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Isoleucine at position 150 of Cyt2Aa toxin from *Bacillus thuringiensis* plays an important role during membrane binding and oligomerization

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Cyt2Aa2 is a mosquito larvicidal and cytolytic toxin produced by *Bacillus thuringiensis* subsp. *darmstadiensis*. The toxin becomes inactive when isoleucine at position 150 was replaced by alanine. To investigate the functional role of this position, Ile150 was substituted with Leu, Phe, Glu and Lys. All mutant proteins were produced at high level, solubilized in carbonate buffer and yielded protease activated product similar to those of the wild type. Intrinsic fluorescence spectra analysis suggested that these mutants retain similar folding to the wild type. However, mosquito larvicidal and hemolytic activities dramatically decreased for the I150K and were completely abolished for I150A and I150F mutants. Membrane binding and oligomerization assays demonstrated that only I150E and I150L could bind and form oligomers on lipid membrane similar to that of the wild type. Our results suggest that amino acid at position 150 plays an important role during membrane binding and oligomerization of Cyt2Aa2 toxin. [BMB Reports 2013; 46(3): 175-180]

INTRODUCTION

Bacillus thuringienesis is a rod shape, Gram-positive, soil bacterium that can produce insecticidal crystal proteins specifically toxic to various insect larvae (1). The crystal proteins could be divided into 2 major families, Cry and Cyt toxins, which have different molecular structures and target insects (2). Cyt toxins are synthesized by some strains of *B. thuringiensis* and highly toxic against larvae of dipteran insects such as mosquitoes and blackflies (1, 3, 4). Toxins in this group are produced in the form of inactive crystalline inclusion or protoxin. Upon ingestion by susceptible larvae, the inclusion is solubilized in alkaline condition of the midgut and proteolytic processed by gut proteases to yield the active toxin (5). Inclusions could be solubilized using alkaline buffer and activated by proteases *in vitro*. In addition, the activated toxin exhibits cytolytic activity against broad range of cells including erythrocytes (5-7).

The mechanism of action of the Cyt toxin is thought to involve a cascade of several events leading to insect death after ingestion. There are two possible models currently proposed for the Cyt toxin action. The pore-forming model suggests that binding of the active toxin to cell membrane induces conformational changes and oligomerization of the toxin molecules which finally leads to osmotic imbalance and cell lysis (8, 9). Alternative model proposes that the toxin may bind to the cell membrane and accumulate until reaching a critical number, and then the toxin complex would perturb cell membrane via a detergent-like mechanism (10, 11). Although the mechanism of Cyt toxin is still a controversy, the site of action of both models is the cell membrane that definitely involves membrane binding, conformational changes and oligomerization processes.

To date, more than 30 Cyt toxins have been identified (2, 12). Amino acid sequence alignment of Cyt toxins showed that they share high homology and therefore are expected to have similar 3D structures. In deed, X-ray crystallographic analyses revealed that structures of the protoxin of Cyt2Aa, Cyt1Aa and the activated Cyt2Ba are highly similar in which the toxin is a single domain protein consisting of two outer layers of α-helix hairpins flanking a core of mixed β-sheets (13-15). Membrane binding motif could not be identified from these structures. However, the motif could be formed during the toxin undergoes conformational changes upon approaching the membrane.

Previous studies showed that substitution at Ile150 in Cyt2Aa toxin with Ala resulted in a total loss of activity although the mutant protein retains similar overall structure, solubility and proteolytic processing similar to the wild type (16). This suggested that amino acid at this position plays important role during in-

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toxication either at the step of membrane binding, conformational changes or oligomerization. To investigate the functional role of this position, Ile150 was replaced by Leu, Phe, Glu and Lys. Comparative biochemical and biological studies of mutants to the wild type suggested that Ile150 in Cyt2Aa plays significant role during membrane binding and oligomerization.

MATERIALS AND METHODS

Bacterial strain, plasmid and oligonucleotides

Escherichia coli K12 JM109 was used throughout the experiment. The recombinant plasmid pGEM-Cyt2Aa2 (4), containing full-length *cyt2Aa2* gene in the pGEM-Teasy vector (Promega), was used as DNA template. The oligonucleotide primers were obtained from Sigma Proligo Co, Ltd. (Singapore). Primer sequences and additional information are shown in supplement 1.

Site-directed mutagenesis

The basic procedure was based on Stratagene's QuikChange™ Site-directed mutagenesis. PCR reactions were performed using pGEM-Cyt2Aa2 as a template. Nucleotides encoding Ile at position 150 was substituted with codons for Ala, Glu, Lys, Leu and Phe using appropriate oligonucleotide primers. Position and model structure of each mutant protein was shown in supplement 2. Recombinant plasmids containing mutated genes were transformed into *E. coli* JM109. Transformants were screened by restriction endonuclease and DNA sequences of all mutant genes were verified by automated DNA sequencing (Biodesign, Thailand).

Protein preparation

The culture of *E. coli* harboring mutant plasmid was induced with 0.1 mM IPTG during exponential growth (OD_{600} of the culture about 0.4-0.5). Cells were harvested by centrifugation and toxin inclusions were extracted as described previously (4). The toxin inclusions were solubilized in 50 mM Na_2CO_3 buffer pH 10.5 with 10 mM DTT at 37°C for 1 hour. After centrifugation at 12,000 × g for 5 min, soluble protoxins in supernatant were collected. For proteolytic activation, the soluble protoxins were incubated with 1% (w/w) proteinase K at 37°C for 1 hour.

Intrinsic fluorescence spectroscopy

Protoxin and processed toxin were purified by size-exclusion chromatography using Superdex200, 10/300 GL column. The overall structure of purified toxin was monitored by spectrofluorometry. Emission spectra were recorded from 300 to 500 nm using excitation wavelength at 280 nm. Both excitation and emission slits were set at 3.0 nm. Each sample was scanned three times and spectrum from buffer was used as background.

Mosquito larvicidal assay

Mosquito larvicidal activity of mutants was tested against 3rd-instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* in 24-well tissue culture plates. One ml of two-fold serial dilutions of toxin inclusions was added into 1 ml of distilled water containing 10 larvae in each well. The assay was carried out at room temperature. Mortality was recorded after the larvae were fed with toxin for 24 hours. LC_{50} (50% lethal concentration) were analyzed by Probit analysis (17).

Hemolytic assay

Hemolytic activity of the proteinase K activated toxin was performed against sheep red blood cell suspension. The assay was slightly modified from Promdonkoy and Ellar (8). Ten μg of activated toxins were mixed with 1 ml of 1% red blood cell suspension in PBS buffer pH 7.4. After incubation at room temperature for 1 hour, the mixture was centrifuged at 3,000 × g for 5 min. Hemoglobin released in the supernatant was measured by spectrophotometer using absorbance at 540 nm. Red blood cells treated with 0.1% Triton-X100 were used as 100% lysis and red blood cells incubated in PBS without toxin were used as blank.

Liposome preparation

Synthetic lipid vesicles or liposomes were prepared from the lipid mixture of egg-yolk phosphatidylcholine (PC), cholesterol and stearylamine in a molar ratio of 4 : 3 : 1 in chloroform:methanol (2 : 1, v/v) as described by Thomas and Ellar (18). Liposomes were purged with nitrogen gas and stored at −80°C until required.

Toxin-membrane interaction

To test membrane binding and oligomerization of the toxin, 5 μg of the proteinase K activated toxins were incubated with 200 μg liposomes in 200 μl PBS buffer. Reactions were incubated for 2 hours at room temperature. Liposomes carrying membrane-bound toxin were separated from unbound toxin by centrifugation at 20,000 × g for 20 min then analyzed on SDS-PAGE.

RESULTS AND DISCUSSION

Production level and structure of mutant proteins

Gene encoding Cyt2Aa2 toxin from *B. thuringiensis* subsp. *darmstadiensis* has been previously cloned and expressed in *E. coli* (4). The toxin is highly produced as inclusion bodies inside the cells. The inclusion could be solubilized in carbonate buffer and the protease processed product exhibits high cytolytic activity (4). Cyt2Aa2 shares identical amino acid sequence with Cyt2Aa1 from *B. thuringiensis* sp. *kyushuensis* that its crystal structure has been resolved (19). Therefore, Cyt2Aa2 should have the same 3D structure as Cyt2Aa1. Previous study on Cyt2Aa1 found that substitution at Ile-150 by Ala completely abolished larvicidal and hemolytic activities. However, those activities could be recovered after introducing additional mutations at other sites of the toxin (16). These results emphasized the importance of amino acid at position 150, although it is not know how this amino acid contributes to the toxin action. By modeling Cyt2Aa2 with Cyt2A1 strucuture, the Ile-150 is found to locate in

the αD–β4 loop on the outer surface of the molecule (Fig. 1). It may involve with any critical steps in intoxication including membrane binding, oligomerization and conformational changes. In order to further investigate the functional role of Ile-150, this position was substituted with 5 amino acids with different properties (I150A, I150L, I150F, I150K and I150E). All mutants were highly produced and formed inclusion bodies at comparable level to the wild type. Partially purified inclusions of all mutants were found to be soluble in 50 mM Na_2CO_3 pH 10.5 plus 10 mM DTT similar to the wild type. Soluble protoxin of all mutants could be activated by proteinase K and yielded 23-kDa processed product similar to that of the wild type (Fig. 2). Therefore, solubilization and protease activation were not affected by these substitutions. Solubilization and protease activation are critical prerequisite steps for intoxication. Toxin activity is usually lost or severely reduced if mutation affects these important steps. Evidences have been previously reported such as in Cyt2Aa2-W132F and W154F (20) and some truncated forms of Cyt2Aa2 (21). These mutants were unable to be solubilized in carbonate buffer and showed no larvicidal and hemolytic activities.

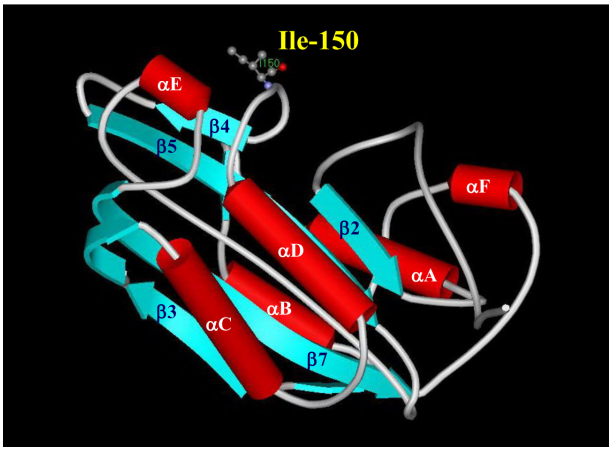

Fig. 1. Model structure of Cyt2Aa2 (wild type). Model was generated based on coordinate of Cyt2Aa1 (PDB accession no. 1CBY). Cyt2Aa1 and Cyt2Aa2 share identical amino acid sequences therefore both toxins should have the same 3D structure.

Intrinsic fluorescence spectroscopy was performed to monitor structural conformation of the soluble toxin either protoxins or activated toxins in comparison with that of the wild type. If the mutation affects the overall structure of the toxin, emission spectra would be different from the wild type as demonstrated for Cyt2Aa2-L33A mutant (22). Here we found that emission spectra of all mutants (I150A, I150L, I150F, I150K and I150E) were identical to the wild type (supplement 3). This result indicated that all mutants had adopted similar overall structure to the wild type, suggesting that substitution of Ile150 with Ala, Glu, Lys, Leu and Phe did not affect the overall structure of protoxin and activated toxin.

Larvicidal and hemolytic activities

Mutant protein inclusions were fed to *A. aegypti* and *C. quinquefasciatus* larvae to determine their larvicidal activities. It was observed that I150E and I150L mutants were toxic to *A. aegypti* and *C. quinquefasciatus* larvae whereas I150A and I150F mutants were not toxic to both types of larvae. The mutant I150K showed very low toxicity to *C. quinquefasciatus* larvae and inactive against *A. aegypti* (Table 1). An agreement with larvicidal activity results was also observed from hemolytic assay against sheep red blood cells. Hemolytic activity of I150E and I150L mutants was comparable to the wild type, The I150K mutant showed dramatic reduction in hemolytic activity while I150A and I150F mutants were completely lost their hemolytic activity even when tested at very high dose up to 250 μg/ml (Table 1). Results from both assays clearly showed substitution of Ile150 with Leu and Glu had no effect on protein toxicity, whereas substitution with Ala, Phe, and Lys severely affect protein toxicity.

Loss of toxicity of I150A, I150F, and I150K mutants did not result from inability of the inclusions to be solubilized and activated in the larvae gut since *in vitro* solubilization and protease processing by proteinase K demonstrated that these mutants

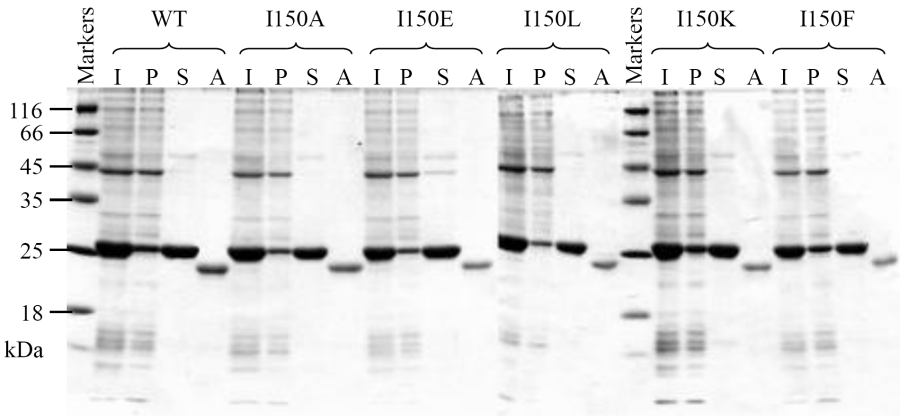

Fig. 2. Solubility and proteinase K activation of Cyt2Aa2 wild type (WT) and its mutants. Inclusion bodies (I) were solubilized in 50 mM Na_2CO_3 buffer pH 10.5 plus 10 mM DTT at 37°C for 1 hour. Soluble fraction (S) was separated from insoluble materials or pellet (P) by centrifugation. Soluble proteins were activated by 1% (w/w) proteinase K at 37°C for 1 hour (A). All samples were subjected to SDS-PAGE and Coomassie blue stain. Protein standard markers were shown alongside in kDa.

Table 1. Biological activities of Cyt2Aa2 wild type and its mutants

Protein	Mosquito larvicidal activity; LC_{50} (ng/ml)		Hemolytic activity (% hemoglobin release ± SD)
	A. aegypti	*C. quinquefasciatus*	
Wild type	286 (261-314)	313 (271-363)	100 ± 0
I150A	Non toxic	Non toxic	No lysis
I150F	Non toxic	Non toxic	No lysis
I150K	Non toxic	47,507 (27,121-118, 283)	5 ± 2
I150E	562 (503-628)	359 (315-408)	94 ± 6
I150L	707 (635-789)	377 (329-431)	96 ± 5

Hemolytic activity of Cyt2Aa mutant toxins against sheep red blood cells was measured after 1 hour incubation of 10 μg of activated toxin with 1 ml of 1% sheep red blood cells in PBS pH 7.4. Mosquito larvicidal activity of mutant toxins against *A. aegypti* and *C. quinquefasciatus* larvae were recorded after feeding the toxin for 24 hours. Three independent experiments were performed and figures in parentheses indicate fiducial limits at 95% confidence.

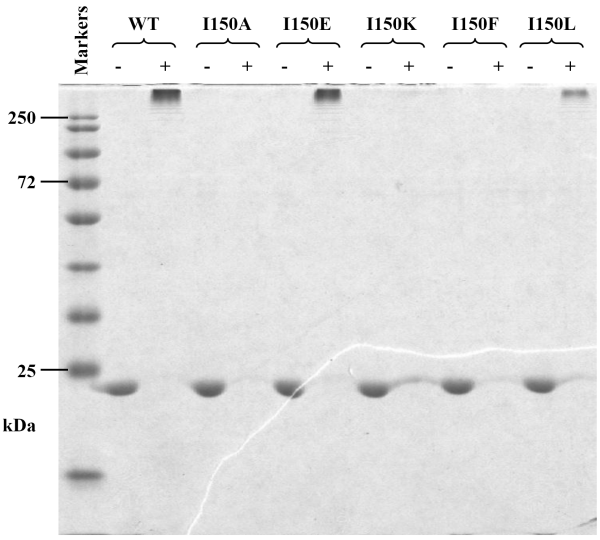

Fig. 3. Membrane binding and oligomerization of the toxin. Proteinase K activated toxins; Cyt2Aa2 wild-type (WT), I150A, I150E, I150K, I150F and I150L, were incubated with (+) or without (−) liposomes at room temperature for 2 hours. Unbound toxins were removed by centrifugation and the membrane-bound toxins were analyzed on SDS-PAGE.

were readily solubilized and yielded similar activated product to that of the wild type (Fig. 2). Moreover, intrinsic fluorescence spectroscopy suggested that these mutants retained similar overall structure to the wild type. Therefore, failure to exhibit larvicidal activity of I150A, I150F, and I150K mutants could due to inability of the processed toxin to bind the target cell membrane, oligomerization and insertion into the membrane.

Membrane binding and oligomerization

Since amino acid replacements at position I150 did not affect overall structure, crystal formation, solubility and protease processing, the role of Ile150 is possibly involved in membrane binding and perturbation during toxin function. Membrane interaction experiment was performed to validate the effect of alteration at Ile150 on toxin binding to the membrane and oligomerization. Previous reports demonstrated that activated Cyt toxins can bind and form oligomers on the synthetic membrane prepared from pure lipid without a specific receptor (11, 23, 24). To access membrane binding and oligomerization ability of Cyt2Aa2 mutants, activated toxins were incubated with liposomes. Unbound toxin in supernatant was removed after centrifugation and the membrane-bound toxin was analyzed by SDS-PAGE. If the toxin can bind and form oligomers on the membrane, a ladder pattern of protein bands could be observed on SDS-PAGE as shown in Fig. 3. Results demonstrated that I150E and I150L mutant toxins could bind and form oligomers on lipid membrane similar to that of the wild type toxin. A ladder pattern was detected at very low amount for the less active mutant I150K. The inactive I150A and I150F mutants were unable to bind and develop oligomers on the lipid membrane.

Results from membrane binding and oligomer formation are consistent with mosquito-larvicidal and hemolytic assays. This suggests that amino acid at position 150 is a critical residue for membrane binding and oligomerization. Substitutions with smaller side chain (I150A) or larger side chain (I150F) resulted in a total loss of biological activities whereas substitutions by a highly conserved amino acid (I150L) or a negatively charged side chain (I150E) preserved the toxin activity. However, substitution by a positive charged amino acid (I150K) significantly reduced toxin activity. This suggests that an appropriate size of the amino acid at this position is required and hydrophobic or negatively charged amino acid is more favorable. Replacement of a highly hydrophobic (isoleucine) by a smaller and less hydrophobic (alanine) or substitution with aromatic residue (phenylalanine) that has intermediate polarity could affect hydrophobic interaction between the toxin and lipid membrane. Substitution by a negatively charged residue (I150E) might enable the mutant toxin to make interaction to stearylamine in liposomes and to

phosphatidylethanolamine that is generally found in red blood cell and mosquito larval gut cell membranes. This interaction is severely interrupted if a positively charged amino acid is introduced into this position (I150K). X-ray structures of the protoxin (13) and protease activated form (14) showed that amino acid at this position is located in the αD-β4 loop and sticking out to the environment. It is expected that this residue plays essential role during membrane binding and oligomer formation. However, Ile-150 is not a single position responsible for membrane binding and oligomerization. Reversion mutagenesis of an inactive mutant Cyt2Aa1-I150A has shown that activity could be recovered by introducing additional mutation at other positions (16). Amino acids in αA and αC were reported to involve with membrane binding and oligomerization (7, 25). These amino acids contribute to some extend and function co-operatively.

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