Role of cysteine at positions 67, 161 and 241 of a *Bacillus sphaericus* binary toxin BinB

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Binary toxin consisting of BinA and BinB from *Bacillus sphaericus* is toxic to mosquito larvae. BinB is responsible for specific binding to the larval gut cell membrane while BinA is crucial for toxicity. To investigate functional role of cysteine in BinB, three cysteine residues at positions 67, 161, and 241 were replaced by alanine or serine. Mutations at these positions did not affect protein production and overall structure of BinB. These cysteine residues are not involved in disulfide bond formation between BinB molecules. Mosquito-larvicidal assays revealed that C67 and C161 are essential for toxicity, whereas C241 is not. Mutations at C67 and C161 resulted in weaker BinA-BinB interaction. The loss of toxicity may be due to the reduction of interactions between BinA and BinB or BinB and its receptor. C67 and C161 could also play a part during conformational changes or internalization of the binary toxin into the target cell. [BMB reports 2010; 43(1): 23-28]

INTRODUCTION

Binary toxin is produced by some strains of *Bacillus sphaericus* during sporulation phase as parasporal crystal protein, which is specifically and highly toxic to Culex and Anopheles larvae but mildly or non-toxic to Aedes species (1). It consists of BinA (~42 kDa) and BinB (~51 kDa) proteins and both of them are required for full toxicity, although BinA alone at high concentration is toxic to mosquito larvae and cultured Culex cells (2-4). Recent report showed that BinA inclusion alone did not have larvicidal activity but its soluble form produced in *Escherichia coli* at low temperature exhibited high toxicity against *Culex* larvae (5). However, most of naturally produced BinA and BinB in *B. sphaericus* and those cloned in *E. coli* and *Bacillus thuringiensis* are existed as inclusions (1). Once ingested by susceptible larvae, the protein crystal is solubilized and proteolytically activated in the alkaline condition of the larvae gut (1, 6). BinA protein is responsible for toxicity via unknown mechanism, while BinB is a receptor binding component which binds to a single class of receptor on brush border membrane of the larvae (7, 8). The binary toxin receptor in *Culex pipiens* larvae is a 60 kDa protein identified as an α-glucosidase anchored on the membrane via a glycosyl-phosphatidylinositol (GPI) moiety (9, 10). Upon binding of BinB to a receptor, BinA binds to BinB or BinB-receptor complex, subsequently the binary toxin is internalized by endocytosis process (8).

Amino acid sequences of binary toxin show no homology to any other protein with known 3D structure thus it constitutes a distinct family of insecticidal toxins (4). Preliminary studies have been performed on crystallization of binary toxin, however its 3D structure has not yet been elucidated (11, 12). Therefore, clues on mode of action of binary toxins come from genetics, biochemistry and biophysics analyses. It is suggested that binary toxin produced by *B. sphaericus* exists as heterotetramer consisting of 2 molecules each of BinA and BinB (13).

Studies on BinB deletion derivatives suggested that the N-terminal region is important for receptor binding, whereas the C-terminal region is responsible for formation of BinA-BinB complex (8). In the same report, it was suggested that both N- and C-terminal regions of BinA are important for BinA-BinB interaction (8). Site-directed mutagenesis of amino acid positions 93, 99, and 104 of BinA revealed that these residues are crucial for toxicity and it was proposed that residues in this region might play a role in the formation of BinA-BinB complex (14, 15). Interestingly, mutagenesis of charged residues (R97, E98, R101, and E114) in the same region showed that these charged residues are also essential for biological activity of binary toxin, but not involved in BinA-BinB interaction (16).

These amino acids may play other roles in other function of binary toxin such as conformational change or internalization of toxin.

BinA and BinB share high homology with 25% identity and 40% similarity to each other. BinA contains three conserved cysteine residues at positions 31, 47, and 195 which are required for full toxicity of the binary toxin (17). BinB also contains three conserved cysteine residues in the active core at po-

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sitions 67, 161, and 241. In this work, directed-mutagenesis was conducted on these cysteine residues to investigate their effects on protein production, inclusion formation, solubilization, overall structure, and biologically activity of the toxin. Here, it was shown that C67 and C161 are crucial for toxicity of binary toxin, possibly involved in BinA-BinB interaction.

RESULTS AND DISCUSSION

Mutations at C67, C161, and C241 did not affect protein production, inclusion formation and the overall conformation of BinB

Previous studies revealed that BinB functions as a receptor binding component probably having the N-terminal region binds to the receptor and the C-terminal region interacts with BinA, thus redirecting BinA to the target cells (8-10). However, amino acids in BinB participating in these interactions have not been identified. It was reported that cysteine residues in BinA are required for full toxicity of binary toxin, although it is unclear which step these cysteines may be involved in (17). BinB also contains 3 cysteine residues in the active core at positions 67, 161, and 241. To investigate the role of these cysteine residues on function of binary toxin, C67, C161, and C241 were changed to alanine or serine. BinB wild type and mutants were constructed as truncated protein containing amino acids 33-408 and expressed in E. coli BL21(DE3)pLysS. This BinB derivative was previously shown to have molecular weight similar to processed BinB in the mosquito larvae gut that still retains maximum toxicity, thus considered as an active form of toxin (18). BinB wild type and variants (C67A, C67S, C161A, C161S, C241A, and C241S) were expressed as inclusion bodies at comparable levels, and SDS-PAGE analysis showed that the expressed proteins had molecular weight about 43 kDa as expected (Fig. 1). This suggests that the mutations of cysteine residues did not affect protein production and inclusion formation of the toxin. Subsequently, the inclusion bodies of BinB mutants were extracted from E. coli cells, solubilized in 25 mM NaOH, and extensively dialyzed against 50 mM Na2CO3 pH 9.2. Again, SDS-PAGE analysis showed that all mutants gave a band at 43 kDa indicating that the mutants were able to stably solubilize in alkaline condition as same as the wild type (Fig. 1).

Disulfide bonds formed between two cysteines are important for structural stability and function of many proteins (19, 20), such as Cry4Aa (21) and grass carp growth hormone (22). To investigate whether C67, C161, and C241 are involved in disulfide bond formations between BinB, solubilized proteins were analyzed on SDS-PAGE with and without reducing agent. The results demonstrated that majority of proteins from both wild type and mutants migrated at the same distance corresponding to a monomeric form of BinB regardless of whether DTT was present or not, while a faint band corresponding to BinB dimer was observed only in samples without reducing agent (Fig. 2). In addition, proteins in both conditions (plus and minus reducing agent) migrated to the same distance in SDS-PAGE and no band shift was observed. These results suggest that there is neither intra- nor intermolecular disulfide bond formation within or between BinB molecules. A faint band of BinB dimer observed in non-reducing condition was likely the result of random intermolecular disulfide bond formation. Previous studies on cysteine mutagenesis in BinA also showed similar results in which the cysteine residues are not involved in disulfide bond formation within the BinA molecules (17). It is proposed that in solution binary toxin exists as tetramer composing of 2 molecules of BinA and 2 molecules of BinB, but the oligomer is not held together by disulfide bonds (13). However, dimers of both BinA and BinB withstood SDS-PAGE sample preparation to some degree so that they could be observed as bands of molecular masses of 110 kDa and 125 kDa, respectively, but these dimer bands disappeared after trypsin digestion (13, 15). In agreement with those findings, our results indicate that the active form of BinB does not contain disulfide bond. It is most likely that the dimers observed in protoxin form derived from interaction between N- and C-terminal regions that are still present, but would be subsequently cleaved off by trypsin activation.

Additionally, an intrinsic fluorescence spectroscopy was conducted on these cysteine residues to investigate their effects on protein production, inclusion formation, solubilization, overall structure, and biologically activity of the toxin. Here, it was shown that C67 and C161 are crucial for toxicity of binary toxin, possibly involved in BinA-BinB interaction.

**Fig. 1.** Protein production and inclusion formation of BinB and its mutants in E. coli. E. coli cells harboring binB gene and its mutants were induced by 1 mM IPTG for 5 h. Cells were lysed using a French Pressure cell and ultrasonication and partially purified by repeated washing and centrifugation. Total cell lysate (T), soluble protein after centrifugation (S) and partially purified inclusion bodies (I) were analyzed by 12% SDS-PAGE. Inclusions obtained from all mutants were similar to the wild type. Arrow indicates position of BinB protein. Molecular weight markers (M) are shown alongside in kDa.
performed in all mutants in order to detect the conformational changes that might have occurred upon amino acid substitutions. Solubilized proteins in sodium carbonate buffer were further purified using gel filtration chromatography before performing intrinsic fluorescent spectrum analysis. Proteins of both wild type and mutants were eluted from the column as oligomeric form suggesting that BinB aggregates in solution when it is present alone without BinA. All mutants showed similar spectra as that of the wild type with the $\lambda_{\text{max}}$ at 338-340 nm after the excitation at 280 nm, albeit C67A and C161S displayed slightly decreased fluorescence intensity compared to the wild type that might be a result of variation in protein concentrations (Fig. 3). This result illustrates that there is no significant change of overall structure of the protein. Therefore, mutations at C67, C161, and C241 did not affect protein production, inclusion formation, solubilization, and overall conformation of the toxin.

C67 and C161 are required for toxicity of binary toxin

Biological activity of BinB mutants was determined by performing larvicidal assays against the second-instar C. quinquefasciatus larvae using 1 : 1 molar ratio of BinA and BinB from partially purified inclusions. It should be noted that the LC$_{50}$ against C. quinquefasciatus larvae produced by truncated BinB protein in this study is similar to that report of previous work (18), implying that this truncated form of BinB is effectively functional. The results showed that mutations at C67 and C161 (C67A, C67S, C161A, and C161S) rendered toxin inactive, while the mutants C241A and C241S showed comparable activity to that of the wild type (Table 1). Although cysteine and serine are very similar amino acids, replacement of cysteine at positions 67 and 161 in BinB by serine caused total loss of toxicity indicating that cysteine is absolutely required for toxicity of the binary toxin. However, C241 is not essential for binary toxin activity. Since mutations at these positions did not affect protein structure as described earlier, loss of toxicity of C67A, C67S, C161A, and C161S could involve other factors rather than structural change. C67 and C161 may be required for BinA-BinB interaction, or play an important role in conformational changes during membrane insertion or trans-

### Table 1. Mosquito-larvicidal activity of the BinB wild type and mutants against the second-instar Culex quinquefasciatus larvae

<table>
<thead>
<tr>
<th>BinA +</th>
<th>LC$_{50}$ (ng ml$^{-1}$)</th>
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<tbody>
<tr>
<td>BinB (wild type)</td>
<td>22.81 (1.20-45.81)</td>
</tr>
<tr>
<td>BinB-C67A</td>
<td>Inactive</td>
</tr>
<tr>
<td>BinB-C67S</td>
<td>Inactive</td>
</tr>
<tr>
<td>BinB-C161A</td>
<td>Inactive</td>
</tr>
<tr>
<td>BinB-C161S</td>
<td>Inactive</td>
</tr>
<tr>
<td>BinB-C241A</td>
<td>45.59 (22.90-72.12)</td>
</tr>
<tr>
<td>BinB-C241S</td>
<td>62.19 (31.52-99.71)</td>
</tr>
</tbody>
</table>

BinA and BinB inclusions were mixed at 1 : 1 molar ratio. Mortality was recorded after feeding for 48 hours. LC$_{50}$ was calculated using Probit analysis (30) from three independent experiments. The fiducial limit at 95% confidence is shown in parentheses. Samples with no mortality at high concentration (2,000 ng ml$^{-1}$) of toxin are regarded as inactive.
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The location into the cytosol. In the absence of its 3D structure, directed-mutagenesis is one of useful techniques for studying the mode of action of binary toxin. With this technique, earlier studies demonstrated that amino acids in both N-terminal and the C-terminal regions of BinB are important for biological activity of the toxin.Mutants containing successive substitutions with alanine residues (33YNL34 → AAA, 38SKK40 → AAA, 387YRL389 → AAA, and 391IQ393 → AA) totally lost toxicity towards \textit{C. quinquefasciatus} larvae (7, 23). Although the exact roles of these amino acids had not been elucidated, ability for two nontoxic derivatives, one with mutation at the N-terminus and the other with mutation at the C-terminus, to functionally complement each other suggests that binary toxin functions as oligomers (7, 23). This result is in agreement with the finding that in solution binding toxin exists as tetramer (13).

Effect of amino acid substitutions on BinA-BinB interaction
Our results showed that C67 and C161 are essential for binary toxin activity, but the role of these residues contributing to the toxin function is unclear. Interaction between BinA and BinB is crucial for activity of binary toxin and studies of binary toxin with lipid bilayers suggest that BinA and BinB may interact in solution prior to binding to the receptor (24). Significance of this interaction is emphasized in the work showing that amino acid at position 93 of BinA is important for biological activity of the toxin by playing a major role in the formation of BinA-BinB complex (15). Also, amino acids centered around position 100 in BinA which has been proved to be important for host ranges of binary toxin may play a part in BinA-BinB interaction (14). In order to explore the possible role of C67 and C161 of BinB in function of binary toxin, binding between BinA and BinB mutants were tested employing dot blot analysis. Various amounts of BinB ranging from 20 to 2.5 μg were immobilized on membrane and were overlaid with GST-BinA (20 μg m\textsuperscript{-1}). Subsequently, the complexes of GST-BinA-BinB were detected by GST antibody. The dot blot analysis of positive control, interaction between BinA and BinB wild type, showed good sensitivity with the highest BinB concentration displaying the strongest signal (Fig. 4). The results revealed that C67A, C161A, and C161S had signal intensity moderately lower than that of the wild type, while C67S showed considerably lower signal intensity compared to the wild type (Fig. 4). These results imply that mutations at C67 and C161 result in weaker interaction between BinA and BinB that may contribute to loss of toxicity of the toxin. Investigation using N- and C-terminal truncated toxins by Oei et al (8) suggesting that the N-terminal region of BinB is required for specific binding to the larval gut cell membrane and the C-terminal region is important for interacting to BinA. Although C67 and C161 are not in the C-terminus of BinB based on primary amino acid sequence, both residues may locate in a close proximity to the C-terminal part after protein folded into the functional 3D structure. Furthermore, interaction between BinA and BinB may involve several residues including those from C-terminus and other regions of BinB.

Nevertheless, it is uncertain that less interaction between BinA and BinB mutants observed here could solely account for the total loss of toxicity since BinB could be involved in other steps in the action of binary toxin. Conformational change of binary toxin occurs accompanying interaction between BinA and BinB in solution, and again following lipid bilayers association. BinB is responsible for receptor binding which is an initially crucial step for the toxin to take action, and also a basis for toxin resistance. Identifying receptor determinant in BinB would give an insight into binary toxin resistance development in mosquito larvae. However, amino acids essential for receptor binding have not been identified. Some evidences suggest that BinB is inserted into membrane and form pores (24-26), although the precise mechanism in which the toxin kills the target cells is unclear. Therefore, C67 and C161 could also play a part in conformational change, receptor binding, or membrane insertion.

MATERIALS AND METHODS
Bacterial strains, plasmids, and oligonucleotides
Both \textit{binA} and \textit{binB} genes used in this study were derived from \textit{B. sphaericus} 2297 binary toxin gene (Genbank accession no. AJ224478). \textit{E. coli} strain JM109 was used as a host for cloning and expression of GST-binA gene from pGEX-BinA (27). \textit{E. coli} BL21(DE3)pLysS (Novagen, USA) was used as a host for expression of \textit{binB} gene. The truncated \textit{binB} gene encoding amino acids N33-K408 was amplified by PCR from pET51N (28) using forward primer, 5'-CTT TTA CTT TCC GAA ACC TTC ACA GTA ACA AAC-3' and reverse primer, 5'-CTT CAA GAT GTT TTA CTG TAA ACC-3'. The PCR product was digested by 

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
BinB (μg per dot) & 20 & 10 & 5 & 2.5 & 0 \\
\hline
Wild type & & & & & \\
C67A & & & & & \\
C67S & & & & & \\
C161A & & & & & \\
C161S & & & & & \\
\hline
\end{tabular}
\caption{BinA-BinB interaction. Various amounts of purified proteins of BinB and its mutants were immobilized on nitrocellulose membrane and then overlaid with GST-BinA. BinA-BinB complexes were detected by rabbit anti-GST and goat anti-rabbit IgG conjugated with alkaline phosphatases.}
\end{table}
chased from Sigma Proligo (Singapore) and their sequences were as follows: C67Af, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C67Ar, 5′-GCTAGATGCTGGATCGCCTCTGACAGT3′; C161Af, 5′-GATGCTGGATCGCCTCTGACAGT3′; C161Ar, 5′-CCTGCAATGGCCTACTCATCTCTGG3′; C241Af, 5′-GCTAGATGCTGGATCGCCTCTGACAGT3′; C241Ar, 5′-GCTAGATGCTGGATCGCCTCTGACAGT3′; C67Sr, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C67Sf, 5′-GCTAGATGCTGGATCGCCTCTGACAGT3′; C161Sr, 5′-GATGCTGGATCGCCTCTGACAGT3′; C161Sf, 5′-GCTAGATGCTGGATCGCCTCTGACAGT3′; C241Sr, 5′-GATGCTGGATCGCCTCTGACAGT3′; C241Sf, 5′-GATGCTGGATCGCCTCTGACAGT3′; C67Ar, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C67SAr, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C161Ar, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C161SAr, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C241Ar, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C241SAr, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C67Sr, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C67Sf, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C161Sr, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C161Sf, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C241Sr, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′; C241Sf, 5′-TTCTAGATCGAAATGCGCATCTCAAATTTG3′. Restriction enzymes used for mutant screening were BamH (C67A), Paul (C161A), Nael (C241A), EcoRI (C67S), XhoI (C161S), and CiaI (C241S). Plasmid pET-IBinB was used as a template for site-directed mutagenesis using Stratagene’s QuikChange™ Site-Directed Mutagenesis kit according to the manufacturer’s manual. The mutants were selected by restriction endonuclease digestion and were verified by DNA sequencing.

**Protein preparation**

E. coli JM109 cells harboring pGEX-IBinA were grown in LB broth containing ampicillin 100 μg ml⁻¹, while E. coli BL21 (DE3)pLysS harboring pET-IBinB (wild type and mutants) were grown in LB broth containing ampicillin 100 μg ml⁻¹ and chloramphenicol 34 μg ml⁻¹ at 37°C until OD₆₀₀ reached 0.3-0.5. The cultures were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further incubated for 5 h. After centrifugation, harvested cells were lysed with French Pressure Cell and ultrasonication and protein inclusions were partially purified using differential centrifugation as described previously (29). Protein inclusions were solubilized in 25 mM NaOH at 25°C for 1 h. Solubilized protein was then dialyzed against 50 mM Na₂CO₃, pH 9.2 at 4°C overnight. The dialyzed protein was further purified by gel filtration using a Superdex 200, 10/300 column (GE Healthcare). Protein concentration was determined by Bradford’s method using bovine serum albumin (BSA) as a standard.

**Intrinsic fluorescence measurements**

Fluorescence spectra were obtained by emission scanning of a protein solution of purified BinB protein or mutants using a JASCO spectrophotofluorimeter at excitation wavelength at 280 nm. The emission wavelengths were observed from 300 to 500 nm. A scanning rate was set up at 100 nm min⁻¹ with excitation slit of 5.0 nm and emission slit of 2.5 nm. Each spectrum was averaged from three scans and all spectra were subtracted from baselines.

**Mosquito larvicidal assays**

Three independent experiments were carried out in duplicate using the second-instar *Culex quinquefasciatus* larvae supplied by the mosquito-rearing facility of the Institute of Molecular Biosciences, Mahidol University, Thailand. Mosquito larvicidal activity was tested by mixing GST-BinA and BinB inclusions at 1:1 molar ratio to the final concentration of 4 μg ml⁻¹ and diluted as a 2-fold serial dilution. One milliliter of diluted protein was added to 1 ml of water containing 10 larvae in each well of a 24-well tissue culture plate (1.5 cm well diameter). Mortality was recorded after incubation at room temperature for 48 h. LC₅₀ was determined using Probit analysis (30).

**BinA-BinB interaction assay**

Dot-blot analysis was performed to detect BinA and BinB interaction. The purified BinB and its mutants were spotted onto nitrocellulose membrane at 20, 10, 5.0, and 2.5 μg. The membrane was blocked with 5% skim milk in PBS buffer at 4°C for 3 h. Then, it was overlaid with 20 μg ml⁻¹ purified GST-BinA in blocking solution for 1 h and subsequently washed with 0.1% Tween-20 in PBS buffer three times, 5 min each. The bound GST-BinA was detected by incubating with rabbit anti-GST antibody at room temperature for 1 h and washed three times with PBS buffer containing 0.1% Tween-20. The membrane was then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate at room temperature for 1 h. The membrane was washed twice with PBS containing 0.1% Tween-20, 5 min each, and subsequently washed with PBS buffer for 5 min. Signals on the membrane were detected by using the reaction mixture consisting of NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate), pH 9.8.

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