Importance of the Central Region of 130-kDa Insecticidal Proteins of Bacillus thuringiensis var. israelensis for Their Activity in Vivo and in Vitro

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Received September 7, 1992

To delineate the mosquitoicidal regions of the ISRH3 (CryIVB) and ISRH4 (CryIVA) proteins, which are two of the mosquitoicidal 130-kDa proteins contained in the crystalline protein bodies (CPBs) of Bacillus thuringiensis var. israelensis (BTI), a deletion analysis of these protein genes has been done. Based on the evidence that each 130-kDa protein had two mosquitoicidal regions, N-terminal and C-terminal ones, and these two regions shared a common part in the center of the 130-kDa proteins, deleted genes on this region were constructed. As the protein products which lacked the central region had reduced activities, the central region could be important for the mosquitoicidal activity. The mosquitoicidal and non-mosquitoicidal truncated gene products of 130-kDa protein genes were also applied to a cultured lepidopteran cell line, TN-368. The mosquitoicidal proteins caused the swelling and disruption of the cells in spite of the insecticidal specificity of CPBs of BTI, but the non-mosquitoicidal proteins did not. Therefore, TN-368 cells were sensitive to the mosquitoicidal fragments of 130-kDa proteins of BTI under the assay conditions used.

Strains of Bacillus thuringiensis produce crystalline protein bodies (CPBs) during sporulation that are strongly toxic for lepidopterans, dipterans, or coleopterans. The CPBs produced by Bacillus thuringiensis var. israelensis (BTI) are highly toxic for the larvae of dipterans such as mosquitoes and black flies. The CPBs consist of several protein subunits, the molecular weights of which are 130-kDa, 70-kDa, 28-kDa, and so on. Among them, the 130-kDa and 70-kDa protein subunits were reported to have specific toxicity toward dipterans, the 28-kDa protein subunit being cytotoxic for both insect and mammalian cells, and also hemolytic.

Several investigatores have cloned and sequenced the genes for the 130-kDa, 70-kDa, and 28-kDa proteins. We previously reported the cloning the sequencing of the genes for the 130-kDa proteins, ISRH3 and ISRH4, which were systematically named as CryIVB and CryIVA, respectively by Höfte and Whiteley, and have demonstrated the mosquitoicidal activity of the gene products synthesized in Escherichia coli. We also delineated one of the mosquitoicidal regions of one of the 130-kDa proteins, ISRH4, by deletion analysis, which suggested that the central region of the 130-kDa protein might be important for the insecticidal activity. Recently, Ge et al. located the region responsible for the insecticidal specificity toward Bombyx mori in the center of the 130-kDa proteins of B. thuringiensis var. kurstaki (BTK). In the case of the 130-kDa proteins of BTI, the central region might contain the same type of determinant of selective insecticidal activity.

In this paper, we report the delineation of the mosquitoicidal regions of the two 130-kDa proteins (ISRH3 and ISRH4), and demonstrate that the 130-kDa proteins were inactivated by deletion of the amino acid residues in the central regions. On the other hand, we have found that cultured lepidopteran cells, TN-368, became swollen and disrupted on treatment with solubilized CPBs of BTI in spite of the insecticidal specificity of CPBs. So we examined whether or not the 130-kDa proteins of CPBs were involved in the cytotoxicity toward TN-368 cells.

Materials and Methods

Bacterial strains, plasmids, and media. Bacillus thuringiensis var. israelensis 4Q1, which was obtained from the Bacillus Genetic Stock Center, Ohio, U.S.A., was cultured in PY medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) or GNB medium (1% Bacto-tryptone, 0.5% beef extract (dry powder), 0.1% glucose, 0.2% NaCl, pH 7.0) at 30°C. E. coli JM109, plasmid vectors, pUC18 and pUC19, and recombinant plasmids, pUH3 and pUHC19, have already been described. The recombinant plasmids pLH4B3 and pLH4-B2 encoding truncated ISRH4 genes under the control of lac promoter were constructed in our previous study. E. coli strains were cultured in 2 × YT medium (1.8% Bacto tryptone, 1% yeast extract, 0.5% NaCl, pH 7.5) containing appropriate chemicals.

Construction of the recombinant plasmid pUAH3. The 3.5-kb EcoRI fragment of pUC13, which encodes the reading frame for ISRH3, was subcloned into the Acl site of pUC13 by filled-in ligation to provide pUAH3 (Fig. 1A), which encoded the ISRH3 gene transcribed under the control of the lac promoter from pUC13. By this filled-in ligation, the second codon of ISRH3 was connected to the downstream of N-terminal 10 amino acid residues of the fragment of lacZ in frame.

Abbreviations: BTI, Bacillus thuringiensis var. israelensis; BTK, Bacillus thuringiensis var. kurstaki; CPBs, crystalline protein bodies; SDS, sodium dodecyl sulfate.

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Received September 7, 1992
Deletion of the central region of 130-kDa protein genes. A recombinant plasmid, pUAH3G, was constructed, which carried the engineered ISR3H gene lacking the tract between the blunt ended AccI and XbaI sites in the coding region. A BamHI linker (5′-CGGATCCG-3′) was inserted in place of the deleted site. A recombinant plasmid, pH4G, was constructed, which carried the engineered ISR4H gene lacking the nucleotides between the HindII and bunt ended AccI sites in the coding region. In both cases their reading frames were not shifted by the internal deletion introduced in them. Plasmids pUAH3G and pH4G were digested with Clal and Sall, filled in and then ligated with BlpII linker DNA (5′-GCGGATCTCG-3′) to yield pUAH3Glac and pH4Glac, respectively, which carried the truncated 130-kDa protein genes fused with the downstream part of lacZ′.

Purification of CBPs and preparation of protein extracts. CBPs were purified from BTI 4Q1 cells by the method previously described.23 Protein extracts of E. coli cells were prepared by the method previously described.19

Western blot analysis. Protein extracts were electrophoresed on 12.5% SDS-polyacrylamide gel and then transferred to a nylon membrane (Nihon-pall). The membrane was treated with antibodies against CBPs of BTI and signals by a color reaction catalyzed by horseradish peroxidase. The details were given previously.20

Assay of mosquitoidal activity. Protein preparations were mixed with latex beads and then incubated at room temperature for an hour.20,21 The mortality of 3rd instar larvae of the mosquito Culex pipiens pallens was scored after 48 h. The concentration of the crude protein extracts was 50 μg/ml.

Culture method for TN-368 cells and assayng of cytotoxicity. The culture method for the cell line, TN-368, was previously described.16

To estimate the effects of the solubilized CBPs proteins and 130-kDa proteins of BTI on the cells, a cell suspension containing approximately 1.0 x 10⁶ cells/ml was subcultured in 96-well microtiter plates (Falcon) (200 μl/well), the plate being incubated at 28°C for 3 days. Then the cells were rinsed gently with PBS (without Ca²⁺ or Mg²⁺, adjusted to pH 6.0 with 1 N HCl), followed by the addition of the protein preparations in PBS. During further incubation of the cells at 28°C, the response of the cells was observed under a phase-contrast microscope and recorded photographically after 2 h.

Results

Construction of deletion mutant plasmids and expression of the genes coding for 130-kDa proteins

To produce the engineered ISR3H and ISR4H in E. coli cells, the inducible expression system of the lac promoter and cloning sites on the pUC plasmid vectors26 were used.

A recombinant plasmid, pUAH3, which encoded the N-terminal 10 amino acid residues of the α-fragment of β-galactosidase25 fused with the polypeptide of ISR3H, was constructed (Fig. 1A). Transcription of the fused ISR3H gene proceeded under the direction of the lac promoter in pUC13. Two deletion mutants of pUAH3 (pUAH3-C6 and C7 in Fig. 1B) were constructed. The N- and C-termini of these truncated ISR3Hs were fused with the upstream and downstream parts, respectively, of the α-fragment of β-galactosidase, (Fig. 1B). The plasmid pUCXI′ encoded a fusion-protein of the C-terminal 575 amino acid residues of ISR3H and the N-terminal 15 amino acid residues of the α-fragment of β-galactosidase from pUC13 as reported previously.19 The plasmid pH4BX encoded the N-terminal 13-amino acid residues in the pUC19-borne upstream part of the α-fragment of β-galactosidase fused with the polypeptide between the 30th and the 1180th amino acid residues of ISR4H, and pH4-B2, which was derived from pH4BX, encoded the N-terminal toxic fragment of ISR4H fused with the downstream part of lacZ′ (Fig. 1B) as reported previously.20

A truncated gene for the C-terminal half of ISR4H, pLH4C (Fig. 1B), spanning downstream from the central HindII site, was newly constructed. This plasmid showed no β-galactosidase activity, as pLH4C did not carry the downstream part of the α-fragment of β-galactosidase (Fig. 1B).

The plasmid pUAH3Glac was derived from pUAH3 and carried the truncated ISR3H gene lacking the sequences between the XbaI and AccI sites and downstream from the ClaI site (Fig. 1B). The plasmid pH4Glac was derived from pH4BX and carried the truncated ISR4H gene lacking the sequences between the central HindII and AccI sites and downstream from the ClaI site (Fig. 1B). Their truncated genes were connected to the downstream part of lacZ′ in frame. And in both cases, their reading frames were not shifted by the internal deletions introduced in them.

The production of engineered toxin proteins in E. coli JM109 cells harboring recombinant plasmids, pUAH3, pUCXI′, pH4BX and pH4C, was confirmed by Western blot analysis (Fig. 2). The signal strength of the main bands on the Western blot was measured densitometrically to estimate the expression ratios. For the product of pUAH3, several bands were detected which might have been caused by degradation of the product, and the strongest band at the position of 130-kDa was measured (lane 7 in Fig. 2). The ratio of the signal strength of pUAH3/pUCXI′ was 7/4 (lanes 7 and 8), and that of pH4BX/pLH4C was 5/3 (lanes 9 and 10). Based on these ratios and the expected molecular weight of the engineered toxin proteins, their molar ratio in the crude extracts was estimated to normalize the results of the bioassays.

The production of other engineered toxin proteins was confirmed by an alternative procedure, since they lacked the essential regions for immunoreactivity. The 615th to 678th amino acid residues of ISR3H and the 719th to 760th amino acid residues of ISR4H are necessary for immunoreactivity for the antibody raised against the total CBP of BTI.7,26 Therefore, these deletion mutants lacking these regions were not detected by the Western blot (data not shown). Because their truncated C-termini were fused with the downstream part of the α-fragment of β-galactosidase, the production of the truncated toxin proteins in the E. coli cells carrying pUAH3-C6, -C7, pH4-B2, pUAH3Glac, and pH4Glac was confirmed indirectly by assaying the activity of β-galactosidase (Fig. 1B). The β-galactosidase activity of these mutants was used for normalization of the bioassay results.

Though pUAH3, pUCXI′, pLH4BX, and pH4C had weak β-galactosidase activity (Fig. 1B), this was not used for the normalization. Because the engineered toxic protein genes were not fused with the downstream part of lacZ′,
the products did not have β-galactosidase activity. Therefore, their weak β-galactosidase activity was due to read-through in transcription and translation.

**Mosquitocidal activity of 130-kDa proteins**

The bioassay for mosquitocidal activity was done using a single dose (50 μg/ml of the crude extracts). Even at this concentration of the crude extract of *E. coli*, nearly 10% of larvae for negative controls (pUC13 and pUC19) were dead (data not shown) and only 30-50% were killed by the toxic extracts, therefore it was impossible to raise the concentration of the samples and to assay using a full range of doses. The results of assaying for mosquitocidal activity are summarized in Fig. 3. The mortalities were corrected...
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Fig. 2. Production of Deletion Mutant Proteins in E. coli Cells.

Protein extracts of E. coli cells harboring appropriate plasmids were analyzed by SDS-PAGE and Western blotting as described previously. An SDS-polyacrylamide gel electrophoresis of lanes 1–3) and the corresponding Western blot (lanes 6–10) are shown. Lanes 1 and 6 contained the crystal protein of BTI 4Q1 (10 μg). Each of lanes 2–5 and 7–10 contained about 50 μg of protein extract; E. coli JM109 carrying pUAH3 (lanes 2 and 7), pUCX1' (lanes 3 and 8), pLH40B (lanes 4 and 9), and pLH4C (lanes 5 and 10). Lane M contained the size-marker proteins, and a size-marker scale is given on both sides of the panel.

Fig. 3. Insecticidal Activity of the Engineered 130-kDa Proteins.

The names of plasmids are shown. Assay method of mosquitoicidal activity is in the Materials and Methods section. The mortality of the 3rd instar larvae of the mosquito, Culex pipiens pallens, was measured after 48 h. The final concentration of the crude protein extracts was 50 μg/ml. The products of pUC13 and pUC19 were used as negative controls for pUAH3 derivatives and pLH4B derivatives, respectively. The mortalities were corrected for the negative controls, and normalized. The mortality of pUCX1' product was normalized to pUAH3 product, and that of pLH4-B2 was normalized to pLH4BX product on basis of the molar ratios estimated from signal strength on the Western blot (Fig. 2) and their expected molecular weights. The mortalities of pUAH3-C7 and pUAH3Glac products were normalized to pUAH3-C6 product, and that of pLH4Glac product was normalized to pLH4-B2 on basis of the β-galactosidase activity. The data present the average percentages ± mean errors for eight independent assays.

for the control mortality, and normalized on basis of the estimated molar ratios or the β-galactosidase activity.

The recombinant plasmid, pUAH3, carried the engineered ISRH3, which lacked the first methionine residue and was fused with the N-terminal part of the β-galactosidase. The recombinant ISRH3 retained the mosquitoicidal activity. The truncated ISRH3 proteins lacking the C-terminal regions beginning from Val636 carried by pUAH3-C6 retained the insecticidal activity. Another truncated ISRH3 protein lacking the C-terminal region beginning from Ser603 (pUAH3-C7) did not have mosquitoicidal activity (Fig. 3). Thus, amino acid residues between positions 636 and 1135 could be removed without a significant loss of the mosquitoicidal activity. Also, the internal region of ISRH3 consisting of 33 amino acid residues, from Ser603 through Ser635, was essential for the mosquitoicidal activity. The active C-terminal half of ISRH3 (pUCX1') contained the internal region of 33 amino acid residues (Fig. 3 and Sen et al.19), suggesting that two mosquitoicidal regions exist in ISRH3. These two toxic regions overlapped in the central region of 76 amino acid residues (Fig. 1B), in which the essential region of 33 amino acid residues was contained.

The C-terminal half of ISRH4, pLH4C, which spanned Thr539 through Glu1180 (Fig. 1B), was also mosquitoicidal (Fig. 3). The N-terminal region between Pro30 and Ile695 of ISRH4 (pLH4-B2) was mosquitoicidal (Fig. 3). Therefore ISRH4 was also proved to have two mosquitoicidal regions that overlapped in the central region between residues 539 and 695 (Fig. 1B), in which the essential region of 100 amino acid residues from Gln596 to Ile695 was contained.

To confirm the importance of the central regions containing the essential region in the 130-kDa mosquitoicidal proteins, we constructed deletion mutants, pUAH3Glac and pLH4Glac, lacking both the internal 200–300 amino acid residues, which included the central region, and the C-terminal 164 amino acid residues (Fig. 1B). The products proved not to be mosquitoicidal (Fig. 3). The toxicity of pLH4-B2 and pLH4Glac products seemed to be rather higher than that of pLH4BX. This might be due to the fact that we did not normalize the results of pLH4BX and pLH4-B2 and that the result of pLH4Glac was normalized to pLH4-B2, because the pLH4BX product has no β-galactosidase activity while pLH4-B2 and pLH4Glac products were not immunoreactive. Therefore, the actual concentration of pLH4-B2 product might be higher than that of pLH4BX, and the results of pLH4-B2 and pLH4Glac might be overestimated.

Cytotoxicity of the 130-kDa proteins

We examined the cytotoxicity of the solubilized CPBs of BTI toward a cultured insect cell line, TH-368, which were derived from a lepidopteran. Contrary to the prediction based on the insecticidal spectra of the toxin, the cells were swollen and disrupted (Fig. 3, panel A).

To examine whether or not the 130-kDa proteins included in the solubilized CPB proteins were concerned with the cytotoxicity, the 130-kDa proteins and their deletion mutants were applied to TN-368 cells. The bioassay for cytotoxicity was also done using a single dose (3 mg/ml of the crude extract). Because the proteins in the crude extracts precipitated at the concentration over 3 mg/ml at pH 6.0, it was impossible to raise the protein concentration. Even below this high protein concentration, about 60% of the cells were disrupted, while nearly 20% were disrupted in the controls. Therefore assays using a full range of doses were impossible. The cells treated with the mosquitoicidal proteins were disrupted (panels D, E, F, G, I, and J in Fig.
4), but the cells treated with non-mosquitocidal proteins were not (panels H, K, and L in Fig. 4) as well as the negative controls (panels B and C).

To assay the responsiveness of cells to the engineered toxin proteins, the ratio of the cells that were disrupted by mosquitocidal or non-mosquitocidal protein was calculated (Fig. 5). The results were normalized like those of the assay for mosquitocidal activity. The engineered proteins retaining the mosquitocidal activity, such as shown in Fig. 3, were significantly toxic for the TN-368 cells, but the non-mosquitocidal proteins were not. The toxicities of pLH4-B2 and pLH4Glc were high. This was coincident with the in vivo results and might be due to the same reason.

**Discussion**

Expression of the 130-kDa protein genes in *E. coli* cells and mosquitocidal regions of the 130-kDa proteins of *BTI*

Even when the cloned genes encoding the 130-kDa proteins and their deletion mutants were placed under the control of the *lac* promoter, the amounts of produced proteins were not enough for the formation of inclusion bodies in *E. coli* cells. Because of the inefficiency in producing the engineered toxic proteins, it was impossible to do bioassays using purified engineered proteins. Therefore, the in vivo and in vitro bioassays using the crude extracts were done. Ward and Ellar had produced inclusion bodies of the CryIVB protein in *E. coli* cells and the CryIVA protein in *Bacillus subtilis* cells. In our experiments, the ISRH3 (CryIVB) protein was rather more efficiently produced in *E. coli* cells than the ISRH4 (CryIVA) protein, judging from the results of Western blot analysis. Adams
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proteins of BTI appear to be important role in the insecticidal activity as well as the central region of the 130-kDa protein of BTK. The crystal structure of an insecticidal protein has been analyzed and the conserved amino acid sequence blocks were compared among insecticidal proteins. The central regions of BTI 130-kDa proteins presented here correspond to domain III of Li's definition and to blocks 4 and 5 of Höfte's definition.

Cytotoxicity of the 130-kDa proteins of BTI toward lepidopteran cells

The solubilized CPBs of BTI were toxic toward TN-368 cells and the mosquitocidal fragments of the 130-kDa proteins were also toxic toward the cells (Figs. 4 and 5). Though the CPBs of BTI were highly toxic toward dipterans, but not toward lepidopterans, they were toxic toward lepidopteran cells. Moreover, these results showed that the 130-kDa proteins, which had been reported to be responsible for the selective toxicity toward dipterans, are also toxic toward the lepidopteran cells. But the in vitro toxicity of the constructs which produced almost the full length of the 130-kDa proteins was not so high as the in vivo toxicity and was supposed to be caused by the degraded proteins that were seen in the Western blot (Fig. 2), because the 130-kDa proteins (protoxins) were proteolytically activated to show the toxicity. These results imply that the TN-368 cells have receptors for the toxic fragments of 130-kDa mosquitocidal proteins of BTI.

The receptors for the toxic fragments of 130-kDa insecticidal proteins of B. thuringiensis are included in the cell membrane so as to be able to react with the toxic proteins. The receptors might determine the insecticidal specificity. Van Rie et al. reported that the resistance of a laboratory-selected Plodia interpunctella strain to CPBs of BTK was correlated with a reduction in the affinity of the membrane receptors to CPBs, and that the resistant strain was still sensitive to other CPBs of B. thuringiensis var. entomocidus HD110. Therefore, it is conceivable that if insect cells carry high-affinity receptors for a certain toxic protein, the toxic protein would be highly toxic toward the insects, and vice versa. According to this hypothesis, the affinity of receptors for the toxic fragments of 130-kDa proteins of BTI in the cell membrane of TN-368 cells is high enough to react with the toxic proteins under the in vitro assay conditions used.

But Woltersberger reported that the toxicity of CryIA did not correlate with its binding affinity, which was against this hypothesis, and there could be some other factors that determine the insecticidal specificity of toxic proteins of B. thuringiensis. The in vitro assay conditions, which we developed, must be different from the conditions in vivo. The CPBs of B. thuringiensis are solubilized and digested in the midgut of the host insects, and then attack the target cells in vivo. But the insecticidal proteins of BTI were already solubilized in E. coli cells and partially degraded by proteolysis under the in vitro assay conditions. If the insecticidal specificity were determined by the ability of the host insects to digest the CPB proteins, the undigested CPB proteins would not be toxic in vivo. It is also possible that the specificity of the receptors was highly restricted in vivo. Considering these possibilities, the cabbage looper, Trichoplusia ni, might be resistant to the
CPBs of \textit{BTI} because of an inability to digest them or of the highly restricted specificity of the receptors. Anyway, the cells derived from \textit{T. ni} were proved to respond to the toxic fragments of 130-kDa proteins of \textit{BTI}. This suggests that the \textit{in vitro} insecticidal specificity of \textit{B. thuringiensis} toxins is not as strict as predicted from the observed \textit{in vivo} phenomena.

Acknowledgments. The authors wish to thank Dr. Ohkawa of Sumitomo Chemical Co., Ltd., Japan, for providing us with eggs of \textit{Culex pipiens pallens}, and Mr. Y. Matsuhashi for his technical assistance. This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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