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The role of interhelical cleavage for insecticidal activity of Bacillus thuringiensis Cry4A toxin

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The Cry4A toxin is a dipteran-specific insecticidal protein produced by *Bacillus thuringiensis* subsp. *israelensis* as a protoxin of 130 kDa. Its active form is a heterodimer of 20- and 45-kDa fragments which is generated by an interhelical cleavage of a 60-kDa intermediate at the position of Gln236 between α5 and α6 helices in domain I. On the other hand, Cry1Aa, which is also produced as a 130-kDa protoxin but toxic to lepidopteran larvae, was processed into the active 60-kDa fragment with no additional cleavage. To investigate the role of the intramolecular cleavage of Cry4A for its insecticidal activity, the loop between α5 and α6 of Cry4A which includes the cleavage site was substituted for the corresponding region of Cry1Aa. The resulting mutant designated GST-60Loop was expressed as a GST-fusion protein. A difference of the processing profile was observed between GST-60 and GST-60Loop in the *in vitro* digestion assay by trypsin, and the insecticidal activity of GST-60Loop was two-fold lower than that of GST-60. These results suggested that the interhelical cleavage of Cry4A promoted the toxicity against *C. pipiens* larvae.

1. INTRODUCTION

Bacillus thuringiensis is a gram-positive soil bacterium and, during sporulation, produces crystalline protein inclusions consisting of highly specific insecticidal proteins called δ-endotoxins which are toxic, upon ingestion, to lepidopteran, dipteran, and coleopteran insect larvae[1]. There are more recent reports of *B. thuringiensis* isolates active against other insect orders such as hymenoptera, homoptera, orthoptera, and mallophaga, and against nematodes, mites, and protozoa[2].

δ-Endotoxins or Cry proteins show homology in the primary sequence and probably have similar three-dimensional structures comprising three domains. Nevertheless, Cry proteins show a great variety of insecticidal specificity. The lepidopteran-specific δ-endotoxins biosynthesized as protoxins are solubilized and proteolytically activated by gut proteases in the susceptible insect larvae[3]. The activated toxin binds to a receptor in the apical microvilli of epithelial cells of the midgut[4,5,6]. It is believed that specificity is determined largely by the interaction of the toxin with the receptor. The conformational change in the toxin molecule bound to the receptor triggers the insertion of its channel-forming domain into the membrane[7,8]. Colloid-osmotic swelling and lysis of the cell results in the death of the larvae[9].

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The three-dimensional structures of two δ-endotoxins have been determined by X-ray crystallographic analyses, and the presence of three domains has been revealed[10,11]. Domain I consists of seven α helices and is involved in membrane partitioning and ion-channel regulation. Domain II consists of three β-sheets in a so-called Greek key conformation, and is proposed to be involved in the determination of insect specificity and in recognition of receptor molecules on midgut epithelial cells of the target insects. Domain III, which consists of two β-sheets in a jellyroll conformation, has also been assumed to be involved in determining specificity, in addition to ion-channel formation and receptor binding[12].

The mode of action of dipteran-specific δ-endotoxins is rather poorly elucidated. Previously we reported that the 130-kDa Cry4A, a dipteran-specific δ-endotoxin produced by *B. thuringiensis* subsp. *israelensis*, was processed into the 20- and 45-kDa fragments, and that the active form of Cry4A was a complex of the 20- and 45-kDa fragments[13]. The 20-kDa fragment of activated Cry4A includes α1~α5 helices of domain I, and the 45-kDa fragment contains α6 and α7 helices of domain I, in addition to domain II and III.

The 20- and 45-kDa fragments of Cry4A were generated by the intramolecular cleavage of the 60-kDa intermediate at the putative loop region between α5 and α6 helices. Schwartz *et al.* reported that channel formation requires domain I to swing away from domain II and III[14]. Therefore, the cleavage into the 20- and 45-kDa fragments of Cry4A may lead to a major conformational change of the toxin molecule and swinging away of a channel-forming domain moiety to be inserted. To investigate this hypothesis, GST-60Loop mutant of Cry4A was constructed, whose loop region between α5 and α6 helices was substituted by that of Cry1Aa which was not cleaved by gut proteases.

2. EXPERIMENTAL PROCEDURE

2.1 Construction of plasmids

PCR-fragment1 was amplified by PCR. The forward primer was 5'-GGGAGGAACAAATATGAATCC-3', and the reverse primer was 5'-AGCGCTGCGCTTCAAATTTGACGGC-3'. KODdash (TOYOBO) was used as a DNA polymerase enzyme in the reaction. The temperatures and times for annealing, extension, and denaturing were followed by manufacturer's instructions. PCR-fragment2 was also amplified by PCR, where the forward primer was 5'-AGCGCTGGGGATTCGATTATTTAGAGCCTTTGCC-3', and the reverse primer was CGAGTAAGTTCAGATTGGACACC-3'. The strategy of the construction is shown in Figure 1c. These two fragments were TA-cloned into pCR2.1 vector (Invitrogen). The plasmid pBS-frag1+2 was obtained by inserting the 0.71-Kb *Eco*RV-*Eco*RI fragment from pCR2.1-fragment1 into the *Eco*RV-*Eco*RI site of pBluescriptIISK$^+$, and by subcloning the 0.29-Kb *Eco*RI fragment from pCR2.1-fragment2 into the EcoRI site of the same vector. By *Aor*51HI digestion followed by self-ligation, the plasmid pBS-frag was constructed. Finally, the plasmid pGST4A60Loop was obtained by substituting the 0.3-Kb *Spe*I-*Bgl*II fragment of pBS-frag for the corresponding region of pGST4A60[13].

2.2 *In vitro* processing assay

The GST-Cry4A fusion proteins were expressed upon induction for 2 h with 0.1 mM IPTG in *E.coli* BL21 cells harboring a pertinent expression vector in 200 ml of TB medium containing 500

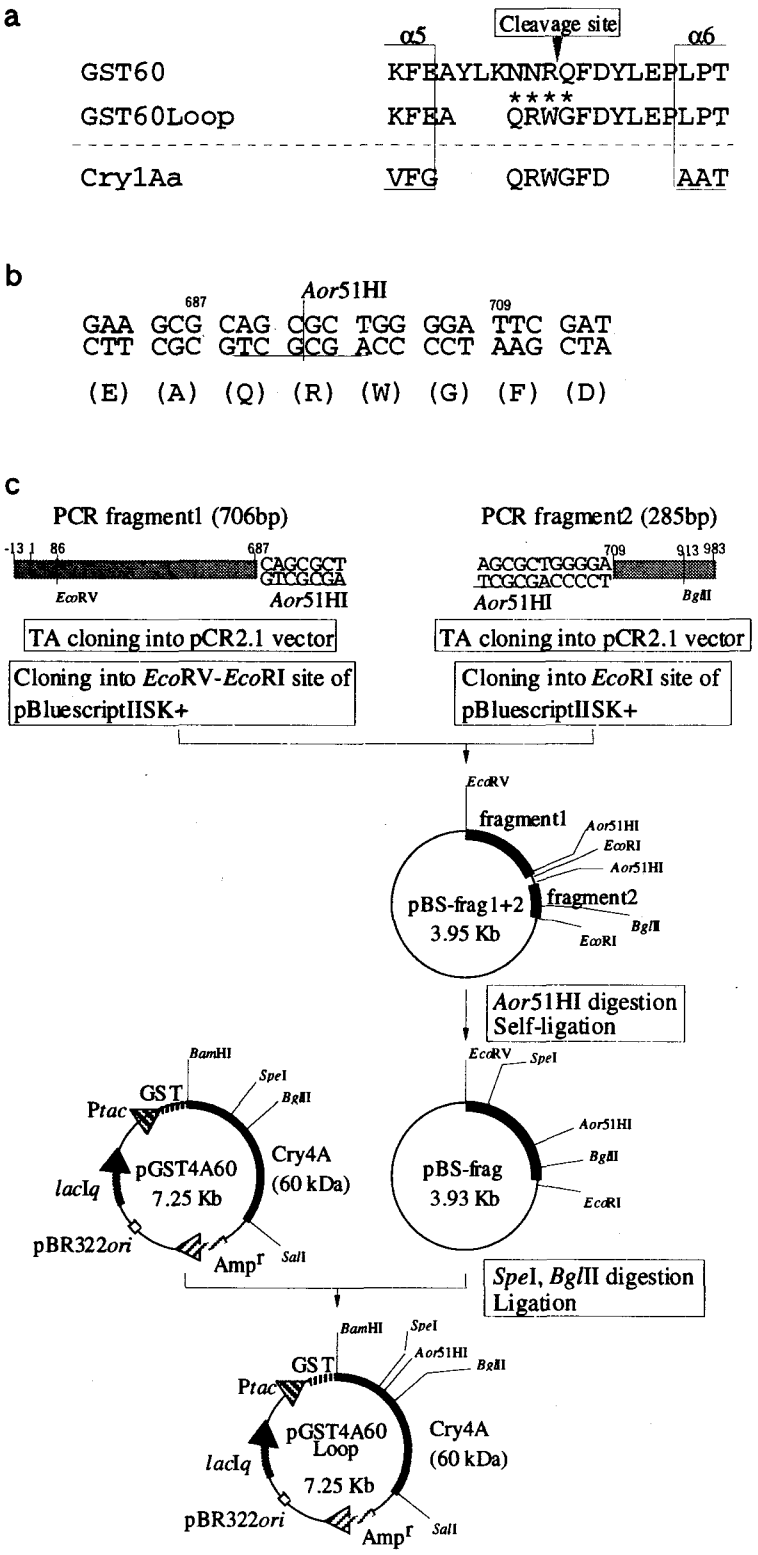

Figure 1 Construction of GST-60Loop.
a, the amino acid sequences of the loop region between the α5 and α6 helices; b, the designed nucleotide sequence corresponding to the loop region between the α5 and α6 helices of GST-60Loop; c, the strategy of the construction of pGST4A60Loop.

μg/ml of carbenicillin. The cells were collected by centrifugation, resuspended in 40 ml of PBS containing Complete (Boehringer Mannheim), the protease inhibitor cocktail, and disrupted by sonication. After centrifugation, the supernatant was obtained. The GST-Cry4A fusion proteins were purified from the supernatant by using Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and eluted with 100 μl of 0.2 M Tris-HCl (pH8.8) containing 30 mM reduced glutathione. Forty micrograms of the purified fusion proteins were digested by different concentrations of trypsin for 3 h at 37°C in a volume of 80 μl.

2.3 Bioassay of the mosquitocidal activities of GST-Cry4A fusion proteins

The GST-Cry4A fusion proteins were expressed in *E. coli* BL21 cells in 200 ml of TB medium. The cells were harvested, resuspended in 40 ml of 100 mM Na_2CO_3 (pH10.5)/ 20 mM β-mercaptoethanol containing 100 μg/ml p-APMSF, and disrupted by sonication followed by centrifugation to obtain the supernatant. To the supernatant was added 200 μl of 50% slurry of Glutathione-Sepharose 4B (Amersham Pharmacia Biotech). After rotating for 2 h at 4°C, the beads were washed three times with the above buffer. The GST-Cry4A fusion proteins were eluted with 100 μl of 0.2 M Tris-HCl (pH8.8) containing 20 mM reduced glutathione. The concentration of the purified protein was determined with the Bio-Rad Protein Assay using bovine serum albumin (Sigma) as a standard. The bioassay of GST-Cry4A fusion proteins was performed essentially by the methods of Schnell *et al*[15]. The proteins were added to 1 ml of 0.1 M Tris (pH7.5), 0.1% latex beads (Sigma) of 0.8 μm in diameter to give a final protein concentration of 10 μg/ml. After a brief vortex, the samples were rotated for 1 h at room temperature. The mosquitocidal activities were assayed on 4th instar larvae of *C. pipiens*. The mosquito larvae were grown in a container (35×25×3 cm) at 25°C. Before the assays, each larva was transferred to 200 μl of distilled water in each well of 96-well plates. After 8 h, the GST-Cry4A fusion proteins adsorbed to latex beads were added. In each experiment, 96 larvae were tested at a protein concentration of 0.5 μg/ml, and the assays were performed more than 3 times. The mortality was scored after 12-h incubation at 25°C. The efficiency of adsorption of protein to the latex beads was almost 100% in a preliminary experiment.

3. RESULTS AND DISCUSSION

Previously we have reported that dipteran-specific Cry4A has a unique activation process of the interhelical cleavage of the 60-kDa intermediate[13]. This cleavage generates the two fragments of 20- and 45-kDa that associate together to form the 60-kDa active complex. On the other hand, Lepidopteran-specific Cry1Aa is activated to the 60-kDa single polypeptides and no additional cleavage has been reported. Therefore, it was speculated that the cleavage of Cry4A molecule was essential for its dipteran-insecticidal activity. To examine this hypothesis, we constructed a mutant designated GST60Loop whose cleavage site was eliminated and the region around cleavage site was substituted for the corresponding region of Cry1Aa. The cleavage site of Cry4A is on the carboxyl side of R^{235} in the loop region between α5 and α6 helices which is AYLKNNRQFDYLEP in Cry4A, and QRWGFD in Cry1Aa (Fig. 1a). In the GST-60Loop mutant, the first four amino acids, AYLK, were deleted, and the next four amino acids, NNRQ, were replaced by QRWG of Cry1Aa. This mutant, therefore, had the Cry1Aa-like loop region, and was expected to acquire the resistance to the cleavage by trypsin. To obtain this mutant, we designed the partial nucleotide sequence including *Aor*51HI restriction site that encodes QRWG of Cry1Aa (Fig. 1b). The expression vector of GST-

60Loop was constructed by the strategy shown in Fig. 1c.

The mutant was expressed in *E. coli* as a GST-fusion protein because of easiness of purification. The insecticidal activities of purified proteins were estimated against *C. pipiens* larvae. As shown in Figure 2, The toxicity of GST-60Loop was lower than that of GST-60 about by half, suggesting that the cleavage was important for exhibiting the insecticidal activity.

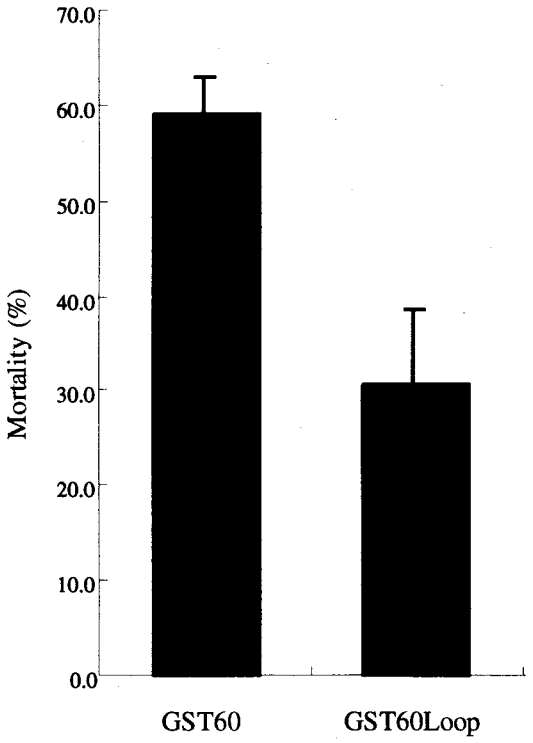

Figure 2 Toxicity of GST-Cry4A fusion proteins against *C. pipiens* larvae.

To investigate the digestion profile of GST-fusion proteins, the *in vitro* tryptic processing assay of the purified proteins was performed. The purified proteins were incubated with different concentrations of trypsin, and the digestion profiles were analyzed by SDS-polyacrylamide gel electrophoreisis followed by CBB staining (Fig. 3). GST-60 was processed into GST and 60-kDa fragment at a ratio of 500:1 (w/w, GST-60/trypsin), and the 45- and 20-kDa fragments appeared at a ratio of 100:1 (w/w, GST-60/trypsin). On the other hand, in the processing of GST-60Loop the processed 45- and 20-kDa fragments were firstly detected at a ratio of 100:1. At a ratio of 50:1, the 45-kDa band of GST-60 was more intense than that of GST-60Loop. Although there was a difference in the processing profile between GST-60 and GST-60Loop, we could not confirm that GST-60Loop acquired the resistance against the digestion by trypsin. The loop region between α5 and α6 helices of Cry1Aa includes arginine residue (QRWGFD), but this arginine residue may not be attacked by trypsin since there has been no report of the cleavage at this residue. However, GST-60Loop, which has the Cry1Aa-like loop region, is cleaved though less effectively than in the case of GST-60 (Fig. 3). This may be partially because the difference of environment around the α5 and α6 helices between Cry1Aa and Cry4A; the loop is buried in the molecule in Cry1Aa, but exposed on the surface of the molecule in Cry4A. Of course, it may be possible that the molecular structure of GST-60Loop was changed, and was attacked more easily by trypsin. To make a clear answer, further investigations are needed.

Figure 2 shows the insecticidal activities of GST-60 and GST-60Loop against *C. pipiens* larvae, where the toxicity of GST-60Loop was two-fold lower than that of GST-60. The data suggested that the interhelical cleavage could promote the toxicity against *C. pipiens* larvae, which was apparently inconsistent with the *in vitro* processing profile shown in Figure 3. Although we could not find the difference of processing pattern between GST-60 and GST-60Loop in the *in vitro* digestion by trypsin (Fig. 3), and by gut extract of *C. pipiens* larvae (data not shown), GST-60Loop might be more resistant than GST-60 to the attack by gut proteases in the midgut of the larvae.

The cleavage in domain I is also detected in Cry3A[16], Cry2A[17], Cry4B[18], and Cry9C[19]. But the results are contradictory. In the case of Cry3A, Carroll *et al.* reported that the interhelical

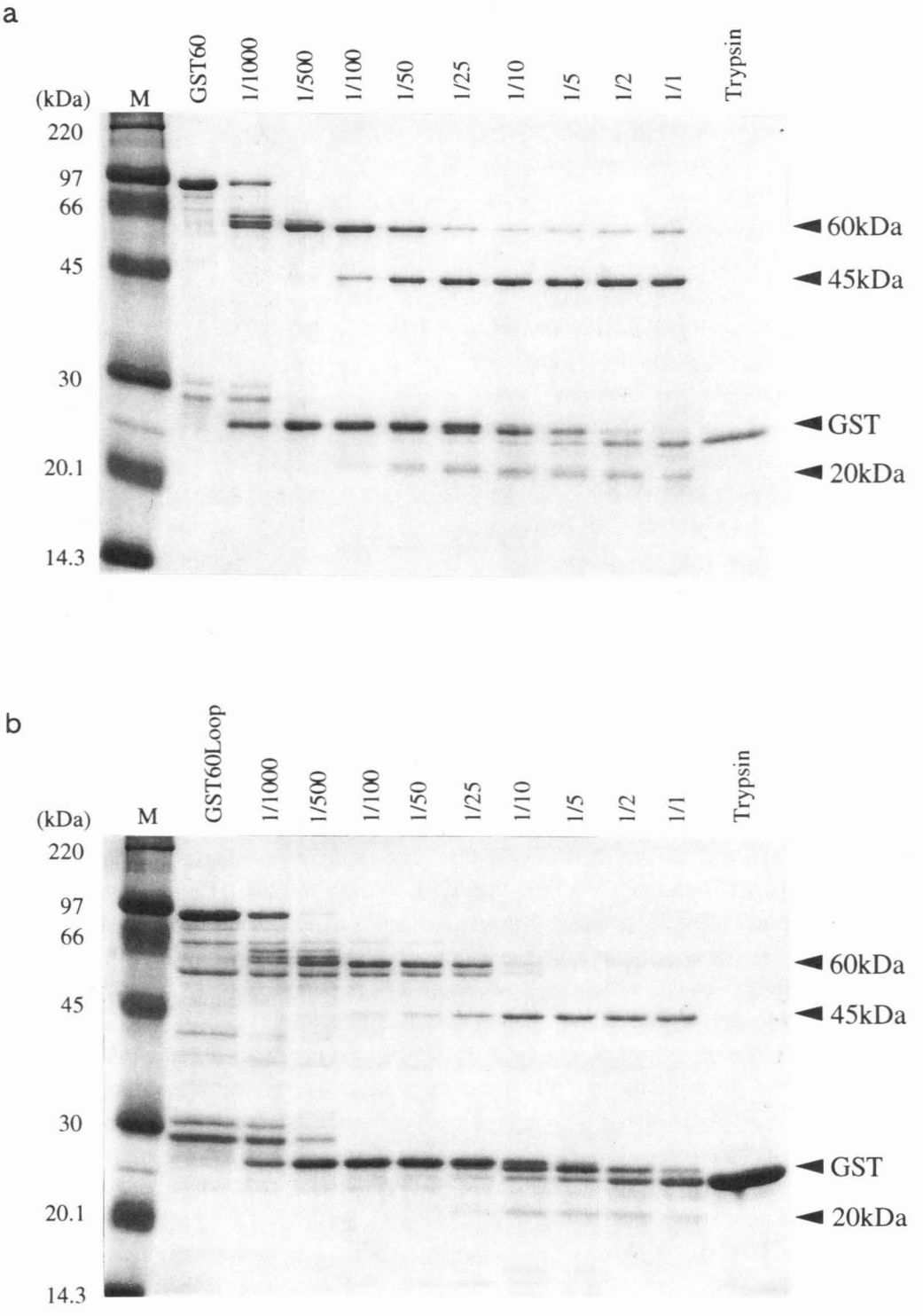

Figure 3 *In vitro* processing of GST-Cry4A fusion proteins by trypsin.
a, the processing pattern of GST-60; b, the proceesing pattern of GST-60Loop.

proteolytic cleavage in domain I might facilitate its coleopteran toxicity[20]. On the contrary, in Cry4B, the blockage of the interhelical proteolytic cleavage in domain I resulted in an increase in toxicity against *Aedes aegypti*[18]. In the case of Cry9C, Lambert *et al.* reported that 130-kDa protoxin of Cry9C was processed into 69-kDa fragment, and that the digestion to a 55-kDa fragment by the intrahelical cleavage resulted in the loss of toxicity[19]. Therefore, the biological significance of the intramolecular cleavage in domain I seems to be different among the target insects. Some unknown factors in the midgut environment of susceptible insects may affect the toxicity.

Thus in this paper we suggested the interhelical cleavage of domain I promoted the toxicity of Cry4A against *C. pipiens* larvae, but we could not obtain the conclusive evidence for the mechanism. Further investigations including the *in vivo* processing assay were needed.

4. SUMMARY

To investigate the role of the intramolecular cleavage of Cry4A for its insecticidal activity, the loop between α5 and α6 of Cry4A which includes the cleavage site was substituted for the corresponding region of Cry1Aa. The resulting mutant designated GST-60Loop was expressed as GST-fusion protein. A difference of the processing profile was observed between GST-60 and GST-60Loop in the *in vitro* digestion assay by trypsin and by gut extract from *C. pipiens* larvae, and the insecticidal activity of GST60Loop was two-fold lower than that of GST-60. These results suggested that the interhelical cleavage of Cry4A promoted the toxicity against *C. pipiens* larvae. But further investigations including the *in vivo* processing assay are needed.

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