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# Crystallization and preliminary crystallographic analysis of poly-γ-glutamate hydrolase from bacteriophage ΦNIT1

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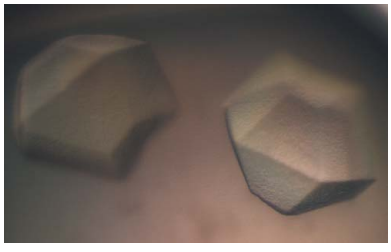




Particular *Bacillus subtilis* strains produce a capsular polypeptide poly-γ-glutamate (γ-PGA) that functions as a physical barrier against bacteriophage infection. Bacteriophage ΦNIT1 can infect *B. subtilis* and produces a novel γ-PGA hydrolase PghP. PghP was overexpressed, purified and crystallized by the sitting-drop vapour-diffusion method. The crystals diffracted to a resolution of 2.4 Å using a synchrotron X-ray source and were found to belong to space group $P3_121$ or $P3_221$.

## 1. Introduction

The bacterial polypeptide poly-γ-glutamate (γ-PGA) is an extracellular polymer consisting of DL-glutamate residues with γ-linkages and is synthesized by the membrane γ-PGA synthetase complex encoded by the *cap*/*pgs* operon (Nagai *et al.*, 1997; Ashiuchi *et al.*, 2001; Tran *et al.*, 2000; Candela & Fouet, 2006). The degree of polymerization of γ-PGA is >10 000. The expression of γ-PGA on the membrane surface protects the cells from the animal host immune system (in pathogenic *Bacillus anthracis* and *Staphylococcus epidermidis*) or from bacteriophage attack (in the industrial strain of *B. subtilis*) (Candela & Fouet, 2005; Uchida *et al.*, 1993; Kocianova *et al.*, 2005; Fouet & Mock, 2006; Kimura & Itoh, 2003). γ-PGA is also known to mediate the formation of biofilms (Stanley & Lazazzera, 2005; Hong *et al.*, 2008). *cap*/*pgs* operons are found in *B. licheniformis*, *Oceanobacillus iheyensis*, *S. haemolyticus* and *S. saprophyticus* (http://blast.ncbi.nlm.nih.gov/), implying that γ-PGA contributes significantly to the survival of these cells, which are found in various environments. The γ-linkage and the unusual D-glutamate of γ-PGA provide a crucial physical barrier to protect the cell from bacteriophage infections and the D-glutamate content differs between species. For example, *S. epidermidis* and *B. subtilis* produce γ-PGAs consisting of both D- and L-glutamate, whereas the γ-PGA from *B. anthracis* consists solely of D-glutamate (Nagai *et al.*, 1997; Candela & Fouet, 2006).

A poly-γ-glutamate hydrolase PghP was found in *B. subtilis* cultures infected with bacteriophage ΦNIT1 (Kimura & Itoh, 2003). Purified PghP has a molecular mass of 25 kDa and degrades γ-PGA into oligomers. Contamination by the phage possessing PghP severely impairs the fermentation of soybeans by *B. subtilis*. Most phages isolated in fermentation factories are PghP-positive and PghP activity facilitates the infection and propagation of the phage in the colony of γ-PGA producing cells. The *pghP* gene encodes a 208-residue protein (with a calculated molecular weight of 22 939). Hypothetical genes (Pfam PF05908) homologous to *pghP* are primarily found in phages and bacterial genomes (Swiss-Prot; http://br.expasy.org/sprot/), but PghP shows no amino-acid similarity to known enzymes. The CapD protein of *B. anthracis* is a well characterized γ-PGA hydrolase and its crystal structure has recently been reported (Wu *et al.*, 2009). The *capD* gene of *B. anthracis* belongs to the γ-glutamyl transferase family (peptidase family T3) and encodes a protein that is involved in the anchoring of γ-PGA to the cell wall (Candela & Fouet, 2005). PghP does not belong to the γ-glutamyl transferase family, nor does it have amino-acid similarity to CapD. Recombinant His-CapD can

degrade *B. anthracis* γ-PGA, indicating that this enzyme possesses both hydrolysis and transferase activities; however, the acceptor molecule in the cell wall for the transferase activity has not been identified. Recently, PghP and CapD proteins have been considered as potential therapeutics because γ-PGA is a virulent factor and removal of γ-PGA from the cell surface can promote phagocytosis (Scorpio *et al.*, 2007). The treatment of *B. anthracis* with CapD enhanced the phagocytotic destruction of this pathogen by neutrophils. However, PghP barely digested *B. anthracis* γ-PGA and was found to be less effective than CapD. The inability of PghP to break down *B. anthracis* γ-PGA reflects differences in the high-order structures of γ-PGA between the two bacteria rather than the substrate-specificity of the enzymes for either D- or L-glutamate. *B. subtilis* γ-PGA contains a large amount of D-glutamate (the D-glutamate content is more than 50%; Nagai *et al.*, 1997) and CapD can digest both *B. anthracis* and *B. subtilis* γ-PGA. Although the amino-acid composition of γ-PGA is rather simple, no three-dimensional structural information is available. The structures of γ-PGA hydrolases with different modes of action should represent a valuable tool for the analysis and determination of the structure of γ-PGA. To elucidate the γ-PGA hydrolysis reaction in detail, a high-level expression system was established. The crystallization conditions of recombinant PghP for structural analysis are presented. In addition, a structural comparison between proteins belonging to the γ-glutamyl hydrolase family is presented and this will aid efforts aimed at dissecting the molecular mechanism of γ-PGA hydrolysis.

## 2. Materials and methods

### 2.1. Protein expression and purification

Bacteriophage ΦNIT1 genomic DNA was extracted and purified as described previously (Kimura & Itoh, 2003). The *pghP* gene (DDBJ/EMBL/GenBank accession No. AB091475) was amplified by PCR using the primer pair pghP5′NdeI (5′-**CATATG**GCACAAACAGACACATATCCA-3′) and pghP3′XhoI (5′-**CTCGAG**GCCATAATACTCTGCCTCTGCTTC-3′; *Nde*I and *Xho*I recognition sequences are shown in bold) and KOD-Plus polymerase (Toyobo, Osaka, Japan). The amplified fragment was cloned into the *Hin*cII site of pUC118. The fragment containing *pghP* was excised as an *Nde*I–*Xho*I fragment and ligated into the same restriction site of pET22b(+) (Novagen, Madison, Wisconsin, USA). This generated the PghP protein-expression plasmid pNAG443 encoding a $His_6$-tagged PghP protein with the sequence LEHHHHHH fused to the C-terminus of PghP.

The pNAG443 plasmid was transformed into *Escherichia coli* BL21 (DE3) cells (Novagen, Madison, Wisconsin, USA). Transformed cells were grown in 1 l LB medium supplemented with 100 μg ml$^{-1}$ ampicillin (Meiji Seika Kaisha, Tokyo, Japan). The cells were grown at 310 K with shaking (120 rev min$^{-1}$) and incubated for ∼3 h until the $OD_{600}$ reached 0.8–1.0. IPTG was added to a concentration of 1 mg ml$^{-1}$ to induce expression of recombinant PghP and culturing was continued for 3 h. The $OD_{600}$ reached ∼3 at the end of the cultivation.

Cells were harvested by centrifugation at 4900*g* for 10 min at 277 K and resuspended in STE buffer (10 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 150 m*M* NaCl). Cells were disrupted using a French press (SLM-Aminco, Rochester, New York, USA) at 15.7 MPa and cell debris was removed by centrifugation at 4900*g* for 10 min at 277 K. Proteins were precipitated by adding ammonium sulfate to 80% saturation. The precipitated protein was dissolved in 40 ml buffer *A* (20 m*M* sodium phosphate pH 7.4, 0.5 *M* NaCl, 1 m*M* 2-mercaptoethanol) and dialyzed against 5 l of the same buffer overnight at 277 K. The dialyzed protein mixture was centrifuged at 100 000*g* for 60 min at 277 K to remove debris and the soluble fraction was collected. The supernatant was filtered using a cellulose acetate membrane (pore size 0.45 μm; Toyo Roshi Kaisha, Tokyo, Japan) to remove precipitated material and was subjected to a nickel-affinity column-chromatography step using a HiTrap Ni-chelating column (GE Healthcare, Uppsala, Sweden; 5 ml column volume). PghP with a C-terminal $His_6$ tag was eluted with buffer *A* containing 100 m*M* imidazole. Fractions containing PghP as judged by SDS–PAGE were collected and dialyzed against buffer *B* (20 m*M* Tris–HCl pH 7.5, 10 m*M* NaCl). The dialyzed sample was loaded onto a DEAE HiPrep column (column volume 20 ml; GE Healthcare) equilibrated with buffer *B*. PghP was eluted using a four column-volume linear gradient of NaCl (0.01–0.4 *M*) in buffer *B* at 2 ml min$^{-1}$. Peak fractions containing the target protein (total volume ∼15 ml) were collected and dialyzed against 2 m*M* Tris–HCl pH 7.4. Purified recombinant PghP was subjected to an enzyme assay. Hydrolyzed products were detected by agarose gel electrophoresis and methylene blue staining (Kimura & Itoh, 2003) and enzymatic activity was confirmed. Unless described otherwise, all reagents were purchased from Wako Pure Chemical (Tokyo, Japan).

### 2.2. Crystallization and X-ray data collection

The solution of the protein in 2 m*M* Tris–HCl buffer pH 7.4 was concentrated to 17 mg ml$^{-1}$ (for examination of the initial screening conditions) by ultrafiltration using an Microcon YM-3 centrifugal filter unit (Millipore, Massachusetts, USA) and filtered through a 0.1 μm membrane (Millipore). Sparse-matrix crystal screening was performed using the crystal screening kits Wizard Screens I and II and Cryo I and II (Emerald BioStructures, Washington, USA). Sitting-drop vapour-diffusion trials were set up in 96-well Intelli-Plates (Art Robbins Instruments, California, USA) at 293 K. The reservoirs contained 50 μl reservoir solution and each drop consisted of 0.3 μl protein solution and 0.3 μl reservoir solution. Within a week, several angular crystals were observed under two conditions using 40%(*v*/*v*) PEG 600 and 0.1 *M* phosphate–citrate buffer pH 4.2 and 40%(*v*/*v*) PEG 300, 5%(*w*/*v*) PEG 1000 and 0.1 *M* Tris pH 7.0 as precipitating agents. Crystals were reproduced using CrystalClear Strips 96-well sitting-drop plates (Douglas Instruments, Berkshire, England). Greater reproducibility of the crystals was achieved when the second condition using PEG 300 (Wako Pure Chemical) and PEG 1000 (Hampton Research, California, USA) as precipitating agents was used.

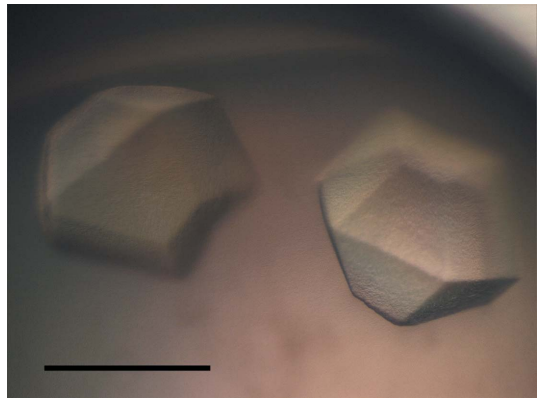
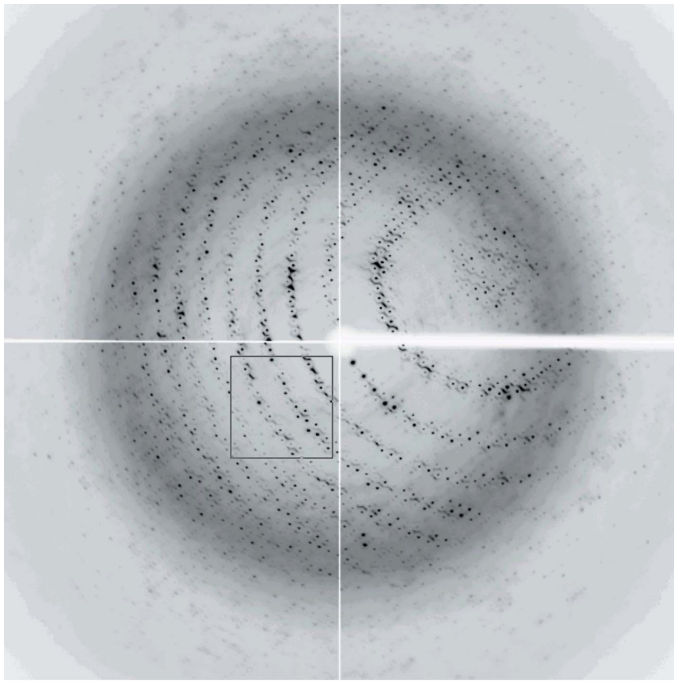
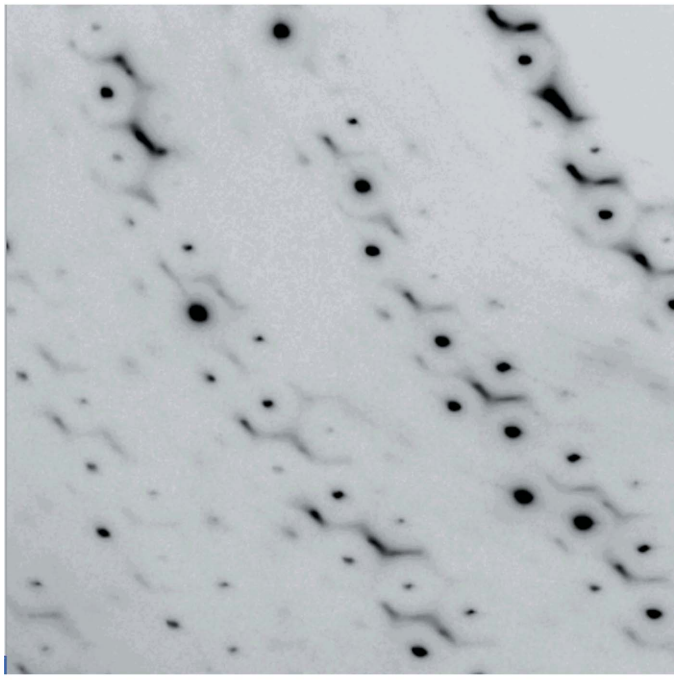


**Figure 1**
Crystals of PghP. The scale bar represents 500 μm.

Native diffraction data were collected from a single crystal on beamline BL-NW12 of the Photon Factory Advanced Ring (PF-AR), High Energy Accelerator Research Organization, Tsukuba, Japan. Since the crystals were grown in cryoprotectant conditions containing 40%(*v*/*v*) PEG 300, it was possible to directly cool the crystals without the requirement for a soaking step. The crystal was scooped into a 0.7 mm nylon loop (Hampton Research) and then flash-cooled in a nitrogen stream at 95 K. Diffraction data were collected at a wavelength of 0.965 Å with a Quantum Q210 CCD detector (ADSC, California, USA) using 5 s exposures and 1° oscillations over a total of 360°. Data were integrated and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL*-2000 program suite (Otwinowski & Minor, 1997).



(*a*)



(*b*)

**Figure 2**
(*a*) Diffraction image of PghP. (*b*) Magnified view of the boxed region in (*a*).

**Table 1**
Data-collection statistics for the PghP crystal.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | $P3_121$ or $P3_221$ |
| Unit-cell parameters (Å, °) | $a = b = 85.9$, $c = 86.7$, $\alpha = \beta = 90$, $\gamma = 120$ |
| Wavelength (Å) | 0.965 |
| Resolution range (Å) | 50.0–2.40 (2.49–2.40) |
| $R_{\rm merge}$† | 8.6 (30.6) |
| Completeness (%) | 98.7 (97.7) |
| Multiplicity | 9.5 (9.6) |
| Average $I/\sigma(I)$ | 42.1 (10.6) |
| Unique reflections | 14780 (1455) |
| Total reflections | 139847 |

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the $i$th observation of reflection $hkl$ and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations $i$ of reflection $hkl$.

## 3. Results

The final crystallization conditions for PghP were as follows. The reservoir solution consisted of 40%(*v*/*v*) PEG 300, 5%(*w*/*v*) PEG 1000 and 0.1 *M* Tris buffer pH 7.0. The refined protein-solution concentration used to obtain good-quality crystals that diffracted to high resolution was 25 mg ml$^{-1}$. A drop consisting of 5 µl protein solution and 3.5 µl reservoir solution was equilibrated against 100 µl reservoir solution at 293 K. The largest crystal grew to a diameter of 700 µm within two weeks (Fig. 1).

Using synchrotron radiation, the crystal diffracted to a maximum resolution of 2.0 Å (Fig. 2). The mosaicity of the crystal was estimated to be 1.5°. A honeycomb pattern was observed around the diffraction spots in some oscillation images (Fig. 2); however, careful selection of the peaks ensured the success of auto-indexing. The data were processed in the trigonal space group *P*321 and inspection of the reflections along the threefold axis indicated the presence of a $3_1$ or $3_2$ screw axis. The crystal therefore belonged to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 85.9$, $c = 86.7$ Å, $\gamma = 120°$. The data were processed to 2.4 Å resolution and the processing statistics of the collected data are summarized in Table 1. Assuming the presence of two PghP molecules in the asymmetric unit of the crystal, the Matthews coefficient was calculated to be 1.92 Å$^3$ Da$^{-1}$, corresponding to a crystal solvent content of 36.0%. In the case that one PghP molecule was present, the Matthews coefficient was calculated to be 3.84 Å$^3$ Da$^{-1}$, corresponding to a crystal solvent content of 68.0% (Matthews, 1968).

We have prepared selenomethionine-substituted PghP and phase determination will be conducted using the single or multiple-wavelength anomalous dispersion (SAD or MAD) techniques.

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