Communication

Purification and Crystallographic Analysis of a Novel Cold-Active Esterase (HaEst1) from Halocynthiibacter arcticus

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Abstract: This report deals with the purification, characterization, and a preliminary crystallographic study of a novel cold-active esterase (HaEst1) from Halocynthiibacter arcticus. Primary sequence analysis reveals that HaEst1 has a catalytic serine in G-x-S-x-G motif. The recombinant HaEst1 was cloned, expressed, and purified. SDS-PAGE and zymographic analysis were carried out to characterize the properties of HaEst1. A single crystal of HaEst1 was obtained in a solution containing 10% (w/v) PEG 8000/8% ethylene glycol, 0.1 M Hepes-NaOH, pH 7.5. Diffraction data were collected to 2.10 Å resolution with P21 space group. The final Rmerge and Rp.i.m values were 7.6% and 3.5% for 50–2.10 Å resolution. The unit cell parameters were a = 35.69 Å, b = 91.21 Å, c = 79.15 Å, and β = 96.9°.

Keywords: esterase; enzyme assay; crystallization; diffraction

1. Introduction

Microbial esterase catalyzes the reaction of formation and hydrolysis of chemical bonds between hydroxyl and carboxylic acid groups, which could be used in the preparation of many biological products such as foods, flavors, cosmetics, drugs, and agrochemicals [1–3]. In addition, they are employed in the degradation of carbanates, pesticides, polymer-based plastics, and industrial wastes. These enzymes share the characteristic α/β hydrolase fold, conserved catalytic triad of Ser-His-Asp/Glu, catalytic strategies, substrate specificities, and a lack of cofactors. It has been shown that esterases perform catalytic reactions via a nucleophilic attack on the substrates [1,4–6].

Cold-active enzymes show relatively high activity at low temperatures compared to their mesophilic or thermophilic proteins [7,8]. Due to high demands, there have been a lot of studies to characterize cold-active enzymes [9]. To date, cold-active esterases were identified from Paenibacillus sp. [10], Pseudomonas mandelii [11], Lactobacillus plantarum [12], and Bacillus halodurans [13]. However, limited information is still available on the structure and function of these enzymes [7–9].

Halocynthiibacter arcticus is a rod-shaped Gram-negative bacteria from the Arctic region, which could be a valuable resource for biotechnological applications [14,15]. Specifically, structural information of enzymes from this bacterium is largely unknown. Here, we report the identification, purification, and preliminary crystallographic analysis of a novel cold-active esterase (HaEst1) from H. arcticus. The recombinant enzyme was purified, characterized, and crystallized for structural studies. Considering that cold-active esterases
have great importance for industrial applications, Halostaphylospora esterase could be an interesting industrial enzyme with cold-active properties.

2. Materials and Methods

2.1. Chemicals and Columns

Nucleic acid modifying enzymes and DNA purification kits were purchased from New England BioLabs (Ipswich, MA, USA) and Intron Korea (Daejon, Korea). PD-10 column for dialysis and His-tag affinity column for purification were obtained from GE Korea (Seoul, Korea). All other reagents of high purity grade were obtained from Sigma-Aldrich (Goyang-si, Korea).

2.2. Gene Cloning and Protein Purification

Microbial culture of Halocynthiaibacter arcticus (Korea Collection of Type Cultures, KCTC 42129) and purification of its chromosomal DNA were carried out as previously described [16]. The open reading frame of the HaEst1 gene was amplified and cloned into a pET-21a vector (pET-HaEst1) using the following primers with NheI and XhoI (forward primer: 5’-GTAACCGCTAGCATGACAGACCCACAG-3’, and reverse primer: 5’-GCTGACTCGAGTCAGAATTTCGCCCG-3’). E. coli BL21(DE3) cells were transformed and grown in LB medium at 18 °C with 1 mM isopropyl-β-D-1-thiogalactoside (IPTG) induction. Then, bacterial cells were centrifuged and resuspended in a cell disruption buffer (20 mM Tris-HCl pH 8.5, 150 mM NaCl, 20 mM imidazole). Following cell lysis by ultrasonication, supernatants were loaded onto a His-Trap affinity column. HaEst1 was eluted by a gradient of imidazole method (from 50 mM to 200 mM). The fractions were buffer-changed with PD-10 column. The final proteins were collected and stored at −20 °C.

2.3. Activity Measurement of HaEst1

Zymographic assay of HaEst1 was performed as previously described [17–19]. Protein purities and concentrations of HaEst1 were determined using SDS-PAGE and Bio-Rad Protein Assay kit. Enzymatic activity of HaEst1 was studied using 4-methylumbelliferyl (4-MU) acetate and 4-methylumbelliferyl (4-MU) phosphate as substrates. The fluorescence of 4-methylumbelliferone was observed in an Eppendorf tube using UV incubation box. Intrinsic fluorescence was measured using a Jasco FP-6200 spectrofluorometer (Easton, MD, USA).

2.4. Crystallization Method

For effective crystallization trials, purified HaEst1 (20.0 mg/mL) was used with crystallization kits including MCSG 1T~4T (Anatrace), JCSG-plus (Molecular Dimensions), and PGA Screen (Molecular Dimensions) [20,21]. The screening process was carried out using an automated crystallization robot (SPT Labtech, San Diego, CA, USA). Initial droplets contained 300 nL of protein solution mixed with 300 nL of reservoir solution by sitting-drop vapor-diffusion method in a 96-well plate. Various crystals of HaEst1 appeared in several conditions within a week. Diffraction-quality single crystals of HaEst1 were observed under JCSG-Plus #16 condition of 10% (w/v) PEG 8000/8% ethylene glycol, 0.1 M Hepes-NaOH, pH 7.5.

2.5. Data Collection and Processing

A single crystal of HaEst1 was removed and transferred to a cryo-protectant solution. After gentle soaking, the crystal was mounted on a synchrotron facility at beamline 5C of the Pohang Light Source (PAL, Pohang, Korea). X-ray diffraction data of HaEst1 were collected at 100 K using the Eiger X 9M detector (Dectris, Switzerland). For complete X-ray diffraction data collection, the cryo-cooled crystal was rotated throughout 360° rotation with 1° oscillation per frame. Finally, collected X-ray data were processed and indexed using HKL2000 (see Table 1). Sequences of HaEst1 and other enzymes were obtained from a public NCBI server, and multiple sequence alignment was prepared using ESPript.
Table 1. Data collection and processing statistics of HaEst1.

| Data Collection |  |
|-----------------|------------------|
| Synchrotron source | PAL-Korea, beamline 5C |
| Wavelength (Å) | 0.9794 |
| Temperature (K) | 100 |
| Detector | Eiger X 9M |
| Crystal to detector distance (mm) | 400 |
| Rotation range per image (°) | 1 |
| Exposure time per image (s) | 1 |
| Rotation range (°) | 360 |

| Data Processing |  |
|-----------------|------------------|
| Crystal parameters |  |
| Space group | P2₁ |
| No. of molecules in asymmetric unit | 2 |
| Unit-cell parameters (Å, °) | A = 35.69, b = 91.21, c = 79.15, α = 90, β = 96.91, γ = 90 |

| Data Statistics |  |
|-----------------|------------------|
| Resolution range (Å) | 50.00–2.10 (2.14–2.10) |
| Total no. of collected reflections | 186,387 (122) |
| No. of unique reflections | 29,269 (1435) |
| Completeness (%) | 99.0 (99.6) |
| Multiplicity | 6.4 (0.1) |
| $\langle I/\sigma(I)\rangle$ | 47.8 (7.67) |
| $R_{merge}$ (%) † | 8.1 (36.3) |
| $R_{meas}$ (%) ‡ | 8.8 (39.8) |
| CC(1/2) (%) | 99.1 (94.7) |
| Overall B-factor from Wilson plot (Å²) | 39.7 |

† $R_{merge} = \sum_{hkl} |I − \langle I\rangle| / \sum_{hkl} I$.
‡ $R_{meas} = \sum_{hkl} [N(hkl) − \langle N(hkl) \rangle − 1]^2 \sum_{hkl} |I(hkl)| − \langle |I(hkl)| \rangle / \sum_{hkl} |I(hkl)|$.

3. Results and Discussion

An open reading frame encoding a novel cold-active esterase (HaEst1, locus tag: WP_039000957, 756 bp) was detected and obtained from the *H. arcticus* chromosome. HaEst1 has 252 amino acids with a pl of 5.21. Multiple sequence alignments of HaEst1 with three related proteins indicated that HaEst1 showed significant sequence identities with a putative hydrolase from *Agrobacterium vitis* (3LLC, 40.0%), a new family of carboxyl esterase with an OsmC domain from *Rhodothermus marinus* (5CML, 19.8%) [22], and a cinnamoyl esterase from *Lactobacillus johnsonii* LJ0536 (3PF8, 20.6%) [23] (Figure 1). Interestingly, HaEst1 showed substantial sequence similarity to an alpha/beta hydrolase domain-containing protein 10 (ABHD10) from *Mus musculus* (6NY9, 23.8%). Catalytic triad of Ser-His-Asp as well as G-x-S-x-G motif were also conserved in these two proteins. This ABHD10 was recently shown to be an S-depalmitoylase affecting reduction/oxidation homeostasis [24].

Highly conserved catalytic residues of Ser101, Asp198, and His228 were identified, with Ser101 located in a typical GXGXSXG motif. Sequence analysis revealed that HaEst1 has a high number of small amino acids such as Gly (11.9%) and Ala (9.9%). In addition, high percentages of Leu (9.1%) and Thr (7.9%) were also observed. The percentage of acidic amino acids (Asp + Glu) was larger than that of basic amino acids (Arg + Lys).

The recombinant HaEst1 was purified using an immobilized His-tag metal-binding column (Figure 2A). The enzymatic activity of HaEst1 was examined using 4-methylumbelliferyl (4-MU) acetate. As shown in Figure 2B, strong fluorescence due to hydrolysis reaction was observed for 4-MU acetate, although little hydrolysis reaction was carried out for control or 4-MU phosphate.
Figure 1. Multiple sequence alignments of HaEst1. Identical and highly conserved amino acids among these proteins are displayed in red and yellow.

Figure 2. Purification and hydrolytic activity of HaEst1. (A) SDS-PAGE analysis of HaEst1. Arrow indicates the position of purified HaEst1. (B) Hydrolysis of 4-MU acetate was examined with HaEst1. Strong fluorescence was observed due to hydrolysis reaction by HaEst1.

The diffraction-quality crystals grew to final dimensions of 0.6 × 0.5 × 0.2 mm within three days at 297 K (Figure 3), which were transferred to a paratone oil, cryo-protectant solution. The diffraction data set of HaEst1 was indexed to P2₁ space group with unit cell parameter of a = 35.69 Å, b = 91.21 Å, c = 79.15 Å, and β = 96.9°. The final data were processed using HKL2000 to 2.10 Å resolution with 99.9% completeness. The final Rmerge and Rp.i.m values were 8.1% and 3.5% for 50–2.10 Å resolution. The final data collection statistics are summarized in Table 1. Assuming two molecules of HaEst1 per asymmetric unit, Matthews coefficient (V_M) and solvent content were calculated to be 2.36 Å³/Da and 47.9% [25].
We tried to solve the HaEst1 structure using the molecular replacement method using MOLREP [26]. The crystal structure of a putative hydrolase from Agrobacterium vitis (PDB code 3LLC) was used as a search model for the cross-rotation search. For the cross-rotation function calculation, we used data in the resolution range of 39.44–2.41 Å. The results of the cross-rotation showed that the highest peak height was above 7.49σ. The highest peak solution of the rotation function was used for the following translation function search. The solution model also gave a strong correlation coefficient value (above 0.52) in the translation function. Rigid body refinements and individual restrained B-factor refinements were performed using REFMAC5 [27]. After these refinement steps, an interpretable electron density map was calculated, as shown in Figure 4. Model building and further refinement are now underway. Furthermore, substrate or product bound HaEst1 structure determination with site-directed mutagenesis experiments will be performed. Thus, a detailed structural analysis of HaEst1 and protein engineering results will be published in an upcoming research paper.

![Crystal Image](image1.png)

**Figure 3.** Representative crystal image (left) and diffraction pattern (right) of HaEst1. The crystal has final dimensions of 0.6 × 0.5 × 0.2 mm.

![Electron Density Map](image2.png)

**Figure 4.** Electron density map of a portion of HaEst1 structure contoured at 2σ. The initially built Cα trace is shown in green and deep teal color for each chain. The figure was made with Pymol.
nological applications. Furthermore, HaEst1 could be further improved/mutated through protein or genetic engineering for its useful applications.

Author Contributions: S.J., W.Y. and J.W.C. identified and purified HaEst1. J.H., H.D. and H.-W.K. got crystals and diffraction data. J.H.L., K.K.K. and T.D.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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