Title: Alanine-162 of *Bacillus thuringiensis* Cyt2Aa2 toxin is essential for membrane binding and oligomerization

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Abstract

Cyt2Aa2 is a cytolytic toxin produced from *Bacillus thuringiensis* subsp. *darmstadiensis*. It is specifically toxic against Dipteran larvae *in vivo*, and also active to several cell types such as erythrocytes**.** The active toxin is proposed to bind to cell membrane, leading to the formation of membrane pore by toxin oligomerization and eventually cell lysis. This study aimed to characterize the role of expected lipid binding residues (I139, S159, L160, S161, A162, D209 and V215) of Cyt2Aa2. Mutants were constructed by site-directed mutagenesis and expressed in *E. coli*. These mutants were expressed as inclusion bodies and solubilized in 50 mM carbonate buffer pH 10.5. All mutants, except I139A and V215A could retain as activated toxins after proteinase K cleavage. Three mutants, S159A, L160A and S161A showed high hemolytic activity but low toxicity against *A. aegypti*. Membrane interaction assays showed that these mutants bound and oligomerized on rat red blood cells (RBC). Two mutants, A162V and D209N, could not completely break RBC even at high concentration and showed no hemolytic activity. The mutant A162V showed no toxicity against *A. aegypti*, but D209N showed low toxicity against *A. aegypti*. Our data suggested that alanine-162 of Cyt2Aa2 toxin was involved in membrane binding and oligomerization. Substitution of this amino acid altered the biological activity. Selectivity of the toxin to certain target cells might be improved by amino acid replacement in this residue.

Keywords: Cyt2Aa2, *Bacillus thuringiensis*, membrane binding residue, *Aedes aegypti*, site-directed mutagenesis

Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive spore-forming bacterium that can produce δ-endotoxins during sporulation (Schnepf et al., 1998). The life cycle of *Bt* is characterized by two phases which include vegetative cell division and spore development, otherwise referred to as the sporulation cycle (Bulla et al., 1980). These toxins are first produced as an inactive protoxin, and then proteolytically processed into an active molecule. The active toxin is proposed to bind to cell membrane, oligomerization of toxin leading to pore-formation at the membrane and eventually cell lysis (Knowles et al., 1987; Li et al., 1996). Cyt2Aa2 is a cytolytic toxin from *Bacillus thuringiensis* subsp. *darmstadiensis* (*Btd*). It is produced as a 29-kDa protoxin and then proteolytic processed to a 25-kDa activated form in alkali condition. It is specifically toxic against dipteran larvae (*Culex sp, Stegomyia sp*.) *in vivo*, and also active to several cell types such as erythrocytes and fibroblasts (Promdonkoy et al., 2003). Cyt2Aa2 protoxin consists of 259 amino acids that show identical sequence to Cyt2Aa1 from *B. thuringiensis* subsp. *Kyushuensis*. These two toxins should adopt a similar α/β structure containing 6 α-helices and 7 β-sheets (Koni et al., 1993; Promdonkoy et al., 2004). The β-sheets are located in the interior, wrapped by helices, αA, αB, αC and αD. It was found that the lengths of these helices are not enough to span the membrane and create the pore, whereas the lengths of β-sheets are more suitable (Li et al., 1996). Li et al. (2001) proposed that β5, β6 and β7 are long enough to span the membrane. These regions should be able to insert into the membrane. The β5, β6 and β7 from several molecules may oligomerize and form "β-barrel" pore similar to that of α-hemolysin (Song et al., 1996) The possible roles of the helices may be involved with binding onto the cell membrane, facilitate the insertion of β-sheets and also the oligomerization of toxin molecules to generate pore (Promdonkoy et al.,

2005; 2000). Cyt toxin is structurally related to a few toxins, volvatoxin A2 (VVA) (Lin et al., 2004) and Evf toxin from *Erwinia carotovora*. For Evf toxin, it was found that C209 was involved with pamitate binding site via thioester linkage. This interaction enhanced the strength of toxin binding to the membrane (Quevillon-Cheruel et al., 2009). Mapping between Cyt and Evf toxins revealed that the lipid binding cavity of Cyt toxin might locate on alpha C (M110), alpha D (I139), loop between alpha-D and beta-4 at the residues, T142, T144, N145, L146 and beta-7 (V215). Also, the electrostatic surface was identified in Cyt and was proposed that these residues, V150, N159, L160, D209 and T221, were involved with lipid binding pocket (Rigden et al., 2009). The hypothesis of this study is the amino acids in αD, loop between β4 and β5, loop between β6 and β7 and β7 of Cyt2Aa2 toxin are involved in membrane binding as well as oligomerization. This study attempts to elucidate the role of expected membrane binding residues. The results from this study reveal more information of membrane binding of Cyt toxin which leads to more understanding of its mechanism.

Materials and methods

1. Bacterial strain, plasmids, and oligonucleotides

The *Escherichia coli* JM109 were used throughout the experiment. The recombinant plasmid pGEM-Cyt2Aa2 (Promdonkoy et al., 2003) containing the full-length *cytAa2* gene in the pGEM-T easy vector (Promega) was used. *E. coli* harboring *cyt2Aa2* wild type cell was provided from B. Promdonkoy (National Center for Genetic Engineering and Biotechnology, Thailand). Oligonucleotide primers for site directed mutagenesis were designed based on *cyt2Aa2* gene (GenBank Accession No. AF472606). DNA sequences of forward and reverse primers for Cyt2Aa2-mutants are shown in Table 1.

Primers were designed based on *cyt2Aa2* gene (GenBank Accession No. AF472606). Mutated nucleotides were underlined. This table showed only the forward primers. DNA sequences of the reverse primers are complementary to their forward primers

2. Site-directed mutagenesis

The mutants, I139A (αD), S159A, L160A, S161A, A162V (loop between β4 and β5), D209 (loop between β6 and β7) and V215A (β7) were constructed by site-directed mutagenesis based on Stratagene's Quik Change Site-Directed Mutagenesis. The recombinant plasmid pGEM-cyt2Aa2 was used as a template for PCR with primers described in Table 1. The PCR products were transformed into *E. coli* JM109. The recombinant plasmids were extracted and verified by automated DNA sequencing.

3. Protein preparation

E. coli cells containing pGEM-Cyt2Aa2 wild type (Promdonkoy et al., 2003) and its mutants were grown at 37◦C for 16–18 h in LB broth containing 100 μg/ml ampicillin. One mM IPTG (isopropyl-b-D-thiogalactopyranoside) was then added to induce protein expression. Cyt2Aa2 toxin inclusion bodies were solubilized in 50 mM bicarbonate buffer (Na_2CO_3/$NaHCO_3$) pH 10.5 at the ratio 1:1 inclusion to the buffer, incubated at 37^{o}C for 1 hour. The supernatant was collected by centrifugation at 12,000 rpm for 10 minutes. The protoxin of Cyt2Aa2 and mutants were digested by proteinase K at the ratio 100:1 toxin to proteinase K, incubated at 37^{o}C for 1 hour. The activity of proteinase K was inhibited by 4 mM phenylmethanesul- fonylfluoride (PMSF). The solubility of protoxins and the activated toxins were analyzed on 12% SDS-PAGE. Protein concentration was determined by Bradford's method using bovine serum albumin (BSA) as a standard.

4. Mosquito-larvicidal assay

The third instar *A. aegypti* larvae were fed with two-fold serial dilutions of toxin inclusions from 0.12 to 250 µg/ml diluted in distilled water. One ml of the serial diluted inclusion was added to a 24-well plate (1.5 cm well diameter) containing 10 larvae in 1 ml of distilled water. The mortality was recorded after 24 hours of incubation at room temperature and LC_{50} value (concentration of toxin that caused 50% mortality) was determined by Probit analysis (Finney, 1971).

5. Hemolysis assay

A suspension of 2% RBC was prepared in PBS buffer. Hemolytic assay was performed by adding the two-fold serial dilutions of the activated Cyt2Aa2 wild type or its mutants in PBS pH 7.4 in a 96-well microtiter plate. One hundred µl of the serial diluted toxin (0.024-50 µg/ml) was mixed with 100 µl of 2% RBC. The hemolytic endpoint was monitored after 24 hours of incubation at room temperature.

6. Hemoglobin release assay

Hemoglobin release assay was performed using the method previously described (Promdonkoy and Ellar, 2003). One hundred µl of 2% rat RBCs in PBS pH 7.4 were incubated with various concentrations of 100 µl activated toxins (0.5–12 µg/ml) for 2 hours at room temperature. Unbroken cells and cell debris were removed by centrifugation at 12,000g for 30 seconds. Hemoglobin in supernatant was determined by measuring absorbance at 540 nm using ELISA plate reader. Supernatant from 2% rat RBCs mixed with 0.1% Triton X-100 was used as a 100% hemolysis control and a supernatant from 2% rat RBCs in PBS buffer were used as a negative control.

7. Binding of Cyt2Aa2 toxin on rat RBC membrane

The 5 µg/ml of activated toxin was mixed with 2% rat RBC in PBS pH 7.4 in total volume 0.4 ml and incubated for 2 hours at room temperature in micro-centrifuge tube.

The mixture was centrifuged at 12,000g for 30 minutes to separate pellet as the membrane-bound complexes from unbound toxin in supernatant. The mixture was mixed with 2X Laemmli sample buffer without reducing agent before subjected to SDS-PAGE without heating. Proteins separated on SDS-PAGE were visualized by Coomassie blue stain and detected by western blotting using anti-Cyt2Aa2. The assays were repeated three times.

Results

All of plasmids were successfully amplified by PCR with designed primers. DNA sequencing was performed to confirm mutant DNA sequences. All mutants were successfully constructed and verified by DNA sequencing

The expression of all Cyt2Aa2 wild type and mutant proteins were induced by 1 mM IPTG. Analysis of the whole cell lysate on SDS-PAGE (data not shown) revealed that all mutants expressed at high level similar to that of wild type toxin except I139A mutant showed low level of protein expression. All mutant proteins were expressed as inclusion bodies in *E. coli* similar to that of the wild type except I139A mutant that showed low protein expression level (Fig. 1).

The solubility of toxins in 50 mM carbonate buffer (Na_2CO_3/$NaHCO_3$) pH 10.5 and the toxins activation by proteinase K (1%, w/w) at 37^{o}C for 1 h were analyzed on 12% SDS-PAGE (Fig. 1). Solubility test revealed that 5 mutants (S159A, L160A, S161A, A162V and D209N) could solubilize in the carbonate buffer (lane S in Fig. 1) but V215A mutant were unable to solubilize. The soluble proteins (protoxin) were detected by western blotting using anti-Cyt2Aa2 (Fig. 2). The mutants, S159A, L160A, S161A, gave the result of protoxin similar to that of wild type. The result showed one major band at 27 kDa which was the protoxin in monomer form and one

minor band at 55 kDa which was the dimer form of the protoxin (Fig. 2A). The mutants, A162V and D209N, only showed one major band at 27 kDa. The mutants, S159A, L160A, S161A, A162V and D209N, were successfully activated by proteinase K. All of them were processed and yielded similar product to that of wild type (Fig. 2B).

The protease activated form of mutant toxins exhibited comparable hemolytic activity to wild type. Replacement at I139A and V215A positions of Cyt2Aa2 yielded the toxin inclusions that are less soluble or insoluble in carbonate buffer. Hemolytic activity of these mutants could not be accessed because no activated toxin was available. These mutants also exhibited no larvicidal activity (Table 2).

Mutant inclusions that were solubilized in carbonate buffer showed different toxicity of hemolytic and larvicidal activity (Table 2). Mutant S159A, L160A and S161A showed high hemolytic activity consistent with hemoglobin release assay that could completely break RBC. However these mutants showed low toxicity against *A. aegypti*. The binding assay with rat RBC of S159A, L160A and S161A mutants showed similar oligomerization pattern to that of wild type (Fig 3). For A162V and D209N mutants, they could not form oligomerization on the rat RBC. Also, the hemoglobin release assay demonstrated that A162V and D209N could not break RBC even at high concentration (Fig 4). These mutants were unable to lyse red blood cells and showed no hemolytic activity.

Discussion

Cyt2Aa2 is specifically toxic against dipteran larvae *in vivo*, and also active to several cell types such as erythrocytes (Promdonkoy et al., 2003). Cyt2Aa2 show identical to Cyt2Aa1 from *B. thuringiensis* subsp. *kyushuensis*, both toxins should adopt a similar

193 α/β structure containing 6 α-helices and 7 β-sheets (Koni et al., 1993; Promdonkoy et
194 al., 2004). It has been proposed that β5, β6 and β7 are long enough to span the
195 membrane and these regions should be able to insert into the membrane (Promdonkoy
196 and Ellar, 2000). The possible roles of amino acids in αA-αD may involve with
197 binding onto the cell membrane, the oligomerization of toxin molecules to generate
198 pore and αD-β4 loop and β6-β7 loop could facilitate conformational changes during
199 membrane insertion of β-sheets (Promdonkoy et al., 2005; 2000). Cyt toxin is
200 structurally related to a few toxins, volvatoxin A2 (VVA) (Lin et al., 2004) and Evf
201 toxin. Mapping between Cyt and Evf toxins revealed that the lipid binding cavity of
202 Cyt toxin might locate on αD (I139), loop between αD and β4, and β7 (V215). Also,
203 the electrostatic surface was identified in Cyt and was proposed that these residues,
204 V150, N159, L160 and D209 were involved with lipid binding pocket (Rigden et al.,
205 2009). This study attempts to elucidate the role of expected membrane binding
206 residues. To investigate this possibility, selected amino acids were substituted (I139A
207 in alpha-D, S159A, L160A, S161A and A162V on loop between β4 and β5, and
208 D209N and V215A on β7). Substitution I139 by alanine in alpha-D may affect protein
209 folding and inclusion formation. This residue is located in the hydrophobic face of
210 helix alpha-D pointing inside the molecule. Replacement this position with smaller and
211 less hydrophobic side-chain may destabilize the van der Waal contact which is
212 required to hold the right conformation. Substitution V215 by alanine on beta-7 may
213 affect protein folding and inclusion formation. Replacement this position with smaller
214 and less hydrophobic side-chain may destabilize the van der Waal contact which is
215 required to hold the right conformation lead to misfolding and alteration of the three
216 dimensional structure. The misfolded protein may not be solubilized in normal buffer.

This result demonstrated that V215 is critical residues playing important role to maintain structural folding of the toxin. Results indicated that the replacement at these positions (S159A, L160A, S161A) did not affect the folding of the toxin that the conformation of these mutants might not different from that of wild type. Substitution at the residues A162V and D209N showed a minor effect to the conformation of this toxin because these two mutants could not retain the dimer form of the protoxin.

Mosquito-larvicidal activity of insecticidal crystal proteins is normally tested by feeding the larvae with the toxin inclusions (Schnepf et al., 1998). It was found that Cyt2Aa2 inclusion is highly toxic to *A. aegypti* larvae (Promdonkoy et al., 2003). The mutants, S159A, L160A and S161A still retained the toxicity against *A. aegypti* and red blood cells. These residues are not important for oligomerization step as indicated from the binding result. The mutant A162V showed no toxicity against *A. aegypti*, but D209N showed low toxicity against *A. aegypti*. The mutants, A162V and D209N, could not completely break the rat red blood cell and could not form oligomerization. For unrelated toxicity results of D209N, it is possible that the different toxicity to cells from various sources might be caused by differences in membrane composition (Chow et al., 1989). The binding and oligomerization mechanism on the red blood cell and mosquito larval cell membranes may not be the same because of the differences of membrane compositions. It could be possible that other membrane components such as glycoproteins and lipoproteins contribute in some ways to the binding of the toxin (Suktham et al., 2013). Major components found in cell membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin and cholesterols, cell membranes contain many other lipids, albeit in lower amounts (Valcarcel et al., 2001). Analysis of phospholipid compositions in *A. aegypti* cells

found PE and PC as major components in membrane fractions (Butters and Hughes, 1981; Jones et al., 1992), whereas PE and sphingomyelin are major phospholipids found in rat red blood cell membranes (Virtanen et al., 1998). A study of lipid polymorphism of erythrocytes found that the erythrocyte membrane contains many different species of lipids more than 100 types. The different lipid compositions could provide appropriate fluidity characteristics as well as presenting the varying lipid physical properties (Cullis and Kruijff, 1979; Cullis et al., 1986). The replacement D209 by asparagine on beta-7 could retain larvicidal activity of the toxin. One of oxygen atoms at the side chain of D209 could form hydrogen bonds with the nitrogen atom of main chain of T206 and the oxygen atom of the side chain of T206. Mutation at this residue by asparagine disrupted the hydrogen bonds leading conformational change. Lower toxicity in the mosquito larvae, no toxicity and no oligomer on rat red blood cell were observed in this mutant. Replacement A162 by valine on loop between beta-4 and beta-5 could not retain membrane binding, oligomerization and activity of the toxin, both hemolytic and mosquitocidal activity. A substitution of this alanine with bigger and more hydrophobic side chain may interrupt their hydrogen bonding between the loop and the beta sheet leading to the conformational change. This alteration also affected an ability to bind and oligomerize on rat red blood cells. In Cyt1Aa, the region helix α-C was important for toxin oligomerization, membrane insertion and toxicity (López-Diaz et al., 2013).

Results presented here clearly demonstrated that alanine-162, located on loop between β4 and β5, of Cyt2Aa2 toxin is also one of the important residues for membrane binding as well as oligomerization. Substitution of this amino acid alters the biological

activity. Selectivity of the toxin to certain target cells might be improved by amino acid replacement in this residue.

References

Bulla, L.A., Bechtel, D.B., Kramer, K.J., Shethna, Y.I., Aronson, A.I. & Fitz-James, A.I. (1980). Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*. *Crit. Rev. Microbiol*., 8, 147–204.

Butters, T.D. & Hughes, R.C. (1981). Phospholipids and glycolipids in subcellular fractions of mosquito *Aedes aegypticells*. *In Vitro Cell Dev. Biol*, 17, 831–838.

Chow, E., Singh, G.J. & Gill, S.S. (1989). Binding and aggregation of the 25-kilodalton toxin of *Bacillus thuringiensis* subsp. *israelensis* to cell membranes and alteration by monoclonal antibodies and amino acid modifiers. *Appl. Environ. Microbiol.*, 55, 2779–2788.

Cullis, P.R. & de Kruijff, B. (1979). Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta*., 559, 399–420.

Cullis, P.R., Hope, M.J. & Tilcock, C.P. (1986). Lipid polymorphism and the roles of lipids in membranes. *Chem. Phys. Lipids*., 40, 127–144.

Finney, D. (1971). Probit Analysis. Cambridge University Press; London, UK.

Knowles, B.H. & Ellar, D.J. (1987). Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxin with different specificity. *Biochem. Biophys. Acta*, 924, 509-518.

Koni, P.A. & Ellar, D.J. (1993). Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic δ-endotoxins. *J. Mol. Biol*., 229, 319-327.

Krissinel, E. & K. Henrick. (2004). Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Biol. Crystallogr*. 60, 2256–2268.

Li, J., Derbyshire, D.J., Promdonkoy, B. & Ellar, D.J. (2001). Structural implication for the transformation of the *Bacillus thurengiensis* δ-endotoxins from water-soluble to membrane-inserted forms. *Biochemical society*., 29, 571-577.

Li, J., Koni, P.A. & Ellar, D.J. (1996). Structure of the Mosquitocidal d-endotoxin from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. *J. Mol. Biol*., 257, 129–152.

Lin, S.C., Lo, Y.C., Lin, J.Y. & Liaw, Y.C. (2004). Crystal structures and electron micrographs of fungal valvotoxin A. *J. Mol. Biol*., 343, 477–491.

López-Diaz, J.A., Cantón, P.E., Gill, S.S., Soberón, M. & Bravo, A. (2013). Oligomerization is a key step in Cyt1Aa membrane insertion and toxicity but not necessary to synergize Cry11Aa toxicity in **Aedes aegypti** larvae. *Environ Microbiol*. 15, 3030-9.

Promdonkoy, B. & Ellar, D.J. (2000). Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis*. *Biochem J*., 350(1), 82–275.

Promdonkoy, B., Chewawiwat, N., Tanapongpipat, S., Luxananil, P. & Panyim, S. (2003). Cloning and characterization of a cytolytic and mosquito larvicidal δ-endotoxin from *Bacillus thuringiensis* subsp. *darmstadiensis*. *Current Microbiology*., 46, 94–98.

Promdonkoy, B., Pathaichindachote, W., Krittanai, C., Audtho, M., Chewawiwat, N. & Panyim, S. (2004). Trp132, Trp154, and Trp157 are essential for folding and

activity of a Cyt toxin from *Bacillus thuringiensis*. *Biochemical and Biophysical Research Communications*, 317, 744–748.

Promdonkoy, B. & Ellar, D.J. (2005). Structure-function relationships of a membrane pore forming toxin by reversion mutagenesis. *Molecular Membrane Biology*., 22(4), 327-337.

Quevillon-Cheruel, S., Leulliot, N., Muniz, C.A., Vincent, M., Gallay, J., Argentini, M., Cornu, D., Boccard, F., Lemaître B. & van Tilbeurgh, H. (2009). Evf, a virulence factor produced by the Drosophila pathogen *Erwinia carotovora*, is an S palmitoylated protein with a new fold that binds to lipid vesicles. *J. Biol. Chem*., 284(6), 3552-3562.

Rigden D.J. (2009). Does distant homology with Evf reveal a lipid binding site in *Bacillus thuringiensis* cytolytic toxins? *FEBS Lett*. 583(10), 1555-60.

Schnepf, H.E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. & Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal proteins. *Microbiol. Mol. Biol. Rev.*, 62, 775–806.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. & Gouaux, J.E. (1996). Structure of Staphylococcal α-hemolysin, a heptameric transmembrane pore. *Science*., 274, 1859–1866.

Suktham, K., Pathaichindachote, W., Promdonkoy, B. & Krittanai, C., 2013. Essential role of amino acids in αD–β4 loop of a *Bacillus thuringiensis* Cyt2Aa toxin in binding and complex formation on lipid membrane. *Toxicon*., 4663, 1–8.

Valcarcel, C.A., Dalla Serra, M., Potrich, C., Bernhart, I., Tejuca, M., Martinez, D., Pazos, F., Lanio, M.E. & Menestrina, G. (2001). Effects of lipid composition on membrane permeabilization by sticholysin I and II, two cytolysins of the sea anemone Stichodactyla helianthus. *Biophys. J*., 80, 2761–2774.

333 Virtanen, J. A., Cheng, K.H. & Somerharju, P. (1998). Phospholipid composition of
334 the mammalian red cell membrane can be rationalized by a superlattice model. *Proc.*
335 *Natl. Acad. Sci.*, 95, 4964–4969.