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THE MOSQUITO LARVICIDAL ACTIVITY OF 130 KDA DELTA-ENDOTOXIN OF BACILLUS THURINGIENSIS VAR. ISRAELENSIS RESIDES IN THE 72 KDA AMINO-TERMINAL FRAGMENT

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Bacillus thuringiensis var. israelensis produces 130 kDa delta-endotoxin which is highly toxic to mosquito-larvae. The mosquito-larvicidal activity was delineated by sequential deletions from ends of the 1136 amino acids delta-endotoxin. A maximum of 459 amino acids could be removed from the carboxy-terminal of the toxin without a significant loss of the larvicidal activity. However, no more than 38 amino acids could be deleted from the amino-terminal without losing the toxicity. The truncated peptide of 72 kDa exhibited similar toxicity to the 130 kDa toxin and was between 39^{th} and 677^{th} amino acids.

Bacillus thuringiensis var. israelensis (B.t.i) produces parasporal crystal proteins, delta-endotoxin, which specifically kills mosquito and blackfly larvae (1). The toxin consists of several protein components with those of 130 kDa, 65 kDa and 25-28 kDa predominating (2-4) ; each of which had been shown to exhibit mosquito-larvicidal activity (5-8). Genes encoding 28 kDa toxin (9,10), 72 kDa (11) and 130 kDa (12,13) had been identified. We recently determined the complete nucleotide sequence of the 130 kDa toxin gene which consisted of 3408 bp encoding 1136 amino acids delta-endotoxin (14). In this report, we attempted to identify the toxic portion of the 130 kDa toxin and found it locating in the amino-terminal peptide of 72 kDa.

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Abbreviations : kDa, kilodalton; bp, base pair; B.t.i., Bacillus thuringiensis var. israelensis ; IPTG, isopropyl β-D-thiogalactoside ; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

I. Bacterial Strains And Plasmids

Bacterial host cell, E. coli JM 107 (15) and plasmid vector, pUC12 (16), have been previously described. All enzymes were obtained from Bethesda Research Laboratories (BRL), Amersham and New England Biolab. ^{125}I-labelled protein-A and [^{32}P]dATP were purchased from Amersham.

II. Construction Of Truncated Plasmids

5'-End deletions

E1 clone: The plasmid pMU388 (Fig 1) which contained the 130 kDa gene (13) was completely digested with EcoRI and then recircularized at low concentration (<10 ng/ul) to eliminate two small EcoRI- fragments. The recircularized plasmid was introduced into host cells (JM 107) by DMSO method (17). The recombinant containing desired plasmid was selected by plasmid extraction and restriction enzyme analysis.

N-Bal-clones: The EcoRI-linearized pMU388 was serially digested by exonuclease Bal31 for a maximum of 30 min. Ends of the digested DNA were repaired by Klenow DNA polymerase. The DNA was mixed with SmaI-predigested pUC12 at 4:1 molar ratio. The DNA mixture was digested with HindIII and further ligated to obtain SmaI to HindIII insert orientation. The ligated products were introduced into E. coli, JM 107. The recombinants expressing part of delta-endotoxin protein were selected by colony immunoassay using antiserum against total B.t.i. crystal. Plasmid size was analysed by agarose gel and corresponding expression products by SDS-PAGE and Western blot analysis (13).

3'-End deletions

Cla2, AccB49 clones: The recombinant plasmid, pMU388, was digested by ClaI-HindIII, and AccI respectively, then repaired the ends. The digested plasmids were recircularized at low concentration (<10 ng/ul) to eliminate 0.6 kb ClaI-HindIII fragment and 1.2 kb AccI fragment, repectively. The recombinants containing desired plasmids (Cla2 and AccB49) were verified by restriction endonuclease analysis.

C-Bal-Clones : The plasmid, AccB-49, was linearized by HindIII, then followed by exonuclease Bal31 digestion (similar to the N-Bal-clones construction). After ethanol precipitation, the digested plasmids were mixed with XbaI-digested pUC12 and then the ends of such DNA mixture were filled. The DNAs were digested by SstI and subsequently ligated to obtain SstI to XbaI insert orientation. After transformation, the recombinants containing desired plasmids were verified by DNA size, restriction enzyme analysis, and the corresponding products by SDS-PAGE and Western blot analysis (13).

Both 5'-and 3'-End deletions.

PvuII fragment subclone (PvX1) : The 2.3 kb PvuII-fragment of delta-endotoxin gene from pMU388 was isolated from agarose gel and subsequently ligated to repaired ends XbaI-digested pUC12. After transformation, the recombinants expressing part of delta-endotoxin were verified by positive signal from colony immunoassay using antiserum against total B.t.i. crystal.

III. Mosquito-Larvicidal Assay

The assay followed the previously described procedure (13), except the media for bacterial growth included addition of 1mM IPTG after 6 h growth.

IV. DNA Sequencing And Peptide Sequences

The DNA sequence of each truncated gene was determined by the Dideoxy method following subcloning into M13 vector (16). The total DNA sequence of the 130 kDa gene has been described (14). The peptide sequences were deduced from DNA sequences of truncated genes.

V. Other Methods

Preparation of plasmids, analysis by restriction enzyme, ligation, exonuclease Bal 31 deletions, fill-in reaction, and DNA fragment isolation from gel used the methods described in Maniatis et al (18).

RESULTS AND DISCUSSION

130 kDa delta-endotoxin from B.t.i. has been shown to specifically kill mosquito larvae (4,7). A B.t.i. gene encoding the 130 kDa toxin, highly toxic to A. aegypti larvae, has been cloned in E.coli (13). Its complete nucleotide sequence reveals 3408 bp coding region which encodes a protein with a calculated molecular weight of 127, 863 Da (14). A restriction endonuclease map of the gene is drawn from the nucleotide sequence and shown in the Figure 1. Several deletions of 3'-end of the gene were carried out using ClaI, PvuII, AccI and XbaI. E. coli clones containing truncated plasmids were assayed for A. aegypti larvicidal activity and protein products as shown in the Figure 2. The ClaI (Cla2), PvuII (PvX1) and AccI (AccB49) deletions produced immunoreactive proteins smaller than 130 kDa. However, the smaller

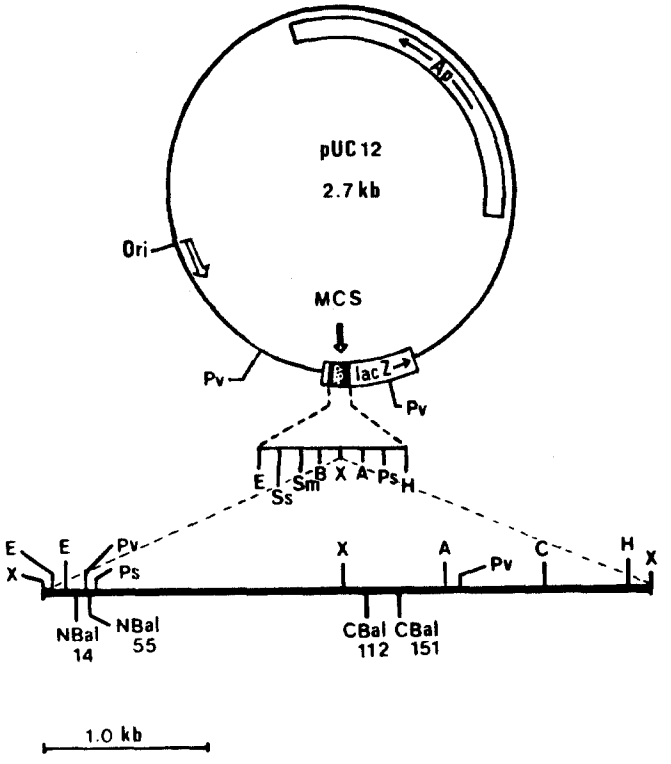

Figure 1 Restriction endonuclease map of pMU388 showing the map of the 130 kDa gene. The map was constructed from the complete nucleotide sequence of the gene (14). X,E,Pv,Ps,A,C Ss, Sm, B and H stands for the restriction enzyme XbaI, EcoRI, PvuII, PstI, AccI, ClaI, SstI, SmaI, BamHI and HindIII respectively. N-Bal 14 and N-Bal 55 represents positions in the gene of the 5'-end deletion clone which was toxic and non-toxic to mosquito larvae respectively. C-Bal 112 and C-Bal 151 represents the 3'-end deletion clone which was non-toxic and toxic respectively.

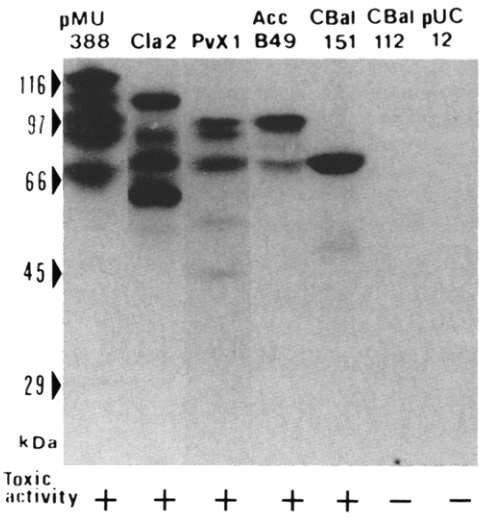

Figure 2 Autoradiographic pattern from Western blot analysis of protiens from the 3'-end deletion clones. Proteins were extracted from recombinant clones (pMU388, Cla2, PvXI, AccB49, C-Bal 151 and C-Bal 121) or pUC12 vector, separated on SDS-PAGE, transferred to nitrocellulose membrane and probed with antiserum to B.t.i. toxin followed by detection with ^{125}I-Protein-A. "+" and "-" represents mosquito larvae toxic activity and non-toxic activity of each clone respectively.

products still maintained the mosquito-larvicidal activity. The XbaI deletion did not yield any immunoreactive product nor larvicidal activity. It became evident that the region between AccI and XbaI (Figure 1) was essential for the toxicity. The AccI-truncated plasmid (AccB49) was further deleted by Bal31 exonuclease digestion which produced E. coli clones which were toxic as well as non-toxic to the A. aegypti larvae. Among the toxic E. coli clones C-Bal 151 contained the smallest truncated gene which produced a major protein of 72 kDa. Among the clones which were not toxic to the larvae, C-Bal 112 contained the largest truncated gene. Although it did not give immunoreactive product, a protein of 65 kDa was observed by ^{35}S-labelling of the minicell containing the C-Bal 112 plasmid (result not shown). Deduced amino acid sequence of the proteins from the nucleotide sequences of C-Bal 151 and C-Bal 112 (as shown in the Figure 3) revealed that the C-Bal 151 contained 677 amino acids from the N-terminal, whereas the C-Bal 112 contained 614 amino acids. The results suggested that the 63 amino acids fragment was essential for expressing both mosquito-larvicidal activity and immunoreactivity.

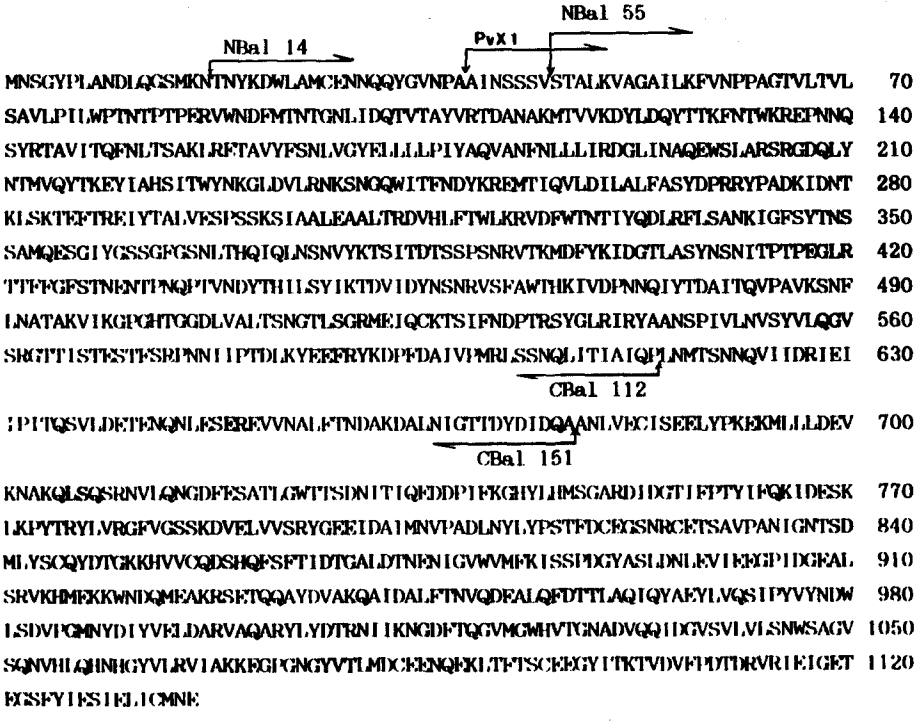

Figure 3 Amino acids sequence of the 130 kDa toxin and truncated proteins from the clones N-Bal 14, PvXI, N-Bal 55, C-Bal 112 and C-Bal 151. The sequences were deduced from the DNA sequences. Downward or upward arrow-points indicate positions of deletion. The toxic portion is between the PvX1 and the C-Bal 151.

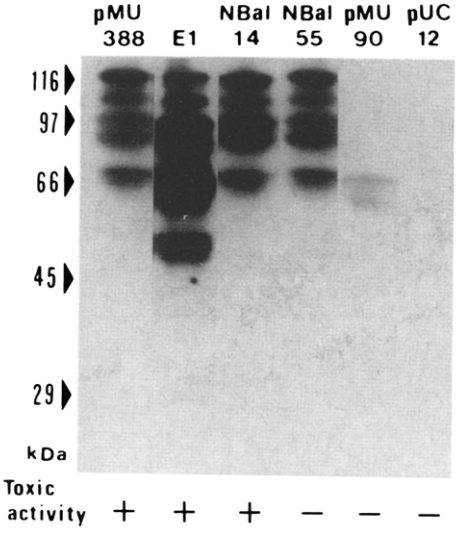

Figure 4 Autoradiographic pattern from Western blot analysis of proteins from the 5'-end deletion clones. Proteins were extracted from recombinant clones (pMU388, E1, N-Bal 14, N-Bal 55, pMU90) or pUC12 vector and analysed as in the Figure 2.

Deletions from the 5'-end of the gene were performed by EcoRI, PvuII and XbaI digestions of pMU388. The EcoRI deletion gave similar protein products which resembled those from the intact gene as shown in the Figure 4. The PvuII deletion (PvX1 in the Figure 2) which resulted in the deletion of both ends of the gene, produced smaller peptide than that of intact gene, however its larvicidal activity was maintained. Deletion from the 5'-end to the XbaI site gave protein of 65 kDa which was not toxic (Figure 4, pMU90). The result indicated a stringent requirement of N-terminal part of the 130 kDa protein for expressing the larvicidal activity. An exact amino acids sequence at the N-terminal part essential for the toxicity was determined by Bal31 deletions of the EcoRI-digested plasmid. E. coli clones containing the Bal31 truncated plasmids were selected for the smallest plasmid (N-Bal 14) which maintained the toxicity as well as the largest plasmid (N-Bal 55) which lost the toxicity (See Figure 4). The proteins produced by N-Bal 14 clone which was toxic to A. aegypti larvae and those by N-Bal 55, non-toxic, did not exhibit size difference in SDS-PAGE (Figure 4). The amino acids sequences of the proteins (Figure 3) which were deduced from the corresponding nucleotide sequences demonstrated that a maximum of 38 amino acids (from the N-terminal to PvX1) could be deleted from the N-terminal of the 1136 amino acids protein. In addition, the 7 amino acids fragment, Ile-Asn-Ser-Ser-Ser-Leu-Ser, was critical for toxicity expression. Our results demonstrated that the toxic portion of the 1136 amino acids mosquito-larvicidal protein lies between amino acids 39^{th} and 677^{th} (i.e. from PvX1 to C-Bal 151).

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