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PsEst3, a new psychrophilic esterase from the Arctic bacterium *Paenibacillus* sp. R4: crystallization and X-ray crystallographic analysis

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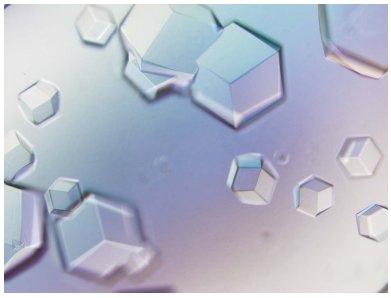
Esterases are very useful biocatalysts in industry: they hydrolyze esters and split them into a carboxylic acid and an alcohol. The psychrophilic esterase PsEst3 was obtained from *Paenibacillus* sp. R4, which was isolated from the active layer of the permafrost in Council, Alaska. PsEst3 was successfully overexpressed using a psychrophilic chaperonin co-expression system and was purified by nickel-affinity and size-exclusion chromatography. Recombinant PsEst3 was crystallized at 290 K using the hanging-drop vapour-diffusion method. X-ray diffraction data were collected to 2.1 Å resolution. The crystal was determined to belong to space group $P4_132$ or $P4_332$, with unit-cell parameters $a = b = c =$ 145.33 Å. Further crystallographic analysis needs to be conducted to investigate the structure and function of this esterase.

1. Introduction

Esterases/lipases/thioesterases (ELTs) form part of the α/β-hydrolase protein family, members of which are widely distributed in plants, animals and microorganisms. They catalyze the hydrolysis of fatty-acid esters, acylglycerols and many other substances. They have a wide range of applications in the medical and pharmaceutical industries, and in bioremediation (Bornscheuer, 2002; Jegannathan & Nielsen, 2013; Panda & Gowrishankar, 2005). Some esterases are also used in the production and processing of food additives (Guglielmetti *et al.*, 2008; Kermasha *et al.*, 2000; Kurita, 2008). Additionally, various esterases are employed as transesterification catalysts in order to transform industrial byproduct waste into more valuable materials.

The rates of reaction of most enzymes decrease at low temperatures, but cold-active enzymes that are produced by organisms inhabiting cold areas exhibit relatively high reaction rates at low temperatures. Cold-adapted enzymes have become prominent as promising catalysts because of their advantages in saving energy compared with mesophilic or thermophilic enzymes (Gerday *et al.*, 2000). Most known cold-adapted enzymes have been isolated from organisms that inhabit cold environments such as the Arctic or Antarctic. Among these enzymes, those from microorganisms have been used as a basis for further enzyme development because of the high genetic diversity of microbes and the ease and convenience of microbial cultivation (Nigam, 2013).

Permafrost is defined as a soil surface layer that has remained frozen for more than two years (Schuur *et al.*, 2008). The frozen soil in this layer contains considerable amounts of organic material from plants and animals. Microorganisms



start degrading organic matter in the soil of the active layer. This layer exerts many effects on the atmosphere as a result of the repeated synthesis and decomposition of organic materials (Pautler *et al.*, 2010). For instance, gas exchange and carbon cycling are achieved through microbial metabolism in the soil. The presence of various metabolic activities indicates the possibility of finding useful enzymes that are involved in the degradation of surplus substances and their conversion into useful substances. Thus, cold-adapted microorganisms in the permafrost may potentially harbour cold-active enzymes.

Paenibacillus sp. R4 was previously isolated from the soil of the active layer in Council, Alaska. The genome of the strain was sequenced and analyzed, and several putative esterase genes were annotated by searching for similar sequences in databases. The psychrophilic esterase PsEst3 was purified in a soluble form, which showed enzymatic activity using 4-nitrophenyl acetate as a substrate. PsEst3 has a low sequence identity compared with proteins that possess characterized structures (Fig. 1). Therefore, a structural approach is needed to understand the function of this protein. In this study, a recombinant form of PsEst3 was crystallized and was analyzed using X-ray diffraction.

2. Materials and methods

2.1. Macromolecule production

The gene encoding PsEst3 was identified by annotating the draft genome sequence of *Paenibacillus* sp. R4 using the *RAST* annotation server (Overbeek *et al.*, 2014). This strain was isolated from the soil in the active layer in Council, Alaska (64° 36′ 24.84″ N, 163° 25′ 31.1″ W). The gene coding for PsEst3 was amplified from the genomic DNA of *Paenibacillus* sp. R4 by PCR using the forward primer 5′-AAGAAGGAGATATACATATGGGAAATGCCGTTGTTGTCAA-3′ (the underlined bases correspond to an NdeI site) and the reverse primer 5′-TGGTGGTGGTGGTGCTCGAGGTAATGTTTGGTTATAAAGC-3′ (the underlined bases correspond to an XhoI site). The PCR product was cloned into the expression vector pET-22b(+) digested with NdeI and XhoI (Table 1). The resulting plasmid was designated pPsEst3. Gene cloning was performed using the one-step sequence- and ligation-independent cloning method (Jeong *et al.*, 2012). A previously reported co-expression system using psychrophilic chaperonin was employed in the present study by transforming the cells with the plasmid pPsyGroELS (Kim *et al.*, 2015). The resulting recombinant *Escherichia coli* BL21(DE3)/pPsyGroELS/pPsEst3 strain was used to express the target protein PsEst3 in a soluble form. *E. coli* DH5*α* cells were used as the host for gene cloning.

The recombinant strain was grown in LB broth (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl) containing 100 mg l^{-1} ampicillin and 50 mg l^{-1} chloramphenicol at 37°C. Expression of the recombinant protein was induced by adding IPTG to a concentration of 0.1 m*M* when the OD_{600} of the culture reached 0.6. The incubation temperature was then reduced to 15°C. After 2 d of cultivation, the cells were harvested *via* centrifugation at 6000*g* for 15 min at 4°C and stored at −80°C

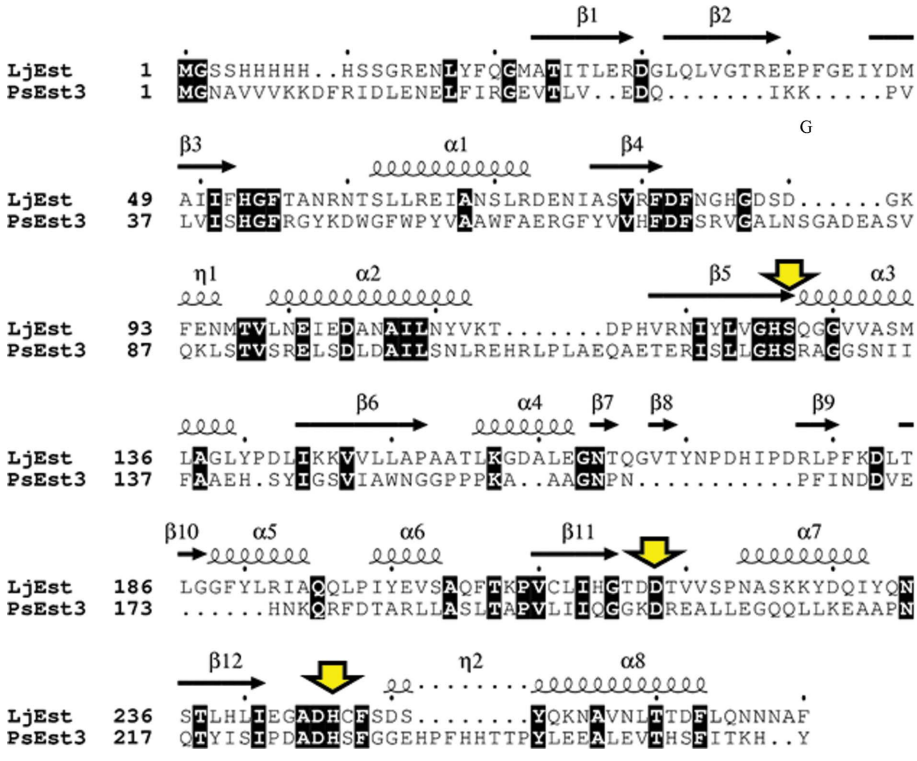


Figure 1
Sequence alignment of PsEst3 and cinnamoyl esterase from *Lactobacillus johnsonii* (PDB entry 3pf8; LjEst). The amino-acid sequences were aligned using *ClustalW*. The catalytic triad residues in LjEst are indicated by yellow arrows.

Table 1
Protein-production information.

Source organism	*Paenibacillus* sp. R4
DNA source	Genomic DNA
Forward primer†	AAGAAGGAGATATACATATGGGAAATGCCG TTGTTGTCAA
Reverse primer‡	TGGTGGTGGTGGTGCTCGAGGTAATGTTTG GTTATAAAGC
Expression vector	pET-22b(+)
Expression host	*E. coli* BL21(DE3)/pPsyGroELS
Complete amino-acid sequence of the construct produced	MGNAVVVKKDFRIDLENELFIRGEVTLVED QIKKPVLVISHGFRGYKDWGFWPYVAAW FAERGFYVVHFDFSRVGALNSGADEASV QKLSTVSRELSDLDAILSNLREHRLPLA EQAETERISLLGHSRAGGSNIIFAAEHS YIGSVIAWNGGPPPKAAAGNPNPFINDD VEHNKQRFDTARLLASLTAPVLIIQGGK DREALLEGQQLLKEAAPNQTYISIPDAD HSFGGEHPFHHTTPYLEEALEVTHSFIT KHYHHHHHH

† The NdeI site is underlined. ‡ The XhoI site is underlined.

until the next experiment. The pelleted cells were suspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl) and lysed *via* sonication. The crude lysate was centrifuged at 20 000*g* for 1 h at 4°C. The supernatant was then loaded onto a nickel-affinity column which had been equilibrated with buffer *B* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 10 m*M* imidazole). The column was washed with buffer *C* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 40 m*M* imidazole) to remove weakly bound proteins. The target protein was eluted with buffer *D* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 300 m*M* imidazole). The eluted sample containing PsEst3 was pooled and concentrated using Amicon Ultra centrifugal filter units (10 000 Da molecular-weight cutoff, Millipore, USA). The eluted fraction was further purified using a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA) which had been pre-equilibrated with buffer *A*. Following size-exclusion chromatography, the fractions were analyzed by SDS–PAGE. The protein was subsequently concentrated to 30 mg ml^{-1} using Amicon Ultra centrifugal filter units. The protein concentration was determined from the absorbance of the sample at 280 nm using a molar extinction coefficient of 32 430 M^{-1} cm^{-1}. Macromolecule-production information is summarized in Table 1.

2.2. Enzyme-activity assay

Stock substrate solutions (50 m*M*) were prepared by dissolving 4-nitrophenyl acetate in acetonitrile and were further diluted to 2.5 m*M* with 50 m*M* Tris–HCl buffer pH 8.0 to create a working solution before use. The enzyme reaction was performed in a mixture consisting of 10 µl properly diluted PsEst3 and 200 µl of the working substrate solution at each temperature for 10 min. The reaction was stopped by adding 200 µl 0.2 *N* NaOH. Esterase activity was determined by measuring the amount of 4-nitrophenol released from 4-nitrophenyl acetate after enzyme reaction at 405 nm. All experiments were performed in triplicate and the extinction coefficient for 4-nitrophenol was determined under every reaction condition. The effect of nonenzymatic hydrolysis of the substrates was subtracted from the value measured by the enzyme reaction.

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion for initial screening, hanging-drop vapour diffusion for crystal optimization
Plate type	96-well plates for initial crystal screening, 24-well plates for crystal optimization
Temperature (°C)	23
Protein concentration (mg ml^{-1})	30
Buffer composition of protein solution	20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl
Composition of reservoir solution†	0.1 *M* bis-tris pH 6.5, 2.0 *M* ammonium sulfate
Volume and ratio of drop	0.5 µl, 1:1 ratio of protein:reservoir solution for initial crystal screening; 1 µl, 1:1 ratio of protein:reservoir solution for crystal optimization
Volume of reservoir	70 µl for initial screening, 500 µl for crystal optimization

† The best condition for data collection.

Table 3
Data collection and processing.

Diffraction source	Beamline 5C, PLS
Wavelength (Å)	0.97957
Detector	ADSC Q315 CCD
Crystal-to-detector distance (mm)	400
Rotation range per image (°)	1
Total rotation range (°)	150
Exposure time per image (s)	1
Space group	$P4_132$ or $P4_332$
Unit-cell parameters (Å)	$a = b = c = 145.33$
Resolution range (Å)	50–2.10 (2.14–2.10)
Total No. of reflections	551590
No. of unique reflections	30940
Completeness (%)	99.1 (93.9)
Average mosaicity (°)	0.249
$CC_{1/2}$	0.969 (0.836)
Wilson *B* factor (Å^2)	24.42
Multiplicity	17.8 (4.3)
$\langle I/\sigma(I)\rangle$	65.711 (8.336)
R_{meas}† (%)	10.8 (38.1)

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

2.3. Crystallization

Crystallization of PsEst3 was performed by the sitting-drop vapour-diffusion method using commercial screening kits including PEG/Ion (Hampton Research), Wizard 1–4 (Rigaku), JCSG-*plus* (Molecular Dimensions), PACT (Molecular Dimensions) and MCSG (Anatrace). Each crystallization drop was prepared by equilibrating a mixture of 0.5 µl reservoir solution and 0.5 µl protein solution against 70 µl reservoir solution. To improve the quality of the crystals, optimization was subsequently performed by altering the buffer, the pH and the salt concentration. Further optimization was performed by replacing the previous method by the hanging-drop vapour-diffusion method. Crystallization using this method was performed in 24-well plates, with each well containing a drop consisting of 1 µl protein solution and 1 µl reservoir solution equilibrated against 500 µl reservoir

solution. In order to confirm the identity of the crystallized protein, the crystals were dissolved in 20 m*M* Tris–HCl pH 8.0 and the esterase activity was performed using 4-nitrophenyl acetate. Crystallization information is summarized in Table 2.

2.4. Data collection and processing

The crystals were transferred into perfluoropolyether oil PFO-X175/08 (Hampton Research, USA) and directly flash-cooled in a nitrogen-gas stream at 100 K. Diffraction data were collected at a wavelength of 0.97957 Å using an ADSC Quantum 315 CCD detector on beamline 5C at the Pohang Light Source (PLS), Pohang, Republic of Korea. A total of 150 images were collected with an oscillation angle of 1°, 1 s exposure per frame and a crystal-to-detector distance of 400 mm. A data set was collected at 2.1 Å resolution from a single crystal. The diffraction data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 3.

3. Results and discussion

The DNA sequence of the full-length gene encoding PsEst3 contains 774 nucleotides and codes for a protein that is 257 amino acids in length. The nucleotide sequence encoding amino acids 1–257 was inserted into a pET-22b expression

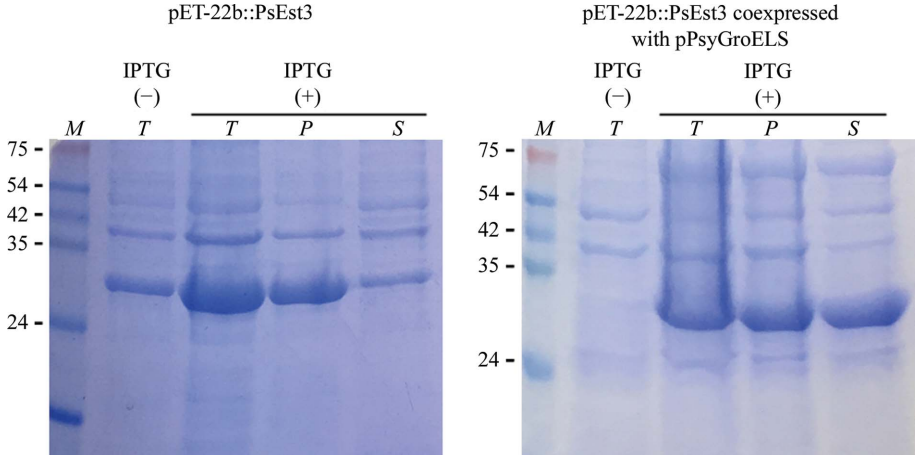


Figure 2
Improvement of the solubility of PsEst3 expressed from recombinant *E. coli* by co-expression of pPsyGroELS as shown by SDS–PAGE analysis. The enzymes from both cell types were induced by adding IPTG to a concentration of 0.1 m*M* when the OD_{600} of the culture reached 0.6. After IPTG induction, the cells were cultured at 15°C for 2 d. The cells were disrupted by sonication. 10 µl of the resulting extract was loaded onto the gel. Lane *M*, Smart Color Protein Marker (Pre-stained, ELPiS; labelled in kDa); lane *T*, total protein; lane *P*, inclusion bodies; lane *S*, soluble fraction.

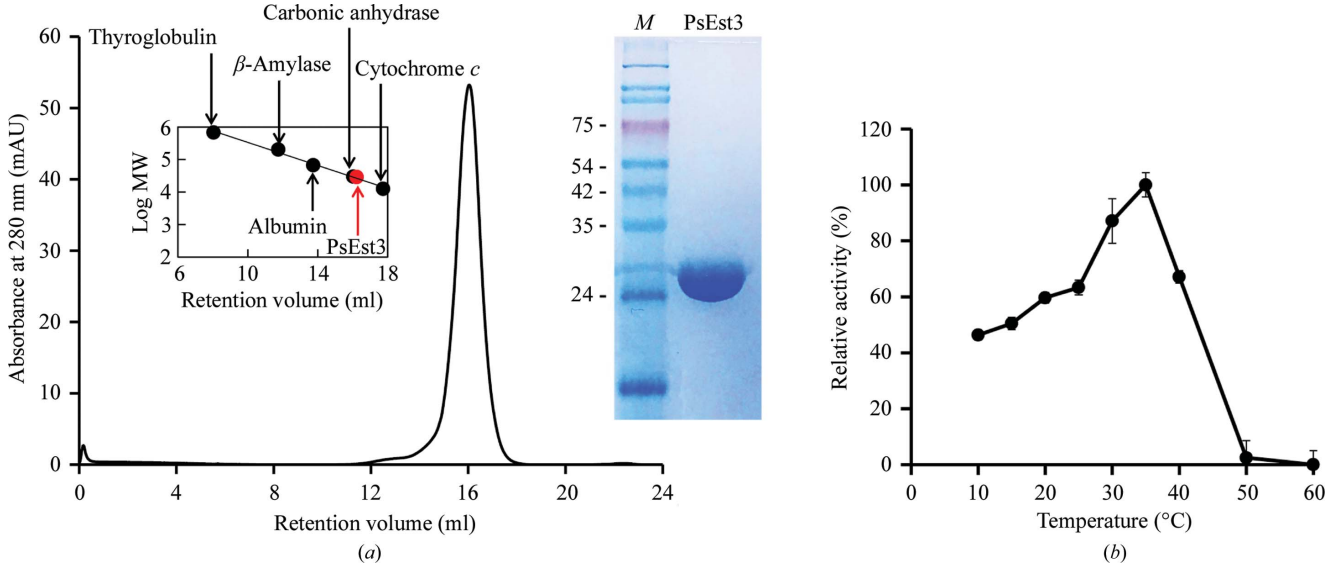


Figure 3
Size-exclusion chromatography and enzyme activity of PsEst3. (*a*) The purified protein was analyzed on a Superdex 200 10/300 GL gel-filtration column which had been pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 with 200 m*M* NaCl. Following size-exclusion chromatography, fractions were analyzed by SDS–PAGE (right). Analytical gel filtration of calibration standards (inset): thyroglobulin, 669 kDa; β-amylase, 200 kDa; albumin, 66 kDa; carbonic anhydrase from bovine erythrocytes, 29 kDa; cytochrome *c*, 12.4 kDa. (*b*) Temperature profile of the enzyme activity of PsESt3.

plasmid and the recombinant protein was overexpressed in *E. coli* BL21(DE3) cells. SDS–PAGE analysis revealed one major protein band with an approximate molecular weight of 29 kDa, but the protein was sequestered into inclusion bodies in the insoluble fraction. In order to obtain a soluble form of the protein, we used the co-expression system described in §2. As shown in Fig. 2, the solubility of the protein was significantly enhanced by the chaperonin in the recombinant host. Therefore, soluble PsEst3 with a C-terminal hexahistidine-containing tag could successfully be obtained for crystallization. The soluble protein was eluted from an Ni–NTA affinity column and purified by size-exclusion chromatography. As shown in Fig. 3(*a*), PsEst3 eluted as the main peak, corresponding to a molecular mass of approximately 29 kDa, suggesting that the protein exists as a monomer.

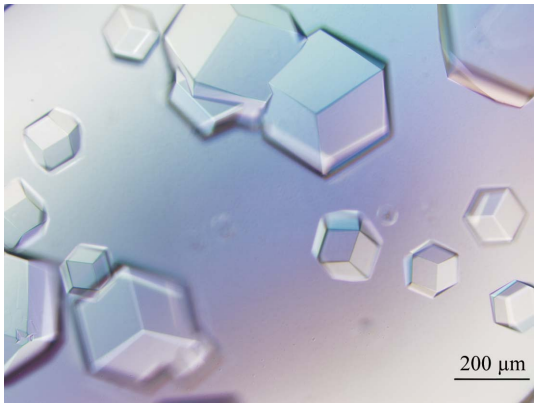
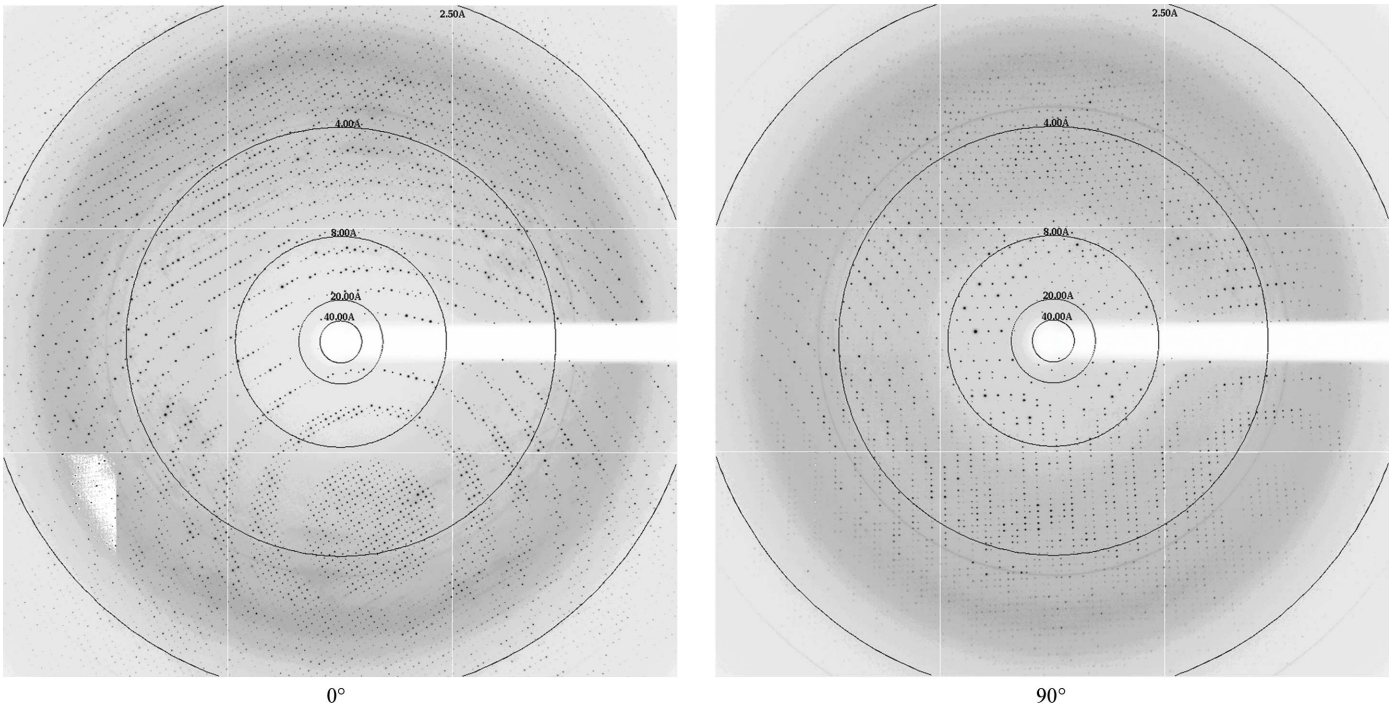


Figure 4
Crystals of PsEst3 grown by the hanging-drop vapour-diffusion method.

In the temperature profile of the enzyme activity, the recombinant PsEst3 had a highest activity at the relatively low temperature of 35°C and retained about 45% of its activity at 10°C. The activity was dramatically decreased at temperatures above 40°C (Fig. 3*b*). This result suggests that PsEst3 has the temperature profile of a typical psychrophilic enzyme.

The protein was initially crystallized using three different buffers: (i) 0.1 *M* Tris–HCl pH 8.5 with 2.0 *M* ammonium sulfate, (ii) 0.1 *M* bis-tris pH 6.5 with 2.0 *M* ammonium sulfate and (iii) 0.1 *M* HEPES pH 7.5 with 2.0 *M* ammonium sulfate. No morphological differences in the protein crystals were observed for the crystals obtained using bis-tris and HEPES buffers, but the reproducibility of protein crystallization in bis-tris buffer was better than that in HEPES buffer. The crystal was optimized by altering the pH and salt concentration of the buffer using the hanging-drop vapour-diffusion method. Large crystals of PsEst3 could be obtained in 1.6–2.0 *M* ammonium sulfate, 0.1 *M* bis-tris pH 6.5 ± 1.0. An enzyme-activity assay of the crystals confirmed that the major component of the crystals was PsEst3. Ammonium sulfate was considered to be a key factor in the crystallization of the protein, as all of the buffers contained a high concentration of ammonium sulfate. The crystals grew to dimensions of 0.2 × 0.3 × 0.05 mm within 3 d (Fig. 4). The protein crystal that was obtained using 0.1 *M* bis-tris pH 6.5 with 2.0 *M* ammonium sulfate was used for diffraction experiments. A total of 551 590 reflections and 30 940 unique reflections were observed at 50–2.10 Å resolution (Fig. 5). Preliminary data processing revealed that the crystal belonged to space group $P4_132$ or $P4_332$, with unit-cell parameters $a = b = c = 145.33$ Å (Table 3). Assuming the presence of two PsEst3 monomers per asymmetric unit, the



0° 90°

Figure 5
Snapshots of diffraction images from a single crystal to a resolution of 2.1 Å.

Matthews coefficient was 2.28 Å^3 Da^{-1} and the solvent content was 46.18%.

Molecular replacement using cinnamoyl esterase from *Lactobacillus johnsonii* (PDB entry 3pf8; 23% sequence identity; Lai *et al.*, 2011) as a template was not successful, indicating that the structure of PsEst3 might be quite different from the previous structures available in the PDB.

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