Video Article

Recombinant Protein Expression, Crystallization, and Biophysical Studies of a *Bacillus*-conserved Nucleotide Pyrophosphorylase, BcMazG

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Abstract

To overcome safety restrictions and regulations when studying genes and proteins from true pathogens, their homologues can be studied. *Bacillus anthracis* is an obligate pathogen that causes fatal inhalational anthrax. *Bacillus cereus* is considered a useful model for studying *B. anthracis* due to its close evolutionary relationship. The gene cluster *ba1554 - ba1558* of *B. anthracis* is highly conserved with the *bc1531-bc1535* cluster in *B. cereus*, as well as with the *bt1364-bt1368* cluster in *Bacillus thuringiensis,* indicating the critical role of the associated genes in the *Bacillus* genus. This manuscript describes methods to prepare and characterize a protein product of the first gene (*ba1554*) from the gene cluster in *B. anthracis* using a recombinant protein of its ortholog in *B. cereus*, *bc1531.*

Video Link

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Introduction

Recombinant protein expression is widely used to overcome problems associated with natural protein sources, such as limited protein quantities and harmful contamination. Moreover, in studies of pathogenic genes and proteins, an alternative laboratory strain that does not require additional safety precautions can be utilized. For example, *Bacillus cereus* is a useful model for studying *Bacillus anthracis* due to their close evolutionary relationship[1].

B. anthracis is an obligate pathogen that causes fatal inhalational anthrax in humans and livestock and can potentially be used as a bioweapon[2]. Thus, laboratory studies on *B. anthracis* are strictly regulated by the US Centers for Disease Control, requiring biosafety level 3 (BSL-3) practices, which mandate that the laboratory area be segregated with negative room pressure. In contrast to *B. anthracis*, *B. cereus* is categorized as a BSL-1 agent and thus has minimal safety concerns. *B. cereus* is an opportunistic pathogen that, upon infection, causes food poisoning that can be treated without medical assistance. However, because *B. cereus* shares many critical genes with *B. anthracis*, the functions of *B. anthracis* proteins can be studied using the corresponding homologs of *B. cereus*[1].

The *ba1554 - ba1558* gene cluster of *B. anthracis* is highly conserved with the *bc1531- bc1535* cluster of *B. cereus*, as well as with the *bt1364-bt1368* cluster of *Bacillus thuringiensis*, in terms of gene organization and sequence. Furthermore, the first genes (*ba1554*, *bc1531*, and *bt1364*) of the respective clusters are absolutely conserved (*i.e.,* 100% nucleotide sequence identity), implying a critical role of the gene product in the *Bacillus* species. Due to its location in these gene clusters, *ba1554* was misidentified as a putative transcription regulator[3]. However, amino acid sequence analysis of the *ba1554* product indicates that it belongs to the MazG family, which has nucleotide pyrophosphohydrolase activity and is not associated with transcription factor activity[4,5]. Although proteins belonging to the MazG family are diverse with respect to overall sequence and length, they share a common ~100-residue MazG domain characterized by an $EXXE_{12-28}EXXD$ motif ("X" stands for any amino acid residue, and the number indicates the number of X residues).

The MazG domain does not always directly account for a certain catalytic activity. A MazG member from *Escherichia coli* (EcMazG) possesses two MazG domains, but only the C-terminal MazG domain is enzymatically active[6]. Moreover, the substrate specificity of MazG enzymes varies from non-specific nucleoside triphosphates (for EcMazG) to specific dCTP/dATP (for integrin-associated MazG) and dUTP (for dUTPases)[6,7,8,9]. Therefore, biophysicochemical analyses of the BA1554 protein are necessary to confirm its NTPase activity and to decipher its substrate specificity.

Here, we provide a step-by-step protocol that most laboratories without a BSL-3 facility can follow to characterize the protein product of the *B. cereus bc1531* gene, which is an ortholog of *B. anthracis ba1554*, at the molecular level. Briefly, recombinant BC1531 (rBC1531) was expressed in *E. coli* and purified using an affinity tag. For X-ray crystallographic experiments, the crystallization conditions of the rBC1531 protein were screened and optimized. To assess the enzymatic activity of rBC1531, NTPase activity was monitored colorimetrically to avoid radioactively

labeled nucleotides that have been conventionally used. Finally, analyses of the obtained biophysicochemical data enabled us to determine the oligomerization state and catalytic parameters of rBC1531, as well as to obtain X-ray diffraction data from the rBC1531 crystal.

Protocol

1. Recombinant Protein Production and Purification of rBC1531

1. **Preparation of a recombinant BC1531 protein (rBC1531)-expression plasmid**
    1. Prepare genomic DNA of *B. cereus*[10].
    2. Amplify the *bc1531* gene from a template of *B. cereus* genomic DNA by polymerase chain reaction (PCR), using forward and reverse primers (see the Materials List) to create *Bam*HI and *Sal*I restriction enzyme sites, respectively[10].
    3. Digest the PCR product and a modified pET49b vector (pET49bm) using *Bam*HI and *Sal*I, as described[11].
    4. Mix the digested PCR product and vector (3:1 ratio) from step 1.1.3 using T4 DNA ligase in a 10 µL reaction, as described[11]. Incubate at 18 °C for 30 min.
    5. Pipette 3 µL of the ligation reaction into 50 µL of chemically competent *E. coli* DH5α cells in a tube. Mix gently by pipetting up and down and place on ice for 30 min. Heat shock for 45 s at 42 °C. Add 1 mL of Luria-Bertani (LB) medium and grow the cells while vigorously shaking (250 rpm, 37 °C, 45 min).
    6. Take 100 µL of the transformed cells from step 1.1.6 and spread them onto the LB-agar plates with 100 µg/mL kanamycin (Kan). Incubate for ~18 h at 37 °C.
    7. Pick colonies using a sterile tip and grow the cells in 3 mL of LB medium with 100 µg/mL Kan; shake vigorously (250 rpm, 37 °C, 18 h).
    8. Prepare plasmid DNA using an alkaline-SDS lysis method, as described[10].
    9. Confirm the nucleotide sequence of the rBC1531-expression construct using DNA sequencing[12].

2. **Overexpression of the rBC1531 protein**
    1. Re-transform the *E. coli* BL21 (DE3) strain with rBC1531-expression plasmid, as described in steps 1.1.5-1.1.6, for overexpression.
    2. Select a colony and grow it in 10 mL of LB medium containing 50 µg/mL Kan (LB+Kan); shake vigorously for 18 h at 37 °C.
    3. Inoculate 10 mL of overnight culture in 1 L of LB+Kan medium and grow at 37 °C until the OD_{600} (optical density at 600 nm) reaches ~0.7.
    4. Submerge the culture in ice-cold water for ~15 min to cool down the temperature to 18 °C and add isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture at a final concentration of 1 mM for recombinant protein expression. Grow the culture at 18 °C for an additional ~16-18 h.

3. **Purification of rBC1531**
    1. Harvest the cells by centrifugation (5,000 x g, 4 °C, 30 min). Discard the supernatant carefully so as to not disturb the cells. Re-suspend the cells in 50 mL of phosphate-buffered saline (PBS) solution containing 10 mM imidazole.
    2. Lyse the cells twice by sonication on ice to prevent the over-heating of the cell lysates (time: 2 min 30 s; cycle on: 5 s; cycle off: 10 s; amplitude: 38%).
    3. Clear the cell lysate by centrifugation (~25,000 x g, 4 °C, 30 min).
    4. Mix 3 mL of nickel beads and 60 mL of PBS containing 10 mM imidazole and gravity-flow the extra buffer to settle the equilibrated nickel beads in a 2.5 x 10 cm glass chromatography column.
    5. Pipette to transfer the supernatant from step 1.3.3 to the column with pre-equilibrated nickel beads and incubate at 4 °C for 2 h on a spinning wheel at a low speed (~20 rpm).
    6. Allow the supernatant to drain by gravity and wash the nickel beads in the column three times with 100 mL of PBS containing 10 mM imidazole.
    7. Elute rBC1531 protein by applying 4 x 5 mL of PBS containing 250 mM imidazole.
    8. Take 15 µL of the elution from step 1.3.7 and run it on 15% SDS-PAGE. Visualize the protein bands on the gel using Coomassie blue staining[10].

4. **Removal of the affinity tags of the rBC1531 protein by thrombin proteolysis**
    1. Pipette the fractions into dialysis tube (molecular weight cut off: 3 kDa) and place the tube in a beaker containing 4 L of thrombin cleavage-compatible buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, and 1.5 mM β-mercaptoethanol) at 4 °C overnight.
    2. Pipette the fractions and transfer them to a conical tube.
    3. Measure the absorbance at 280 nm and estimate the rBC1531 protein concentration, as described[13].
    4. Take 50 µg of rBC1531 in a 0.5 mL tube and add various amounts of thrombin (*i.e.,* 2, 1, 0.6, and 0.3 units). Incubate the rBC1531 and thrombin reactions at 20 °C for ~3 h to determine the least amount of thrombin required to cleave the N-terminal affinity tag.
    5. Run the rBC1531 and thrombin reactions on a 15% SDS-PAGE and determine the lowest amount of thrombin (*e.g.,* 0.3 units of thrombin per 50 µg of rBC1531) that completely digests the N-terminal affinity tag and produce a tag-free rBC1531.
    6. Add 10 mg of rBC1531 protein and 60 units of thrombin to a 15 mL tube and incubate at 20 °C for ~3 h for the thrombin-proteolysis reaction.
    7. Apply gel-filtration standards to a size-exclusion chromatography (SEC) column to prepare a standard curve of molecular weights and elution volumes, as described[13].
    8. Place the thrombin-digested rBC1531 protein from step 1.4.6 in a centrifugal filter tube (molecular weight cut off: 3 kDa) and centrifuge at 3,000 g at 4 °C to reduce to a volume of less than 5 mL.
    9. Apply the concentrated rBC1531 protein to the SEC column and collect 60 eluted fractions in 0.5 mL per tube[13].
    10. Take 15 µL of each fraction, run them on a 15% SDS-PAGE, and stain the gel using Coomassie staining solution (0.15% (w/v) Coomassie brilliant blue, 40% (v/v) methanol, and 10% (v/v) glacial acetic acid) as described[10].
    11. Pipette the fractions that contain rBC1531 and collect them in one tube.

2. Crystallization Screening and Optimization of rBC1531

1. **Screening conditions for rBC1531 protein crystallization**
    1. Concentrate the rBC1531 from step 1.4.11 up to ~18.5 mg/mL using a centrifugal filter tube, as described in step 1.4.8.
    2. Add 50 µL of each crystallization screening solution (see section 2.2)[14] to the wells of 96-well sitting-drop crystallization plates. Place 0.5 µL of the 18.5 mg/mL rBC1531 protein solution on a sitting bed. Mix the protein solution with 0.5 µL of the well solution, repeating the process for the entire plate.
    3. Cover the plate with clear adhesive film. Place the 96-well plates at 18 °C and allow for vapor diffusion.
    4. Scan the drops at 20-40X magnification using a light microscope to monitor crystal formation daily for 2-3 weeks.
    5. Collect X-ray diffraction data[12] using the obtained rBC1531 protein crystals.

2. **Optimization of rBC1531 crystallization conditions**
    1. Prepare 500 µL of the selected initial crystallization condition solution (*e.g.,* 0.1 M sodium cacodylate, pH 6.5 and 1.0 M sodium citrate) and fill a 24-well crystallization plate for the sitting drop.
    2. Add 0.5 µL of the 18.5 mg/mL rBC1531 protein solution to a sitting bed, mix with 0.5 µL of the well solution, and cover the plate immediately with clear adhesive film. Place the plate at 18 °C.
    3. Observe crystal growth under a light microscope for a week.
    4. Optimize various crystallization conditions by varying the pH (*i.e.,* pH 5.5-6.8) of 0.1 M cacodylate and by altering the salt concentration (*i.e.,* 0.9-1.2 M) of sodium citrate to obtain singular crystals. Monitor crystal growth for ~1 week and collect X-ray diffraction data[12].

3. Characterization of the Nucleoside Triphosphatase (NTPase) Activity of rBC1531

1. **Validation of the inorganic phosphate-dependent colorimetric study**
    1. Prepare a stock of pyrophosphate (100 mM) and dilute it to final concentrations of 0.1, 0.25, 0.5, 1.0, 5.0, 10, 50, 100, and 250 µM in 200 µL of reaction buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; and 2 mM $MgCl_2$) in duplicate 96-well plates. Use the first plate as a control (this does not contain pyrophosphatase). Ensure that the second plate contains pyrophosphatase as a working plate, as directed in step 3.1.2.
    2. Add 0.01 unit of *Saccharomyces cerevisiae* inorganic pyrophosphatase (1 µL) to the wells of the working plate and incubate at 20 °C for 30-60 min.
    3. Prepare molybdate acid solution by mixing ammonium molybdate (0.86% v/v) and ascorbic acid (14% v/v) solutions in a 7:3 ratio. Add 16 µL of molybdate acid solution to both the working and control wells and wait for 15 min.
    4. Read the optical density at 690 nm (OD_{690nm}).

2. **Colorimetric NTPase assay**
    1. Prepare a stock of 100 mM nucleoside triphosphate (NTP) substrate and dilute to the desired concentration (*e.g.,* 0, 0.2, 4.4, 11, 22, 44, 88, or 132 µM) in 180 µL of assay buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; and 2 mM $MgCl_2$).
    2. Add 20 µg of rBC1531 (1.5 mM) and the NTP substrates from step 3.2.1 up to 200 µL per reaction in a 96-well plate and pipette up and down to mix well. Cover the plate with its lid and place it in a 37 °C incubator for 30 min to perform the substrate hydrolysis reaction.
    3. Transfer the plate to a 70 °C water bath and allow to stand for 15 min to stop rBC1531-mediated catalytic reactions.
    4. Add 0.01 unit of *S. cerevisiae* inorganic pyrophosphatase (1 µL) to each well of the reaction plate (from step 3.2.3) and incubate at 20 °C for 30 min. Add 16 µL of molybdate acid solution to the reaction plate to develop the color (~15 min). Read at OD_{690nm}.
    5. Analyze using the Michaelis-Menten equation, as described[12].

Representative Results

Characterization of the protein of interest in this study began by preparing a sufficient quantity of recombinant *B. cereus bc1531* (rBC1531) protein, preferably more than several milligrams. The DNA fragment encoding the BC1531 protein was prepared by PCR using the genomic DNA of *B. cereus* as a template, as it contains orthologous genes identical to *ba1554*. rBC1531 was overexpressed as a soluble protein in *E. coli* cells. The rBC1531 protein was expressed with a 6x-Histidine tag and a thrombin cleavage site at its N-terminus[12]. As a first step of purification, rBC1531 was purified by nickel-affinity chromatography (**Figure 1A**). Next, a thrombin digestion trial was performed to determine the lowest amount of thrombin required for the complete digestion of the affinity tag from rBC1531 (**Figure 1A**). In our hands, 0.3 units of thrombin was sufficient to completely remove the affinity tag from rBC1531. Thus, when scaling, 10 mg of rBC1531 was treated with 60 units of thrombin for tag removal. After thrombin digestion, the tag-free rBC1531 was applied to a SEC column to remove any soluble aggregates and higher- or lower-molecular weight contaminants. The rBC1531 fractions were analyzed by SDS-PAGE, the results of which suggested that rBC1531 was ~99% pure (**Figure 1A**). Overall, 1 L of culture yielded ~8 mg of rBC1531 protein.

To estimate the oligomeric status of rBC1531, SEC was performed. A standard linear curve that correlates the log value of the molecular weights of the samples with their corresponding elution volumes was plotted using the peaks of the protein standards (**Figure 1B**). rBC1531 was eluted at an elution volume of ~84 mL, and its apparent molecular weight was estimated as ~55 kDa. Given that the calculated molecular weight of the rBC1531 monomer is ~13 kDa, these results indicate that rBC1531 assembles as a tetramer.

Purified rBC1531 was screened for crystallization using ~400 conditions in a sitting-drop vapor diffusion method. The rBC1531 crystals appeared in ~2 days at two conditions: condition-A, 0.1 M sodium citrate (pH 5.5) and 20% PEG 3000 (**Figure 2A**) and condition-B, 0.1 M sodium cacodylate (pH 6.5) and 1.0 M sodium citrate (**Figure 2B**). The crystallization conditions were optimized by varying the pH (0.1 M sodium citrate, pH 5.0-6.0) and the concentration of PEG 3000 (18-21% PEG 3000) from condition-A and by modifying the pH (0.1 M sodium cacodylate, pH 5.5-6.8) and salt concentration (0.9-1.2 M sodium citrate) from condition-B. Diffraction-suitable crystals were obtained only for condition-B, whereas the crystals from condition-A tended to be inter-grown rather than singular. Condition-B crystals diffracted X-rays to a resolution of 2.74 Å (**Figure 2C**) and were used for rBC1531 structure determination.

The observation of a conserved MazG domain in the BA1554 protein sequence suggested the presence of NTPase activity capable of hydrolyzing NTPs. NTP hydrolysis generally yields nucleoside diphosphate (NDP) and inorganic phosphate (Pi), or nucleoside monophosphate (NMP) and pyrophosphate (PPi). PPi requires additional pyrophosphatase activity to yield Pi. Levels of Pi can be directly measured using molybdate, which forms a blue-colored complex with Pi (molybdate-Pi) that can be monitored using the optical density at a wavelength of 690 nm (OD_{690nm}) (**Figure 3A**). In our study, after NTP hydrolysis by rBC1531, no visible blue color was produced after adding molybdate (**Figure 3B**). However, when pyrophosphatase was added to the rBC1531-mediated NTP hydrolysis reaction, the color changed to deep blue (**Figure 3B**), indicating that rBC1531 has NTP pyrophosphohydrolase activity. A plot of the OD_{690nm} and NTP concentration was analyzed using a non-linear regression method to determine the kinetic parameters (V_{max} of 0.75 and K_m of 10 µM) for the rBC1531 enzyme (**Figure 3C**).

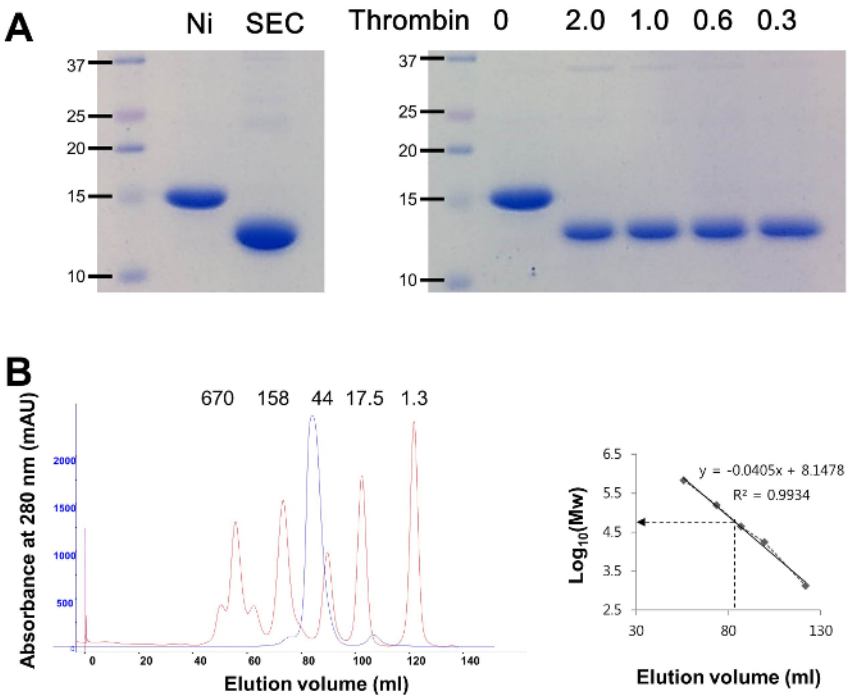

Figure 1: SDS-PAGE and size-exclusion chromatography (SEC) analysis. (**A**) SDS-PAGE analysis of rBC1531. (Left) The rBC1531 elution peaks from nickel affinity chromatography (lane 2, Ni) and SEC (lane 3) were analyzed along with protein standards (lane 1; labeled in kDa). (Right) Analysis of the thrombin digestion of rBC1531 for the removal of the affinity tag. The amounts of thrombin added to 50 µg of rBC1531 in different reactions are indicated in units above the gel. (**B**) Size-exclusion chromatography (SEC) analysis of rBC1531 and the protein standards. (Left) Elution profiles of rBC1531 (blue) and standards (red). Molecular weights of the standard proteins are shown above each peak, in kDa. The vertical (y) and horizontal (x) axes show the milli-absorption units (mAU at 280 nm) and retention volumes (mL), respectively. (Right) The apparent molecular size of rBC1531 was estimated using a linear plot for the molecular weights, in log scale, and elution volumes of protein standards (R^2 = 0.9934). Please click here to view a larger version of this figure.

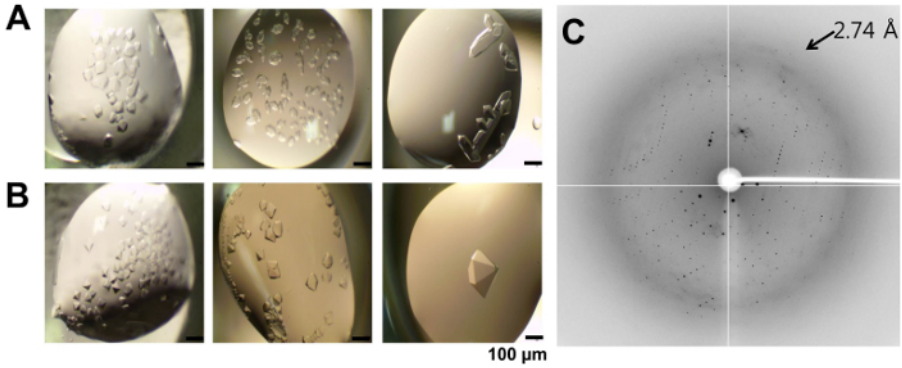

Figure 2: Crystallization and X-ray diffraction. (**A**) rBC1531 crystals in condition-A, 0.1 M sodium citrate (pH 5.5) and 20% PEG 3000. (**B**) rBC1531 crystals in condition-B, 0.1 M sodium cacodylate (pH 6.5) and 1.0 M sodium citrate. Initial (left) and optimized (middle and right) crystals are shown. The optimized rBC1531 crystal (right, ~0.35 mm x ~0.35 mm x ~0.20 mm) was used for X-ray diffraction. Scale bars = 100 µm. (**C**) X-ray diffraction image of the rBC1531 crystal obtained at the PAL beamline BL-7A[12]. The arrow indicates a high-resolution spot. Please click here to view a larger version of this figure.

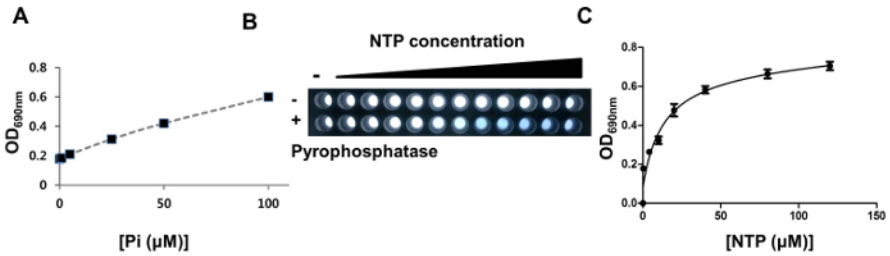

Figure 3: NTPase activity. (**A**) The formation of the molybdenum-Pi complex is dependent on Pi concentration. Optical density at 690 nm is directly associated with increase in Pi levels. The Y-axis and X-axis represent the OD_{690nm} and concentration (µM) of Pi, respectively. (**B**) Color changes upon the addition of pyrophosphatase. In the first row of wells, pyrophosphatase was not added and did not form the blue-colored Pi-complex. However, the second row consists of wells in which the rBC1531-NTP reactions were treated with pyrophosphatase and developed a blue color. OD_{690nm} values increased with increase in NTP concentrations. (**C**) Michaelis-Menten plot of the rBC1531-NTP reactions. The Y-axis and X-axis represent the OD_{690nm} and concentration (0-120 µM) of NTP substrates, respectively. The error bars represent the standard deviation for three separate experiments. Please click here to view a larger version of this figure.

Discussion

Studies of true pathogens are limited due to safety restrictions and regulations. Alternatively, pathogens can be studied using evolutionarily related non-pathogens or less pathogenic species. The *ba1554* gene is considered a critical gene in *B. anthracis*. Fortunately, an identical gene is present in nonclinical *B. cereus.* Thus, without serious safety concerns, recombinant BC1531 protein can be used for biophysical and enzymatic characterization. Here, we described detailed procedures, moving from a gene to a purified recombinant protein. Using recombinant protein, biophysical characterization analyses were conducted to reveal that tetrameric rBC1531 catalyzes NTP hydrolysis into NMP and PPi.

SEC was employed as the last step of protein purification to remove any protein contaminants derived from the cell lysates that have different sizes than rBC1531. A SEC column was also used to determine the oligomerization status of rBC1531, which was a tetramer. The crystal structure of rBC1531 was consistently determined to be tetrameric[12].

Among biophysical techniques, X-ray crystallography is the best technique for generating atomic models of protein. In general, three-dimensional singular crystals with dimensions larger than 0.1 mm are required for X-ray diffraction. To obtain X-ray diffraction-suitable crystals, crystallization conditions were optimized. For rBC1531, two conditions produced crystals in the initial screening, but only one condition was useful for producing diffraction-suitable crystals[12].

The NTP hydrolysis activity of rBC1531 was assessed by monitoring the formation of a blue-colored complex of molybdate and Pi in the rBC1531-mediated enzymatic reaction[15]. It has the great advantage of non-radioactively detecting NTP hydrolysis, since the molybdate-Pi complex can be optically measured. In the rBC1531 reaction, the blue-colored complex was generated only in the presence of pyrophosphatase, demonstrating that rBC1531 hydrolyzes NTP into NMP and pyrophosphate. Furthermore, because the blue color of the molybdate-Pi complex can be detected with the naked eye, Pi levels in the rBC1531 reaction, as well as in other applications, can be easily assessed in pilot experiments. Moreover, kinetic parameters, such as K_m and V_{max} can be calculated by quantifying the intensity of the colorimetric reaction using a visible-light spectrophotometer. However, the presences of free phosphate in the reaction buffer or precipitation during the enzymatic reaction would impair the attainment of accurate results from the NTP hydrolysis reactions. Thus, it is desirable to prepare a Pi standard curve using the identical reaction buffer as in the NTP hydrolysis runs and to ensure an increase in optical density only in the presences of Pi.

Given that the K_m value of the rBC1531-NTP was low (K_m = 10 µM), the true substrate for rBC1531 may be a specific type of canonical or noncanonical (d)NTP. To test this possibility, the experimental conditions can be modified to use a specific NTP (*i.e.,* ATP, GTP, CTP, or UTP)

rather than a mixture of NTPs. By comparing kinetic parameters, substrate specificity can be determined in future applications. Indeed, additional studies indicate that BC1531 prefers ATP[12]. Despite the technical simplicity of the colorimetric assay using the molybdate reaction, this assay has a drawback in that the color can become randomly intense when the blue-colored molybdate-Pi complex is left at neutral pH for an extended period of time. Therefore, a critical step is to perform measurements within 15-30 min after the molybdate is added to the Pi-containing sample. The experiment is repeated using the same time point to avoid variations in OD measurements.

In conclusion, various biophysicochemical methods were used to analyze the oligomerization state and enzymatic activity of rBC1531 and to prepare diffraction-quality crystals for structural studies. These methods can be safely and easily applied to other proteins of interest with minimal experimental equipment requirements.

Disclosures

The authors have nothing to disclose.

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