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Amino acids at N- and C-termini are required for the efficient production and folding of a cytolytic δ-endotoxin from *Bacillus thuringiensis*

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Bacillus thuringiensis **Cyt2Aa toxin is a mosquito-larvicidal and cytolytic δ-endotoxin, which is synthesized as a protoxin and forms crystalline inclusions within the cell. These inclusions are solubilized under alkaline conditions and are activated by proteases within the larval gut. In order to assess the functions of the N-and C-terminal regions of the protoxin, several N- and C-terminal truncated forms of Cyt2Aa were constructed. It was determined that amino acid removal at the N-terminal, which disrupts the β1 structure, might critically influence toxin production and inclusion formation. The deletion of 22 amino acids from the C-terminus reduced the production and solubility of the toxin. However, the removal of more than 22 amino acids from the C-terminus or the addition of a bulky group to this region could result in the inability of the protein to adopt the proper folding. These findings directly demonstrated the critical roles of N- and C- terminal amino acids on the production and folding of the *B. thuringiensis* cytolytic δ-endotoxin. [BMB reports 2008; 41(11): 820-825]**

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive soil bacterium which can synthesize insecticidal crystal proteins during sporulation (1). The toxic proteins have been classified into two major groups, referred to as the Cry and Cyt toxins (2). The receptor-specific Cry proteins are generally toxic to insect larvae in the orders *Lepidoptera, Diptera*, and *Coleoptera*. The toxins in the other group, the Cyt toxins, are specifically toxic to *Dipteran* larvae including mosquito and blackfly larvae, *in vivo*. The toxins in this group also evidence cytolytic activity to a broad range of cells, including erythrocytes, *in vitro* (3-6). The amino acid sequences of all Cyt toxins show a high degree of homology with each other (7), and therefore all of them should evidence similar 3D structures. On the basis of the X-ray crystallographic structure of Cyt2Aa1 (8), all Cyt toxins should be single domain proteins with alpha-beta architectures, comprised of six helices and seven beta sheets, in which the two outer-layers of the alpha helix hairpins are wrapped around a mixed beta sheet (8).

The Cyt toxins are generated as protoxins in the form of crystalline inclusions. The crystal protoxin can be solubilized under alkaline conditions, and then proteolysis is processed by proteases in the mid-gut tracts of susceptible larvae (9). The activated toxin will bind to the epithelial cell membranes and form an oligomeric complex prior to the breakage of the cell. Two possible hypotheses have been currently proposed to explain its mode of action, and these hypotheses are referred to as the detergent-liked and pore-forming models. The detergent-like model proposes that the activated toxin molecules aggregate on the membrane surface, thereby inducing membrane fragmentation and releasing intracellular molecules (10, 11), whereas the pore-forming model suggests that parts of the toxin molecule are inserted into the lipid bilayers in order to form transmembrane pores, thus resulting in osmotic imbalances and cell lysis (12, 13).

Although no model has yet been developed in which the Cyt toxin is employed to disrupt the cell membrane, the toxin inclusions must be solubilized and the soluble protoxin must be activated by gut proteases. For Cyt2Aa, 33-37 and 22-31 amino acids at the N- and C-termini are removed in order to convert the inactive protoxin into the active toxin (9). This clearly demonstrates that amino acids at both termini are not crucial for biological activity. However, they may be required to facilitate the production and folding of the toxin within the host cell. In order to assess the functions of the N- and C-terminal regions, several N- and C-terminal truncated forms of Cyt2Aa were constructed. The truncated fragments and full-length protein were designed to be generated independently (non-fusion), and as N-terminal fusion proteins to glutathione S-transferase (GST) or C-terminal fusion proteins to green fluorescent protein (GFP).

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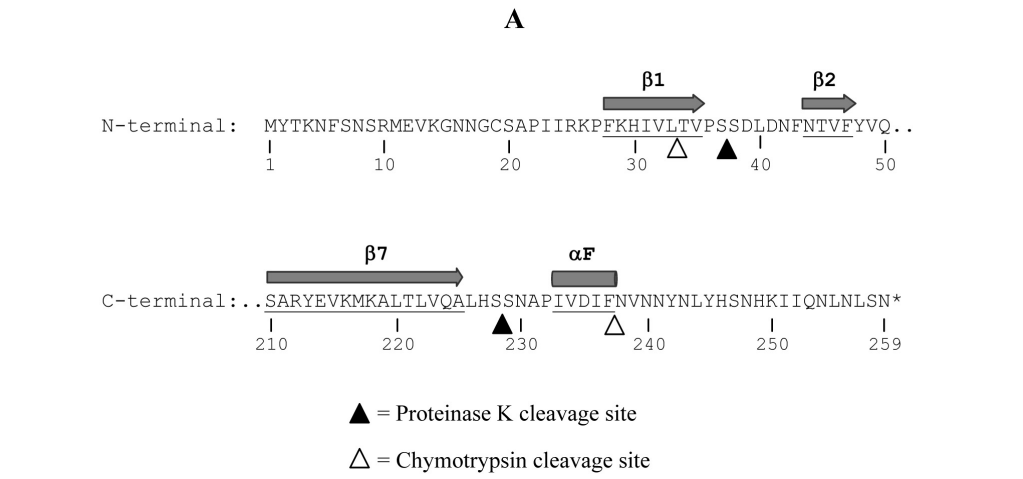

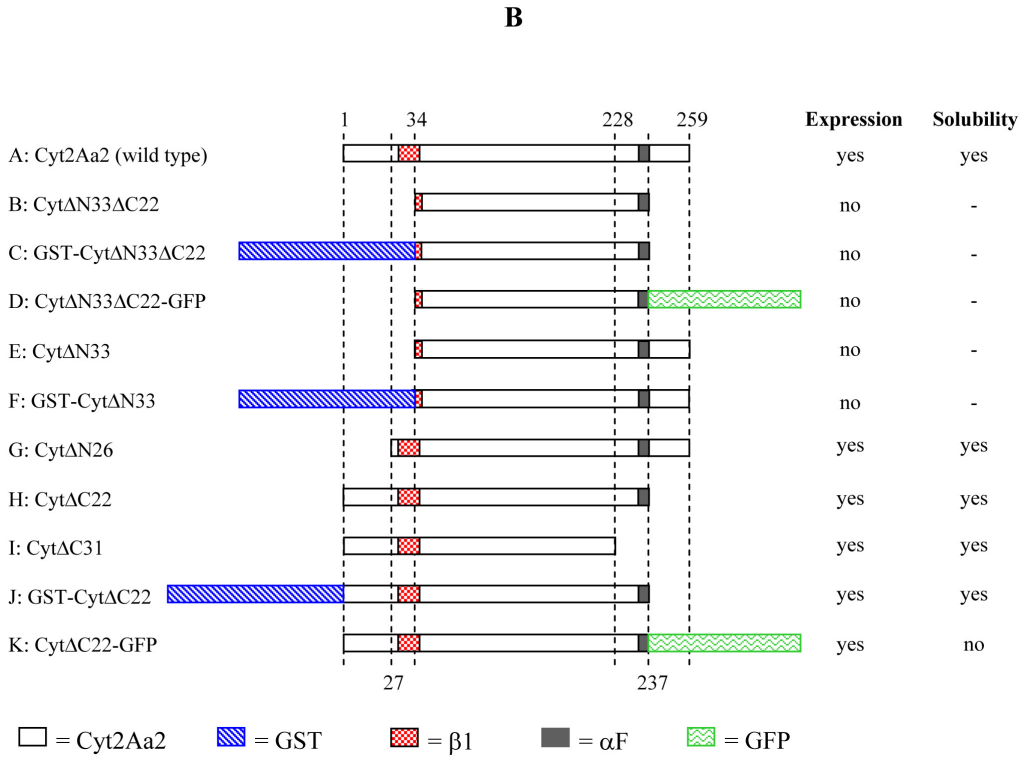

Fig. 1. (A) The deduced amino acid sequence at the N- and C-termini of Cyt2Aa. The secondary structures of the Cyt2Aa protein are shown on top of the sequence. Arrows and rod represent β-sheets and α-helix. Proteinase K and chymotrypsin cleavage sites are shown as filled and open triangles, respectively. (B) Schematic diagram representation of truncated and fusion proteins of Cyt2Aa2 toxin. Upper and lower numbering represents the definite amino acids for the design of truncated toxins. The non-fusion forms of truncated toxins were cloned into pGEM-Teasy to be expressed under the control of the *lac* promoter. Some truncated forms were subcloned into pGEX-4T-3 between the *Bam*HI and *Xho*I sites to be expressed as GST-fusion proteins. The GFP was fused to the C-terminus of truncated toxin genes (CytΔN33ΔC22 and CytΔC22) and cloned in pTZ57R.

RESULTS AND DISCUSSION

Production of truncated Cyt2Aa2 fragment similar to the protease activated toxin

The gene encoding for Cyt2Aa2 from *Bacillus thuringiensis* subsp. *darmstadiensis* has been cloned and sequenced in our laboratory (4). It comprises 259 amino acids, identical to that of Cyt2Aa1 (14). The gene was placed downstream of the *lac* promoter in pGEM-Teasy vector and transformed into *E. coli* JM109, so that it could be induced by IPTG. The full-length Cyt2Aa2 was abundantly generated as inclusion bodies within the *E. coli* cells (4). These inclusions can be readily solubilized in alkaline buffers such as 50 mM Na_2CO_3 pH 10.5. The soluble toxin can be activated *in vitro* by proteinase K or chymotrypsin. The cleavage sites for both enzymes in Cyt2Aa2 are shown in Fig. 1. Digestion with proteinase K removes up to 37 and 31 amino acids from the N- and C-terminal regions, respectively, whereas activation by chymotrypsin deletes 33 and 22 amino acids from the N- and C-terminal regions, respectively (9). Activated products from both enzymes evidenced similar activity. In order to generate truncated proteins similar to the chymotrypsin-activated toxin (CytΔN33ΔC22), the gene encoding for truncated Cyt2Aa2 between Thr34-Phe237 was amplified via PCR with a pair of specific primers. The PCR product was cloned into pGEM-Teasy vector, to be expressed under the control of the *lac* promoter. The production of the truncated fragment was not detected (not shown), although this system has already proven successful for the abundant expression of full-length Cyt2Aa2 (4). This result indicates that amino acids at either the N- or C-terminus, or both, are required for protein folding and inclusion formation. The truncated protein may fail to fold properly or may be unable to form inclusions, and could be completely digested by the proteases of the host. Amino acids at the N- and C-termini of other *B. thuringiensis* crystal toxins, such as Cry4Ba, also perform a crucial function in inclusion formation and solubilization (15, 16).

The production of several proteins in *E. coli* has been improved when expressed as GST-fusion proteins, including mosquitocidal toxin 1 (Mtx1) (17), chitinases (18), and HIV proteins (19). This approach was therefore employed to improve the production of the truncated Cyt2Aa2. The truncated gene was cloned into pGEX-4T-3 between the *Bam*HI and *Xho*I sites in order to be expressed as the GST-CytΔN33ΔC22 fusion protein (Fig. 1, fragment C). Unfortunately, no recombinant proteins were generated in this system. The truncated Cyt2Aa2 gene was also linked to the GFP gene in order to be expressed as the CytΔN33ΔC22-GFP fusion protein (Fig. 1, fragment D). However, this construct proved unable to generate the recombinant protein (not shown). Our results showed that the N- or C-terminus, or both, perform a crucial function in the production of Cyt2Aa2.

Role of N-terminal region of the Cyt toxin

In order to assess the functions of the N-terminal region of the protoxin, 3 different truncated forms of the Cyt2Aa2 toxin were constructed. The first N-terminal truncated toxin, CytΔN33, corresponding to amino acids Thr34 through Asn259 (Fig. 1, construct E), was generated in respect to the chymotrypsin cleavage site at the C-terminus of β1. The production of this truncated fragment could not be detected when the truncated gene was positioned downstream of strong promoters including lacZ (in pGEM-Teasy) and T7 (in pET-17b). The truncated gene was subsequently cloned in pGEX-4T-3, to be expressed as the GST-CytΔN33 fusion protein (Fig. 1, construct F). However, the fusion protein could not be generated in *E. coli* (not shown). The results demonstrated that the removal of 33 amino acids from the N-terminal region of Cyt2Aa2 disrupted the formation of the first β-sheet, which may be required for nucleation and subsequent folding. The misfolded protein is unstable, and could be digested completely by the host cell proteases.

Our results showed that β1 may perform a central function in protein folding. In order to evaluate this possibility, another truncated toxin harboring β1 was constructed via the deletion of the first 26 amino acids from the N-terminus (Fig. 1, construct G). This fragment (CytΔN26) was generated abundantly in *E. coli*. However, the majority of the fragments were produced in soluble form. This result suggested that amino acids at the N-terminal region upstream of β1 are required for inclusion formation, and β1 is required for protein folding or the stabilization of the protein structure.

Helix αF at the C-terminus of Cyt toxin is required for folding

The C-terminal truncated toxins were constructed on the basis of proteolytic cleavage sites (Fig. 1). Activation by chymotrypsin removes 22 amino acids from the C-terminus and leaves the αF helix intact, whereas digestion with proteinase K removes 31 amino acids from the C-terminus, including αF (Fig. 1). In order to generate C-terminal truncated toxins similar to these fragments, nucleotides encoding for Asn and Ser at positions 238 and 229 were mutated to stop codons (Fig. 1, constructs H and I). It was determined that the removal of 22 amino acids from the C-terminus (CytΔC22) exerted no detectable effects on protein production. The truncated protein was generated at a high level, and formed inclusion bodies similar to that of the full-length toxin (Fig. 2). However, protein production was reduced significantly when an additional 9 amino acids were deleted (CytΔC31). This suggested that helix αF at the very end of the molecule performs a crucial function in the efficient folding and crystal packing of the Cyt toxin. The overall conformation of the purified CytΔC31 protoxin was investigated in comparison with that of the full-length toxin via intrinsic fluorescence spectroscopy. It was determined that the CytΔC31 protoxin evidenced significantly different emission spectra as compared to the full-length protein (Fig. 3). The

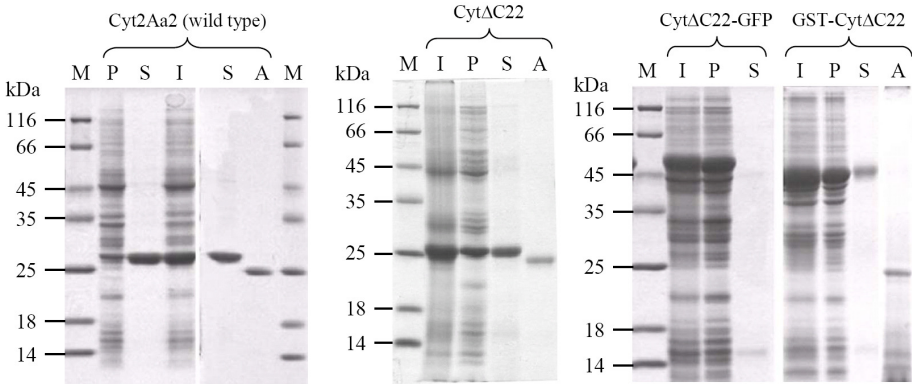

Fig. 2. Coomassie-stained SDS-polyacrylamide gel of inclusions, soluble and protease-activated Cyt2Aa2 and its truncated forms. Toxin inclusions (I) were extracted and partially purified from *E. coli*. The inclusions were solubilized in 50 mM Na_2CO_3 buffer at a pH of 10.5 plus 10 mM DTT, and the soluble fraction (S) was separated from insoluble materials or pellets (P) via centrifugation. Soluble proteins were activated (A) for 1 hour with 1% (w/w) proteinase K at 37°C. Protein molecular weight markers (M) were shown alongside in kDa.

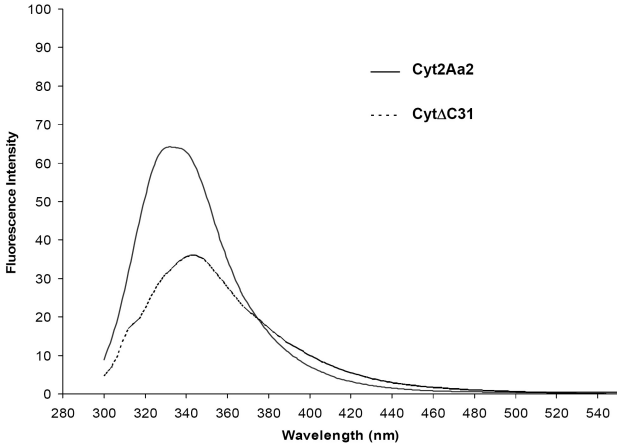

Fig. 3. Intrinsic fluorescent spectra of solubilized toxins. Solubilized toxin in carbonate buffer was excited at 280 nm and the emission spectra were scanned at between 300-500 nm.

emission spectra of the CytΔC31 protoxin evidenced a maximum peak at 342 nm, whereas that of Cyt2Aa2 was observed at 332 nm. This result showed that the overall structure of CytΔC31 differed significantly from the full-length toxin. This result suggested that the abrogation of the αF structure could effectively result in the misfolding and formation of inclusions that are more difficult to solubilize.

The addition of a bulky group, such as GST and GFP, to either end of the C-terminal truncated toxin, CytΔC22 (Fig. 1, constructs J and K), exerted no significant effects on the levels of protein production, but interfered significantly with the solubility of the toxin inclusions. The solubility of the GST-CytΔC22 inclusion was reduced and inclusions of the CytΔC22-GFP fusion protein could not be solubilized (Fig. 2). The solubilized GST-CytΔC22 fusion protein could be processed into an activated form similar to that of the wild-type (Fig. 2). These results showed that a bulky group added to the N- or C-terminus of the Cyt toxin influenced the solubility of the toxin inclusion. This indicated that both termini are involved in inclusion formation.

Biological activity of truncated proteins

Only 5 constructs were determined to be able to generate the truncated proteins, CytΔN26, CytΔC22, GST-CytΔC22, CytΔC22-GFP and CytΔC31. The mosquito larvicidal activity of these proteins was assessed by feeding to *A. aegypti* and *C. quinquefasciatus* larvae, and the results are provided in Table 1. We detected only 2 constructs that evidenced larvicidal activity, namely CytΔC22 and GST-CytΔC22. However, both of these evidenced toxicity approximately 2-5 times lower than that of the full-length toxin. Other constructs were unable to kill larvae at toxin concentrations of up to 250 μg/ml. It should be noted that the 2 truncated forms that retain larvicidal activity were generated abundantly as inclusion bodies, and the inclusions could be solubilized and activated by proteinase K. The reduction in toxicity may be attributable to the lower solubility of both truncated forms as compared to that of the full-length toxin (Fig. 2). The results of the *in vitro* solubilization test showed that CytΔC22-GFP was insoluble, and that only a minute amount of CytΔC31 could be solubilized. This showed that both of them might not be solubilized in the larval gut, and thus should not evidence any activity. The N-terminal truncated form that retains β1 (CytΔN26) exhibited no toxicity to mosquito larvae. Although it was generated at a high level, it exists in soluble form and is incapable of resisting proteinase K digestion. This protein could be digested completely by the larval gut proteases prior to exhibiting any toxicity. Hemolytic activity assays of the proteinase K-activated fragments evidenced a trend similar to that of larvicidal toxicity (Table 1).

In summary, amino acids at both termini of Cyt2Aa2 perform a crucial function during protein folding and inclusion formation. The first 26 amino acids at the N-terminal region upstream of β1 may be required for intermolecular interaction and inclusion formation. The deletion of this part might disrupt such interactions and the truncated protein could be generated in soluble form. Amino acids in β1 could be responsible for initial folding or nucleation. The truncated protein lacking β1 may not be capable of folding properly, and the misfolded protein is unstable and degraded. The final 22 amino acids at the

Table 1. Biological activities of the truncated proteins. Mosquito larvicidal activity of the mutant toxins against *A. aegypti* and *C. quinquefasciatus* larvae were reported as LC_{50}. The fiducial limits at 95% confident were shown in parentheses. Hemolytic activity against sheep red blood cells was recorded after 24 hours

Toxin	Mosquito larvicidal activity; LC_{50} (ng/ml)[a]		Hemolysis end-point (μg/ml)
	A. aegypti	*C. quinquefasciatus*	
Full-length	286 (261-314)	313 (271-363)	0.25-0.50
CytΔC22	491 (430-559)	1,075 (941-1,230)	0.25-0.50
GST-CytΔC22	717 (570-904)	1,433 (1,172-1,755)	1.0-2.0
CytΔC22-GFP	Inactive	Inactive	ND[b]
CytΔC31	Inactive	Inactive	2.0-4.0
CytΔC26	Inactive	Inactive	2.0-4.0

[a] inactive means no mortality was observed when using toxin up to 250,000 ng/ml.
[b] ND means not determined (no activated toxin available)

C-terminus of Cyt2Aa2 downstream of the αF helix should not perform a major function in protein production and biological activity. However, the removal of 31 amino acids from the C-terminal region (including αF) or the addition of a bulky group to this region have resulted in the production of inclusion bodies that are insoluble within the larvae gut. The αF helix is, therefore, required for the formation of a uniform or crystalline inclusion, in order to be solubilized in the larvae gut.

MATERIALS AND METHODS

Bacterial strain, plasmid, and oligonucleotides

Escherichia coli JM109 was utilized for the cloning and expression of the full-length and truncated Cyt2Aa2 throughout this experiment. The recombinant plasmid pGEM-Cyt2Aa2 (4), containing the full-length of the *cyt2Aa2* gene from *B. thuringiensis* subsp. *darmstadiensis* 73E10-2, was utilized as a template for the construction of the truncated *cyt2Aa2* gene. The recombinant plasmid pBCgfp (20) was utilized as a source for the *gfp* gene. The plasmid pGEM-Teasy (Promega), pTZ57R (Fermentas) and pGEX-4T-3 (GE Healthcare) were utilized for the cloning and expression of truncated and fusion proteins. The oligonucleotide primers employed in this experiment were obtained from Sigma Proligo, Singapore.

Construction of recombinant plasmids expressing truncated *Cyt2Aa2* and fusion proteins

Genes encoding for truncated proteins were generated via polymerase chain reaction (PCR) using a high fidelity *Pfu* polymerase. The PCR reactions were conducted using pGEM-Cyt2Aa2 as a template, together with specific primers designed for each of the constructs. The PCR products of the gene encoding for truncation at the N-terminal region, CytΔN26 and CytΔN33, as well as the active core protein, CytΔN33ΔC22, were cloned into pGEM-Teasy vector. The construction of the C-terminal truncated toxin was based on Stratagene's QuikChange™ Site-directed mutagenesis Kit. Codons encoding for Asn at position 238 and Ser at position 229 were substituted with stop codons to generate the C-terminal truncated mutants (CytΔC22 and CytΔC31). Genes encoding for CytΔN33, CytΔC22, and the active core proteins were fused to the C-terminus of the GST gene in pGEX-4T-3 vector between the *Bam*HI and *Xho*I sites, to be expressed as GST-CytΔN33, GST-CytΔC22, and GST-CytΔN33ΔC22, respectively. The GFP gene was also tagged to the end of genes encoding for CytΔC22 and CytΔN33ΔC22 using SOE-PCR and cloned into the pTZ57R vector. The DNA sequences of all constructs were confirmed via automated DNA sequencing (Macrogen Inc, Korea). Schematic diagrams representing protease cleavage sites and the construction of truncated and fusion proteins are provided in Fig. 1.

Expression, purification, and solubilization

E. coli cells harboring mutant plasmids were inoculated in LB broth containing 100 μg of ampicillin/ml and were grown at 37°C until the OD_{600} of the culture reached 0.4-0.5. One mM IPTG was added in order to induce the expression of the toxin gene, and the culture was grown for at least an additional 5 hr. Toxin inclusions were released from cells using a French press and ultrasonication, as previously described (21). Inclusions were solubilized for 1 hour in 50 mM Na_2CO_3 pH 10.5 containing 10 mM DTT at 37°C. After 5 min of centrifugation at 12,000xg, soluble protoxin in the supernatant was collected. For proteolytic activation, the soluble protoxin was processed for 1 hour with 1% proteinase K (w/w) at 37°C.

Intrinsic fluorescence spectroscopy analysis

The comparative overall conformations of the truncated and full-length toxins were analyzed via fluorescent spectroscopy. One μM of purified toxin in carbonate buffer was added to a quartz cuvette, then placed in a Jasco FP-6500 fluorospectrometer. The protein sample was excited at 280 nm and the emission spectrum was monitored between 300-550 nm using an excitation and emission slit width of 3 nm. All emission spectra were subtracted with buffer prior to comparison with that of the full-length Cyt2Aa2.

Hemolysis activity assay

The hemolysis assay was conducted as previously described (21), with minor modifications. Sheep red blood cells were washed twice in PBS at a pH of 7.4, and diluted to a 2% suspension in the same buffer. Proteinase K-activated toxin was placed in each well of a 96-well microtiter plate as a two-fold serial dilution (100 μl/well). An equal volume of the diluted red blood cells was added to each well and incubated for 24 h at room temperature. The hemolysis end-point was judged at the last well at which coloration remained visible.

Mosquito larvicidal activity assay

Toxin inclusions were diluted in distilled water as two-fold serial dilutions from 500 to 0.25 μg/ml. One milliliter of diluted inclusion was fed to ten 3rd-instar larvae (*Culex quinquefasciatus* and *Aedes aegypti*) in 1 ml of water. The mortality of larvae in each well was recorded after 24 h of feeding with the toxin. The LC_{50} (50% lethal concentration) was calculated via Probit analysis (22).

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