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Received 22 December 2009

Accepted 26 April 2010

Crystallization and preliminary X-ray crystallographic analysis of a full-length active form of the Cry4Ba toxin from *Bacillus thuringiensis*

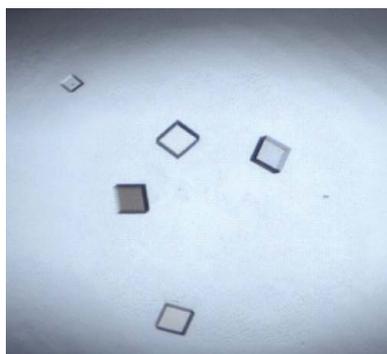
To obtain a complete structure of the *Bacillus thuringiensis* Cry4Ba mosquito-larvicidal protein, a 65 kDa functional form of the Cry4Ba-R203Q mutant toxin was generated for crystallization by eliminating the tryptic cleavage site at Arg203. The 65 kDa trypsin-resistant fragment was purified and crystallized using the sitting-drop vapour-diffusion method. The crystals belonged to the rhombohedral space group *R*32, with unit-cell parameters $a = b = 184.62$, $c = 187.36$ Å. Diffraction data were collected to at least 2.07 Å resolution using synchrotron radiation and gave a data set with an overall R_{merge} of 9.1% and a completeness of 99.9%. Preliminary analysis indicated that the asymmetric unit contained one molecule of the active full-length mutant, with a V_M coefficient and solvent content of 4.33 Å³ Da⁻¹ and 71%, respectively.

1. Introduction

Bacillus thuringiensis is a rod-shaped aerobic spore-forming Gram-positive bacterium that is widespread in nature (Schnepf *et al.*, 1998). Insecticidal proteins (known as δ -endotoxins) that are produced as cytoplasmic inclusions during sporulation have been classified *via* sequence similarities into two main families: the Cry (crystal) and the Cyt (cytolytic) toxins (Crickmore *et al.*, 1998). The three-domain Cry toxins are of particular interest as they have been shown to be toxic towards a wide variety of insect larvae (Schnepf *et al.*, 1998). For instance, the 130 kDa Cry4Ba toxin produced from *B. thuringiensis* subsp. *israelensis* is specifically toxic to mosquito larvae of the genera *Aedes* and *Anopheles*, which are the most important vectors of life-threatening human diseases such as dengue haemorrhagic fever and malaria (Federici *et al.*, 2007).

B. thuringiensis Cry toxins are synthesized as inactive protoxins found within inclusions and are transformed into cytolytic pore-forming toxins *via* a multistep process (Gomez *et al.*, 2007). Upon ingestion by susceptible insect larvae, the protoxins are solubilized in the midgut lumen and activated by larval gut proteases to yield ~65 kDa toxic fragments comprising three domains: a bundle of α -helices, a three- β -sheet domain and a β -sandwich (Schnepf *et al.*, 1998; Crickmore *et al.*, 1998). The active toxins subsequently bind to specific receptors in the luminal plasma membrane, allowing the insertion of their pore-forming portion into the target cell membrane and leading to the eventual death of the larvae (Soberon *et al.*, 2009). However, the actual underlying mechanisms of the toxic process are still not completely understood.

The protoxins of the 130 kDa mosquito-specific Cry4Aa and Cry4Ba toxins are proteolytically activated by trypsin *in vitro* to two protease-resistant fragments of ~47 and ~20 kDa that remain non-covalently associated, forming a 65 kDa active toxin (Angsuthanasombat *et al.*, 2004). These two trypsin fragments are produced by cleavage at Arg235 and Arg203, which are located in the exposed loop connecting helices 5 and 6 within the pore-forming domain of Cry4Aa and Cry4Ba, respectively (Angsuthanasombat *et al.*, 2004). As reported previously, glutamine substitution of Arg235, resulting in blockage of the tryptic cleavage site, in Cry4Aa has been shown to lead to retention of larvicidal activity (Boonserm, Poonwiroon *et al.*, 2004) and the 65 kDa toxin has successfully been crystallized and



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modelled (Boonserm, Angsuthanasombat *et al.*, 2004; Boonserm *et al.*, 2006). An incomplete structure of the 65 kDa chymotrypsin-treated Cry4Ba toxin that lacked the N-terminal helices owing to proteolysis has also been reported (Boonserm *et al.*, 2005). It is possible that chymotrypsin treatment produced heterogeneous protein fragments by cleavage at alternative N-terminal sites of the Cry4Ba native toxin. Thus, glutamine substitution of Arg203 in Cry4Ba, which results in resistance to further proteolytic digestion, should be of great use in determining the full-length three-dimensional structure. In this study, we therefore generated a full-length Cry4Ba-R203Q mutant that retains biological activity. The purification and crystallization of the 65 kDa trypsin-activated mutant protein are reported here.

2. Materials and methods

2.1. Construction of a trypsin-site elimination mutant

The pMU388 plasmid encoding the 130 kDa Cry4Ba protoxin under control of the *lacZ* promoter (Angsuthanasombat *et al.*, 1987) was used as a template for generating a trypsin-site elimination mutant (R203Q) using a pair of mutagenic oligonucleotides (forward primer, 5'-GGTCTTTAGCACAGTCTGCAGGTGACCAACTATA-TAAC-3'; reverse primer, 5'-GTTATAGTTGGTCACCTGCAGACTGTGCTAAGACC-3'; changed nucleotides are indicated in bold and *Pst*I restriction sites are indicated in italics). The trypsin site at Arg203 was deleted by substitution with glutamine *via* PCR-based site-directed mutagenesis following the procedure of the Quik-Change Mutagenesis Kit (Stratagene).

2.2. Protein expression, tryptic activation and purification

Escherichia coli strain JM109 harbouring the Cry4Ba-R203Q plasmid was grown at 310 K in Luria-Bertani medium containing ampicillin (100 µg ml⁻¹) and protein expression was induced with isopropyl β-D-1 thiogalactopyranoside (IPTG; 0.1 mM) for 4 h. *E. coli* cultures that overexpressed the mutant protein as cytoplasmic inclusions were harvested by centrifugation, resuspended in distilled water and disrupted using a French pressure cell (69 MPa). After centrifugation, the inclusion pellets were washed once with wash buffer consisting of 100 mM KH₂PO₄ pH 6.5, 0.8 M NaCl and 0.1%

Triton X-100 and washed twice with cold distilled water by sonication. The partially purified inclusions (5–10 mg ml⁻¹) were solubilized in 50 mM sodium carbonate pH 9.0 by incubation at 310 K for 1 h and insoluble materials were removed by centrifugation at 12 000g for 15 min.

The 130 kDa solubilized protoxin was activated with trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated, Sigma) at a 1:20(*w:w*) enzyme:toxin ratio in 50 mM sodium carbonate pH 9.0 for 16 h at 310 K. Proteolysis was stopped by the addition of 1 mM phenylmethanesulfonyl fluoride. The trypsin-treated fraction was subjected to size-exclusion chromatography on Superdex 200 (GE Healthcare) equilibrated with 50 mM sodium carbonate pH 9.0 at a flow rate of 0.4 ml min⁻¹. The eluted peak fractions containing the 65 kDa mutant protein were pooled and concentrated to ~10 mg ml⁻¹ by ultrafiltration using a Centriprep column (30 kDa cutoff, Amicon) prior to crystallization trials.

A mosquito-larvicidal assay of the toxin inclusions was performed using two-day-old *Aedes aegypti* larvae. The assay was carried out in 1 ml distilled water containing a serial dilution of toxin inclusions (0.2, 1.0, 2.5, 5, 10 and 25 µg ml⁻¹) in a 48-well microtitre plate at room temperature (298–303 K) with ten larvae per well and a total of 100 larvae per toxin concentration. Mortality was recorded after 24 h incubation. LC₅₀ values and 95% confidence were calculated using the Probit method of Finney analysis.

2.3. Protein crystallization

Crystals were grown by the vapour-diffusion method in sitting drops. 0.5 µl purified protein solution (~10 mg ml⁻¹) was mixed with an equal volume of reservoir solution consisting of 0.05 M ammonium acetate, 0.01 M MgCl₂, 5–10% (*v/v*) (±)-2-methyl-2,4-pentane-diol (MPD) in 0.05 M Tris-HCl buffer pH 7.0–8.0 (modified from Natrix screening kit condition No. 43, Hampton Research). The drop was then equilibrated against 100 µl reservoir solution at 293 K. Crystals appeared after a period of between 15 d and four weeks and grew to their maximum dimensions in three months.

2.4. Sequence analysis of Cry4Ba-R203Q by LC/MS/MS

Purified Cry4Ba-R203Q protein and dissolved Cry4Ba-R203Q crystals were subjected to SDS-PAGE (12% gel); they were subsequently eluted from the excised gel and digested with trypsin according to the standard protocol. Trypsin-generated peptide fragments were separated on a C18 column (0.18 × 100 mm, Thermo Electron, USA) and analyzed by liquid chromatography-tandem mass spectrometry (Finnigan LTQ Linear Ion Trap Mass Spectrometer) as performed by the Genome Institute, BIOTEC, Thailand.

2.5. Crystallographic data collection and processing

The crystal was transferred from the crystallization drop into a cryoprotectant solution (5 µl) consisting of 0.05 M ammonium acetate, 0.01 M MgCl₂, 10% (*v/v*) MPD and 20% (*v/v*) glycerol in 0.05 M Tris buffer pH 7.5 for a few seconds, mounted on a synthetic nylon loop (0.1–0.2 mm, Hampton Research) and flash-cooled in liquid nitrogen. X-ray diffraction data were collected on beamline BL13B1 equipped with a CCD detector (Q315, ADSC) at the National Synchrotron Radiation Research Center (Hsinchu, Taiwan). For complete data collection, 90° of rotation was measured with 1.0° oscillation using an X-ray wavelength of 1.00 Å, an exposure duration of 10 s and a crystal-to-detector distance of 300 mm from a crystal cooled to 110 K in a nitrogen stream using an X-Stream cryosystem

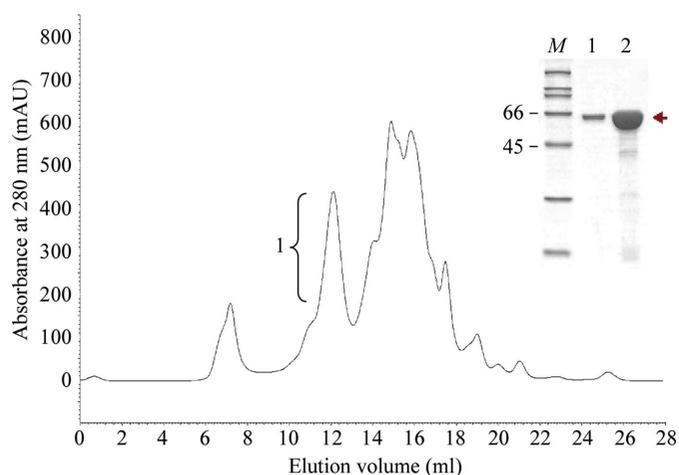


Figure 1 Protein purification of the 65 kDa trypsin-activated Cry4Ba-R203Q mutant using Superdex 200 size-exclusion chromatography. The inset shows SDS-PAGE analysis of the pooled (lane 1) and concentrated (10 mg ml⁻¹; lane 2) fractions of peak 1 containing the 65 kDa mutant protein. Lane M contains molecular-mass standards (with values in kDa).

Table 1

 Comparison of the toxicity of Cry4Ba and its mutant inclusions towards *A. aegypti* larvae.

Toxin inclusion	LC ₅₀ † (µg ml ⁻¹)
Wild-type Cry4Ba	0.50 (0.36–0.71)
Cry4Ba-R203Q	0.59 (0.37–0.93)

† LC₅₀ represents the toxin inclusion concentration that leads to 50% mortality after 24 h exposure to a serial dilution of each toxin inclusion. 95% confidence limits are indicated in parentheses.

(Rigaku/MS). All data were indexed, integrated and scaled using the *HKL-2000* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

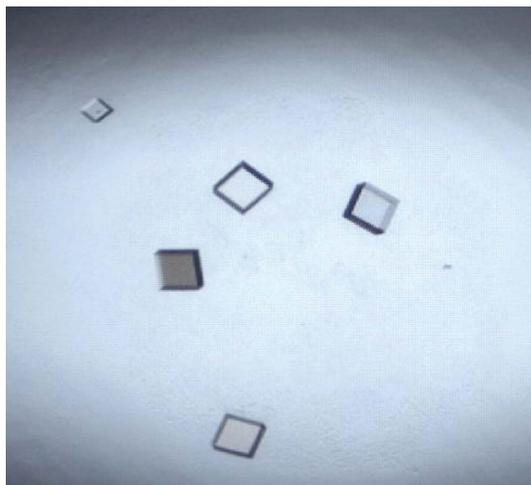
3.1. Protein preparation of the 65 kDa Cry4Ba-R203Q mutant toxin

Upon IPTG induction, the 130 kDa Cry4Ba-R203Q mutant protoxin was predominantly produced as a crystalline inclusion that was partially purified from the *E. coli* lysate and solubilized in carbonate buffer pH 9.0; it had a solubility that was >70% of that of the wild-type inclusion under similar conditions. After activation by trypsin, the activated mutant toxin was successfully purified by gel filtration as a single peak at an elution volume corresponding to a 65 kDa monomer, which was obtained with a purity greater than 90% as analyzed by SDS-PAGE (Fig. 1).

To assess the effect of the mutation on toxicity, the Cry4Ba-R203Q mutant toxin inclusion was tested for its relative biological activity against *A. aegypti* mosquito larvae. The results given in Table 1 show that the mutant toxin retained a high larvicidal activity that was comparable with that of the wild-type toxin. This indicated that the elimination of the tryptic cleavage site at Arg203 by glutamine substitution (R203Q) had no detrimental effect on the toxicity of Cry4Ba.

3.2. Crystallization of the trypsin-activated Cry4Ba-R203Q mutant toxin

In analogy to our previous study of Cry4Aa (Boonserm, Angsuthanasombat *et al.*, 2004), we successfully crystallized the 65 kDa trypsin-activated Cry4Ba-R203Q protein. Well formed crystals were obtained by using 0.05 M ammonium acetate, 0.01 M MgCl₂ and


Figure 2

Crystals of the 65 kDa trypsin-activated Cry4Ba-R203Q protein. The approximate dimensions of the largest crystal are 0.07 × 0.07 × 0.02 mm.

Table 2

Diffraction statistics for Cry4Ba-R203Q.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.00
Temperature (K)	110
Resolution range (Å)	30.0–2.07 (2.15–2.07)
Space group	R32
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 184.62, <i>c</i> = 187.36
Unique reflections	74138 (8174)
Completeness (%)	99.9 (100)
<i>I</i> / <i>σ</i> (<i>I</i>)	19.8 (4.3)
Average redundancy	6.2
<i>R</i> _{merge} † (%)	9.1 (40.4)
Mosaicity (°)	0.25
No. of molecules per asymmetric unit	1
Matthews coefficient (Å ³ Da ⁻¹)	4.33
Solvent content (%)	71.0

† $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, 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*.

10% (v/v) MPD in 0.05 M Tris–HCl pH 7.5 (Fig. 2). It is noteworthy that when the dissolved crystals were verified by LC/MS/MS in order to confirm the presence of the intact 65 kDa Cry4Ba-R203Q protein, all parts of the trypsin-generated peptide sequences were found to perfectly match the corresponding Cry4Ba mutant sequence (residues Lys57–Ile633). This indicated that the crystals of the activated Cry4Ba-R203Q toxin still contained the complete molecule containing the N-terminal fragment, in contrast to those of the 65 kDa chymotrypsin-treated native toxin, which lacked the N-terminal α1–α2b helices (Boonserm *et al.*, 2005).

3.3. X-ray diffraction analysis of the Cry4Ba-R203Q activated toxin

Analysis of the X-ray diffraction data indicated that the crystals belonged to the rhombohedral space group R32, with unit-cell parameters *a* = *b* = 184.62, *c* = 187.36 Å. A complete data set was collected to a resolution of 2.07 Å and consisted of 74 138 unique reflections with an *R*_{merge} of 9.1%. Assuming the presence of one activated toxin molecule (molecular weight of ~65 000) in the asymmetric unit, the Matthews coefficient (*V*_M) of the crystal (Matthews, 1968) was calculated to be 4.33 Å³ Da⁻¹, corresponding to an estimated solvent content of 71%. The data statistics are summarized in Table 2. Structure determination of the Cry4Ba-R203Q mutant (602 residues) by the molecular-replacement method using the atomic coordinates of the truncated Cry4Ba structure (558 residues; PDB code 1w99; Boonserm *et al.*, 2005) as a search model is in progress.

This work was generously funded by the Thailand Research Fund (TRF) in cooperation with the Commission of Higher Education. The Royal Golden Jubilee PhD scholarship from TRF (to NT) is gratefully acknowledged. We thank Yuch-Cheng Jean and supporting staff for technical assistance at the synchrotron-radiation facility during data collection at BL13B1 of NSRRC in Taiwan. This study was also supported by the National Synchrotron Radiation Research Center (973RSB02 to C-JC).

References

- Angsuthanasombat, C., Chungjatupornchai, W., Kertbundit, S., Luxanil, P., Settasatian, C., Wilairat, P. & Panyim, S. (1987). *Mol. Gen. Genet.* **208**, 384–389.
- Angsuthanasombat, C., Uawithya, P., Leetchewa, S., Pornwiroon, W., Ounjai, P., Kerdcharoen, T., Katzenmeier, G. R. & Panyim, S. (2004). *J. Biochem. Mol. Biol.* **37**, 304–313.

- Boonserm, P., Angsuthanasombat, C. & Lescar, J. (2004). *Acta Cryst.* **D60**, 1315–1318.
- Boonserm, P., Davis, P., Ellar, D. J. & Li, J. (2005). *J. Mol. Biol.* **348**, 363–382.
- Boonserm, P., Mo, M., Angsuthanasombat, C. & Lescar, J. (2006). *J. Bacteriol.* **188**, 3391–3401.
- Boonserm, P., Pornwiroon, W., Katzenmeier, G., Panyim, S. & Angsuthanasombat, C. (2004). *Protein Expr. Purif.* **35**, 397–403.
- Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. & Dean, D. H. (1998). *Microbiol. Mol. Biol. Rev.* **62**, 807–813.
- Federici, B. A., Park, H. W., Bideshi, D. K., Wirth, M. C., Johnson, J. J., Sakano, Y. & Tang, M. (2007). *J. Am. Mosq. Control Assoc.* **23**, 164–175.
- Gomez, I., Pardo-Lopez, L., Munoz-Garay, C., Fernandez, L. E., Perez, C., Sanchez, J., Soberon, M. & Bravo, A. (2007). *Peptides*, **28**, 169–173.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. & Dean, D. H. (1998). *Microbiol. Mol. Biol. Rev.* **62**, 775–806.
- Soberon, M., Gill, S. S. & Bravo, A. (2009). *Cell. Mol. Life Sci.* **66**, 1337–1349.