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## *Research article*

# Proteolytic Sites at Protein Termini of BT Cytolytic Cyt2Aa2 Protein Affect its Expression in *Escherichia coli*

**Chontida Tangsongcharoen[1], Boonhiang Promdonkoy[2], Chartchai Krittanai[3] and Sudarat Tharad[4]***

*[1]Faculty of Allied Health Sciences, Burapha University, Chonburi 20131, Thailand*
*[2]National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani 12120, Thailand*
*[3]Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand*
*[4]Department of Biology, Faculty of Science, Burapha University, Chonburi 20131, Thailand*

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## Abstract

*Bacillus thuringiensis* (Bt) is well-known for its bio-larvicidal properties. Cytolytic (Cyt) protein is one of Bt larvicidal proteins. This protein requires proteolytic activation to remove partial N- and C-terminal regions. In this study, to improve proteolytic cleavage specificity, proteinase K cleavage sites at both end termini of Cyt2Aa2 protein were substituted by a trypsin cleavage sites L33R, S37K, S228R, and F237K. Afterward, the engineered Cyt2Aa2 proteins were heterologous expressed in *Escherichia coli*. Most of the mutants were produced as inclusion proteins similar to the wild type but their capability of solubilization and trypsin activation was significantly reduced, particularly for L33R, S37K, and F237K. The S228R mutant could be partially solubilized and activated. Moreover, the new trypsin cleavage site at N-terminus of L33R resulted in an aberrant toxic Cyt2Aa2 protein against *E. coli.* The L33R mutant limited *E. coli* growth during protein synthesis. Remarkably, although the capability of solubilization of the mutant proteins was reduced, their mosquito larvicidal activity (except L33R and L33R/F237K) was comparable to the wild type. These findings demonstrate that although the amino acid residues at N- and C-terminal regions of Cyt2Aa2 are eliminated from active protein, they are necessary for protein production and for preventing toxicity against *E. coli* during heterologous expression.

**Keywords:** *Bacillus thuringiensis*; Cyt2Aa2 protein; protein expression; trypsin cleavage site; *Escherichia coli*; N-terminal protein

## 1. Introduction

*Bacillus thuringiensis* (Bt) is a Gram-positive aerobic bacterium that produces insecticidal proteins during the sporulation phase (Schnepf et al., 1998). Insecticidal proteins are synthesized as parasporal crystals and can be classified into two families of delta-

*Corresponding author: E-mail: sudarat.td@go.buu.ac.th
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endotoxins, Crystal (Cry) protein and Cytolytic (Cyt) protein (Hofte & Whiteley, 1989). Although, both Cry and Cyt proteins exhibit larvicidal activity, their amino acid sequences and conformational configurations are different (Li et al., 1991; Li et al., 1996). Moreover, the larvicidal mechanisms of Cry and Cyt proteins differ in that Cry protein requires a particular protein receptor on bacterial cell membrane (Bravo et al., 2007; Pigott & Ellar, 2007), whereas Cyt protein attaches directly to cell membrane phospholipids (Thomas & Ellar, 1983). These proteins are solubilized in alkaline conditions and activated by larval gut proteases (Angsuthanasombat et al., 1993; Al-yahyaee & Ellar, 1995). After solubilization and proteolytic activation, the active protein is liberated and exerts its cytolytic activity.

Cyt proteins have broad in vitro cytolytic action against eukaryotic cells but specific in vivo toxicities toward dipteran insect larvae (Maddrell et al., 1989; Knowles et al., 1992). The ambiguous evidence of cytolytic mechanisms has led to two proposed models; the pore-forming model, in which each monomer comes together to form a pore (Thomas & Ellar, 1983; Li et al., 1996; Du et al., 1999; Li et al., 2001; Promdonkoy & Ellar, 2000, 2005), and the detergent-like model, which involves proteins that aggregate on the target cell membrane surface acting as a detergent (Thomas & Ellar, 1983; Butko, 2003; Manceva et al., 2005). Recent evidence reveals that Cyt proteins have different manners depending on protein concentration (Tharad et al., 2015) and target lipid membranes (Onofre et al., 2020). Cyt proteins are classified into Cyt1, Cyt2, and Cyt3 types based on a similarity of their amino acid sequences (Crickmore et al., 2016). *Bacillus thuringiensis* subsp. *darmstadiensis* produces Cyt2Aa2 protein, which is a protoxin with 259 amino acids. The entire *cyt2Aa2* gene is able to highly express in *E. coli* and Cyt2Aa2 protein is substantially produced as an inclusion protein (Promdonkoy et al., 2003). This has facilitated studies of the amino acid mutation effect on Cyt2Aa2 structure; studies that were problematic in the original *B. thuringiensis*.

Proteinase K, a non-specific cleaving protease, can activate Cyt2Aa resulting in two sizes of active protein (Figure 1). For the larger size ~23 kDa (T34 to F237), N- and C-termini are cleaved at the residues L33 and F237, respectively. On the other hand, the smaller fragment (~20 kDa; residues S38 to S228) is produced when proteinase K cleaves at residues S37 and S228 (Li et al., 1996). In this study, the proteinase K cleavage sites (L33, S37, S228, and F237) were substituted by trypsin recognition amino acids, arginine (R) or lysine (K), to achieve more specific proteolytic cleavage and yielding a homogeneity of active Cyt2Aa2 with either 20 kDa or 23 kDa. The proteolytic cleavage sites at N- and C-termini of Cyt2Aa2 are eliminated from active protein, therefore it is not supposed to affect the property of protein. Unexpectedly, the new trypsin site at N- and C-terminals Cyt2Aa2 has an impact on protein production, protein solubilization, and host cell toxicity. The findings indicate that although the amino acid residues at N- and C-termini of Cyt2Aa2 do not participate in cytolytic action due to their removal from the active protein, they play a crucial role in protein synthesis.

## 2. Materials and Methods

### 2.1 Bacterial strain and plasmid

The *cyt2Aa2* gene from *Bacillus thuringiensis* subsp. *darmstadiensis* (GenBank accession no. AF472606) was subcloned into the pGEM-T-easy vector (Promega, USA), known as pGEM-T-*cyt2Aa2*, as described previously (Promdonkoy et al., 2003). *Escherichia coli* strain JM109 was used for the heterologous protein expression of Cyt2Aa2 protein under *lac* promoter.

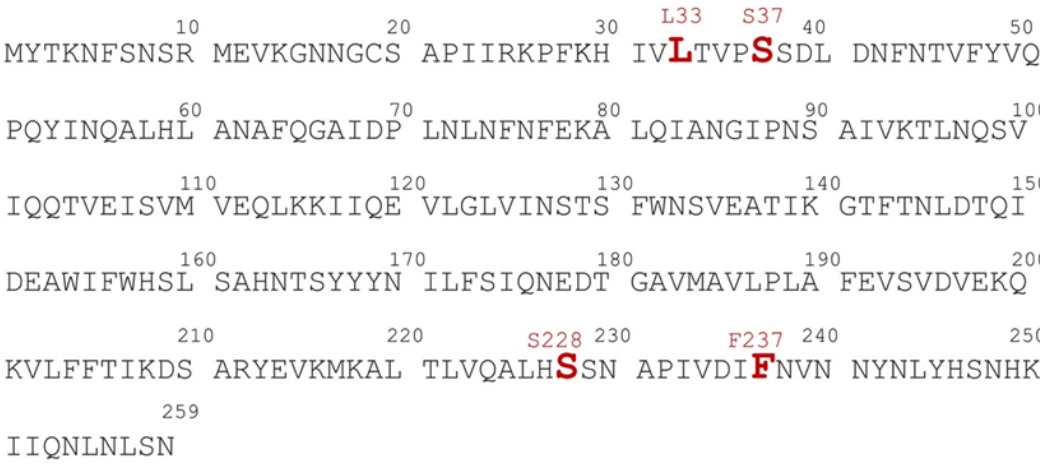


**Figure 1.** The amino acid sequence of Cyt2Aa2 protein. The full length of Cyt2Aa2 protein is 259 amino acids. The number marked above the amino acid residues indicates the position of amino acids. The bold red letters indicate the proteinase K cleavage sites, L33, S37, S228, and F237, which were changed to the trypsin site in mutants.

## 2.2 Construction of Cyt2Aa2 mutants

The mutated *cyt2Aa2* genes were constructed by PCR-based site-directed mutagenesis with following PCR conditions; 0.4 pmole forward and reverse primers, 0.2 mM dNTPs, 50 ng DNA template, and 2 U Phusion DNA polymerase (Thermo Scientific, USA). The PCR temperature profile for each mutant is shown in Table 1. For single mutations, pGEM-T-*cyt2Aa2* was employed as a template, with specific primers for L33R, S37K, S228R, and F237K (Table 2). Subsequently, the prior single mutated *cyt2Aa2* (L33R and S228R) was used as a template for the construction of the double mutated *cyt2Aa2*, L33R/F237K and S37K/S228R, respectively. Prior to DNA transformation into *E. coli* JM109, the DNA templates were removed by *Dpn*I digestion (Thermo Scientific, USA). The heat shock method was employed for DNA transformation. The transformed cells were selected on Luria-Bertani (LB)-agar plate containing 100 µg/mL ampicillin (Thermo Scientific, USA). Each mutant plasmid was preliminary selected using restriction enzyme digestion. The DNA sequencing method (1st Base, Malaysia) was used to confirm the nucleotide changes in the mutated *cyt2Aa2* genes.

## 2.3 Protein expression and inclusion protein preparation of Cyt2Aa2 protein

*E. coli* JM109 carrying the pGEM-T-*cyt2Aa2* was cultured for 16 h in LB broth containing 100 µg/mL ampicillin at 37°C with 250 rpm shaking. The culture was inoculated (1% inoculum) into LB broth (plus 100 µg/mL ampicillin) and grown until the OD.600 reached 0.4-0.6. Protein expression was induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific, USA) and maintained continuously at 37°C with 250 rpm shaking for 5 h. *E. coli* carrying the empty pGEM-T-easy vector (without *cyt2Aa2* gene) was employed as a negative control due to it being unable to produce the Cyt2Aa2 protein.

**Table 1.** The PCR temperature profile for *cyt2Aa2* gene mutagenesis

| Mutant / PCR Step | L33R | S37K | S228R | F237K | Time (s) |
|---|---|---|---|---|---|
| Initiation temperature (°C) | 95 | 95 | 95 | 95 | 60 |
| Denaturing temperature (°C) | 95 | 95 | 95 | 95 | 30 |
| Annealing temperature (°C) | 50 | 50 | 50 | 45 | 30 |
| Extension temperature (°C) | 68 | 68 | 68 | 68 | 120 |
| Final extension temperature (°C) | 68 | 68 | 68 | 68 | 120 |

Note: The PCR is performed for 20 cycles.

**Table 2.** Mutagenic primers for site-directed mutagenesis

| Primers | Sequence (5'→ 3') | Restriction Enzymes |
|---|---|---|
| **L33Rf** | TAAACATATTGT**GCGC**ACGGTTCCATCCAG | *Fsp*I |
| **L33Rr** | TGGAACCGT**GCGC**ACAATATGTTTAAATGG | *Fsp*I |
| **S37Kf** | TTAACGGTTCC**TAAG**AGTGATTTAGATAAT | *Dde*I |
| **S37Kr** | ATCTAAATCACT**CTTA**GGAACCGTTAATAC | *Dde*I |
| **S228Rf** | GCTCTACAT**CGA**TC**G**AATGCCCCAATTGTA | *Pvu*I |
| **S228Rr** | TGGGGCATT**C**GA**TCG**ATGTAGAGCTTGAAC | *Pvu*I |
| **F237Kf** | ATTGTAGATAT**CAAG**AATGTTAATAACTAT | *Eco*RV |
| **F237Kr** | GTTATTAACATT**CTTG**ATATCTACAATTGG | *Eco*RV |

Note: Mutated nucleotides are shown in bold and restriction enzyme recognition sites are underlined.

For inclusion protein preparation, the 1% inoculum was cultured in 500 mL of LB-medium containing 100 µg/mL ampicillin, and protein expression was stimulated with 1.0 mM IPTG. The cultures were centrifuged at 7,000xg for 10 min at 4°C and the cell pellets were resuspended in 30 mL of sterile distilled water before disruption by ultrasonication. After centrifugation at 10,000xg for 10 min at 4°C, the inclusion protein was resuspended in 30 mL of ice-cold gradient buffer (50 mM Tris-HCl and 10 mM KCl, pH 7.5) plus 0.1% (v/v) Triton-X 100 (Bio Basic, Canada). The remaining intact cells were repeatedly broken. After collecting the inclusion protein, it was washed twice with 30 mL of ice-cold sterile distilled water. Finally, the inclusion protein was resuspended in 3 mL of sterile distilled water and kept at 4°C in 1 mL aliquots until use.

Protein concentration was measured by the Bradford method. The protein samples were mixed with Bradford reagent in a flat bottom to 96-well plate and incubated for 5 min. A serial dilution of bovine serum albumin (BSA) was used as a standard protein concentration. The absorbance was measured at wavelength of 595 nm.

### 2.4 Protein solubilization and proteolytic cleavage of Cyt2Aa2 protein

The inclusion protein was solubilized in 50 mM carbonate buffer (pH 10.5) at 37°C for 1 h. Soluble protein was isolated from the insoluble fraction (pellet) by centrifugation at 10,000xg for 10 min at 4°C. For proteolytic cleavage, the inclusion proteins in 50 mM carbonate buffer (pH 10.5) were treated with 1:10 (w/w) trypsin (Sigma, Germany) at 37°C for 1 h. Subsequently, the soluble fraction was collected by centrifugation at 10,000xg for 10 min at 4°C and determined the proteins by SDS-PAGE.

### 2.5 Growth monitoring of *Escherichia coli* during Cyt2Aa2 protein expression

*E. coli* JM109 harboring pGEM-T-*cyt2Aa2* either the wild type or mutants were cultured for 16 h in LB broth containing 100 µg/mL ampicillin at 37°C with 250 rpm shaking. The 1% inoculum culture were initially inoculated in 50 mL LB-medium containing 100 µg/mL ampicillin to obtain an OD at 600 nm (OD.600) of about 0.05. The cultures were grown at 37°C with 250 rpm shaking. Protein overexpression was induced with 1.0 mM IPTG at the OD.600 of approximately 0.4. The culture was continuously grown and OD.600 was measured every 30 min for 9 h.

### 2.6 Mosquito larvicidal activity assay

The 2$^{nd}$ instar *Culex quinquefasciatus* larvae were starved for 3 h before used in the experiments. Proteins were serially diluted two-fold in distilled water. Ten larvae in 1 mL of distilled water in each well of 24-well plate were fed with 1 mL of each protein concentration for 24 h and recorded for mortality. The distilled water was used as negative control.

## 3. Results and Discussion

### 3.1. Protein expression of Cyt2Aa2 mutants in *Escherichia coli*

The Cyt2Aa2 mutants with substituted trypsin cleavage sites at N- and C-termini were successfully constructed. The native proteinase K cleavage sites were at the positions of L33 and S37 for the N-terminus and S228 and F237 for the C-terminus, where the recognition amino acids of trypsin either arginine (R) or lysine (K) were introduced instead of the original proteinase K sites in the mutants. The change of DNA codon was confirmed by DNA sequencing for four single mutants (L33R, S37K, S228R, and F237K) and two double mutants (L33R/F237K and S37K/S228R) (Figure 2). For protein extraction, the cells were broken and fractionated to obtain the pellet and supernatant. Subsequently, the formation of protein inclusion of the mutants was compared to the wild type. The Cyt2Aa2 wild type was abundantly detected in the pellet and inclusion protein fractions. Similarly, three of four single mutants, L33R, F237K (Figure 3a), and S228R (Figure 3b), were detected in the pellet and inclusion protein similar to the wild type. Whereas the S37K mutant was unclearly presented in the cell lysate, and the 25-kDa protein band appeared

faint in both the pellet and inclusion protein fractions (Figure 3b). These findings imply that the protein production in host cells of the S37K mutant was lower than the other mutants and the wild type. For a double mutation, the L33R/F237K (Figure 3a) and S37K/S228R mutants (Figure 3b) were found in the lysate, pellet, and inclusion protein fractions. The L33R/F237K exhibited a resemble expression to its single variants, the L33R and F237K mutants, forming the inclusion protein. In comparison to the former, the S37K/S228R was able to produce as much as the single S228R mutant rather than less produce as the S37K mutant. This demonstrates that the S228R may contribute to the S37K/S228R synthesis being restored over the effects of the S37K mutation. This is intriguing since the N-terminal amino acids are translated earlier than the C-terminal amino acids, but it can counterbalance the effect of N-terminal amino acids. It is possible that improper protein folding of the mutants during synthesis makes them more accessible to proteolytic digestion by host cell proteases, hence lower amounts of mutants were produced compared with the wild type, and this was notable for the S37K mutant. According to a previous report, Cyt2Aa2 lacking either 10 amino acids (unpublished data) or 33 amino acids at N-terminus (Thammachat et al., 2008) was unable to produce, indicating the relevance of amino acid sequence for protein production.

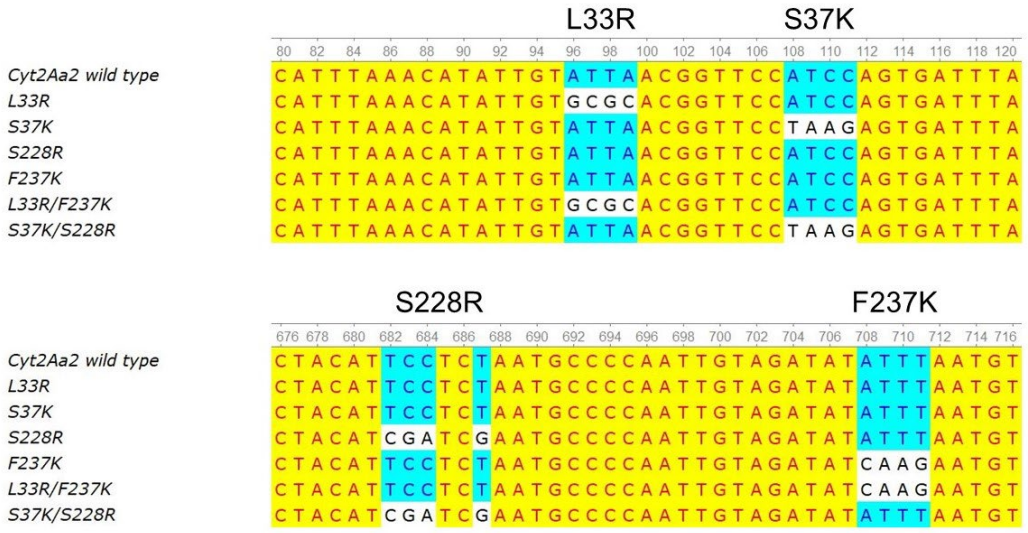


**Figure 2.** The alignment of DNA sequences between the mutated *cyt2Aa2* genes and the wild type *cyt2Aa2* gene. *E. coli* harboring mutated *cyt2Aa2* genes was selected by restriction enzyme digestion. The changes of nucleotides of the desired mutated *cyt2Aa2* gene were confirmed by DNA sequencing. The DNA sequence alignment was performed by Unipro UGENE program with Clustal Omega algorithm. The identical nucleotides are highlighted with yellow. The positions of mutation are highlighted with blue while the mutated nucleotides are not highlighted.

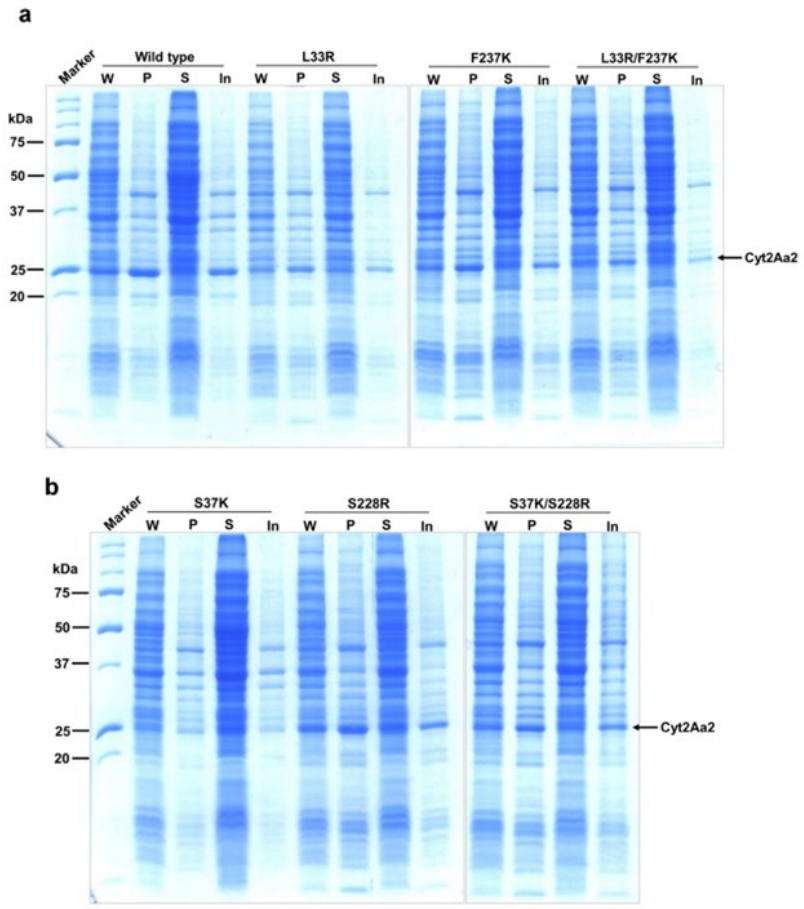


**Figure 3.** Analysis of protein forms of Cyt2Aa2 wild type and mutants by SDS-PAGE. The cell lysate was fractionated by centrifugation, and protein fractions were analyzed by SDS-PAGE. W, whole cell lysate (0.1 OD); P, pellet fraction (0.2 OD); S, soluble fraction (0.2 OD); In, inclusion protein. (a) Cyt2Aa2 wild type, L33R, F237K, and L33R/F237K mutants. (b) S37K, S228R, and S37K/S228R mutants.

## 3.2. Capability of solubilization of Cyt2Aa2 mutants

Regularly, Cyt2Aa2 protein is solubilized in alkaline conditions, which are the physiologic pH of a midgut of insect larvae (Al-yahyaee & Ellar, 1995). Therefore, the solubility of all mutants in carbonate buffer pH 10.5 was determined compared to native protein. Figure 4 shows that after alkaline buffer resuspension of the wild type inclusion protein, the ~25 kDa Cyt2Aa2 protein was dominantly presented in the soluble fraction and was faintly detected in the pellet fraction. In contrast, almost mutants except for the S228R remained predominantly in the pellet fraction indicating either a poor or ineffective solubilization. For S228R mutant, the 25 kDa-protein was found in both the pellet and soluble fractions indicating that this mutant was partially solubilized up to 50%. On the other hand, the S37K/S228R double mutant was unable to solubilize due to its presence in the pellet.

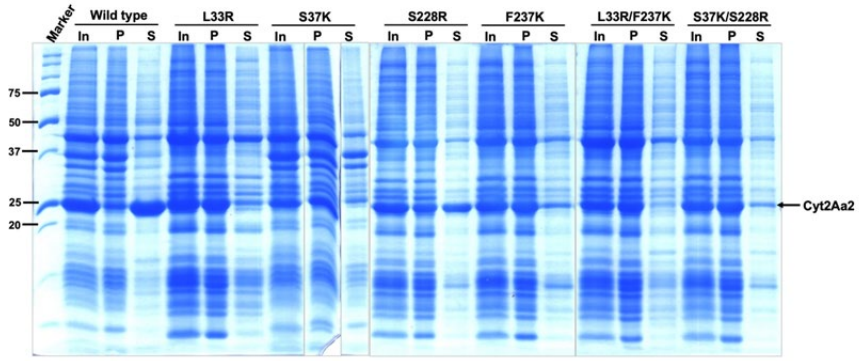


**Figure 4.** Protein solubilization of Cyt2Aa2 mutants in alkaline buffer. The 20 µg inclusion protein of the Cyt2Aa2 wild type and mutants were solubilized in carbonate buffer pH 10.5 at 37°C for 1 h. The soluble proteins and the insoluble proteins (pellet) were separated by centrifugation. Each protein fraction was determined by SDS-PAGE and stained by Coomassie blue G250. In, inclusion protein; P, pellet fraction; S, soluble fraction.

According to conformational structure of Cyt2A (Li et al., 1996), three of four mutated points (L33, S37, and F237) are located at the dimer's intermolecular interface, whereas S228 is located on the molecule's surface (Figure 5). The large positively charged lysine or arginine replacement at those mutated points may interfere in the protein dimer formation, leading to irregular packing of inclusion protein. On the other hand, it is possible that at pH 10.5 of solubilizing buffer, arginine/lysine cannot deprotonate due to their high pKa values (R-side chain, pKa = 12.5 and K-side chain, pKa = 10.5). Thus, each protein mutant molecule still likely forms an ionic bond in the inclusion protein.

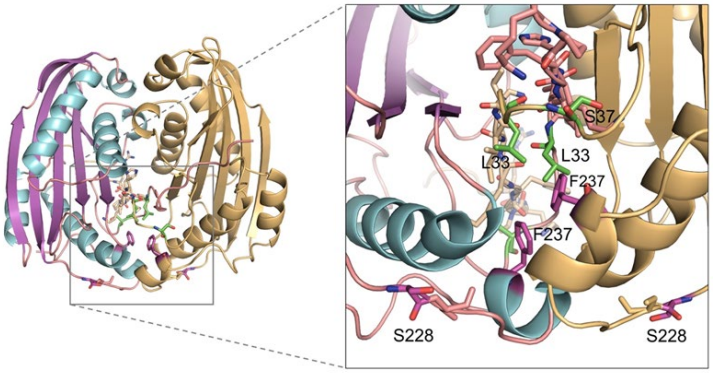


**Figure 5.** Location of mutated residues in three-dimensional structure of Cyt2A protein. The crystal protein structure of Cyt2A (PDB: 1CBY) reveals a dimeric molecule of Cyt2A protein. Each monomeric molecule is presented in distinct colors. The N-terminal mutated residues, L33 and S37, are visualized as the green stick residues, whereas C-terminal mutated residues are shown in the magenta stick residues. The images were generated by the PyMOL program.

### 3.3. Accessibility of trypsin into the cleavage site in the mutant inclusion protein

According to the solubilization results, the Cyt2Aa2 mutants were supposed to exhibit regular protein folding like the wild type, but the additional arginine and lysine in the mutants impede inclusion protein solubilization. If trypsin is able to cleave at either R or K mutated residues, the mutant proteins may be released from the inclusion protein. All mutant inclusion proteins were treated with trypsin in carbonate buffer pH 10.5. Following that, the insoluble and soluble fractions were separated, and trypsin-cleaved Cyt2Aa2 was expected to be found in the soluble fraction (Figure 6). For the Cyt2Aa2 wild type, it was greatly solubilized in which the soluble fraction clearly shows with ~25 kDa. An addition of trypsin shows the molecular weight shift to the ~20 kDa. This indicates that the wild type was solubilized as protoxin prior to cleavage by trypsin.

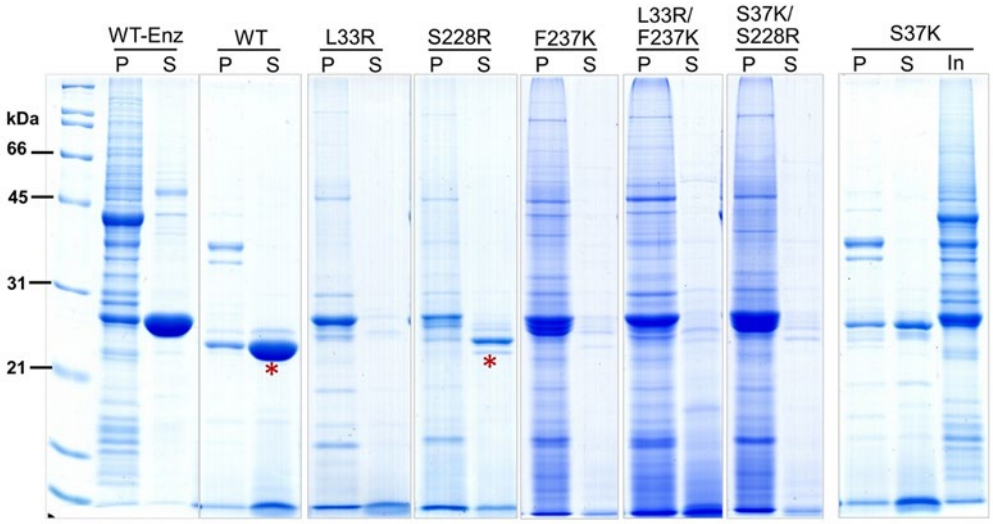


**Figure 6.** Trypsin cleavage of Cyt2Aa2 inclusion protein. Inclusion protein of 20 µg of the Cyt2Aa2 wild type and mutants were incubated with trypsin in carbonate buffer pH 10.5 at 37°C for 1 h. The soluble and insoluble (pellet) fractions were separated by centrifugation. Each fraction was separated by SDS-PAGE and stained by Coomassie blue G250. P, pellet fraction; S, soluble fraction; In, inclusion protein. The red asterisk indicates the size of trypsin-digested Cyt2Aa2.

Although the Cyt2Aa2 wild type can be cleaved by trypsin, but its hemolytic activity dramatically decreases compared to proteinase K cleavage (Figure 7). Based on the amino acid sequence of Cyt2Aa2, trypsin possibly cleaves at positions K29 and K250 whereas proteinase K cleaves at L33 and F237 residues. Therefore, it is proposed that the extended residues at both termini of trypsin-cleaved Cyt2Aa2 interfere with the Cyt2Aa2 hemolytic activity. For the mutants, the L33R, F237K, L33R/F237K, and S37K/S228R were mainly found in the pellet fraction. Furthermore, the molecular weight of the mutants was closed to the protoxin (~25 kDa), indicating the trypsin was incapable of reaching its cleavage sites on the Cyt2Aa2 inclusions and releasing the protein into the solution. On the other hand, the S37K and S228R single mutants partially dissolved in the buffer. The S37K was

detected in both soluble and pellet fractions with molecular weight smaller than the protoxin (inclusion protein) but larger than the active wild type (red asterisk). This implies that although trypsin is capable to cleave this mutant, the overall protein conformation may fold improperly for the proteolytic process. In contrast, the S228R mutant showed the molecular weight of trypsin-cleaved protein comparable to the wild type. Together with solubilization result, the S228R may exist in two conformations; (i) a regular folding protein that can be dissolved and cleaved by trypsin and (ii) an irregular form that lacks both features. These findings indicate that even though the mutants could be produced, the proteins were unable to solubilize and were infrequently cleaved by trypsin.

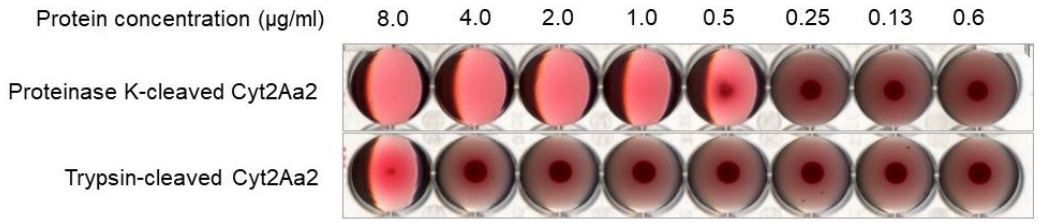


**Figure 7.** Hemolytic end point of protease K- and trypsin-cleaved Cyt2Aa2 protein. Sheep red blood cell (RBC) was separated from blood serum by centrifugation at 3,000 xg at 4°C for 5 min. The red blood cell was washed with PBS, pH 7.4 for 3 times. Then, the RBC was re-suspended with PBS, pH 7.4 to 2% RBCs. Cyt2Aa2 protein was 2-fold serial diluted with PBS, pH 7.4. Each protein concentration of 100 µl of Cyt2Aa2 solution was incubated with 100 µl of 2% RBCs in round bottom 96-well plate at room temperature for 2 h and then the hemolytic end point was determined.

### 3.4. Bacterial growth inhibition of L33R mutant

Due to the less production of the mutants, the toxicity against host cell was proposed. The growth of *E. coli* was monitored during Cyt2Aa2 expression. Initially, *E. coli* containing the wild type *cyt2Aa2* or mutated *cyt2Aa2* genes were cultured until the OD.600 reached approximately 0.4. At this point, the L33R showed a slower growth rate than the others. Protein overexpression was triggered by adding 1.0 mM IPTG. For the wild type, the bacterial host could grow normally with the maximum OD.600 = 1.8 at a stationary phase. The overexpression of S37K, S228R, F237K, and S37K/S228R mutants did not affect bacterial growth in which their growth curves were similar to the wild type. In contrast, overexpression of the L33R and L33R/F237K mutants decreased the growth rate which showed the maximum OD.600 of about 0.8 and tended to drop after this point (Figure 8). Because the C-terminal F237K single mutant did not reduce cell growth, it seems likely that the toxicity of L33R/F237K was due to the L33R. Similarly, the trypsin cleavage site at the C-terminal S228R was not toxic as well. This suggests that the N-terminal sequence is more important for Cyt2Aa2 activity than the C-terminal sequence. Interestingly, S37K did not limit cell proliferation despite being located at the N-terminus. It is possible that the four amino acids, T34 to S37, play an essential function in protein folding and bactericidal activity.

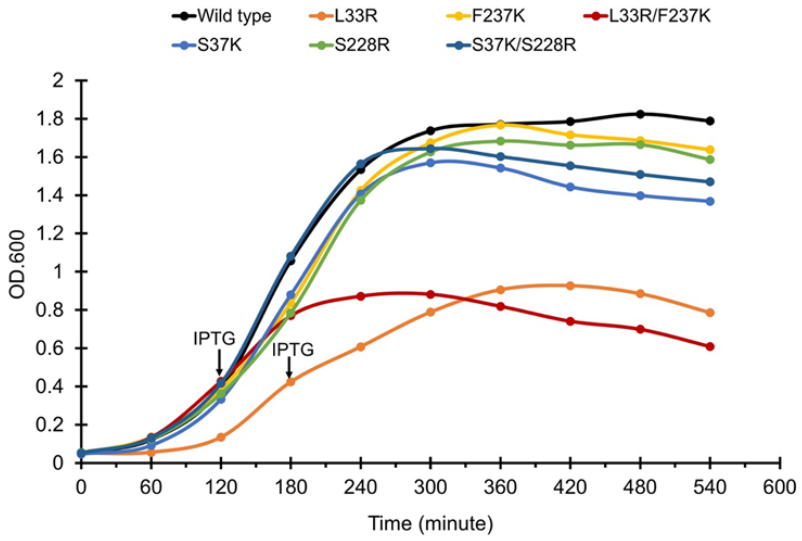


**Figure 8.** Growth monitoring of *Escherichia coli* during Cyt2Aa2 protein expression. *E. coli* containing wild type *cyt2Aa2* gene or mutated *cyt2Aa2* gene were cultured until the mid-log phase. Cyt2Aa2 protein expression was then induced by IPTG. The cell growth was monitored by means of cell density at OD.600 every 30 min.

Similarly, expression of the Cyt1Aa protein in *E. coli* without a p20 helper protein is harmful to the host cell (Visick & Whiteley, 1991; Manasherob et al., 2003; Sazhenskiy et al., 2010). However, the bactericidal activity of Cyt1Aa1 against *E. coli* during protein expression has been unclear until now. The toxicity of Cyt2Aa2-L33R to *E. coli* can provide clues to the mechanisms by which Cyt1Aa1 is toxic during protein expression. In previous cytolytic studies, Cyt1Aa1 (Al-yahyaee & Ellar, 1995) and Cyt1Ab1 (Escobar et al., 2000) activated by trypsin with arginine R30 is able to exert hemolytic activity. Analysis of amino acid alignment (Figure 9) revealed that the N-terminal R30 of Cyt1Aa1 and Cyt1Ab1 were located close to the mutated L33 of Cyt2Aa2. Therefore, substitution of L33 with arginine (L33R) may render Cyt2Aa2 proteins susceptible to activation by trypsin-like proteinase of host cell during expression, leading to inhibition of cell growth. For future work, the trypsin cleavage site at R30 of Cyt1Aa1 will be changed to alanine (L) as Cyt2Aa2 wild type to prove its effect on toxicity reduction of Cyt1Aa1 in *E. coli* expression.

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                                                 L33
Cyt2Aa    (1) ----------MYTKNFSNSRMEVKGNNGCSAPIIRKPFKHIVLTVPSSDLDNFNTVFYVQPQYINQALHLANAFQGAIDPLNLNFN-------FEKALQ
Cyt1Aa    (1) --------MEN-----LNHCPLEDIKVNPWKTPQSTARVITLRVEDPNEINNLLSINEIDNPNYILQAIMLANAFQNALVPTSTDFG-DALRFSMAKGLE
Cyt1Ab    (1) --------MEN-----PNHCPLEDIQVNPWKTPQSKARVITLRIDDPNEINNLLSINEIENTNYLLQAIMLANAFQKALVPTSTEFAEDALQFSMTKGLE
Cyt1Ba    (1) MKESIYYNEENEIQISQGNCFPEELGHNPWRQPQSTARVIYLKVKDPIDTTQLLEITEIENPNYVLQAIQLAAAFQDALVPTETEFG-EAIRFSMPKGLE
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**Figure 9.** The alignment of N-terminal protein sequences between Cyt2 protein and Cyt1 proteins. The protein sequence alignment was performed by Clustal Omega program. The L33 residue of Cyt2Aa2 is an underlined red letter which was changed to arginine (trypsin cleavage site). The native trypsin cleavage sites of Cyt1 proteins are bold green letters.

### 3.5. Mosquito larvicidal activity of the mutants

In susceptible larvae, the inclusion protein is solubilized in the alkaline conditions of the larval midgut and subsequently cleaved by gut proteases (Koni & Ellar, 1994). The mosquito larvicidal activity of the mutant proteins was proposed to be abolished because of inability of in vitro solubilization and activation. Figure 10 shows unexpectedly larvicidal activity where the mutant proteins (except L33R and L33R/F237K) had larvicidal activity similar to the wild type. At the lowest protein concentration (2.5 µg/mL), the S37K, S228R, F237K, and S37K/S228R remained at least 50% activity. In contrast, the toxic mutants, L33R and L33R/F237K completely lost their larvicidal activity. At the highest protein concentration (80 µg/mL), the treated larvae remained alive in distilled water (negative control). Remarkably, these larvicidal mutants showed poor solubilization and proteolytic cleavage in vitro. These findings suggest that the gut environment of *Culex quinquefasciatus* provides conditions more suitable for Cyt2Aa2 action. In vitro condition of buffer pH 10.5 may possibly be the minimum requirement for Cyt2Aa2 solubilization as at least the Cyt2Aa2 wild type can be solubilized. For the mutants, the other factors in the larval gut (such as ions, type of protease, and other enzymes) may be additionally required for the protein solubilization. As a result of larvicide of the mutants, the core protein appears to be a protease-resistant protein that can exert the activity implying that the S37K, S228R, and F237K residues affect inclusion protein formation but not the core protein folding. On the other hand, the L33R and L33R/F237K might be synthesized in form of incorrect conformation because of no larvicidal activity. According to the decreased bacterial growth rate, the overexpression of L33R and L33R/F237K toxic proteins could trigger cellular stress, prompting the host cell to survive and attempt to reduce protein toxicity (James et al., 2021).

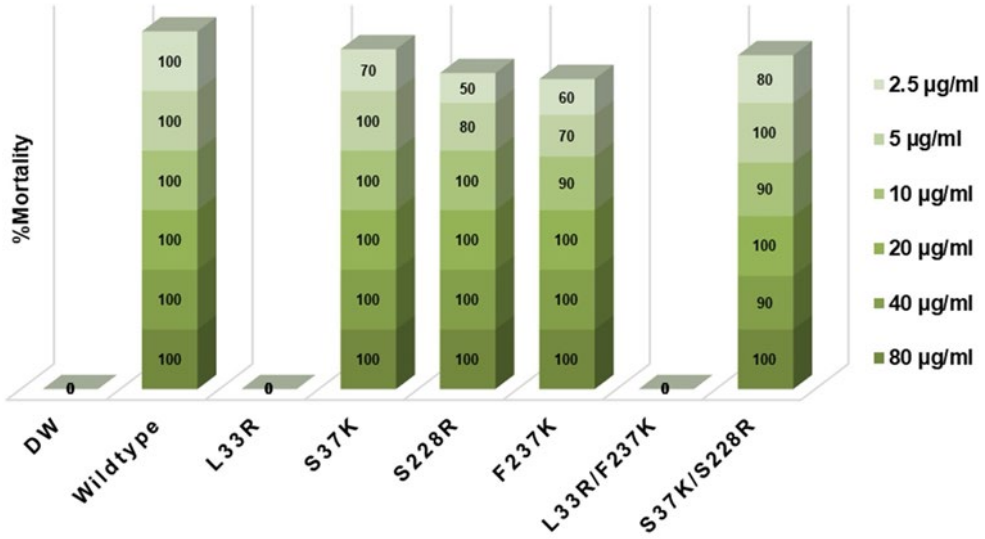


**Figure 10.** Mosquito larvicidal activity of Cyt2Aa2 mutants. The 2nd instar *Culex quinquefasciatus* larvae were treated with different protein concentrations of either Cyt2Aa2 wild type or mutants for 24 h. Then, the death larvae were recorded and calculated for the %mortality. Distilled water was used as the negative control.

## 4. Conclusions

The current study highlights the critical function of trypsin cleavage sites for *Bacillus thuringiensis* Cyt2Aa2 protein heterologous expression in *E. coli*. Protein synthesis and solubilization were reduced when trypsin cleavage sites were introduced into the Cyt2Aa2 protein in place of proteinase K cleavage sites. The significance of this study is the bacterial growth inhibition by L33R and L33R/F237K. It is possible that N-terminus of Cyt2Aa2 was removed by *E. coli* proteases, thereby enabling its toxicity against *E. coli* host cells. The extra Cyt2Aa2 trypsin cleavage site at the N-terminus proved to be more critical for bactericidal activity than the cleavage sites at the C-terminus. Although the mutants (except L33R and L33R/F237K) could not be solubilized in vitro, they showed the larvicidal activity similar to the wild type. In contrast, the host toxic mutants, L33R and L33R/F237K, lost their larvicidal activity.

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## 6. Authors' Contributions

Chontida Tangsongcharoen: coordinated research; analyzed data; drafted manuscript; approved final manuscript. Boonhiang Promdonkoy: conceptualized; designed experiments; materials; revised manuscript; approved final manuscript. Chartchai Krittanai: conceptualized; materials; revised manuscript; approved final manuscript. Sudarat Tharad: conceptualized; designed experiments; performed research; analyzed data; drafted manuscript; approved the final manuscript

**ORCID**
Chontida Tangsongcharoen https://orcid.org/0000-0002-3934-843X
Boonhiang Promdonkoy https://orcid.org/0000-0002-0584-5016
Chartchai Krittanai https://orcid.org/0000-0003-4697-3118
Sudarat Tharad https://orcid.org/0000-0003-2248-3034

## 7. Conflicts of Interest

All authors declare no conflicts of interest in this paper.

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