Scientific Research Publishing

Advances in Bioscience and Biotechnology, 2018, 9, 215-223
http://www.scirp.org/journal/abb
ISSN Online: 2156-8502
ISSN Print: 2156-8456

Expression, Purification and Crystallization of Thermostable Mutant of Cutinase Est1 from *Thermobifida alba*

Kengo Kitadokoro[1*], Shingo Matsui[1], Ryouhei Osokoshi[1], Kensuke Nakata[1], Shigeki Kamitani[2]

[1]Departmentof Biomolecular Engineering, Graduate School of Science and Technology, Kyoto Institute of Technology, Kyoto, Japan
[2]Graduate School of Comprehensive Rehabilitation, College of Health and Human Sciences, Osaka Prefecture University, Osaka, Japan
Email: *kengo@kit.ac.jp

How to cite this paper: Kitadokoro, K., Matsui, S., Osokoshi, R., Nakata, K. and Kamitani, S. (2018) Expression, Purification and Crystallization of Thermostable Mutant of Cutinase Est1 from *Thermobifida alba*. *Advances in Bioscience and Biotechnology*, **9**, 215-223.
https://doi.org/10.4236/abb.2018.95015

Received: March 17, 2018
Accepted: May 27, 2018
Published: May 30, 2018

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Abstract

A double mutantEst1, which is a plastic degrading cutinase-type esterase in *Thermobifida alba*, has been over-expressed in *Escherichia coli*. The recombinant protein was purified by a two-step protocol involving immobilized metal affinity chromatography and cation-exchange chromatography, yielding 120 mg of protein per liter of bacterial culture. Crystals have been obtained by using the sitting-drop vapor-diffusion technique. Native diffraction data to 1.37 Å resolution were obtained at the BL44XU beam line of SPring-8 from a flash-frozen crystal at 100 K. The crystals belong to space group *C*2, with unit-cell parameters $a = 127.2$ Å, $b = 42.1$ Å, $c = 63.2$ Å, $\beta = 114.7°$, likely containing one Est1 double mutant (296 residues) per asymmetric unit.

Keywords

Cutinase, *Themobifida alba*, Plastic Degrading Esterase, Crystallization, High Resolution Crystallographic Data

1. Introduction

Plastic degrading enzymes were very important for reducing plastic waste and various cutinases were capable to degrade plastic [1]. The genes for polyester-hydrolyzing enzymes from *Thermobifida alba* [2], *Thermobifida fusca* [3], and *Thermobifida cellulosilytica* [4] have been cloned and determined to encode serine hydrolases that belong to the lipase/esterase family. The genus *Thermobifida* possesses two tandem cutinase genes in general [5]. *Thermobifida alba* also produces two cutinases from different genes, *est*1 and *est*119 [6]. Est119, re-

named cutinase2, was first investigated and characterized as polyester-hydrolyzing type esterase from *Thermobifida alba* [2]. Lately, it was found that Est1 also has the ability to degrade ester type plastics [5]. The two cutinases from *Thermobifida alba* showed different activities and thermostabilities, although they share 95% identity and 98% similarity. Est1 showed approximately 2-fold higher activity than Est119 [5].

We first determined the crystal structure of Est119 as the polyester hydrolyzing type cutinase [7]. Other groups also determined the crystal structures of cutinases from *Thermobifida fusca* [8] and *Thermobifida cellulosilytica* [9].

Est1 mutants were constructed for increasing thermostability and enzymatic activity. Interestingly, it was found that Est1 (A68V/T253P) double mutant (Est1DM) had higher enzymatic activity than wild type Est1 and Est119, and was stable for 1 hour below 333 K and even at 338 K, more than 70% and 50% activities were maintained after 30 and 60 min, respectively [6]. It is also reported that Est1DM degraded various aliphatic and aliphatic-co-aromatic polyesters and PET film [6].

We wanted to shed light on the so far undiscovered structural bases of *Thermobifida* cutinases, and to unveil the relationships between high activities and thermostable differences from comparing cutinase 3D structures of Est1DM with Est119. Toward this goal, we present here the overexpression, purification, crystallization and preliminary X-ray diffraction analysis of the thermostable mutant of cutinase Est1 from *Thermobifida alba*.

2. Materials and Methods

2.1. Protein Expression and Purification

Twenty mililiters of an overnight culture of *E. coli* cells Rosetta-gami B (DE3) transformed with pQE80L-est1 was inoculated to 400 mL of LB medium with 50 $\mu g \cdot ml^{-1}$ ampicillin (**Table 1**). Different culture conditions were tested with 10 mL small scale broth for expression check. The culture broth was grown at 310 K with vigorous shaking until the Abs_{600nm} reached 0.6. Then protein expression was induced by the addition of isopropyl-1-thio-*β*-D-galactopyranoside (IPTG, Nacalai Japan) to the culture to give a final concentration of 0.1 mM. Two types temperature was tested with 310 K and 298 K, respectively. For the 310 K, both six hours and overnight (18 hours) culture after induction were tested. For 298 K culture were tested with overnight (18 hours). And then the cells were harvested by centrifugation at 4000 rpm for 30 min at 277 K, washed with ice-cold deionized distilled water, suspended in lysis buffer (50 mM Tris-HCl buffer pH 8.0 including 0.3 M NaCl and 1mM imidazole), and then disrupted by sonication with Ultrasonic Disruptor UD-211 (TOMY) at 273 K. The disrupted cell suspension was centrifuged at 8000 rpm for 40 min at 277 K to pellet insoluble material. The supernatant was purified by immobilized metal affinity chromatography (HIS-Select Nickel Affinity Gel, Sigma-Aldrich, Germany), which was equilibrated with lysis buffer, the sample was loaded and washed 5 times with lysis

Table 1. Macromolecule production information.

Source organism	*Thermobifida alba*
DNA source	Genbank AB445476
Forward primer	5’-CGCGGATCCAACCCCTACGAACGCGGC-3’
Reverse primer	5’-GCGAAGCTTGAACGGGCAGGTGGAGCGGT-3’
Cloning vector	pGEM-T
Expression vector	pQE80L-est1
Expression host	*E. coli* Rosetta-gami (DE3)
Complete amino acid sequence of the construct produced	MRGSHHHHHHGSNPYERGPNPTESMLEARSGPFSVS EERVSRLGADGFGGGTIYYPRENNTYGAIAISPGYTG TQSSIAWLGERIASHGFVVIAIDTNTTLDQPDSRARQL NAALDYMLTDASSSVRNRIDASRLAVMGHSMGGGG TLRLASQRPDLKAAIPLTPWHLNKSWRDITVPTLIIGA DLDTIAPVSSHSEPFYNSIPSSTDKAYLELNNATHFAPN IPNKTIGMYSVAWLKRFVDEDTRYTQFLCPGPRTGLLS DVDEYRSTCPF

buffer, then the soluble recombinant protein was eluted with an imidazole concentration gradient (1 - 500 mM) with lysis buffer. The Est1DM fraction was collected and dialyzed to buffer A (0.1 M NaCl 10 mM Tris-HCl pH 8.0) to remove imidazole. Further purification was carried out by MonoS (GE Healthcare, Sweden) column chromatography, which was equilibrated with buffer A, then the dialyzed protein was loaded and washed 5 times with buffer A, then the protein was eluted with a NaCl concentration gradient (0 - 1.0 M). The fractions containing the Est1DM protein were combined and dialyzed to 0.2 M NaCl 10 mM Tris-HCl pH 8.0. The Est1DM protein was concentrated to 15 mg·ml^{-1} by using a VIVASPIN 15 turbo concentrator (Sartorius, Germany) and sterile-filtrated by 0.1 μm Ultrafree-MC (Merck Millipore, Germany) device. The homogeneity of the purified preparation was confirmed by 15% SDS-PAGE and native PAGE.

Determination Specific Activity

Enzymatic activities were determined by using *p*-nitrophenyl butyrate (pNFB) ester substrates as previously described. For inhibition studies, the concentration of enzyme was adjusted to 0.001 mM, inhibitors to 0.2 mM, and pNFB to 1 mM. Each assay was performed at 37˚C in 50 mM Tris-HCI pH 8.0 containing 10% dimethyl-formamide in 1-ml cuvettes. Reactions were started by the addition of substrate after pre-incubation at 37˚C for 5 min with various inhibitors. The absorbance of liberated *p*-nitrophenol was monitored continuously at 405 nm and the initial linear velocity was used to calculate the specific activities of cutinases, which was expressed as an amount (μmol) liberated of *p*-nitrophenol using 1.0 mg cutinase in 1 min under above conditions.

2.2. Crystallization

For crystallization experiments, a 15 mg·ml^{-1} solution of the recombinant

Est1DM, in 0.2 M NaCl, 10 mM Tris-HCl, pH 8.0, was employed. Crystallization trials were set up at 295 K as sitting-drop vapor-diffusion experiments on Crys-chem™ crystallization plates. Initial screening was performed using the sparse-matrix method [10] with commercial crystal screening kits (Hampton Research). Several condition optimizations were performed by changing protein concentration, precipitant concentration and pH.

2.3. Data Collection and Processing

X-ray diffraction data on Est1DM crystals were collected at 100 K in a nitrogen stream, supplementing the mother liquor solution with 30% PEG400 as cryo-protectant. X-ray preliminary data sets were collected from a single crystal on beamline BL44XU at SPring-8. The X-ray wavelength was 0.9 Å, the angle oscillation range was 1.0˚ and the crystal-to-detector distance was 180 mm. The diffraction images were integrated and scaled using the program packages HKL2000 [11].

3. Results and Discussion

Est1DM was expressed in *E. coli* and purified by affinity-resin and cation-exchange chromatography. Different temperature and shaking time of growth conditions were tried several times for obtaining a large number of recombinant protein. The overnight culture at 310 K gave very few amount of proteins (**Table 2**), but the six hours culture after induction with IPTG, which was enough even 0.1 mM final concentration, gave a lot of cutinase after immobilized metal affinity chromatography (IMAC) with batch methods (**Figure 1(a)**). But the overnight culture at 298 K gave a twice amount of Est1DM than 310 K with six hours (**Table 2**). Results of growth conditions and the protein amounts by IMAC batch methods are given in **Table 2**. It has been successfully improved to obtain a large amount of purified recombinant Est1DM; the protein yield was more than 120 mg per liter of *E. coli* culture (**Figure 1(b)**). This condition is suitable for protein crystallization experiments including crystallization

Table 2. Improving of growth conditions.

	Est1-dm	Est1-dm	Est1-dm
Culture conditions	25˚C O/N	37˚C·6h	37˚C O/N
Culture volume (mL)	10	10	10
IPTG concentration (mM)	0.1	0.1	0.1
Harvested cells (g)	0.3	0.2	0.4
Eluted volume (mL)	5	5	5
Protein concentration (mg/mL)	2	1	0.12
Total protein (mg)	10	5	0.6

Cell were suspended 30 mL with lysis buffer after loading sample on Ni column, the columns were washed 12 mL with lysis buffer. (lysis buffer; 1 mM imidazole 10 mM Tris-HCl pH8, 0.3 M NaCl) Then the columns were eluted by batch method with 250 mM imidazole 10 mM Tris-HCl pH8, 0.3 M NaCl.

Table 3. Crystallization.

Method	Sitting-drop vapor diffusion
Plate type	24-well
Temperature (K)	295
Protein concentration ($mg \cdot mL^{-1}$)	15
Buffer composition of protein solution	10 mM Tris-HCl pH8.0, 200 mM NaCl
Composition of reservoir solution	7% PEG 4000, 0.2 M Sodium Acetate 0.1 M MES pH6.5
Volume and ratio of drop	1:1
Volume of reservoir (mL)	0.5

screening. The final purified protein was confirmed by SDS-PAGE as shown in Figure 1(c) and the enzymatic activity was also confirmed by using *p*-nitrophenyl butylate as a hydrolyzing substrate (data not shown).

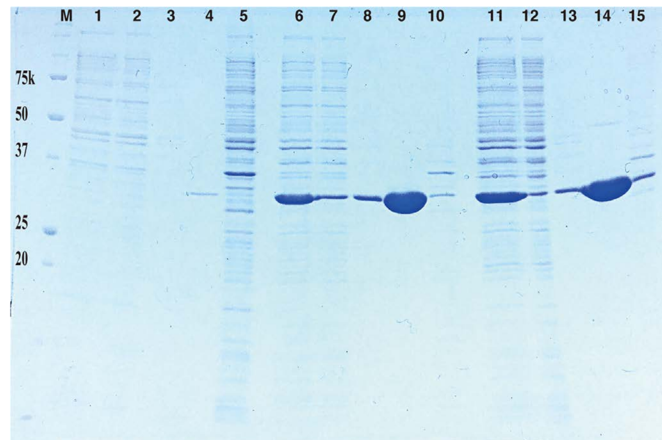
Crystallization screenings were repeated using various crystallization kits. Crystals of the recombinant Est1DM proteins were readily grown from polyethylene-glycol solutions. The best crystals were obtained through equilibration against a solution containing 5% - 8% PEG3350, 0.1 M sodium acetate and 0.1 M MES buffer, pH 6.5, 293 K, in sitting-drop vapor-diffusion setups (Table 3). The crystallization droplets consisted of 1 μl protein and 1 μl reservoir solutions, with 500 μl of reservoir solution, plate like crystals appeared within a few weeks and grew to maximum dimensions of about $1.5 \times 0.2 \times 0.1\ mm^3$ (Figure 2).

X-ray diffraction data were collected at a resolution of 1.37 Å (Figure 3). The crystals belonged to the centered monoclinic space group *C*2, with unit-cell parameters $a = 127.2$ Å, $b = 42.1$ Å, $c = 63.2$ Å, $\beta = 114.7^{\circ}$ (see Table 4). A total of 250,545 reflections in the resolution range 50.0 - 1.37 Å were collected with 98.3% completeness and an *R* merge of 4.7%. The data-collection and processing statistics are summarized in Table 4. Assuming a M_r of 31.7 kDa (296 residues) for the expressed Est1DM domain, packing density calculations indicate the most probable value for V_M as 2.42 $Å^3\ Da^{-1}$, with one Est1DM chain per asymmetric unit. This corresponds to a solvent fraction of about 49.2%, a typical value for protein crystals [12].

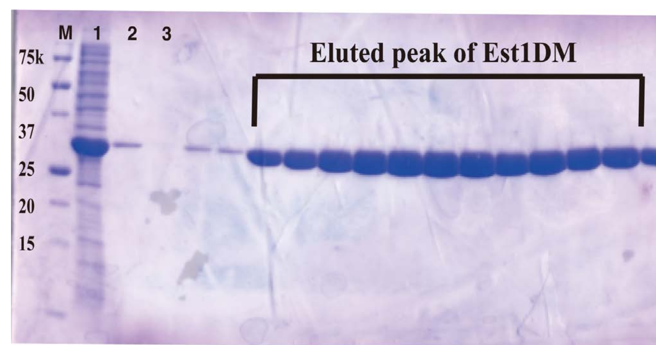
We are currently attempting phase determination by the molecular-replacement method using the crystal structure of Est119 from *Thermobifida alba* (PDB entry 3VIS) [7] as a search model with the program [13] included in the *CCP*4 program package [14]. A single correct solution was found and complete structure determination and refinement are in progress.

Acknowledgements

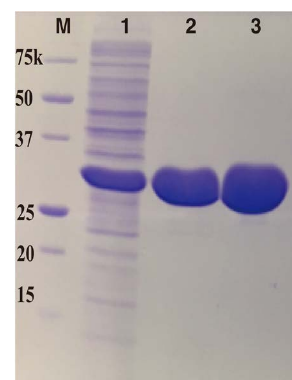
We are grateful to all members of beamline BL44XU at SPring-8 for their help in collecting data. Use of the synchrotron beamline BL44XU at SPring-8 was obtained through the Cooperative Research Program of the Institute for Protein



(a)



(b)



(c)

Figure 1. (a) Small scale expression check. M, corresponds to the molecular weight standards (kDa), lane 1, cell lysate; lane 2, flow through; lane 3, washed elution with lysis buffer; lane 4, Ni chromatography elution; lane 5, cytosol (37˚C, 18 hours); lane 6, cell lysate; lane 7, flow through; lane 8, washed elution with lysis buffer; lane 9; Ni chromatography elution; lane 10, cytosol (37˚C, 6 hours); lane 11, cell lysate; lane 12, flow through; lane 13, washed elution with lysis buffer; lane 14, Ni chromatography elution; lane 15, cytosol (25˚C, 18 hours); (b) Purification by Mono S chromatography. SDS-PAGE analysis of peak fractions indicates a purity of >99%: M, corresponds to the molecular weight standards (kDa), lane 1, cell lysate; lane 2, Flow through, lane 3, washed elution with lysis buffer; lane 4 to the end results of the Mono S column elution. (c) SDS-PAGE gel (15%) stained with Coomassie Brilliant Blue. M, molecular-weight markers (labelled in kDa); lane 1, cell lysate; lane 2, protein after nickel-NTA chromatography; lane 3, the final purified protein.

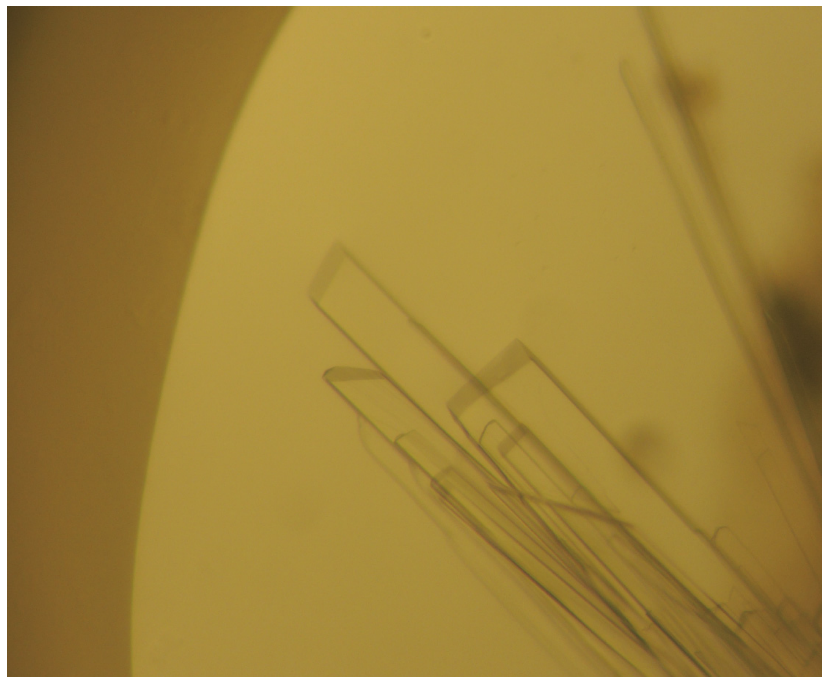


Figure 2. Results of a typical Est1DM crystals growth droplet. The crystals displayed approximately maximum size with $1.0 \times 0.3 \times 0.1$ mm^3.

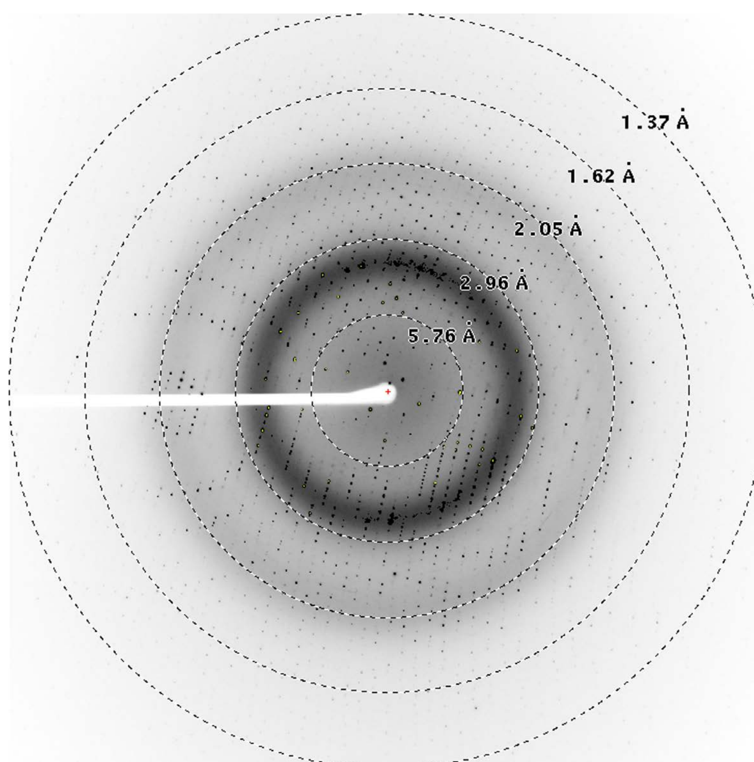


Figure 3. Diffraction image of Est1DM crystal. The circles indicate 5.76, 2.96, 2.05, 1.62 and 1.37 Å resolution, respectively.

Research, Osaka University. We also thank to Prof. Fusako Kawai, for kindly giving us the est1 mutant genes. This work was supported in part by

Table 4. Data collection and processing values for the outer shell are given in parentheses.

Diffraction source	BL44XU, SPring-8
Wavelength (Å)	0.9
Temperature (K)	100
Detector	MX300HE
Crystal-detector distance (mm)	190
Rotation range per image (˚)	1
Total rotation range (˚)	200
Exposure time per image (s)	1
Space group	*C*2
a, *b*, *c* (Å)	127.2, 42.1, 63.2
α, *β*, *γ* (˚)	90.0, 114.7, 90.0
Mosaicity (˚)	0.33
Resolution range (Å)	50 - 1.37 (1.39 - 1.37)
Total No. of reflections	250,545
No. of unique reflections	62,727
Completeness (%)	98.3 (99.7)
Redundancy	4.0 (4.0)
$\langle I/\sigma(I)\rangle$	25.3 (10.1)
$R_{r.i.m.}$	0.058 (0.194)
Overall *B* factor from Wilson plot (Å^2)	15.2

Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Science and Technology of Japan.

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Abbreviations Used

Est1DM (cutinase est1 double mutant), PET (Polyethylene Terephthalate).