BMB
reports

Amino acid substitution on β1 and αF of Cyt2Aa2 affects molecular interaction of protoxin

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Cyt2Aa2 is a mosquito-larvicidal protein produced as a 29 kDa crystalline protoxin from *Bacillus thuringiensis* subsp. *darmstadiensis*. To become an active toxin, proteolytic processing is required to remove amino acids from its N- and C-termini. This study aims to investigate the functional role of amino acid residues on the N-terminal β1 and C-terminal αF of Cyt2Aa2 protoxin. Mutant protoxins were constructed, characterized and compared to the wild type Cyt2Aa2. Protein expression data and SDS-PAGE analysis revealed that substitution at leucine-33 (L33) of β1 has a critical effect on dimer formation and structural stability against proteases. In addition, amino acids N230 and I233-F237 around the C-terminus αF demonstrated a crucial role in protecting the protoxin from proteolytic digestion. These results suggested that β1 and αF on the N- and C-terminal ends of Cyt2Aa2 protoxin play an important role in the molecular interaction and in maintaining the structural stability of the protoxin. [BMB reports 2010; 43(6): 427-431]

INTRODUCTION

Cyt2Aa2 is a crystal delta-endotoxin produced during a sporulation stage of a Gram-positive, spore-forming bacterium *Bacillus thuringiensis* subsp. *darmstadiensis* (1, 2). This protein family is recognized as a promising bio-insecticide with a large number of applications. It is highly toxic to the larvae of dipteran insects such as blackflies and mosquitoes (3, 4), the latter of which are a major insect vector for the dengue virus which causes dengue hemorrhagic fever, a serious disease in tropical countries.

The Cyt toxin family shows a broad range of activity *in vitro*. However, *in vivo* it is specifically toxic to dipteran larvae (4-7). Based on amino acid sequence similarity, the toxins can be divided into two classes: Cyt1 and Cyt2. They share 39% and 70% amino acid sequence identity and similarity, respectively (8-11). Expression of Cyt1 toxin requires P20 helper protein, while Cyt2 toxin can be expressed by itself. An X-ray crystal structure of Cyt2 revealed a single domain in α/β architecture. The structural folding is composed of six α-helices and seven β-sheets (12). Even though Cyt2 is produced as a crystal protein in *Bacillus thuringiensis*, it can be cloned and expressed in *E. coli* as a protein inclusion (10). These crystal and inclusion proteins are solubilized in an alkaline condition of the larval midgut. Proteolytic digestion by insect gut proteases is generally required for a 29 kDa protoxin to become a 23-25 kDa active toxin. For example, a 259-residue Cyt2Aa2 undergoes proteolytic processing by proteinase K to remove N- and C-terminal fragments at the cleavage sites after serine 37 and serine 228 to become an active form (10, 12, 13). This active molecule then binds to the brush border membrane, resulting in cell lysis (1). Its mode of action is thought to act as either a detergent-like or a pore-forming toxin. In a detergent-like model, the activated toxins are locally aggregated on the surface of lipid bilayers causing membrane disruption, releasing protein and lipid complexes (14, 15). In the pore-forming model, the activated toxins are inserted into the lipid bilayers, forming an oligomeric assembly and generating transmembrane pores. The leakage through these pores then leads to a colloidal osmotic imbalance, lysing the target cells (16).

Since Cyt toxin does not require the N- and C-terminal fragments for its activity (9, 13), this study aims to investigate the significance of its terminal ends - especially the N-terminal β1 and C-terminal αF on the production and structural folding of the protoxin. Selected mutations of amino acids on N- and C-terminals were introduced using site-directed mutagenesis. The mutant toxins were expressed in *E. coli* and characterized for their biochemical properties, structural conformation, molecular interactions and biological activities.

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Received 25 November 2009, Accepted 18 March 2010

Keywords: *Bacillus thuringiensis*, Cytolytic toxin, Mutagenesis, Protein folding, Toxicity

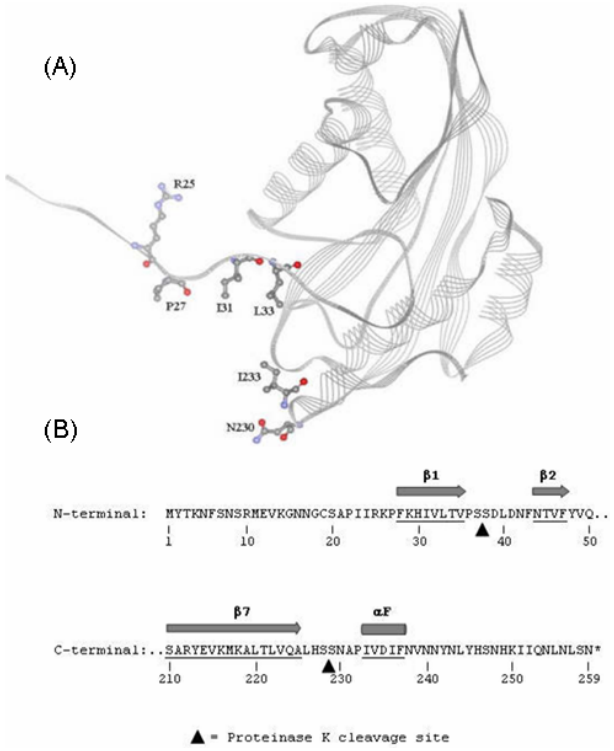

Fig. 1. Mutated residues of Cyt2Aa2: R25, P27, I31, L33 are located around β-1 sheet on N terminus, while N230 and I233 are in α-F helix on C-terminus of Cyt2Aa2 (A). The cleavage site of proteinase K digestion are located after serine 37 and serine 228 (B).

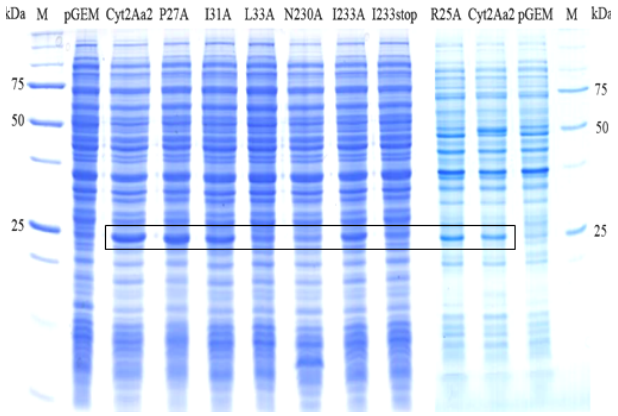

Fig. 2. Expression of mutants and Cyt2Aa2 are analyzed by SDS-PAGE. The toxins were cloned into pGEM-T Easy and expressed under *lac* promoter. The rectangle represents the position of Cyt2Aa2 protein.

RESULTS AND DISCUSSION

Mutational effect of selected amino acids on toxin production and folding

Based on the crystal structure of Cyt2 toxin, site-directed mutagenesis was designed for alanine substitution on the targeted amino acid residues: R25, P27, I31 and L33, located on N-terminus β1 (R25-L33) (12); and N230 and I233, located on C-terminus αF (N230-I233) (Fig. 1). According to their structural folding, the N- and C-terminal fragments are removed via proteolytic processing at cleavage sites at serine 37 and serine 228, yielding the 23-25 kDa active toxin. However the amino acid residues located in these removed fragments are suggested to be important, and may be involved in intermolecular interactions between the protoxins (12).

We have successfully constructed these mutant toxins in an *E. coli* system and confirmed the correct gene sequences by automated DNA sequencing. The constructed mutants were: R25A, P27A, I31A, L33A, N230A, I233A and I233stop (with an introduced stop codon). After IPTG induction, all the prepared cell lysates from each mutant were verified for Cyt2Aa2 expression by SDS-PAGE analysis. The mutants - R25A, P27A, I31A, I233A and wild type - showed a high expression level of protoxin products, while the expression of L33A, N230A and I233stop were found to be slightly lower (Fig. 2). This differential expression could be due to the perturbation of substituted amino acids on structural folding and molecular stability of these mutants. Such a mutation and production of truncated protein, leading to structural alteration, can be found in many other proteins, for example in mutated cytochrome cbb3 oxidase (19) and Cry4Ba toxins (20).

Biochemical characterization of toxin solubility revealed that R25A, P27A and I31A were well soluble in carbonate buffer pH 9.5, similar to the wild type. However L33A, N230A, I233A and I233stop showed lower solubility. The reduced solubility also leads to a lower yield of active toxin after proteinase K processing (data not shown).

In routine SDS-PAGE analysis of Cyt2Aa2, two protein bands corresponding to monomeric and dimeric protoxins are generally detected and confirmed by Western blot at 25 and 50 kDa, respectively. In addition, the 50 kDa dimeric band disappears when the toxin is treated in conditions with reducing agents. In this work, we prepared an equal amount of each expressed inclusion protein in carbonate buffer with and without DTT, and loaded on SDS-PAGE. The wild type and other mutants (P27A, I31A and I233A, without DTT) were found to be present as dimers and monomers. Under DTT treatment, their dimeric forms disappeared as expected (Fig. 3). In contrast, we did not detect the dimeric form of L33A, N230A, and I233stop mutants in conditions without DTT. Their monomers were also found to be unstable, and were present as a very faint band. Destabilization of these mutants, especially on the dimeric form, could be due to the loss of inter- and intramolecular interaction by substitution of these residues.

This result was found to be in good agreement with the construction of the N- and C-terminal truncated toxins CytΔN26,

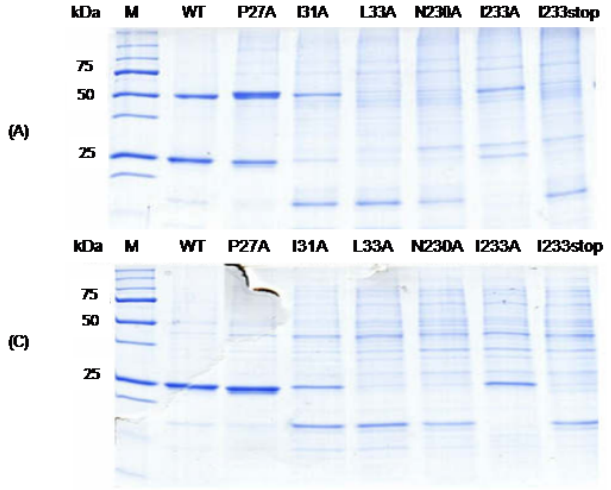

Fig. 3. Coomassie blue stained SDS-PAGE of inclusion protoxins solubilized in 50 mM Na_2CO_3/$NaHCO_3$ pH 9.5 at 37°C for 1.5 h without DTT and heating (A), compared with conditions with DTT and heating (B).

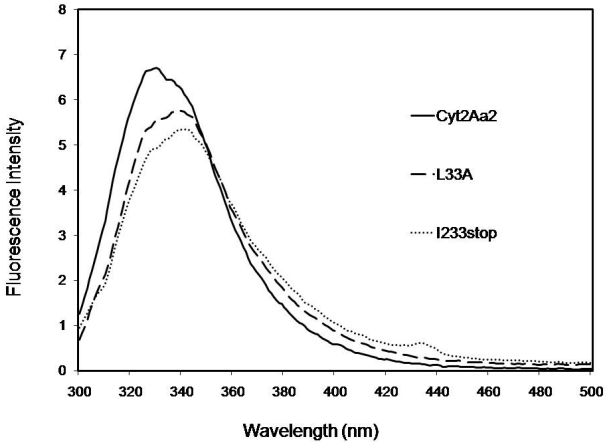

Fig. 4. Intrinsic fluorescence spectra of selected mutants are compared with Cyt2Aa2 wild type. Solubilized protoxins in Na_2CO_3 buffer were excited at 280 nm and emission spectra were scanned from 300 to 500 nm.

CytΔN33 and CytΔC31 (21), indicating the critical roles of amino acids located on N- and C-termini in the initial step of protein folding, and in maintaining structural integrity and molecular interaction (21).

L33 is critical for β1 in controlling appropriate conformation

From SDS-PAGE analysis, the L33A mutant toxin was expressed at a lower level, but it can be solubilized and processed by proteinase K. A very low yield of Cyt2Aa2 resulted from proteinase K processing. Moreover, the intrinsic fluorescence spectrum of L33A demonstrated a red shift emission, suggesting that L33A has adopted a different conformation compared to that of the wild type (Fig. 4). This amino acid substitution on β1 could lead to a significant perturbation of toxin folding, causing conformational variation and multiple populations of protoxins in solution (12). As a consequence, the misfolded or partially unfolded L33A protoxin was observed to be more sensitive to proteinase K digestion than the native wild type. This finding is in good agreement with a recent report on production of a truncated toxin, lacking β1 (21). The product of this truncated mutant cannot be detected, and it was suggested that the β1-sheet of Cyt2Aa2 may be involved in nucleation and protein folding. Consequently, residual L33 on the β1-sheet is recognized as a critical residue for protein conformation and structural folding.

Role of αF and the loop residues between β7 and αF

From our previous study (21), a C-terminal truncation protein (N238stop) showed comparable expression and characteristics to the wild type. However when the S229stop mutant was constructed to include a truncation of amino acids in αF and the loop between β7 and αF regions, a significantly low amount of protein expression was observed (21). Further characterization of this mutant by intrinsic fluorescence also showed unfolded conformation different from the wild type.

In this work, a truncation of αF in the I233stop mutant also showed a lower yield of protein, reduced toxin solubility, and non-native toxin conformation (Fig. 4). The critical role of amino acid residues in αF for protoxin folding and stability was confirmed by N230A and I233A mutants. These mutants produced low amounts of protoxins, and the expressed protoxins showed reduced solubility and high sensitivity to proteinase K digestion.

According to the crystal structure of Cyt2 protoxin, the residues N230 and I233 are located on αF and on the connecting loop to β7 of the C-terminal end (12). Thus, they may be involved in intermolecular contacts between αF of dimeric protoxins. Analysis of their bond lengths and bond angles suggested possible interactions between N230 and I233 of one protoxin and A231 and V234 of another protoxin molecule through hydrogen bonding. Replacement of N230 and I233 may perturb interactions that help maintain the proper conformation of αF for dimer formation.

Biological activity

Biological activity of these mutant toxins was investigated using *Aedes aegypti* mosquito larvae and sheep red blood cells for *in vivo* and *in vitro* activity assays, respectively (supplemental material). As expected, larvicidal and hemolytic activities of L33A, N230A and I233stop were found to be much lower than that of the wild type. Reduced toxicity of these toxins may be due to a decrease in protoxin solubility and incorrect conformation. Since these toxins were found to be highly sensitive to proteolytic digestion, they may exist in the non-native

conformation, becoming more susceptible to protease digestion. Moreover, the truncated C-terminal protoxin I233stop may exist in monomeric rather than dimeric form, causing the toxin to be more sensitive to gut proteases, resulting in loss of active toxin. In addition, the mutation at these positions may change the conformation of Cyt2Aa2 protoxin, resulting in exposing extra proteolytic cleavage sites.

In conclusion, we have demonstrated that the amino acids on β1 and αF of the terminal fragments of Cyt2Aa2 protoxin play a crucial role in protein expression, solubilization, folding and stability. The L33 residue located in the β1-sheet of the N-terminus may be an important residue in controlling the β1-sheet in the right conformation and protoxin dimerization. The substitution of L33 with alanine residue may affect the protein structure and inclusion formation. On the other hand, amino acids at the C-terminal part of Cyt2Aa2 which are in the loop (β7 and α-F), and α-F helix are responsible for dimer contacts and maintaining correct protein folding. The alanine substitutions or truncation of this part may destroy the interaction between the two monomers, resulting in misfolded structure of protein. Therefore β1-sheet, loop (β7 and α-F), and α-F of Cyt2Aa2 are essential for protein folding, production, dimerization, solubilization, and proteinase K activation.

MATERIALS AND METHODS

Site-directed mutagenesis

In vitro site-directed mutagenesis using a PCR-based method (QuikChange^{TM}) was carried out to construct mutant toxins. Mutagenic primers were designed using Vector NTI software. The pGEM-T_{Easy} plasmid vector containing the *Cyt2Aa2* gene (10) was amplified with appropriate primers using high-fidelity *Pfu* DNA polymerase in the PCR reaction. The restriction enzymes were used to screen for mutants. The nucleotide sequences for all mutants were then confirmed by automated DNA sequencing (Macrogen Inc., Korea).

Protein expression, solubilization and activation

E. coli cells harboring wild type and mutant plasmids were cultured at 37°C in LB broth containing 100 μg/ml ampicillin. When OD_{600} of the culture reached 0.3-0.4, protein expressions were induced by adding 0.1 mM IPTG. After 5 h, *E. coli* cells expressing protein were collected by centrifugation and lysed by French pressure cell to obtain inclusion toxin. Partially purified inclusions were solubilized in 50 mM Na_2CO_3/$NaHCO_3$ pH 9.5 at 37°C for 1.5 h. Concentrations of solubilized protoxins were determined by the Bradford method (17) using a Bio-Rad protein assay kit and bovine serum albumin (BSA) as a standard curve. In case that proteolytic processing was required, solubilized protoxins were added with 1% (w/w) proteinase K enzyme, and incubated at 37°C for 1 h.

Mosquito larvicidal activity assay

Inclusion toxin was diluted with distilled water as a two-fold serial dilution in 1 ml (containing 500 to 0.25 μg of toxin/ml). The 2nd instar *Aedes aegypti* larvae (10 larvae/well) in 1 ml water were fed with 1 ml of diluted inclusions in each well. Mortality of mosquito larvae was recorded after 24 h, and LC_{50} (50% lethal concentration) was determined using a Probit program (18).

Hemolytic activity assay

Sheep red blood cells (RBC) were collected by centrifugation at 3,000 rpm, 4°C for 5 min, and then washed twice with PBS buffer (pH 7.4) to make a 2% RBC preparation (4, 10). Toxin samples (500 μg/ml) were diluted in twofold serial dilutions with PBS buffer (100 μl/well). A 100 μl sample of 2% sheep RBC was mixed and incubated with the diluted toxin at room temperature. The end-point of hemolysis was monitored and recorded after 24 h.

Intrinsic fluorescence spectrometry

A 400 μl fixed volume of 10-40 μg/ml of purified toxin in carbonate buffer was added into a quartz cuvette and analyzed by a Jasco FP-6300 spectrofluorometer. An intrinsic excitation wavelength was set at 280 nm and the emission spectra were scanned from 300 to 550 nm. Excitation and emission slit widths were 2.5 nm. The fluorescence emission spectra of protein samples were recorded, and the baseline spectrum subtracted from the carbonate buffer.

Acknowledgements

This work was supported by the Thailand Research Fund (TRF), the National Center for Genetic Engineering and Biotechnology, and the Thailand Center of Excellence for Physics (Integrated Physics Cluster). We also would like to thank K. Pootanakit for his contribution in critical reading of the manuscript, and a scholarship received from the Royal Golden Jubilee Ph.D. Program (to S. Thammachat).

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