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Crystallization of surface mutated Penicillin G acylase from *Baillus megaterium*

Nattawut Buasri[1], Thepparit Traisaranakul[1], Ketsaraphorn Channgam[1], Jirundon Yuvaniyama[1,*]

[1] Department of Biochemistry and Center for Excellence in Protein and Enzyme Technology, Faculty of Science, Mahidol University, Rama IV Road, Ratchathewi, Bangkok 10400, Thailand
*E-mail: jirundon.yuv@mahidol.ac.th

Abstract:

Bacillus megaterium Penicillin G acylase (*Bm*PGA) catalyzes the hydrolysis of penicillin G to 6-aminopenicillanic acid and phenylacetic acid. This enzyme has been extensively studied in our laboratory as a model for protein engineering investigations for its application in semi-syntheses of antibiotic compounds. A major obstacle in the development of *Bm*PGA is the intrinsic electrostatic aggregation of this enzyme and its mutants under certain conditions, which limits its industrial utilization. Multiple mutations have been designed and generated to tackle this problem. One of the mutants demonstrated a significant solubility improvement when compared with the wild-type enzyme. Moreover, this mutant exhibits similar behaviors to the wild-type enzyme in terms of expression, purification, and kinetics. The three dimensional structure of this mutant is being pursued as a template for subsequent engineering designs.

In this study, Thermofluor screening was conducted to explore effects of small molecules on the thermal stability of the *Bm*PGA mutant. Additives that elevated the melting temperature (T_m) of the mutant will be further studied whether they can facilitate its crystal growth. Obtained crystals were exposed with X-rays and its diffraction data were collected.

Introduction

Penicillin acylases (PAs, E.C. 3.5.1.11) have been extensively studied for its potential in the pharmaceutical industry. They are widely distributed among microorganisms, including bacteria, filamentous fungi and yeast[1]. These enzymes catalyzed the deacylation of natural penicillins to give the active pharmaceutical intermediate 6-aminopeniillilanic acid (6-APA), which is widely used in the production of semisynthetic antibiotics[2].

Baillus megaterium penicillin G acylase (*Bm*PGA) has been extensively studied in our laboratory. Its active matured form composes of an α-subunit (24.4 kDa) and a β-subunit (61.4 kDa). This enzyme expressed as a single-polypeptide precursor, which consisted of a signal peptide (2.9 kDa), the α-subunit, a spacer peptide (3.4 kDa) the β-subunit[2]. Experimental observation revealed that *Bm*PGA could also be found in a form whose spacer peptide still jointed to the α-subunit, namely the α'-subunit (27-28 kDa).

One of our goals in this research project is to utilize this enzyme as a model for protein engineering investigations, in order to produce novel enzymes that enable a single-step process hydrolysis of its related substrate for the application in semi-syntheses of antibiotic compounds. Furthermore, we aim to improve several properties of this enzyme such as solubility, stability and reusability for the purpose of its industrial applications.

However, one of the major obstacles in this study is the aggregation of this enzyme and its mutants under certain conditions. The mechanism of this phenomenon is most likely due to the combined effects of both intrinsic properties of the enzyme and the extrinsic factors toward the enzyme[3]. From the structure of the wild-type *Bm*PGA determined in our laboratory, it was revealed that this enzyme contains two hemispheres of opposite polarities. This caused the formation of electrostatic aggregates of *Bm*PGA in certain conditions, specifically at pH closed to 6.0 and low ionic strength. Based on the already existed wild-type PGA structure, multiple mutations were designed and generated to tackle this problem by interrupting the enzymes' two hemisphere of opposite polarities.

One of the mutants demonstrated a significant solubility improvement when compared with the wild-type enzyme. The N5 mutation was designed to disrupt the electronegative surfaces in an area further away from the active site with an expectation to reduce the enzymes' ionic aggregation. The mutation was also designed to retain compatibility of intermolecular interaction within the wild-type crystal lattice. The *Bm*PGA-N5 mutant is more soluble than the wild-type PGA in low salt condition. Moreover, this mutant exhibits similar behaviors to the wild-type enzyme in terms of expression, purification, and kinetics. The three dimensional structure of this mutant is being pursued as a template for subsequent engineering designs.

In this study, Thermofluor-based screening was conducted to explore effects of small molecules on the thermal stability of the *Bm*PGA-N5. Additives that elevated the melting temperature (T_m) of this mutant were further studied whether they can facilitate its crystal growth. Obtained crystals were exposed with X-rays and its diffraction data were collected.

Methodology

Protein preparation

The expression vector pET24a(+) (Novagen) containing the *Bm*PGA-N5 gene was transformed into *Escherichia coli* Rosetta 2 (DE3) (Novagen). The cells were grown in 2x YT medium supplemented with 5 mM $CaCl_2$ at 37°C with 250 rpm agitation. Protein expression was induced at an OD_{600} of 0.5 by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 hours at 16°C. The cells were collected by centrifugation with the speed of 12,000 × g for 15 min at 4°C. The harvested cells were then resuspended with cold lysis buffer (300 mM NaCl in 50 mM Tris·HCl pH 8.0).

In order to extract the cells' periplamic contents, sodium deoxycholate was slowly added to the final concentration of 0.05% (w/v). The mixture was vortexed 6 times for a few seconds each every 10 minutes. Later, the protein crude extract was retrieved by centrifugation with the speed of 12,000 × g for 30 min at 4°C and partially purified using ammonium sulfate precipitation at the concentration of 40% (w/v). The precipitate materials were pelleted by centrifugation at the speed of 13,000 × g for 30 min, and discarded. Then, more ammonium sulfate was added to the remaining solution to the concentration of 100% (w/v) to precipitate nearly all of the remaining proteins. The precipitate was collected and then resuspended in the lysis buffer containing 300 mM NaCl.

Trypsin was utilized to digest impurities and trim the Penicillin G Acylase. It was dissolved in 1 mM HCl solution and was added to the solution from the previous step in the ratio of 1 mg trypsin per 50 mg protein. The digestion was incubated at 37°C for 30 minutes. After that, the solution was dialyzed overnight against the solution of 50 mM sodium acetate pH 5.7 with 300 mM of NaCl at 4°C.

Cation exchanger CM Sepharose Fast Flow (GE Healthcare) was used in the purification process. The media was equilibrated with 5 column volume of 150 mM NaCl in 50 mM sodium acetate pH 5.7. The dialyzed protein sample was diluted with 50 mM sodium acetate pH 5.7 solution to reduce the NaCl concentration to 150 mM. The protein solution was then centrifuged at the speed of 13,000 × g for 20 min at 4°C, and the supernatant was loaded to the column. The column was then washed and proteins were eluted from the column using

the equilibration buffer with NaCl concentration gradient. Fractions were collected and analyzed for protein concentration, purity, and enzymatic activity. The fractions containing *Bm*PGA-N5 as a major protein were pooled together and subjected to Butyl Sepharose Fast Flow (GE Healthcare) pre-equilibrated with 3.0 M NaCl in 50 mM sodium acetate buffer pH 5.7. Prior to the sample loading, its NaCl concentration was adjusted to 3.0 M. The proteins were eluted with 3.0-1.0 M step NaCl gradient in the 50 mM sodium acetate buffer. Fractions containing *Bm*PGA-N5 with high purity were pooled, concentrated, dialyzed with the storage buffer (600 mM NaCl in 50 mM HEPES pH 7.5), and kept at 4°C.

Thermal shift assay

Thermofluor assay was performed using a real-time PCR system (CFX Connect™). The stabilizing effect of 12 additives at the concentration of 10 mM was tested. Ionic salts and amino acids used in this experiment were dissolved in distilled water and 10 mM sodium hydroxide, respectively. In the assay, the mixture of protein at the concentration of 4 µM, 5x SYPRO orange dye (Thermo, from 5000x stock), 50 mM HEPES pH 7.5, 600 mM NaCl and the additives in the total reaction volume of 25 µL in the 0.1 mL 8-strip PCR tubes (NEST) sealed with 8-strip flat caps (NEST). The samples were subjected to a temperature gradient from 25°C to 95°C with a rate of 1°C/min. The melting temperature values (T_m, mid-point temperature of the protein-unfolding transition) corresponding to each experimental conditions were determined. Those values from each conditions were compared with the T_m from the condition which does not contain any additives, in order to determine whether that specific additives can increase the thermal stability of *Bm*PGA-N5 or not.

Crystallization

The purified *Bm*PGA-N5 sample was concentrated to 10 mg/mL at 4°C. Prior to the crystallization setup, precipitates were removed by centrifugation at the speed of 12,000 × g for 5 minutes at 4°C. A crystallization drop was set up from 1 µL of the purified *Bm*PGA-N5 solution and 1 µL of the precipitant solution on a siliconized-glass cover slip, which was then inverted over the reservoir in a hanging-drop vapor equilibration with 500 µL of the precipitant solution. The glass cover slip and the rim of the well were sealed together with a high-vacuum grease (Dow Corning®). All crystallization trials were setup in 24-well plates at 22 °C.

Crystallographic data collection

Crystal was transferred from a crystallization drop to a cryo-protectant solution for more than 8 hours based on our previous studies[4]. It was flash-frozen in liquid nitrogen and exposed to X-ray radiation (CuK_α, λ = 1.5418 Å) from the rotating-anode generator (Rigaku/MSC) with R_AXIS IV^{++} image plate detector at Center for Excellence in Protein and Enzyme Technology, Faculty of Science, Mahidol University, Thailand. Complete X-ray diffraction data of crystal samples were automatically acquired with the *CrystalClear*® (Rigaku/MSC). The space group was determined from Laue symmetry and systematic absences analysis.

Results and Discussion

For the preparation of the interested protein, after the purification scheme as mentioned, a sufficient amount of purified *Bm*PGA-N5 with ~90% purity was achieved. The SDS-PAGE result shows interested protein bands, which are the α-subunit (~24 kDa) and the β-subunit (~61 kDa) bands (**Figure 1.**). This protein was used for thermal stability studies and crystallization trials.

In order to find small molecules which might facilitate the crystallization of *Bm*PGA-N5, a thermofluor-based additive screening was performed. Additives which affect the interested protein thermal stability of the protein may increase the likelihood of its

crystallization. **Figure 2.** illustrates the obtained T_m values of *Bm*PGA-N5 in the presence of various additives. The condition which contained only the storage buffer (50 mM HEPES pH 7.5, 600 mM NaCl) was designated as a control. Four additives that increase the protein Tm were discovered (calcium chloride, copper (II) sulfate, zinc acetate and tryptophan). The remaining five additives affect the interested protein in an opposite manner. However, several unforeseen technical difficulties occurred during this experiment. For example, when copper (II) sulfate and zinc acetate were introduced to the mixtures, the solution suddenly precipitated. Moreover, tryptophan and tyrosine was not completely dissolved in the 10 mM sodium hydroxide solution, due to its low water solubility which in turn made its concentration debatable. Therefore, only calcium chloride was left as an additive that increases the *Bm*PGA-N5 melting temperature. It was incorporated in to the crystallization trials of *Bm*PGA-N5.

While most of the crystallization conditions resulted in precipitates, there were some conditions which promote *Bm*PGA-N5 crystallization. Multiple plate-like crystals were discovered in the condition containing 0.1 M Tris·HCl pH 8.5, 22.5% PEG4000 and 250 mM NaCl (**Figure 3.a**). These thin plate-like crystals were jointed together at the center of itself, which was difficult to separate and therefore made it impossible to dissect these crystal. However, in the presence of calcium chloride, the crystallization of *Bm*PGA-N5 seems to be different. Monoclinic crystals were observed instead of thin plate-like crystals.

The optimized crystal with the dimension of 200 × 500 × 100 μm was found in the condition containing 0.1 M Tris·HCl pH 8.5, 27 % PEG4000, 200 mM NaCl and 10 mM $CaCl_2$. It was transferred to a cryo-protectant solution for several hours, mounted and flash-freezed (**Figure 4.a**). Diffraction patterns were collected. **Figure 4.b** shows an example of an oscillation image. Statistics of data collection are summarized in **Table 1.** The crystal belongs to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 58.5, b = 77.9, c= 86.0 Å, β = 102.8° at 2.15 Å resolution, which is similar to our previous study with the wild-type *Bm*PGA[4].

The diffraction data will be used to determine the structure of *Bm*PGA-N5 by the molecular replacement using the available structure of the wild-type *Bm*PGA.

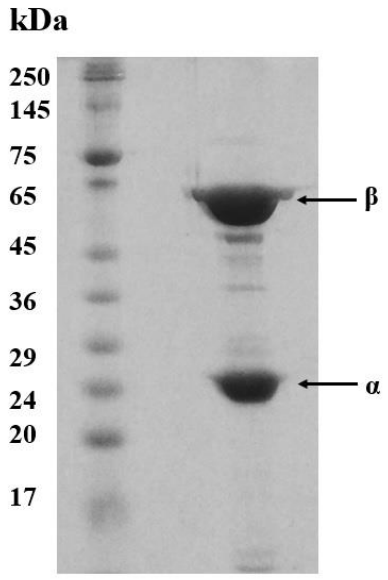


Figure 1. The 12% SDS-PAGE analysis of the purified *Bm*PGA-N5 protein. Positions of both α and β subunits of *Bm*PGA-N5 are marked with arrows.

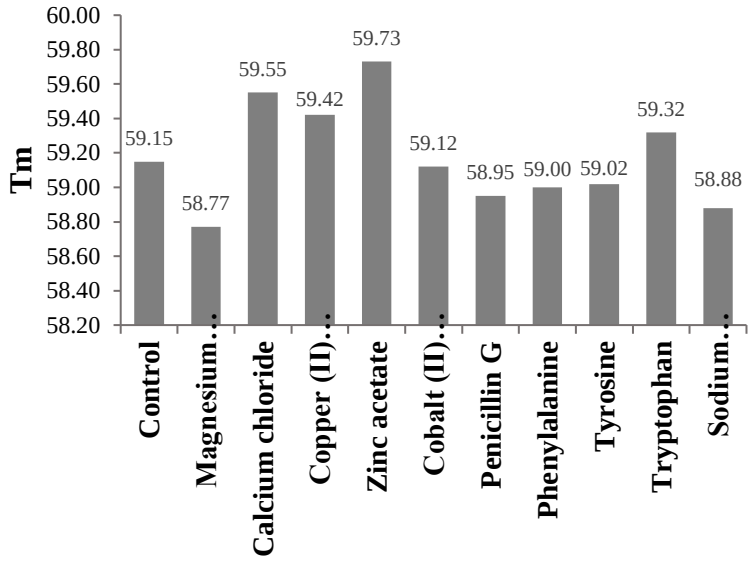
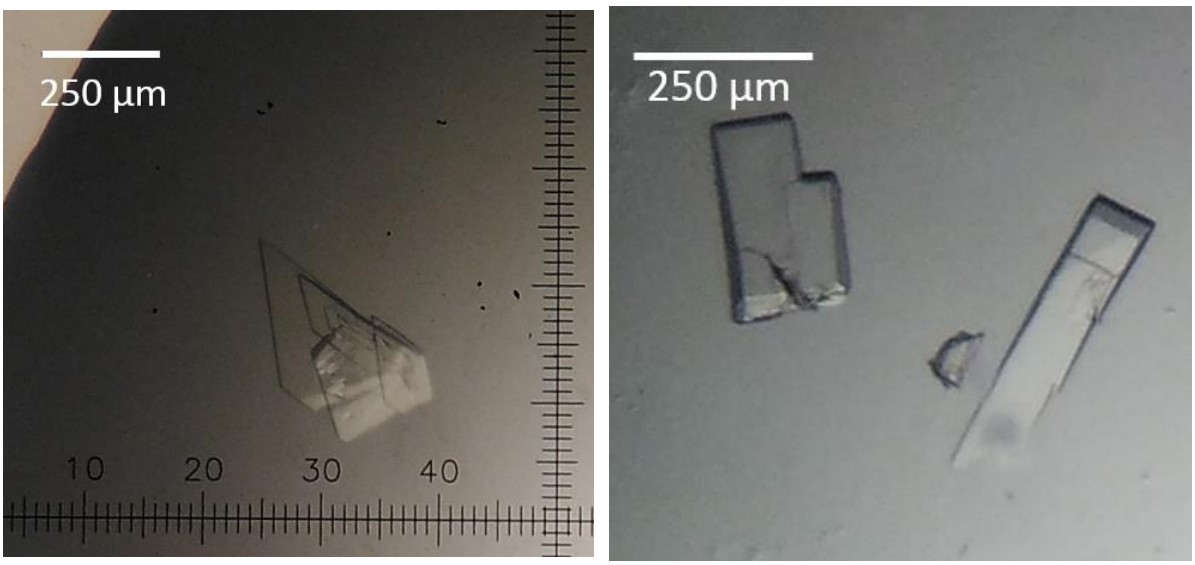
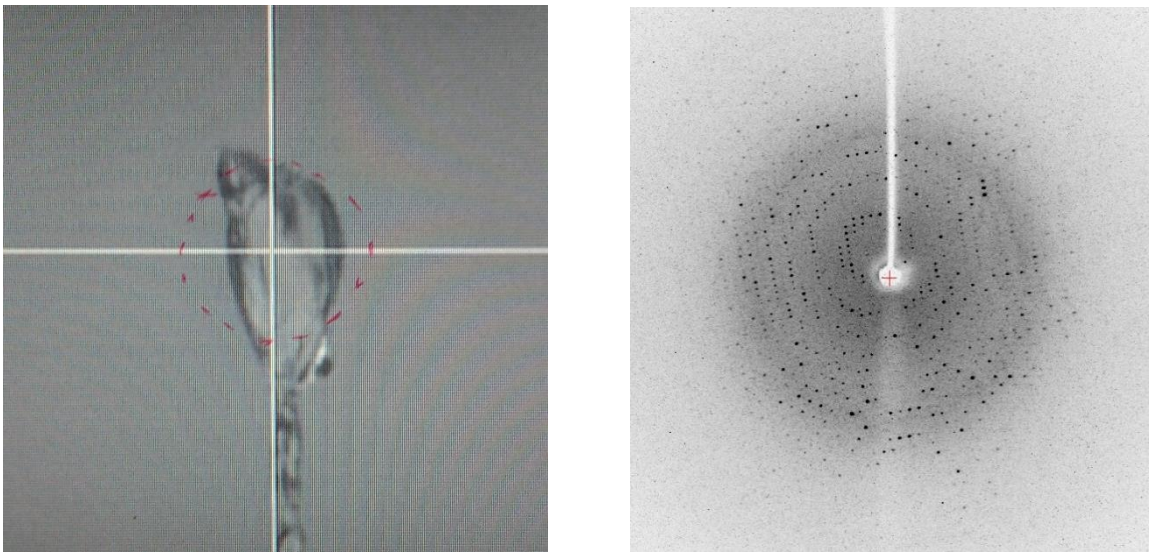


Figure 2. Melting temperature (T_m) of *Bacillus megaterium* penicillin G acylase N5 (*Bm*PGA-N5) in the presence of various additives. The condition which contains only the storage buffer is specified as control. T_m in Celsius are indicated above each condition.



(a) **(b)**

Figure 3. Crystals obtained from crystallization trials **(a)** A plate-like multiple crystal of *Bm*PGA-N5 received from the precipitation solution containing 0.1 M Tris·HCl pH 8.5, 22.5% PEG4000 and 250 mM NaCl **(b)** Monoclinic crystals of *Bm*PGA-N5 obtained from the precipitation solution containing 0.1 M Tris·HCl pH 8.5, 27.5% PEG4000, 150 mM NaCl and 5 mM $CaCl_2$



(a) **(b)**

Figure 4. *Bm*PGA-N5 crystal alignment for data collection and its X-ray diffraction patterns **(a)** A close-up view of the crystal mounted on the MicroMount™ at 0°-orientation during X-ray data collection **(b)** Photograph of X-ray diffraction patterns at crystal orientation of 0°

Table 1. Data-collection statistics for a *Bm*PGA-N5 crystal. Values in parentheses are for the highest resolution shell.

Resolution range (Å)	38.04 – 2.15 (2.23 – 2.15)
Space group	$P2_1$
Unit-cell parameters (Å; °)	58.5, 77.9, 86.0; 90.0, 102.8, 90.0
No. of total reflection	139170
No. of unique reflection	40012 (3788)
Redundancy	3.48 (3.31)
Completeness (%)	97.4 (92.6)
I/σ(I)	7.3 (1.7)
R_{merge}	0.081 (0.508)

Conclusion

The *Bm*PGA-N5 protein with ~90% purity was achieved via cation-exchange and hydrophobic interaction chromatography. Thermal stability of this protein in various additives was explored. It was found that the presence of calcium chloride elevates the melting temperature of *Bm*PGA-N5 protein. The optimized crystal was found in the condition containing 0.1 M Tris·HCl pH 8.5, 27 % PEG4000, 200 mM NaCl and 10 mM $CaCl_2$. The crystal belongs to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 58.5, b = 77.9, c= 86.0 Å, β = 102.8° at 2.15 Å resolution. The diffraction data will be used to determine the structure of *Bm*PGA-N5 by the molecular replacement using the available structure of the wild-type *Bm*PGA.

References

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