

Communication

Sequence Analysis and Preliminary X-ray Crystallographic Analysis of an Acetylcysteine Esterase (LgEstI) from *Lactococcus garvieae*

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Abstract: A gene encoding LgEstI was cloned from a bacterial fish pathogen, *Lactococcus garvieae*. Sequence and bioinformatic analysis revealed that LgEstI is close to the acetyl esterase family and had maximum similarity to a hydrolase (UniProt: Q5UQ83) from *Acanthamoeba polyphaga mimivirus* (APMV). Here, we present the results of LgEstI overexpression and purification, and its preliminary X-ray crystallographic analysis. The wild-type LgEstI protein was overexpressed in *Escherichia coli*, and its enzymatic activity was tested using *p*-nitrophenyl of varying lengths. LgEstI protein exhibited higher esterase activity toward *p*-nitrophenyl acetate. To better understand the mechanism underlying LgEstI activity and subject it to protein engineering, we determined the high-resolution crystal structure of LgEstI. First, the wild-type LgEstI protein was crystallized in 0.1 M Tris-HCl buffer (pH 7.1), 0.2 M calcium acetate hydrate, and 19% (*w/v*) PEG 3000, and the native X-ray diffraction dataset was collected up to 2.0 Å resolution. The crystal structure was successfully determined using a molecular replacement method, and structure refinement and model building are underway. The upcoming complete structural information of LgEstI may elucidate the substrate-binding mechanism and provide novel strategies for subjecting LgEstI to protein engineering.

Keywords: esterase; *Lactococcus garvieae*; X-ray crystallography



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1. Introduction

Esterases (E.C. 3.1.1.X) catalyze the hydrolysis of various substrates containing ester groups. Esterases are serine hydrolases that contain a conserved Ser-Asp/Glu-His catalytic triad with an α/β hydrolase fold. Although esterases harbor the same α/β hydrolase fold and have high sequence homology, they have different substrate specificities and perform varying biological functions. Recently, esterases of microbial origin have gained significant interest in scientific research because of their potential applications in the biotechnology industry [1,2]. Therefore, extensive efforts are being made to identify unique esterases with higher activity, improved stability, and broad substrate specificity from newly found microorganism genomes as well as metagenomes. Such esterases can be further subjected to protein engineering to generate esterases with a precise structure and desirable functions [3].

To date, many microbial esterases have been discovered and characterized [4,5]. In particular, pathogenic bacteria have been well-known for producing various extracellular esterases. These secreted esterases may be important for the virulence and pathogenesis

of some bacteria. In addition, some pathogenic bacterial esterases are involved in the drug resistance mechanism by enzymatic cleavage of antibiotics [6,7]. However, detailed enzymatic activity differences and the structural information of pathogenic bacterial esterases are still unclear. Recently, the complete genome sequence information of a major fish pathogen bacteria, *Lactococcus garvieae*, has been published. *Lactococcus garvieae* is a Gram-positive bacterium with a sphere-shape (cocci) [8].

In this study, several putative esterase encoding genes of *Lactococcus garvieae* were identified as targets and sequentially tested. In this study, we cloned the LgEstI of *L. garvieae* into a plasmid vector to overexpress the LgEstI protein in *Escherichia coli*. To obtain structural information, we crystallized LgEstI to perform initial X-ray crystallographic experiments and successfully determined its structure. Further structural refinements and model building are underway. We believe that structural analysis of LgEstI in the near future will add further value to our biochemical analysis and facilitate a better understanding of the potential application of LgEstI, as well as provide new insights for further engineering of this protein.

2. Materials and Methods

2.1. Protein Clustering

ProtBLAST/PSI-BLAST was used to detect the remote homologs [9]. Initially, the LgEstI sequence was blasted against the Protein Data Bank (PDB) database, and the result (E-value cutoff for reporting = 1×10^{-10}) was reloaded for performing a second blast against the Uniprot_sport database. Finally, the data with full-length sequences were forwarded to CLANS [10] for clustering by sequence similarity. The data were visualized with connecting lines, and shorter lines indicated higher sequence similarity.

2.2. Esterase Activity

p-Nitrophenyl esters were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA) and used as substrates to assay LgEstI activity. LgEstI activity with different lengths of acyl carbon chains was determined by monitoring the production of *p*-nitrophenol (PNP) spectrophotometrically as previously described, with minor modifications [11].

2.3. Gene Cloning, Expression, and Purification of Recombinant LgEstI Protein

Genomic DNA of *L. garvieae* was isolated using a DNA extraction kit according to the manufacturer's instructions (Bioneer, Daejeon, Korea). The CDS of LgEstI (NCBI accession number: WP_042219410.1) was amplified by performing PCR using appropriate primers. The PCR product was cloned into the pET21a vector between the *Nde*I and *Xho*I restriction sites. For protein expression, cells of the *E. coli* BL21 (DE3) strain were transformed with the plasmid harboring LgEstI (Table 1). Fresh transformants were grown at 37 °C in 4 L of Luria–Bertani medium containing 50 µg/mL ampicillin. When the O.D.₆₀₀ reached 0.5, protein overexpression was induced by adding 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cell culture, which was then continued for 20 h at 20 °C. Next, cells were pelleted by 20 min of centrifugation at 4000 rpm, resuspended in a lysis buffer (NPI-20, 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole), and disrupted by ultrasonication at 30% amplitude. An ice bath was used to maintain the temperature below 40 °C. The cell debris was removed by 1 h of centrifugation at 16,000 rpm and 4 °C, and the supernatant was used for the purification step.

The recombinant LgEstI was purified using gravity-flow purification as the initial step. To eliminate the remaining free nickel, a pre-packed column with His-tagged resin was washed with 5 mL of NPI-20. The supernatant containing recombinant LgEstI was slowly loaded onto the column and then washed by applying 10 CV with NPI-20. Next, the purified recombinant LgEstI was eluted with NPI-300 (50 mM NaH₂PO₄, 300 mM NaCl, and 300 mM imidazole). The elution was concentrated to 5 mL and incubated with thrombin for 2 days in a rotating incubator at 4 °C to cleave the His tag. For the second purification, the tag-removed sample was loaded onto a Superdex prep grade column

(HiLoad[®] 16/600 Superdex[®] 200 pg) equilibrated with 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) for SDS-PAGE. The Bradford protein assay was performed to confirm the purity and concentration of the recombinant *LgEstI*.

Table 1. Recombinant *LgEstI* protein production information.

<i>LgEst</i>	
Source organism	<i>Lactococcus garvieae</i>
DNA source	Genomic DNA
Cloning vector	pET21a
Expression host	<i>Escherichia coli</i> BL21 (DE3)
Amino acid sequence	MVERISLEKAALEFSEANAPHPRIYELPVEEGRSLNEV QDSPVVKEDVDIEDIAVDTGEWGEINVRFIRPLHQEKKL PVIFYIHGAGWVFGNAHTHDKLIRELAVRTNSVVFSE YLSPEAKYPTAIEQNYAVLQQLKDFANDKKFDVNHLT VAGDSVGGNMATVMTLLTKQRGGQKIGQQVLYYPVT DANFDTDSYNEFAENYFLTKEGMIWFDQYTTSQEER HQITASPLRATKEDLADLPAALIITGEADVLRDEGEAYA RKLREADVEVTQVRFQAIHDFVMVNSMNETHATRAA MSLSTQWINEKNRK

2.4. *LgEstI* Crystallization, Data Collection, and Phasing

More than 1000 different crystallization screening solutions were screened for the initial crystallization trial using recombinant *LgEstI* (25 mg/mL) by the sitting-drop vapor diffusion method. The crystallization conditions established from the initial screening were further optimized to generate diffraction-quality crystals (Table 2). Diffraction data were collected at the 5C beamline of the PLS, Korea. A single square pillar crystal was mounted on a goniometer equipped with a nitrogen stream with perfluoropolyether cryo-oil (Hampton Research, Laguna, CA, USA) as a cryoprotectant, with an oscillation range of 1°. Native data diffracting to 2.0 Å were collected, and processing and reduction were performed using XDS [12]. The *LgEstI* phase was obtained by molecular replacement using the I-TASSER model [13]. Next, the coordinates of *LgEstI* were built by iterative model building using a combination of Coot and Autobuild. Refinement of *LgEstI* was performed using phenix.refine from Phenix. The details of selected X-ray data collection statistics are listed in Table 3.

Table 2. Crystallization details.

Method	Vapor Diffusion
Plate type for screening	96-well sitting drop MRC plate (Molecular Dimension, Suffolk, UK)
Plate type for optimization	24-well hanging drop plate (Molecular Dimension, Suffolk, UK)
Temperature (°C)	22
Protein concentration (mg/mL)	25
Composition of protein solution	20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1 mM TECP
Composition of reservoir solution	0.1 M Tris: HCl (pH 7.1), 0.2 M calcium acetate hydrate, and 19% (w/v) PEG 3000
Volume and ratio of drop	2 µL, 1:1
Volume of reservoir (µL)	500

Table 3. X-ray diffraction data collection statistics.

Data Collection	LgEstI
Wavelength (Å)	0.97949
X-ray source	BL-5C beamline
Rotation range per image (°)	1
Exposure time (s)	0.1
Space group	$I2_1$
Unit-cell parameters (Å, °)	$a = 54.41, b = 92.77, c = 218.11, \alpha = \gamma = 90.0, \beta = 96.61$
Resolution range (Å) ^a	28.49–2.0 (2.05–2.0)
No. of observed reflections ^a	494,404
No. of unique reflections ^a	71,847 (4652)
Completeness (%) ^a	98.7 (99.8)
Redundancy ^a	6.9 (7.2)
R_{sym} ^{a,b}	0.059 (0.540)
I/σ ^a	16.1 (2.8)
Mosaicity	0.36
$CC\frac{1}{2}$ ^{a,c}	(99.7%)

^a Values in parentheses correspond to the highest resolution shell. ^b $R_{\text{sym}} = \sum_i \sum_j |I(h)_i - \langle I(h) \rangle| / \sum_i \sum_j I(h)_i$, where I is the intensity of the reflection h , \sum_i is the sum over all reflections, and \sum_j is the sum over i measurements of reflection h . ^c Percentage correlation between intensities from random half-datasets [14].

3. Results

3.1. Clustering Analysis of LgEstI

Clustering analysis of LgEstI was performed against the PDB and UniProt databases for primary and secondary blasting, respectively, using the MPI Bioinformatics Toolkit. Available online: <https://toolkit.tuebingen.mpg.de> (accessed on 6 December 2021) [9]. In total, 170 proteins were detected and aligned to visualize these relationships. Four main clusters were identified from the clustering analysis. The acetyl esterase family is tightly clustered and shows a high degree of sequence similarity. The second cluster included a combination of carboxylesterase, acetylcholinesterase, and para-nitrobenzyl esterase. Arylacetamide deacetylases and tuliposide A-converting enzymes were grouped into separate clusters. Interestingly, LgEstI could not be assigned to any of these clusters and showed maximum similarity to a hydrolase (UniProt: Q5UQ83) of *Acanthamoeba polyphaga mimivirus* (APMV) within the UniProt database. Based on the length of the connection and the color indicating sequence similarity, LgEstI appeared close to the acetyl esterase group but was not part of it. The clustering analysis confirmed that LgEstI has a unique sequence and may have a distinctive function from the other enzyme clusters (Figure 1).

3.2. Purification and Biochemical Characterization of LgEstI

To conduct a preliminary study for understanding the detailed functional mechanisms of LgEstI, we cloned the LgEstI of *L. garvieae* into a plasmid vector and overexpressed the LgEstI protein in *E. coli*. Purified LgEstI protein was used to perform preliminary X-ray crystallography. Two conventional purification steps, namely tag-affinity purification and size-exclusion chromatography, were applied to purify the recombinant LgEstI protein (>95%), and the final protein purity was checked on SDS-PAGE. The molecular weight of LgEstI was approximately 37 kDa, which is similar to the calculated molecular weight based on the amino acid sequence (Figure 2A). The enzyme activity assay using purified LgEstI protein with various *p*-nitrophenyl esters showed that LgEstI had a narrow substrate specificity, as it was active only against *p*-nitrophenyl acetate (Figure 2B). Thus, the clus-

tering and enzyme activity in combination indicated that *LgEstI* exhibits specific activity against analogs of acetate.

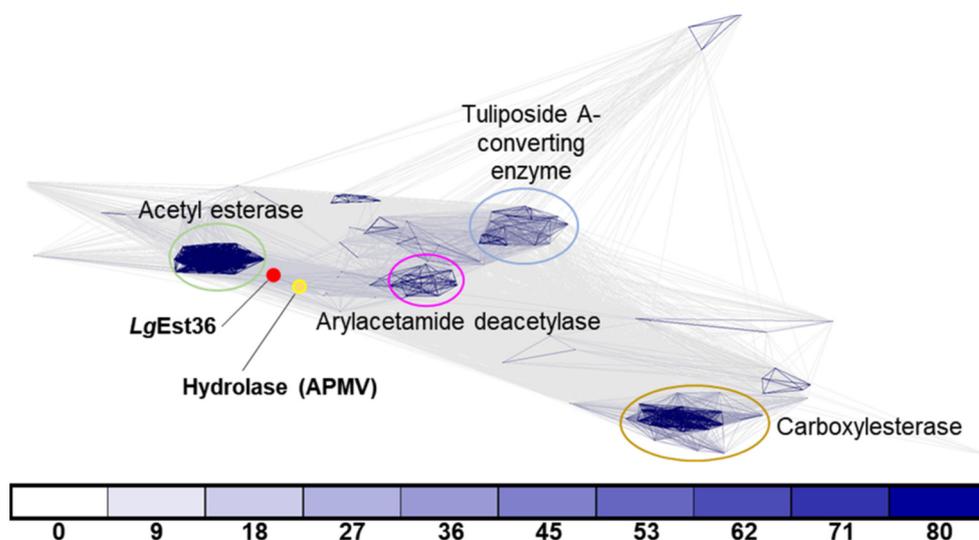


Figure 1. Sequences of orthologs from different species were used for clustering by CLANS clustering. ProtBLAST/PSI-BLAST was used to detect distantly related proteins using the Protein Data Bank (PDB) and Uniprot_sport databases. *LgEstI* and the hydrolase (UniProt: Q5UQ83) from *Acanthamoeba polyphaga mimivirus* (APMV) within the UniProt database are indicated by red and yellow dots, respectively. Connecting lines of darker intensity and shorter length indicate higher sequence similarity. Connections with a p -value better than 1×10^{-x} are drawn in the corresponding color (x = number below).

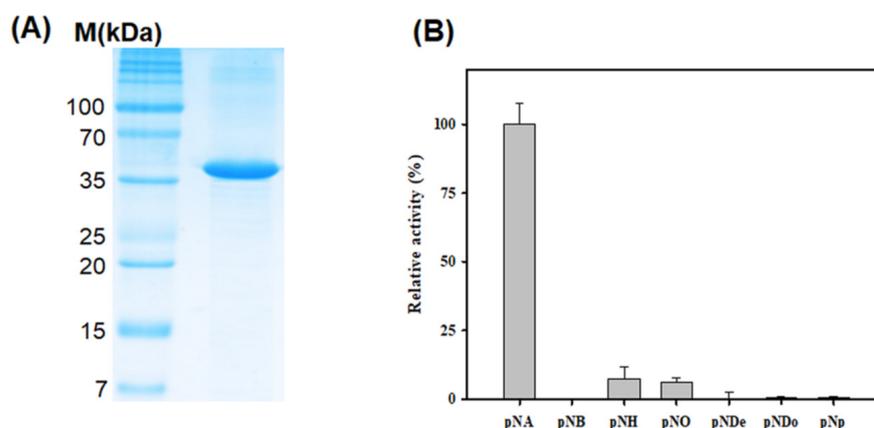


Figure 2. Purification and activity analysis of *LgEstI* using various p -nitrophenyl esters. (A) SDS-PAGE analysis of *LgEstI* purified by tag-affinity purification and size-exclusion chromatography. (B) The relative activities of *LgEstI* against p -nitrophenyl esters, expressed as percentages. This activity assay was carried out using various substrates (p NA: p -nitrophenyl acetate; p NB: p -nitrophenyl butyrate; p NH: p -nitrophenyl hexanoate; p NO: p -nitrophenyl octanoate; p NDe: p -nitrophenyl decanoate; p NDo: p -nitrophenyl dodecanoate; p Np: p -nitrophenyl phosphate). The activity of *LgEstI* with p -nitrophenyl acetate was defined as 100%.

3.3. X-ray Crystallographic Study of *LgEstI*

To obtain the factual structural information of *LgEstI*, we performed crystallization and initial X-ray crystallographic experiments. The crystallization conditions for *LgEstI* protein were screened using more than 1000 different crystallization buffers. After optimizing the crystallization conditions by changing the precipitant concentration and pH in the reservoir solution and drops, rhombus-shaped crystals (approximately $100 \times 200 \mu\text{m}$)

were obtained using 0.1 M Tris-HCl (pH 7.1), 0.2 M calcium acetate hydrate, and 19% (*w/v*) PEG 3000 (Figure 3A). However, X-ray diffraction of *LgEstI* crystals resulted in a poor diffraction pattern with a resolution of approximately 4 Å. Instead of searching for new crystallization conditions where *LgEstI* could be better diffracted, we aimed to optimize the cryoprotectant conditions. *LgEstI* crystals soaked in solutions containing perfluoropolyether cryo-oil (Hampton Research, Laguna, CA, USA) for 10 s exhibited a dramatic improvement in diffraction quality (~ 2 Å), whereas the crystals cryoprotected with general cryoprotectants (e.g., glycerol and PEG) showed low-resolution diffraction patterns (Figure 3B). Briefly, we tested more than 50 *LgEstI* crystals to find the optimal cryoprotectant solution. *LgEstI* crystals were very unstable in glycerol or PEG-containing cryoprotectant solutions, resulting in the *LgEstI* crystals melting and cracking. However, *LgEstI* crystals were more stable for a long time (more than 5 min) without melting or cracking in oil-based cryoprotectant solutions (Paratone-*N* oil and perfluoropolyether cryo-oil). We used the same conditions and similar sized crystals grown under the same crystallization conditions for this experiment. Finally, we obtained the best *LgEstI* crystals for X-ray diffraction data collection under the conditions with perfluoropolyether cryo-oil. During X-ray diffraction data collection, flash cooling the crystal in a nitrogen gas stream at 100 K prevented crystal cracking. We inferred that an oil-based solution could be a suitable cryoprotectant for *LgEstI*. All the diffraction data (completeness: 98.7%) were collected at a resolution of 2.0 Å. The data processing program XDS was used to index, integrate, and scale the acquired diffraction data [12].

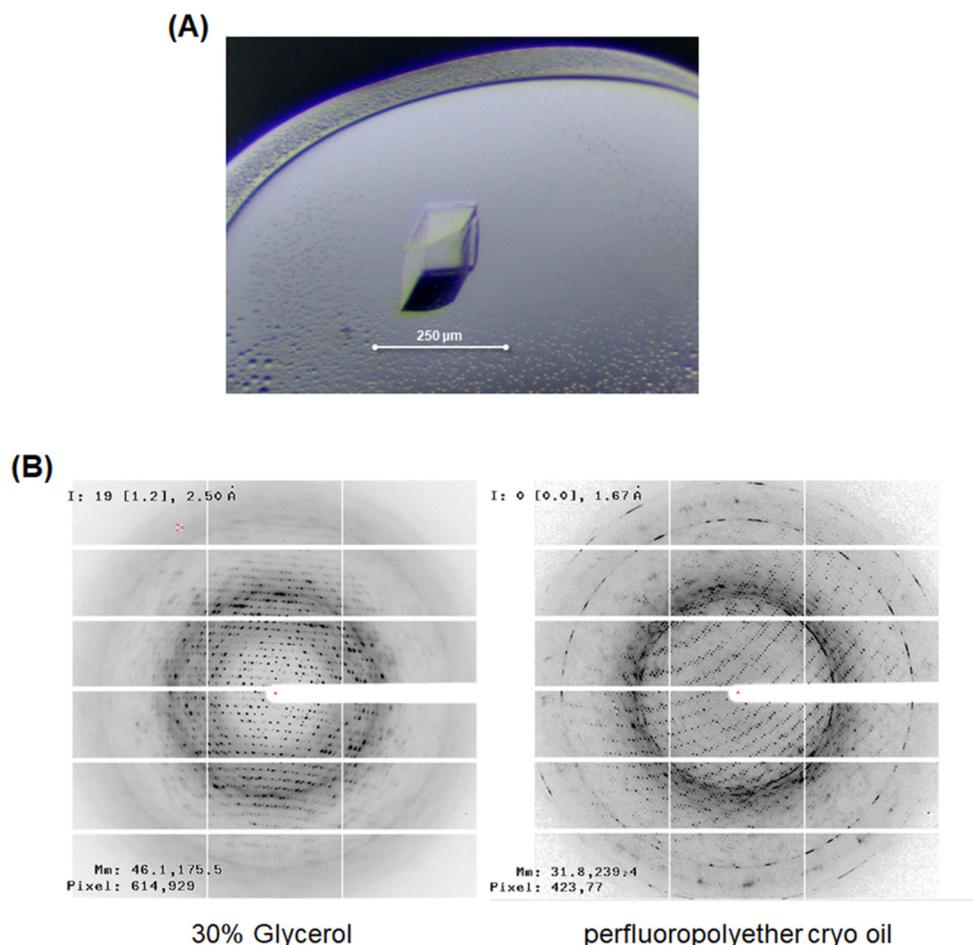


Figure 3. X-ray crystallographic study of *LgEstI*. (A) Crystal of *LgEstI* protein grown in 0.1 M Tris-HCl (pH 7.1), 0.2 M calcium acetate hydrate, and 19% (*w/v*) PEG 3000. (B) Cryoprotectant optimization for the X-ray diffraction study. *LgEstI* crystals soaked in solutions containing perfluoropolyether cryo-oil for 10 s and flash-frozen under a nitrogen stream are shown (right).

The initial structure of LgEstI was successfully determined. For phasing LgEstI, we first generated the LgEstI model based on the homologous structures using the protein structure prediction server I-TASSER [13]. The quality of the model was confirmed to have a C-score of 1.40 and a TM-score of 0.91 ± 0.06 , respectively. Next, the LgEstI model was used to overcome the phase problem using the molecular replacement method. Further structural refinements and model building are underway in our laboratory. We believe that a structural analysis of LgEstI in the near future will facilitate a better understanding of our biochemical analysis results, as well as providing valuable insights for further engineering of LgEstI protein.

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