a six-histidine affinity tag at the C-terminus. The recombinant plasmid with the *aci* gene was transformed into *E. coli* BL21

Table 3

Data-collection statistics for *Vej*ACI.

Diffraction source	Beamline 7A, PAL
Wavelength (Å)	0.97934
Temperature (K)	100
Detector	ADSC Quantum 270r
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	360
Space group	<i>P</i> ₄ ₁ ₂ ₁ 2 or <i>P</i> ₄ ₃ ₂ ₁ 2
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 87.9, <i>c</i> = 143.5, $\alpha = \beta = \gamma = 90$
Resolution (Å)	50.00–2.20 (2.24–2.20)
<i>R</i> _{merge} † (%)	13.7 (38.4)
Completeness (%)	99.9 (100)
Total No. of reflections	397120
No. of unique reflections	29525
Multiplicity	13.5 (8.4)
$\langle I/\sigma(I) \rangle$	15.8 (4.5)
Overall <i>B</i> factor from Wilson plot (Å ²)	20.0

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\sum_{hkl}$ denotes the sum over all reflections and $\sum_i$ is the sum over all equivalent and symmetry-related reflections.

(DE3) cells for overexpression. Recombinant *Vej*ACI protein expressed in *E. coli* BL21 (DE3) cells was purified by a two-step purification comprising Ni²⁺-affinity chromatography and anion-exchange chromatography. The protein eluted from the HisTrap HP column at 130 mM imidazole and from the HiTrap Q FF anion-exchange column at 250 mM NaCl. For crystallization, the buffer was exchanged to 20 mM Tris–HCl pH 8.0 using a HiPrep 26/10 desalting column (GE Healthcare, USA). The molecular weight and purity of the *Vej*ACI protein were verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Fig. 2). The activity of *Vej*ACI has been reported previously (Yun, Lee *et al.*, 2015). *Vej*ACI was confirmed to be present in a monomeric form by size-exclusion chromatography (data not shown).

We performed crystallization screening by the sitting-drop vapour-diffusion method using approximately 500 conditions.

Figure 2

SDS–PAGE analysis of purified *Vej*ACI. Lane *M*, molecular-weight marker (labelled in kDa). Lane 1, lysate from non-induced cells. Lane 2, supernatant (soluble fraction). Lane 3, purified *Vej*ACI from the HisTrap HP column. Lane 4, purified *Vej*ACI from the HiTrap Q FF column

Figure 3

Crystals of *Vej*ACI obtained by the hanging-drop vapour-diffusion method at 295 K.

Figure 4
X-ray diffraction image of a *VejACI* crystal recorded on beamline 7A at Pohang Accelerator Laboratory (PAL).

Needle-shaped crystals of *VejACI* were obtained from Crystal Screen condition No. 18 [0.2 M magnesium acetate tetrahydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, 20% (w/v) polyethylene glycol 8000]. Bipyramid-shaped crystals were obtained from Index HT condition No. 83 consisting of 0.2 M magnesium chloride hexahydrate, 0.1 M bis-tris pH 6.5, 25% (w/v) polyethylene glycol 3350 (Fig. 3). Attempts were made to optimize the crystallization conditions for *VejACI* by varying the concentration of PEG 3350 (15, 20, 25 and 30%) and the pH of the buffer (6.0–7.0) based on the initial crystallization conditions. The best crystal of *VejACI* was obtained from the initial crystallization conditions.

The bipyramid-shaped crystals diffracted to 2.20 Å resolution using synchrotron radiation at PAL (Fig. 4). The crystals belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 87.9$, $c = 143.5$ Å. The Matthews coefficient (Matthews, 1968) was $2.3 \text{ Å}^3 \text{ Da}^{-1}$, with a solvent content of 47%, suggesting that the asymmetric unit contained one *VejACI* molecule. A total of 397 120 reflections in the

resolution range 50.00–2.20 Å were merged into 29 525 unique reflections with an R_{merge} of 0.137. The ACI enzyme has substrate specificity towards the novel metabolite AHGA. The crystal structure of the ACI enzyme involved in the molecular function of AHGA is unknown. Therefore, the crystal structure of ACI is required to investigate the reaction mechanism of ACI on AHGA at the molecular level. Structural analysis of ACI will provide a guide for the design of an efficient biocatalyst for use in the production or bioconversion of AHGA. Structure determination of *VejACI* by molecular replacement is in progress.

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