Israel Journal of Entomology Vol. XXXH (1998) pp. 185-198

ACTIVATION PROCESS OF THE INSECTICIDAL PROTEINS CrylVA AND CryTVB PRODUCED BY *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS*

TOHRU KOMANO,[1] MASASHI YAMAGIWA,' TOMOYUKINISHIMOTO,[1] HAJIME YOSHISUE,' KOUICHI TANABE,[1] KIKUO SEN[2] AND HIROSHI SAKAI[1] *[1] Laboratory of Biochemistry, 2 Department of Agricultural Chemistry, Kyoto University, Kyoto 606-01, Japan ^Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano 399-45, Japan*

ABSTRACT

We have changed the amino acid residues in block 5, one of the conserved sequences of CryIVA produced by *Bacillus thuringiensis* subsp. *israelensis,* and found that intracellular production of the altered CrylVA protein was markedly reduced. It is strongly suggested that the decrease was caused by the unstable conformation of the altered CrylVA protein as shown by digestion with trypsin and thermolysin. An *in vitro* processing experiment of CrylVA and CrylVB by the gut extract of the larvae of *Culex pipiens pallens* showed that the 130-kDa protoxin of CrylVA was converted to a 60-kDa intermediate, which was subsequently cleaved into the final and protease- resistant 45-kDa and 20-kDa fragments, while the 130-kDa protoxin of CrylVB was converted to protease-resistant70-kDa, 46-kDa, and 18-kDa fragments. The W-terminal amino acid of each fragment was identified; the 60-kDa and 20-kDa fragments of CrylVA had Gly58 at the /V-termini in common, and the //-terminus of the 45-kDa fragment of CrylVA was Gln236, while in CrylVB, the 18-kDa fragment had Gly34 on the Af-terminus, and the 46-kDa and 70-kDa fragments had Ser204 on the /V-termini in common. These results indicate that the processing pattern is different between CrylVA and CrylVB, and that the proteolytic processing has different biological significance for activating CrylVA and CrylVB.

KEY WORDS. *Bacillus thuringiensis* subsp. *israelensis, ciyJVA* and *crylVB* genes, 8-endotoxin, thermal stability, processing of CrylVA, gut protease.

INTRODUCTION

Bacillus thuringiensis subsp. *israelensis (Bti)* is a Gram-positive bacterium that produces 8-endotoxin, proteinous crystalline inclusions composed of CrylVA, CrylVB, CrylVD, and CytA during sporulation. CrylVA and CrylVB are highly specifically toxic to the larvae of dipteran insects (Aronson et al, 1986; Hofte and Whiteley, 1989).

It is believed that 8-endotoxin is solubilized in the alkaline conditions of the midgut and processed by gut proteases, producing the activated toxin. This activated toxin binds a receptor

---

2The Department has been renamed in 1997 and is now: Laboratory of Biochemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University.

on the brush border membrane to form a leakage channel, causing cell lysis and ultimately the death of the larvae (Hofmann et al., 1988a,b).

The three-dimensional structure of CryIIIA has been analyzed (Li et al., 1991), indicating that it comprises three domains. Domain I consists of a seven-helix bundle that penetrates the membranes to form pores, but a major conformational change of the CryIIIA molecule must occur for this seven-helix bundle to form these pores in the membrane.

Angsuthanasombat et al. (1992) showed that CryIVB is activated by gut proteases *in vitro* to produce 47- to 48-kDa and 16- to 18-kDa peptide fragments. In this activation, proteolytic cleavage occurs in the exposed loop joining helices 5 and 6 in the seven-helix bundle (Li et al., 1991; Angsuthanasombat et al., 1993). Similarly, CryIIA (Nicholls et al., 1989) and CryIIIA (Carroll et al., 1989) are cleaved in the exposed loop joining helices 3 and 4 (Li et al., 1991). These results indicate that the cleavages in the seven-helix bundle cause major conformational changes in the toxin molecules, and that the activated toxins become capable of forming the pores in the membranes. Domain II is considered to be a receptor-binding domain, and domain III consists of a β-sandwich that plays a critical role in retaining the protein structure. Chen et al. (1993) proposed that block 4, one of the five conserved regions, which is contained in domain III, affects the structural integrity of the protein molecule and the function of ion channels.

Block 5 is another highly conserved region in domain III and is considered to be at the *C*-terminus of the activated toxin (Adang et al., 1985; Schnepf and Whiteley, 1985; Höfte et al., 1986) precluding further proteolytic processing of the *C*-terminus (Li et al., 1991). The aim of this work is to discover the function of block 5 in CryIVA by the substitution of amino acid residues (Nishimoto et al., 1994), and the *in vitro* processing of CryIVA and CryIVB with mosquito gut extracts.

MATERIALS AND METHODS

Bacterial strains

A pBTI-6-cured acrystalliferous *B. thuringiensis* subsp. *israelensis* strain HD522 C37-21 (Spo^+ Cry^-) was used as a host for the production of inclusions (Himeno et al., 1985). *Escherichia coli* strain JM109 was used as an indicator bacteria for transfection and DH5α was used for the other procedures.

Construction of plasmids

A recombinant plasmid, pIS422, was obtained by inserting the 4.29-kb *Pma*CI-*Xba*I fragment containing the entire *cryIVA* gene from pBGH4 (Sen et al., 1988) into the *Sma*I-*Xba*I site of a shuttle vector, pHY300PLK (Takara Shuzo Co., Ltd.) (Ishiwa and Shibahara-Sone, 1986). We subcloned the 2.78-kb *Sna*BI-*Xba*I fragment from pIS422 into the *Sma*I-*Xba*I site of the phage vector M13mp19 to obtain the recombinant phage MIS278. Site-directed mutagenesis of *cryIVA* in MIS278 was done with the oligonucleotide-directed *in vitro* mutagenesis system version 21 (Amersham). After mutagenesis, the nucleotide sequences of the mutant genes were confirmed. A 2.75-kb *Bgl*II-*Xba*I fragment that contained an altered *cryIVA* gene was excised from the replicative form DNA of a mutagenesis derivative of MIS278, was substituted for the BglII-XbaI segment of *cryIVA* in pIS422, and the mutation sites were re-confirmed by sequencing. The pIS422 mutants were introduced into HD522 C37-21 by electroporation (Bone and Ellar, 1989).

Protein analysis

B. thuringiensis HD522 C37-21 harboring pIS422 mutants was cultured in 10 mL of Luria-Bertani medium containing tetracycline (20 μg/mL) until the end of exponential growth, and the cells were harvested and resuspended in the same volume of Schaeffer medium (Schaeffer et al., 1965) to induce sporulation. After the cells reached stage VI, the final period of spore development, whole cells were disrupted by sonication and analyzed by SDS-7% PAGE/Coomassie brilliant blue staining. The sporulation process was microscopically monitored.

Preparation of inclusions

B. thuringiensis HD522 C37-21 harboring pIS422 or pIS422 mutants was cultured in Schaeffer medium containing tetracycline (20 μg/mL) until cells were lysed completely, and the resultant CryIVA inclusions contaminated by spores were purified on discontinuous sucrose gradients as described previously (Thomas and Ellar, 1983).

Measurement of protein concentration

To measure the concentration of wild type CryIVA inclusions, the purified inclusions were solubilized in 50 mM Na_2CO_3 (pH 10.5)–10 mM DTT for 1 h at 37°C (Angsuthanasombat et al., 1992) and assayed for the protein concentration with a Bio-Rad Protein Assay using bovine serum albumin (Bio-Rad) as a standard.

Mutant CryIVA inclusions were analyzed by SDS-7% PAGE/Coomassie brilliant blue staining, and the 130-kDa band intensities were measured with a densitometer (Pharmacia LKB). Then the concentration of mutant CryIVA inclusions was calculated by comparing the 130-kDa peak area of mutant CryIVA inclusions with that of the wild type inclusion.

Protease digestion

To detect alterations in the conformation of the CryIVA protein molecule, changes in the resistance to proteolytic cleavage were analyzed according to a modification of the described procedure (Almond and Dean, 1993). For the test with trypsin digestion, the inclusions were solubilized in 50 mM Na_2CO_3 (pH 10.5)–10 mM DTT for 1 h at 37°C, and treated with a high concentration of trypsin (1:2, w/w, trypsin/protoxin) for 3 h at 37°C. For the test with thermolysin digestion, the inclusions were solubilized in 50 mM CAPS (pH 10.5)–10 mM DTT for 1 h at 37°C. After adding $CaCl_2$ to give a final concentration of 10 mM, the CryIVA proteins were incubated with thermolysin (1 : 50, w/w, thermolysin/protoxin) at various temperatures. After 30 min, the digestion was stopped by adding EDTA to give a final concentration of 20 mM. Samples were analyzed by SDS-12% PAGE/Coomassie brilliant blue staining, and the 130-kDa band intensities were measured with a densitometer as above. The *Ts* value is the temperature at which 50% of the intact toxin remains.

Thermolysin digestion was also done to measure the structural alteration in the 45-kDa protein molecules produced by the treatment with gut extracts of *Culex pipiens pallens* (1 unit : 10 mg toxin) for 6 h at 37°C. The gut extracts were prepared as described previously (Angsuthanasombat et al., 1991). Proteolytic activities of the gut extracts were measured as described previously (Harwood et al., 1990; Angsuthanasombat et al., 1992).

Assays of the mosquitocidal activities of inclusion body

The mosquitocidal activities of the toxins were assayed on the 2nd to 3rd instar larvae of *Cx. pipiens pallens*. The mosquito larvae were grown in a container (35 × 25 × 3 cm) at 23°C. Before the assays, the larvae were transferred in groups of twenty to 25 mL of distilled water in a cup (3 cm in diameter). After 12 h, the inclusion preparations were added. The mortality was scored after 24 h. Three cups of the larvae, sixty larvae in total, were tested at each toxin concentration.

***In vitro* processing of CryIVA and CryIVB**

The inclusions were solubilized in 50 mM Na_2CO_3 (pH 10.5)–10 mM DTT for 1 h at 37°C, and treated with gut extract of *Cx. pipiens pallens* for various times at 30°C. Samples were analyzed by SDS-14% PAGE with Coomassie brilliant blue staining.

Assays of the mosquitocidal activities of processed fragments

In vitro processed toxin solution was adsorbed to latex beads (Sigma). Before the assays, ten larvae were transferred to 10 mL of distilled water in each well of a six-well plate. After 12 h, the processed toxin adsorbed to latex beads was added. The mortality was scored after 24 h. Fifty larvae were tested at the toxin concentration of 0.1 μg/mL. In a preliminary experiment, the efficiency of adsorption of toxin to the latex beads was examined and it was confirmed that the adsorption was complete.

N-terminal amino acid sequencing

The processed toxin was fractionated in SDS-14% PAGE and transferred to PVDF membrane (Bio-Rad). The *N*-terminal amino acid of each fragment band were sequenced using an Applied Biosystems model 476 pulsed-liquid sequencer.

RESULTS

Production of mutant CryIVA proteins in *Bti*

To investigate the functional contribution of block 5, each of the ten amino acid residues (Fig. 1A) in block 5 of CryIVA was replaced by alanine (Fig. 1B), the resultant genes were expressed in HD522 C37-21 (Spo^+ Cry^-), and the obtained crude inclusions were analyzed by SDS-PAGE (Fig. 1C). The mutant CryIVA–Asp670Ala protein was poorly produced and the mutant CryIVA–Glu673Ala protein was only slightly produced. The amount of production of the other eight mutant inclusions was similar to that of the wild type (Fig. 1C). When Asp670 was replaced by additional amino acids, the production of the resultant mutant CryIVA proteins was reduced, especially that of the four mutants CryIVA–Asp670Tyr, –Asp670Leu, –Asp670Pro and –Asp670Arg was reduced markedly (Nishimoto et al., 1994). Additional amino acid residues with charged side chains in block 5, Lys671 and Glu673, were replaced by glutamic acid and lysine residues, respectively, markedly decreasing the production of the CryIVA protein (data not shown). Judging from the result of agarose gel electrophoretic analysis, the plasmid DNAs of pIS422 mutant derivatives were stably maintained in HD522 C37-21 cells, and did not undergo major structural changes (data not shown).

(A)

```
5'-ATGTATATACAAACACAACAGTACTTATTGATAAAATTGAATTTCTGCCAATTACTCGTTCTATAAGA-3'
                        V  L  I  D  K  I  E  F  L  P
```

(B)

```
V667A  CAAACACAACAGCACTTATTGATA
L668A  ACACAACAGTAGCTATTGATAAAA
I669A  CAACAGTACTTGCTGATAAAATTG
D670A  CAGTACTTATTGCTAAAATTGAAT
K671A  ACTTATTGATGCTATTGAATTTCT
I672A  TTATTGATAAAGCTGAATTTCTGC
E673A  TTGATAAAATTGCGTTTCTGCCAA
F674A  ATAAAATTGAAGCTCTGCCAATTA
L675A  AAATTGAATTTGCGCCAATTACTC
P676A  TTGAATTTCTGGCAATTACTCGTT
```

(C)

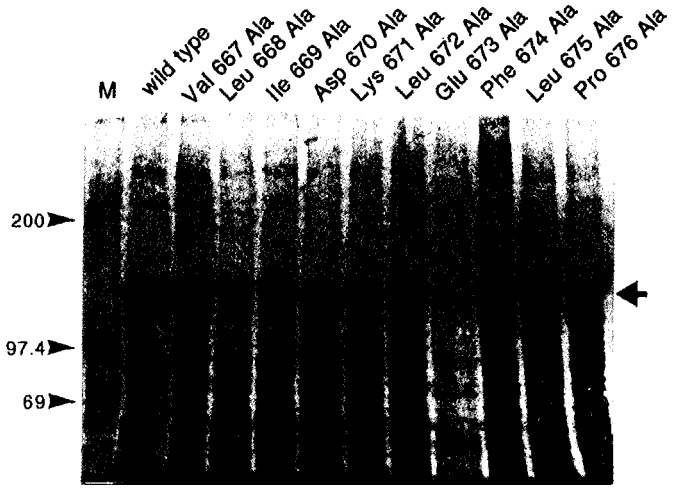

Fig. 1. (A) The nucleotide sequence of CryIVA and deduced amino acid sequence of block 5 depicted in a box. (B) Oligonucleotide primers used for the amino acid replacements in block 5 of CryIVA. The changed nucleotides are indicated by (*). (C) Polyacrylamide gel electrophoretic analyses of the production of the mutant CryIVA proteins in *B. thuringiensis* subsp. *israelensis*. Protein extracts from *B. thuringiensis* subsp. *israelensis* cells harboring the mutant derivatives of pIS422 were analyzed by SDS-7% PAGE/Coomassie brilliant blue staining. The arrow indicates the 130-kDa proteins. The amino acid replacements in each of the mutant CryIVA proteins is indicated. Lane M represents the molecular mass standards.

Conformational stability of the mutant CryIVA protein structure

To examine whether the mutant CryIVA proteins carried conformational alterations that made the protein molecules unstable, we tested them with protease digestion methods (Almond and Dean, 1993). Thus, the five mutant CryIVA proteins that carried the amino acid replacements at Asp670 were treated with high concentrations of trypsin (data not shown). Proteins of the three mutants CryIVA–Asp670Tyr, -Asp670Leu, and -Asp670Pro, the production of which was significantly decreased (Fig. 2A), were slightly more susceptible to proteolytic digestion

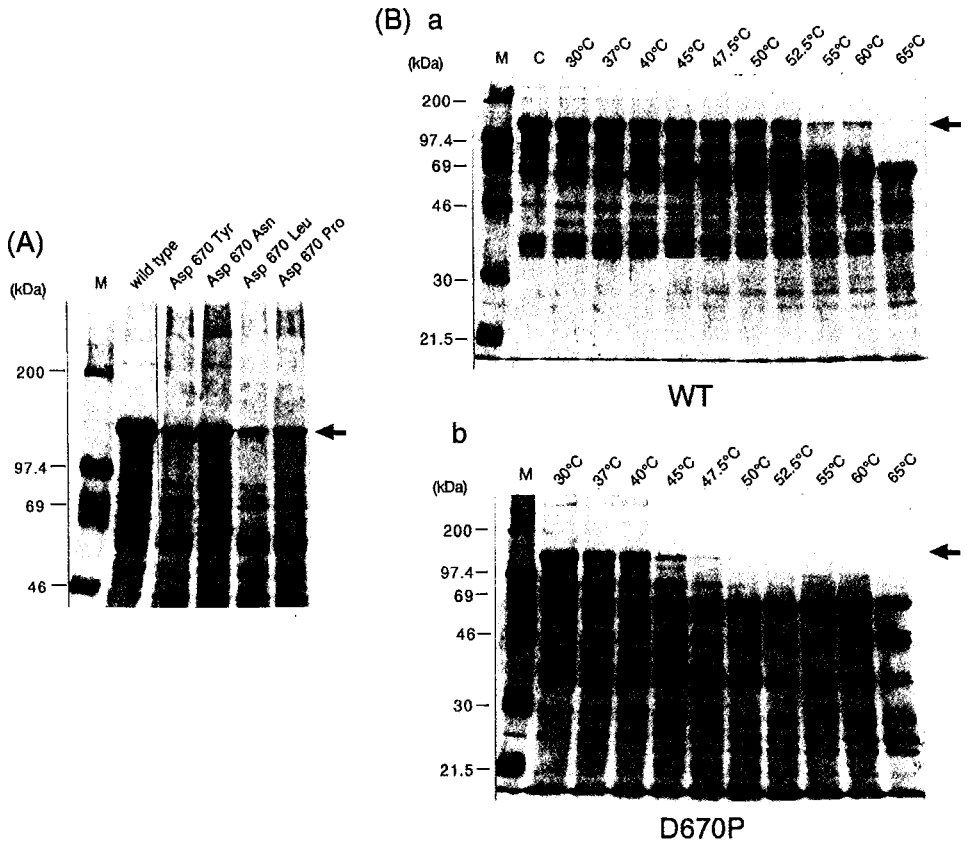

Fig. 2. (A) Polyacrylamide gel electrophoretic analyses of the production of Asp670 mutant CryIVA proteins in *B. thuringiensis* subsp. *israelensis*. Protein extracts from *B. thuringiensis* subsp. *israelensis* cells harboring the mutant derivatives of pIS422 were analyzed by SDS-7% PAGE/Coomassie brilliant blue staining. The arrow indicates the 130-kDa proteins. Lane M represents the molecular mass standards. (B) Comparison of thermal stability of the 130-kDa protein molecules of the wild type CryIVA and the mutant CryIVA–Asp670Tyr. The purified crystals were solubilized and proteolytically digested with thermolysin at various temperatures by the methods described in Materials and Methods. The resultant digested proteins were analyzed by SDS-12% PAGE/Coomassie brilliant blue staining. Panel a: the wild-type CryIVA. Panel b: the mutant CryIVA–Asp670Pro. The reaction temperatures are shown above the electrophoregrams. The arrow indicates the 130-kDa proteins. Lanes M and C represent the molecular mass standards and the CryIVA protein as controls, respectively.

than the proteins of the wild-type CryIVA and the mutants CryIVA–Asp670Asn and –Asp670His. Three of the five mutant CryIVA proteins that had been tested above were further digested with thermolysin at various temperatures. As shown in Fig. 2B, the critical temperature for the efficient proteolytic degradation of the mutant CryIVA–Asp670Pro protein was lower than that of the wild-type protein. That is, the *T*s value of the mutant CryIVA–Asp670Pro protein was about 42°C, while that of the wild-type was about 51°C. This indicates that the mutant CryIVA–Asp670Pro protein is more susceptible to thermolysin digestion. Similarly, the proteins of the mutants CryIVA–Asp670Leu and -Asp670Tyr (Fig. 3) were markedly susceptible to proteolytic digestion with thermolysin (*T*s values about 47°C and 42°C, respectively).

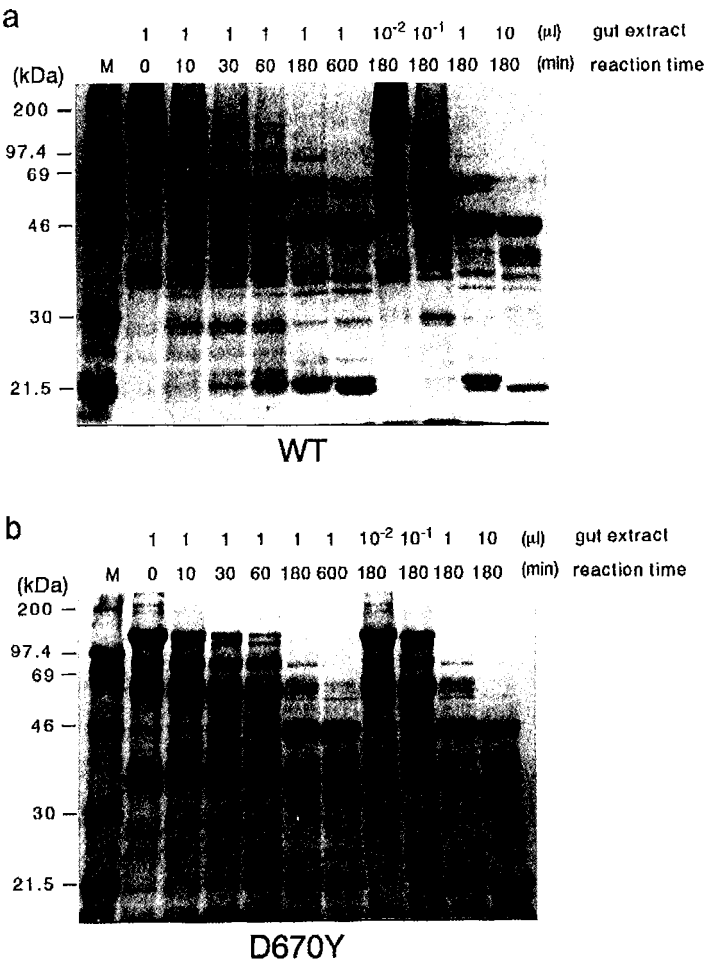

Fig. 3. Processing pattern of the wild-type CryIVA and the mutant CryIVA–Asp670Tyr by the gut extract of *Cx. pipiens pallens*. The solubilized crystals were treated with gut extracts at 37°C. The resultant digested proteins were analyzed by SDS-12% PAGE/Coomassie brilliant blue staining. Panel a: the wild type CryIVA. Panel b: the mutant CryIVA–Asp670Tyr. The reaction time and the volume of gut extract are shown above the electrophoregrams. Lanes M represent the molecular mass standards.

To investigate whether the *in vitro* processing pattern of the mutant CryIVA–Asp670Tyr protein changed the activation process, the mutant CryIVA–Asp670Tyr and the wild-type CryIVA proteins were treated for activation by gut extracts of *Cx. pipiens pallens*. They were processed similarly; 130-kDa proteins of the mutant and the wild-type CryIVA were processed to 45- and 20-kDa fragments *via* a 60-kDa fragment (Fig. 4). The resultant 45-kDa proteins were tested for susceptibility to proteolytic digestion with thermolysin. The 45-kDa toxin proteins of the wild-type and the mutant were very heat-stable, and had the same profiles of susceptibility to proteolytic degradation with thermolysin (Nishimoto et al., 1994). That is, these two 45-kDa toxin proteins had the same *T*s values, about 68°C, which is higher by about 17°C than the *T*s value of the wild-type 130-kDa protein of CryIVA (Nishimoto et al., 1994).

Assay of the mosquito larvicidal activity

To examine the effects of these amino acid substitutions in block 5 on the toxicity of the corresponding CryIVA mutants, their mosquito larvicidal activities were assayed. The LC50 values of the mutants and the wild-type inclusions were about the same. As far as the mutations examined in this work are concerned, they have no direct effect on the insecticidal activity of CryIVA. The production of the inclusions of the three mutants CryIVA–Asp670Arg, –Lys671Glu, and –Glu673Lys, was too poor to prepare a large enough amount protein for the assays (Nishimoto et al., 1994).

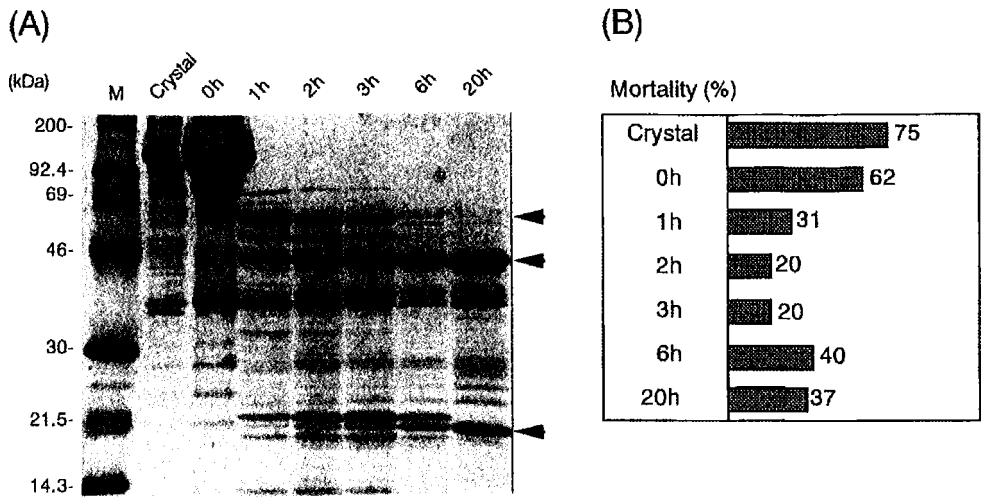

Fig. 4. (A) Processing pattern and insect toxicity of CryIVA by the gut extract of *Cx. pipiens pallens*. The solubilized crystals were treated with gut extracts at 30°C. The processing was stopped by adding p-APMSF at each time (0, 1, 2, 3, 6, and 20 hours after adding gut extract). The resultant processed fragments at each time were analyzed by SDS-14% PAGE/Coomassie brilliant blue staining. (B) Mortality of the insect with processed fragments of CryIVA at each time. Bioassays were performed as described in Materials and Methods at 0.1 μg/mL of total toxin concentration.

In vitro processing pattern of CryIVA and CryIVB

As shown in Fig. 3, we found that CryIVA was, unlike CryI-type proteins that were processed to about 60- to 70-kDa fragments, processed to 45- and 20-kDa fragments *via* a 60-kDa fragment. To examine the processing profile of CryIV-type toxin, the 130-kDa protoxins of CryIVA and CryIVB were solubilized and processed *in vitro* by the gut extract of the larvae of *Cx. pipiens pallens*. CryIVA was first processed to a 60-kDa intermediate at t_1~t_3 (1~3 hours after the onset of processing), and finally to protease-resistant 45- and 20-kDa fragments (Fig. 4A). On the other hand, in the case of CryIVB, a 70-kDa fragment appeared at t_1~t_3 and protease-resistant 46- and 18-kDa fragments were the final processed fragments (Fig. 5A).

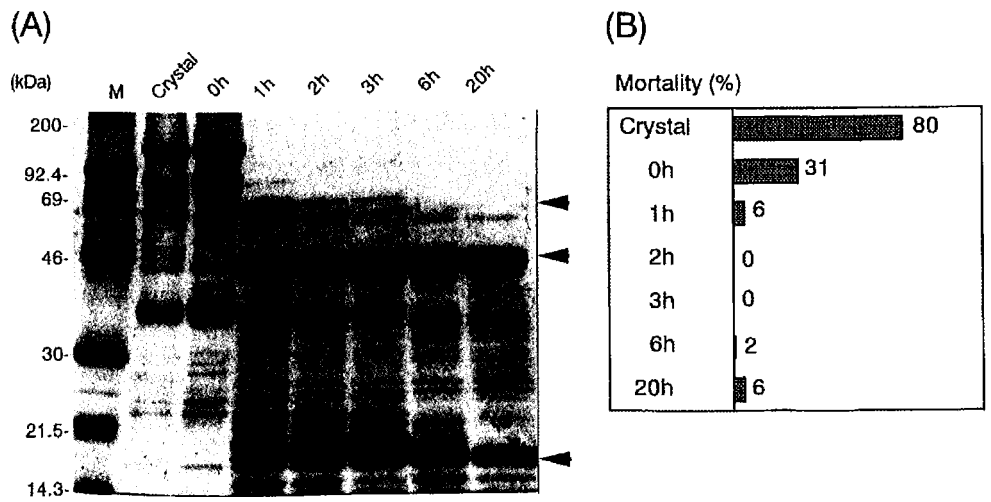

Fig. 5. (A) Processing pattern and insect toxicity of CryIVB by the gut extract of *Cx. pipiens pallens*. (B) Mortality of the insect with processed fragments of CryIVB at each time.

Mosquito larvicidal activity of processed fragments of CryIVA and CryIVB

The mosquito larvicidal activity of each of the gut extract-treated samples was assayed against *Cx. pipiens pallens*. The toxicity of the t_1 to t_3 samples of CryIVA decreased to about 1/2~1/3 of that of the t_0 sample, but the toxicity of the t_6 and t_{20} samples recovered to become twice as high as the t_2 and t_3 samples (Fig. 4B). Unlike CryIVA, each sample of CryIVB except t_0 had virtually no larvicidal activity (Fig. 5B).

N-terminal amino acid sequencing of processed fragments of CryIVA and CryIVB

To identify the site at which the protoxin polypeptides are cleaved to generate the fragments upon treatment with the gut extract, we analyzed the *N*-terminal amino acid residue of each fragment of CryIVA and CryIVB. Both the 60- and 20-kDa fragments of CryIVA had the *N*-terminal amino acid residue of Gly58. The *N*-terminus of the 45-kDa fragment of CryIVA was Glu236. On the other hand, the *N*-terminal amino acid residue of the 18-kDa fragment of CryIVB was Gly34 and the 46- and 70-kDa fragments had Ser204 in common at their *N*-termini (Fig. 6).

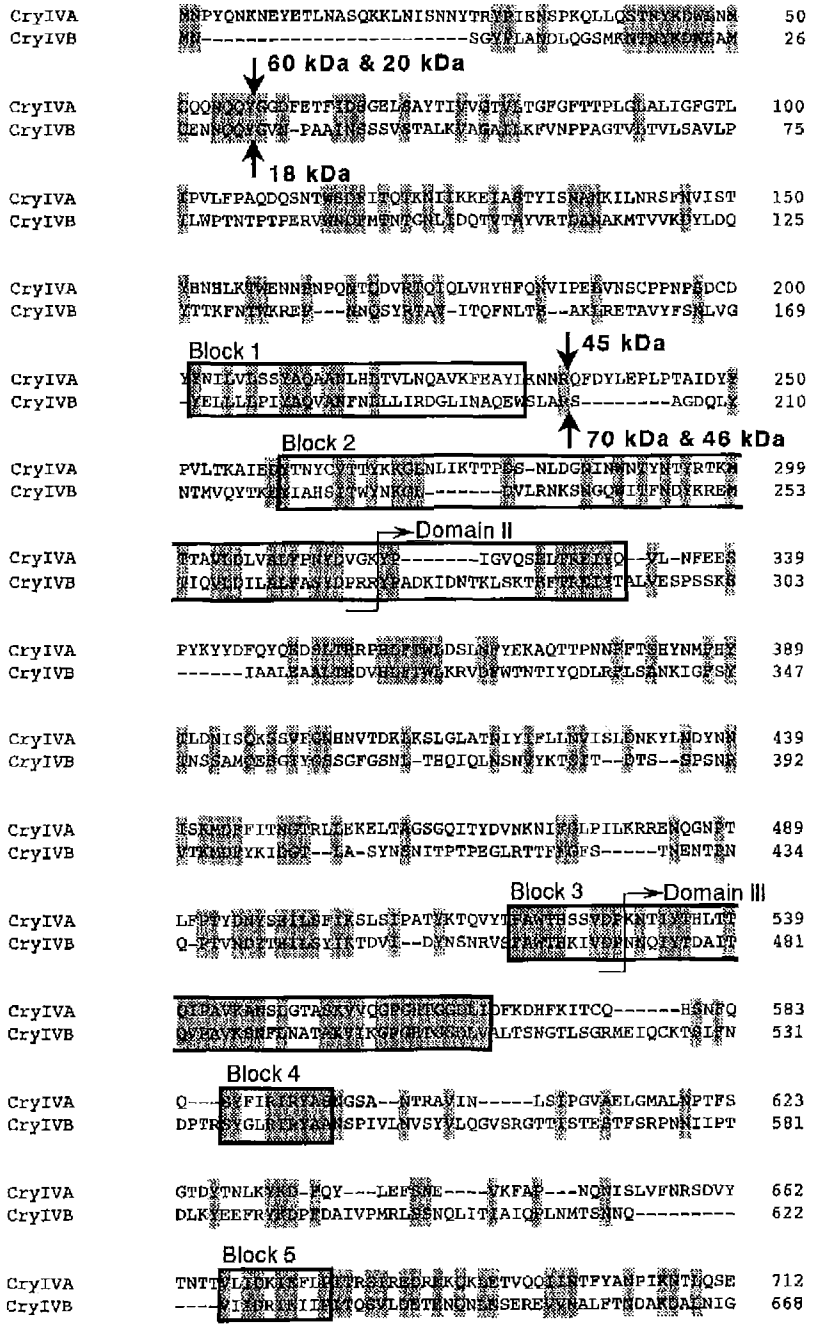

Fig. 6. Amino acid sequence alignment of CryIVA and CryIVB. The amino acid residues conserved between CryIVA and CryIVB are shaded. The five highly conserved regions among many δ-endotoxins are depicted in a box. The cleavage site by gut extract is indicated by vertical arrows. Rectangular arrows indicate boundaries that separate the domain.

DISCUSSION

Mutational analysis of the CryIVA toxin indicates that changes of some amino acid residues with charged side chains in block 5 greatly decrease their production. The mutant CryIVA proteins were digested with proteases to see whether the decreases in their intracellular production resulted from the structural instability of the protein molecules. The 130-kDa protein molecules of the mutant CryIVA–Asp670Tyr, -Asp670Leu, and -Asp670Pro, the production of which was poor in *B. thuringiensis* subsp. *israelensis*, greatly decreased in thermal stability (Fig. 2) and slightly decreased in resistance to trypsin digestion. These results suggest that the poor intracellular production of the mutant CryIVA toxins are primarily caused by the conformational alterations of the 130-kDa protein molecules that increase susceptibility of proteolytic degradation. Since block 5 of CryIVA is supposed to be in the core of the 130-kDa protein molecule, based on the three-dimensional structure of the CryIIIA protein molecule (Li et al., 1991), it is not surprising that changes of the amino acid sequence of block 5 cause critical conformational alterations. Recently, the three-dimensional structure of CryIA(a) has been analyzed by Grochulski et al. (1995), and structurally important salt bridges between the conserved amino sequences were identified. In addition to the amino acid sequence alignment in block 5 shown in Fig. 7, Arg589 and Arg591 in block 4 of CryIVA correspond to Arg525 and Arg427 in block 4 of CryIA(a), respectively. Since, in CryIA(a), Asp525 and Glu602, Arg527 and Asp599, and Arg600 and Glu602 are linked by salt bridges, it is strongly suggested that, in CryIVA, Arg589 and Glu673, Arg591 and Asp670, and Lys671 and Glu673 are linked by salt bridges. Therefore, the mutations at Asp670 and Glu673 in block 5 may destroy the salt bridges to cause conformational changes in the 130-kDa proteins, so that the 130-kDa proteins become more susceptible to proteolytic degradation, resulting in the poor accumulation in the *B. thuringiensis* subsp. *israelensis* cells. Thus, we conclude that block 5 of CryIVA is one of the major elements that determine the structural stability of toxin protein molecules.

Since the mutant and the wild type CryIVA proteins gave the same processing pattern upon digestion by the *Cx. pipiens pallens* gut extract (Fig. 3), it is strongly suggested that the mutations in block 5 have no effect on the activation by the midgut proteases.

Lepidopteran-specific δ-endotoxins are proteolytically processed to produce 60- to 70-kDa activated toxins in the midguts of target insect larvae (Höfte and Whiteley, 1989). On the contrary, Angsuthanasombat et al. (1992) got the 48-kDa protein from the 130-kDa protein of dipteran-specific CryIVA through treatment with the gut extracts of *Aedes aegypti, Ae. gambiae,* and *Cx. quinquefasciatus*, and demonstrated that the 48-kDa protein is active

CryIVA	667	VLIDKIEFLP	676
CryIVB	623	VIIDRIEIIP	632
CryIAa	596	VYIDRIEFVP	605
CryIAc	598	VIIDRFEFIP	607
CryICa	605	LYIDKIEIIL	614
CryIIIA	633	VYIDKIEFLP	642

Fig. 7. Amino acid sequence alignment of block 5. The amino acid residues conserved among several δ-endotoxins are enclosed in a box.

against *Anopheles gambiae* and *Cx. quinquefasciatus* cells. Now we have shown that, upon treatment with the gut extracts of *Cx. pipiens pallens*, the 45-kDa protein is produced from the 130-kDa protein of CryIVA (Fig. 3). The 45-kDa protein may be an active toxin because its size agrees with the size of 48 kDa of the activated toxin of CryIVA as shown by Angsuthanasombat et al. (1992).

In the case of CryIA(b), a mutant carrying the mutation at Arg601, which corresponds to Lys671 in block 5 of CryIVA, is not converted to the protease-resistant 60-kDa fragment upon proteolytic digestion (Nakamura et al., 1992). On the other hand, the 45-kDa fragment can be produced from the 130-kDa protein of the mutant CryIVA–Lys671Ala upon treatment with trypsin or the gut extract (data not shown). This suggests that the mechanism of activation of CryIVA is different from that of CryIA(b).

Interestingly, the 45-kDa protein that is produced upon proteolytic processing of the mutant CryIVA–Asp670Tyr has the same thermal stability as the wild type (Nishimoto et al., 1994). This suggests that block 5 of CryIVA is not a major determinant of the conformation of the activated 45-kDa protein molecule. Possibly, the 45-kDa protein molecules of the activated CryIVA–Asp670Tyr take the same conformation as the wild type. Considering this and the insecticidal activities of the CryIVA mutants, we may conclude that the mutations in block 5 of CryIVA have no direct effect on the insecticidal activities (Nishimoto et al., 1994).

To obtain further information about the processing of CryIVA, the proteolytic digestion of CryIVA by the gut extract from the larvae of *Cx. pipiens pallens* was analyzed. The 130-kDa protein of CryIVA was processed to the protease-resistant 45- and 20-kDa fragments *via* the 60-kDa intermediate (Fig. 4A). When samples of fragments processed for different times of CryIVA was assayed for the insecticidal activity on *Cx. pipiens pallens*, all samples had the toxicity; t_1~ t_3 fractions were less toxic and the activity was partially restored in the t_6 and t_{20} fractions (Fig. 4B). On the other hand, CryIVB, another CryIV type protein, was converted to the 70-, 46-, and 18- kDa fragments upon digestion with the gut extract (Fig. 5A). No fraction of CryIVB had significant insecticidal activity (Fig. 5B). Angsuthanasombat et al. (1993) reported that CryIVB is processed to 47- to 48-kDa and 16- to 18-kDa fragments upon treatment by gut extract of *Ae. aegypti*, and that the cleavage site was between Arg203 and Ser204. This is consistent with our result that the 46- and 70-kDa fragments have Ser204 at their *N*-termini. However, the insecticidal activity of CryIVB is lost when the 18-, 46-, and 70-kDa fragments are produced upon digestion with gut extract. This bewildering result may be because CryIVB is more susceptible to proteases than CryIVA; in the assay, adsorption of the processed fragments of CryIVB to latex beads causes instability, leading to rapid degradation by the midgut proteases. In fact, CryIVB was much more rapidly processed *in vitro* than CryIVA by gut extract of *Cx. pipiens* (data not shown).

In the processing of both CryIVA and CryIVB, the proteolytic cleavage sites of the internal regions are in predicted loops between helices 5 and 6 in the bundle of a-helices (Fig. 6). We now propose a model for the processing of CryIVA and CryIVB (Fig. 8), where CryIVA is processed to 45- and 20-kDa fragments *via* the 60-kDa intermediate. On the other hand, CryIVB is converted to 18- and 70-kDa and finally 18- and 46-kDa polypeptides, suggesting the difference of the processing patterns between CryIVA and CryIVB.

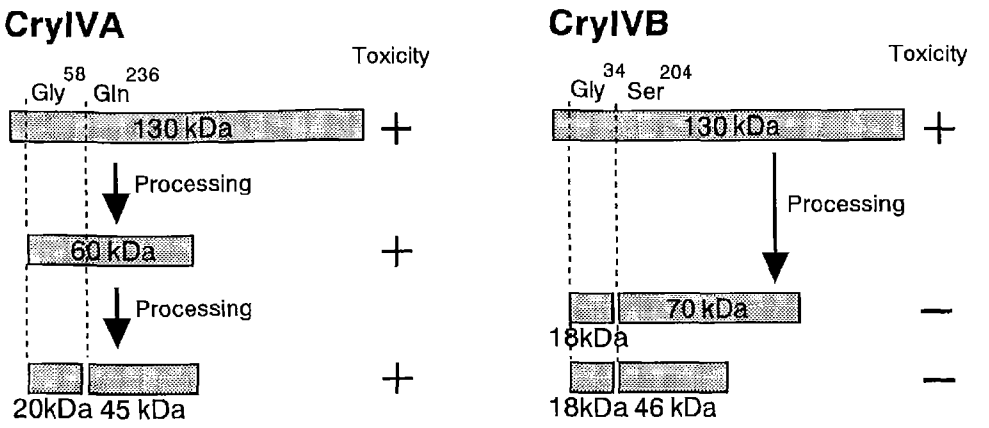

Fig. 8. The model of the processing pathway of CryIVA and CryIVB by the *Cx. pipiens pallens* gut extract. CryIVA is processed to 45- and 20-kDa fragment *via* the 60-kDa intermediate. On the other hand, CryIVB is converted to 18- and 70-kDa and finally 18- and 46-kDa polypeptides.

REFERENCES

Adang, M.J., Staver, M.J., Rocheleau, T.A., Leighton, J., Barker, R.F., and Thompson, D.V. 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. *Gene (Amsterdam)* 36:289-300.

Almond, B.D., and Dean, D.H. 1993. Structual stability of *Bacillus thuringiensis* δ-endotoxin homolog-scanning mutants determined by susceptibility to proteases. *Applied and Environmental Microbiology* 59:2442–2448.

Angsuthanasombat, C., Crickmore, N. and Ellar, D.J. 1991. Cytotoxicity of a cloned *Bacillus thuringiensis* subsp. *israelensis* CryIVB toxin to an *Aedes aegypti* cell line. *FEMS (Federation of European Microbiological Societies) Microbiology Letters* 83:273–276.

Angsuthanasombat, C., Crickmore, N., and Ellar, D.J. 1992. Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism *in vivo*. *FEMS (Federation of European Microbiological Societies) Microbiology Letters* 94:63–68.

Angsuthanasombat, C., Crickmore, N., and Ellar, D.J. 1993. Effects on toxicity of eliminating a cleavage site in a predicted interhelical loop in *Bacillus thuringiensis* CryIVB δ-endotoxin. *FEMS (Federation of European Microbiological Societies) Microbiology Letters* 111:255–262.

Aronson, A.I., Beckman, W. and Dunn, P. 1986. *Bacillus thuringiensis* and related insect pathogens. *Microbiological Review* 50:1–24.

Bone, E.J., and Ellar, D.J. 1989. Transformation of *Bacillus thuringiensis* by electroporation. *FEMS (Federation of European Microbiological Societies) Microbiology Letters* 58:171–178.

Carroll, J., Li, J. and Ellar, D.J. 1989. Proteolytic processing of a coleopteran-specific δ-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochemical Journal* 261:99–105.

Chen, X.J., Lee, M.K. and Dean, D.H. 1993. Site-directed mutations in a highly conserved region of *Bacillus thuringiensis* δ-endotoxin affect inhibition of short circuit current across *Bombyx mori* midguts. *Proceedings of the National Academy of Sciences of the United States of America* 90:9041–9045.

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwaltz, J., Brousseau, R. and Cygler, M. 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: Crystal structure and channel formation. *Journal of Molecular Biology* 254:447–464.

Harwood, C.R., Coxon, R.D. and Hancock, I.C. 1990. Protease assay. In: Molecular Biological Methods for *Bacillus*. Edit. C.R. Harwood and S.M. Cutting. Wiley, Chichester. p. 383.

Himeno, M., Ikeda, M., Koyama, N., Komano, T., Yamamoto, H. and Nakayama, I. 1985. Plasmids and insecticidal activity of delta-endotoxin crystals from *Bacillus thuringiensis* var. *israelensis*. *Agricultural and Biological Chemistry* 49:573–580.

Hofmann, C., Lüthy, P., Hütter, R. and Pliska, V. 1988a. Binding of the delta endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). *European Journal of Biochemistry* 173:85–91.

Hofmann, C., Vanderbruggen, H., Höfte, H., Van Rie, J., Jansens, S. and Van Mellaert, H. 1988b. Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proceedings of the National Academy of Sciences of the United States of America* 85:7844–7848.

Höfte, H., De Greve, H., Seurinck, J., Jansens, S., Mahillon, J., Ampe, C., Vandekerckhove, J., Vanderbruggen, H., Van Montagu, M., Zabeau, M. and Vaeck, M. 1986. Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis* Berliner 1915. *European Journal of Biochemistry* 161:273–280.

Höfte, H. and Whiteley, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* 53:242–255.

Ishiwa, H. and Shibahara-Sone, H. 1986. New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*: IV, The nucleotide sequence of PHY300PLK and some properties in relation to transformation. *Japanese Journal of Genetics* 61:515–528.

Li, J., Carroll, J. and Ellar, D.J. 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5Å resolution. *Nature (London)* 353:815–821.

Nakamura, K., Murai-Nishioka, R., Shimizu, M., Oshie, K., Mikitani, K., Oeda, K. and Ohkawa, H. 1992. Insecticidal activity and processing in larval gut juices of genetically engineered 130-kDa proteins of *Bacillus thuringiensis* subsp. *aizawai*. *Bioscience Biotechnology and Biochemistry* 56:1–7.

Nicholls, C.N., Ahmad, W. and Ellar, D.J. 1989. Evidence for two different types of insecticidal P2 toxins with dual specificity in *Bacillus thuringiensis* subspecies. *Journal of Bacteriology* 171:5141–5147.

Nishimoto, T., Yoshisue, H., Ihara, K., Sakai, H. and Komano, T. 1994. Functional analysis of block 5, one of the highly conserved amino acid sequences in the 130-kDa CryIVA protein produced by *Bacillus thuringiensis* subsp. *israelensis*. *FEBS (Federation of European Biochemical Societies) Letters* 348:249-254.

Schaeffer, P., Millet, J. and Aubert, J.-P. 1965. Catabolic repression of bacterial sporulation. *Proceedings of the National Academy of Sciences of the United States of America* 54:704–711.

Schnepf, H.E. and Whiteley, H.R. 1985. Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. *Journal of Biological Chemistry* 260:6273–6280.

Sen, K., Honda, G., Koyama, N., Nishida, M., Neki, A., Sakai, H., Himeno, M. and Komano, T. 1988. Cloning and nucleotide sequences of the two 130 kDa insecticidal protein genes of *Bacillus thuringiensis* var. *israelensis*. *Agricultural and Biological Chemistry* 52:873–878.

Thomas, W.E. and Ellar, D.J. 1983. *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *Journal of Cell Science* 60:181–197.