

# 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*

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## Abstract

There are five amino acid sequences highly conserved among *Bacillus thuringiensis*  $\delta$ -endotoxins. We have changed the amino acid residues in block 5, one of the conserved sequences, of CryIVA. When the amino acid residues with charged side chains were replaced by others, the amount of production of the altered CryIVA protein was markedly decreased. It is suggested that the decrease is caused by the unstable conformation of the altered CryIVA protein molecule, as judged by digestion with trypsin and thermolysin. On the other hand, the substitution of amino acid residues in block 5 did not affect the insecticidal activity of CryIVA. These results strongly suggest that block 5 of CryIVA is one of the stability-determining elements of the protoxin molecule.

**Key words:**  $\delta$ -Endotoxin; Insecticidal activity; Thermal stability; *Bacillus thuringiensis*

## 1. Introduction

*Bacillus thuringiensis* is a Gram-positive bacterium that produces proteinous crystalline inclusions during sporulation. The inclusions are composed of polypeptides called  $\delta$ -endotoxins that have highly selective toxicity to the larvae of lepidopteran, coleopteran, and dipteran insects [1]. There are five amino acid sequences, blocks 1–5, which are highly conserved among the *B. thuringiensis*  $\delta$ -endotoxins [2]. It is believed that these regions are important for the insecticidal activity of the  $\delta$ -endotoxins.

Many studies on the mode of action of the  $\delta$ -endotoxins have provided us with the following information. The  $\delta$ -endotoxins are solubilized in the alkaline digestive juice of the larval midgut, and digested by gut proteases to produce the activated toxin. This activated toxin binds to receptors localized in the midgut brush border and creates leakage channels in the cell membranes, causing cell lysis and death of the larvae [3,4].

An analysis of the three-dimensional structure of CryIIIA by Li et al. [5] provided a lot of information about the interdependence of the structure and function of  $\delta$ -endotoxins. It was found that CryIIIA comprises

three domains. Domain I is a seven-helix bundle that is believed to penetrate the membranes and form pores, but a major conformational change of the CryIIIA molecule must occur for this seven-helix bundle to form pores in the membrane. Angsuthanasombat et al. showed that CryIVB is activated by gut proteases in vitro to produce 47–48 kDa and 16–18 kDa polypeptides [6]. In this activation, in addition to removing the C-terminal half, proteolytic cleavage occurs in the exposed loop joining helices 5 and 6 in the seven-helix bundle [5,7]. Similarly, CryIIA [8] and CryIIIA [9] are cleaved in the exposed loop joining helices 3 and 4 [5]. It was proposed in these studies that these cleavages in the seven-helix bundle cause major conformational changes in the toxin molecules, as a consequence of which the activated toxins become capable of forming the pores in the membranes. Domain II is a three- $\beta$ -sheet domain that is believed to function as a receptor-binding region. Domain III is a  $\beta$ -sandwich and is believed to play a critical role in retaining the protein structure. Chen et al. proposed that block 4, which is contained in domain III, affects not only the structural integrity of the protein molecule but also the function of toxins as ion channels [10].

Block 5, which is expected to be at the C-terminus of the activated toxin [11–13], is another highly conserved region in domain III. Li et al. proposed that this region is essential to preclude further proteolytic processing of the C-terminus [5], but the role of block 5 remains obscure. In this article, we show that some amino acids in block 5 of CryIVA made a great contribution to the stability of the protein molecule of the 130 kDa protoxin. On the other hand, even when these amino acids are replaced by others, the 45 kDa activated toxin remained stable. Further, we show that the substitution of amino

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**Abbreviations:** *B.*, *Bacillus*; *E.*, *Escherichia*; *C.*, *Culex*; *A.*, *Aedes*; kDa, kilodalton(s); h, hour(s); bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CAPS, 3-cyclohexylaminopropanesulfonic acid; LC<sub>50</sub> is defined in detail in the text.

acid residues in block 5 did not affect the insecticidal activity of CryIVA.

## 2. Materials and methods

### 2.1. Bacterial strains

A pBTI-6-cured acrySTALLIFEROUS *B. thuringiensis* subsp. *israelensis* strain HD522 C37-21 (Spo<sup>+</sup> Cry<sup>+</sup>) was used as a host for the production of inclusions [14]. *E. coli* strain JM109 was used as an indicator bacteria for transfection and DH5 $\alpha$  was used for the other procedures.

### 2.2. Construction of plasmids

A recombinant plasmid, pIS422, was obtained by inserting the 4.29-kb *PmaCI*–*XbaI* fragment containing the entire *cryIVA* gene from pBGH4 [15] into the *SmaI*–*XbaI* site of a shuttle vector, pHY300PLK (Takara Shuzo Co.) [16]. We subcloned the 2.78 kb *SnaBI*–*XbaI* fragment from pIS422 into the *SmaI*–*XbaI* site of the phage vector M13mp19 to obtain the recombinant phage MIS278. Site-directed mutagenesis of *cryIVA* in MIS278 was performed with the oligonucleotide-directed in vitro mutagenesis system (version 2.1; Amersham). After mutagenesis, the nucleotide sequences of the mutant genes were confirmed. A 2.75 kb *BglII*–*XbaI* 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 [17].

### 2.3. Protein analysis

*B. thuringiensis* HD522 C37-21 harboring pIS422 mutants were cultured in 10 ml of Luria-Bertani medium containing tetracycline (20  $\mu$ g/ml) until the end of exponential growth, and the cells were harvested and resuspended in the same volume of Schaeffer medium [18] 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 with Coomassie brilliant blue staining. The sporulation process was microscopically monitored.

### 2.4. Preparation of inclusions

*B. thuringiensis* HD522 C37-21 harboring pIS422 or pIS422 mutants were cultured in Schaeffer medium containing tetracycline (20  $\mu$ g/ml) until cells were lysed completely, and the resultant CryIVA inclusions contaminated by spores were purified on discontinuous sucrose gradients as described previously [19].

### 2.5. Measurement of protein concentration

To measure the concentration of wild-type CryIVA inclusions, the purified inclusions were solubilized in 50 mM Na<sub>2</sub>CO<sub>3</sub> (pH 10.5), 10 mM DTT for 1 h at 37°C [6] and assayed for 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 with Coomassie brilliant blue staining, and the 130 kDa peak 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.

### 2.6. 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 [20]. For the test with trypsin digestion, the inclusions were solubilized in 50 mM Na<sub>2</sub>CO<sub>3</sub> (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/prototoxin) 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<sub>2</sub> to give a final concentration of 10 mM, the CryIVA proteins were incubated with thermolysin (1:50, w/w, thermolysin/prototoxin) 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 with Coomassie brilliant blue staining,

and the 130 kDa peak intensities were measured with a densitometer (Pharmacia LKB). The *T<sub>s</sub>* value is the temperature at which 50% of the untreated toxin remains.

Thermolysin digestion was also performed to measure the structural alteration in the 45 kDa protein molecules activated by the gut extracts of *Culex pipiens pallens* (1 unit: 10  $\mu$ g toxin) for 6 h at 37°C. The gut extracts were prepared as described previously [21]. Proteolytic activities of the gut extracts were measured as described previously [6,22].

### 2.7. Assay of the mosquitocidal activity

Assays of the mosquitocidal activities of the toxins were performed on the 2nd to 3rd instar larvae of *Culex pipiens pallens*. The mosquito larvae were grown in a container (35  $\times$  25  $\times$  3 cm) at 23°C. Before the assays, the larvae were transferred twenty by twenty in 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.

## 3. Results

### 3.1. Production of mutant 130 kDa CryIVA proteins in *B. thuringiensis* subsp. *israelensis*

To investigate the functional contribution of block 5, each of the ten amino acid residues in block 5 of CryIVA was replaced by alanine. The resultant ten mutant genes were expressed in HD522 C37-21 and the obtained crude inclusions were analyzed by SDS-7% PAGE (data not shown). 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. In a further experiment Asp<sup>670</sup> was replaced by additional amino acids. The production of the resultant mutant CryIVA proteins was decreased (Fig. 1), especially that of the four mutants CryIVA-Asp670Tyr, -Asp670Leu, -Asp670Pro and -Asp670Arg. Additional amino acid residues with charged side chains in block 5, Lys<sup>671</sup> and Glu<sup>673</sup>, were replaced by glutamic acid and lysine residues, respectively, resulting in a marked decrease in the production of the CryIVA proteins (data not shown). The aspartic acid residues outside block 5, Asp<sup>660</sup> and Asp<sup>684</sup>, were replaced by other amino acid residues as control replacements. They caused no decrease in the production of the CryIVA proteins (Fig. 1). 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).

### 3.2. 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 [20]. Thus, the five mutant CryIVA proteins that carried the amino acid replacements at Asp<sup>670</sup> were treated with a high concentration of trypsin (data not shown). The production of the mutant CryIVA-

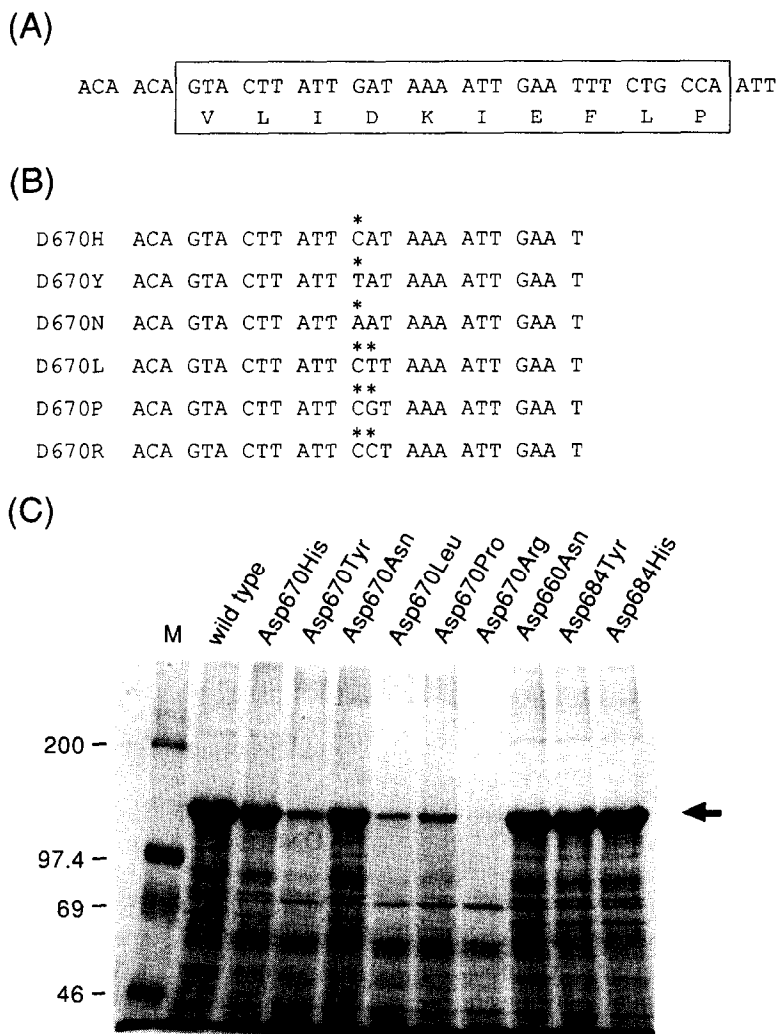


Fig. 1. (A) The nucleotide sequence and deduced amino acid sequence of block 5 is depicted in the box. (B) Oligonucleotide primers used for the amino acid replacements at Asp<sup>670</sup>. The changed nucleotides are indicated by (\*). (C) Polyacrylamide gel electrophoretic analyses of the production of the mutant CryIVA proteins in *B. thuringiensis* subsp. *israelensis*. Protein extracts of *B. thuringiensis* subsp. *israelensis* cells harboring the mutant derivatives of pIS422 were analyzed by SDS-7% PAGE with 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.

Asp670Arg protein was so poor that we failed to prepare enough of the protein sample for the protease digestion test. Proteins of the three mutants CryIVA-Asp670Tyr, -Asp670Leu and -Asp670Pro, the production of which was significantly decreased (Fig. 1), were slightly more susceptible to proteolytic digestion with trypsin than the proteins of the wild-type CryIVA and the mutants CryIVA-Asp670Asn and -Asp670His. Three of the five CryIVA proteins that had been tested above were further digested with thermolysin at various temperatures. As shown in Fig. 2, the critical temperature for the efficient proteolytic degradation of the mutant CryIVA-Asp670Tyr protein was lower than that of the wild-type protein. That is, the  $T_s$  value of the mutant CryIVA-Asp670Tyr protein was about 42°C, while that of the wild-type was about 51°C. This indicates that the mutant CryIVA-Asp670Tyr protein is more susceptible to ther-

molysin digestion. Similarly, the proteins of the mutants CryIVA-Asp670Leu and -Asp670Pro were markedly susceptible to proteolytic digestion with thermolysin ( $T_s$  values about 47°C and 42°C, respectively).

The mutant CryIVA-Asp670Tyr and wild-type CryIVA proteins were treated for activation by gut extracts of *C. pipiens pallens*. 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 highly heat stable, and exhibited the same profiles of susceptibility to proteolytic degradation with thermolysin (Fig. 3). 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.

We observed the purified inclusions by a phase contrast microscope at a magnification of 500. The wild-type

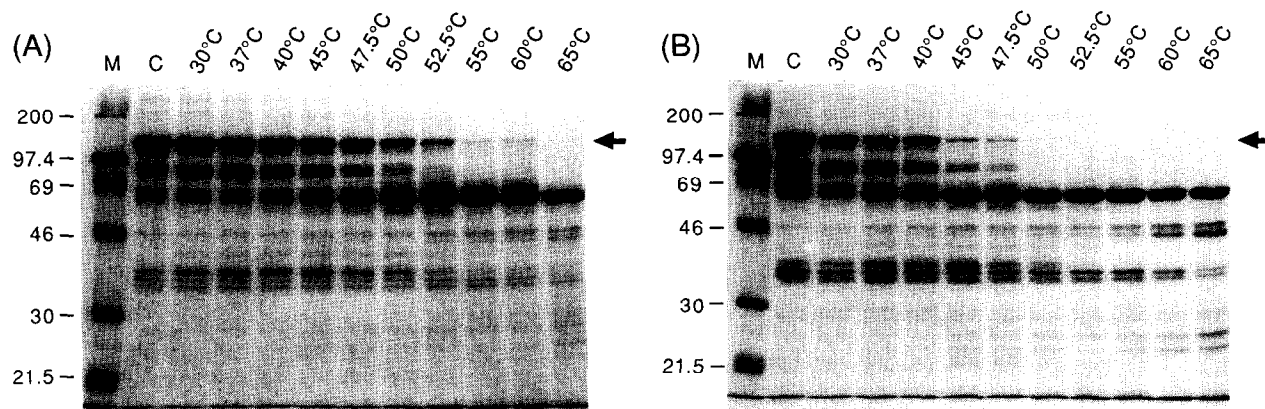


Fig. 2. Comparison of the thermal stability of the 130 kDa protein molecules of the wild-type CryIVA and the mutant CryIVA-Asp670Tyr. The purified crystals were solubilized and proteolytically digested with thermolysin at various temperatures as described in section 2. The resulting digested proteins were analyzed by SDS-12% PAGE with Coomassie brilliant blue staining. (A) The wild-type CryIVA. (B) The mutant CryIVA-Asp670Tyr. 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.

inclusions were large and polyhedral. The mutant inclusions the production of which was poor were 3- to 5-fold smaller in size than the wild-type ones, and their shape had changed into a globular form. However, the mutant inclusions the production of which was as abundant as the wild-type were as large as the wild-type ones, and their shape was unchanged (data not shown).

### 3.3. Assay of the mosquitocidal activity

To examine the effects of these amino acid substitutions in block 5 on the toxicity of the corresponding CryIVA mutants, their mosquitocidal activities were assayed. As shown in Table 1, the  $LC_{50}$  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 ac-

tivity of CryIVA. The production of the inclusions of the three mutants CryIVA-Asp670Arg, -Lys671Glu, and -Glu673Lys, was too poor to prepare big enough protein samples required for the assays.

## 4. Discussion

Mutational analysis of the CryIVA toxin indicates that changes of some amino acid residues with charged side chains in block 5 cause great decreases in 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

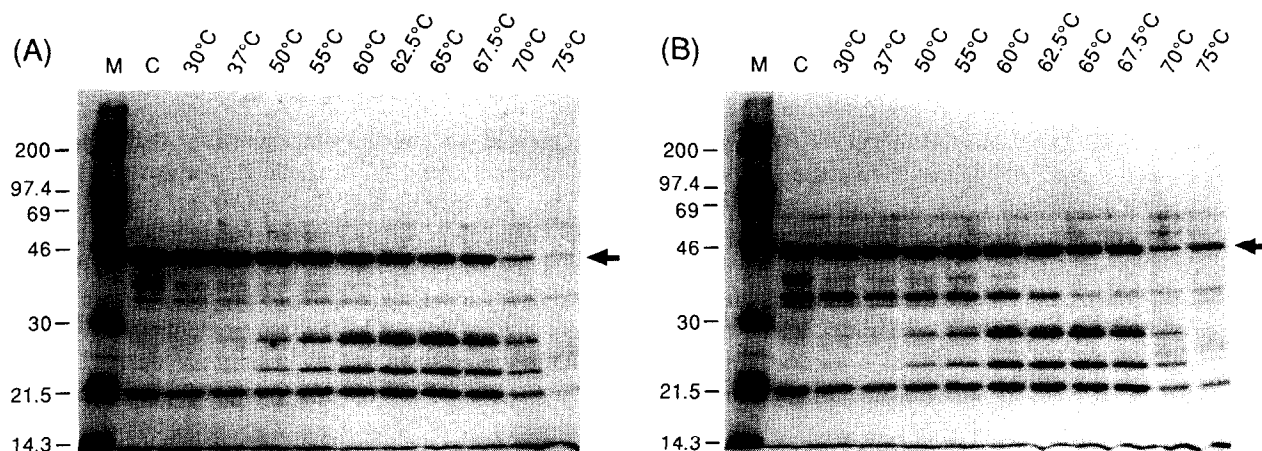


Fig. 3. Comparison of the thermal stability of the 45 kDa protein molecules of the wild-type CryIVA and the mutant CryIVA-Asp670Tyr. The solubilized crystals were treated with gut extracts, and then proteolytically digested with thermolysin at various temperatures as described in section 2. The resulting digested proteins were analyzed by SDS-14% PAGE with Coomassie brilliant blue staining. (A) The wild-type CryIVA. (B) The mutant CryIVA-Asp670Tyr. The reaction temperatures are shown above the electrophoregrams. The arrow indicates the 45 kDa proteins. Lanes M and C represent the molecular mass standards and the activated 45-kDa protein as controls, respectively.

-Asp670Pro, the production of which was poor in *B. thuringiensis* subsp. *israelensis*, exhibit great decreases in the thermal stability (Fig. 2) and slight decreases in the resistance to trypsin digestion. On the other hand, the 130 kDa proteins of the mutants CryIVA-Asp670Asn, and -Asp670His, which are produced intracellularly at the wild-type level, exhibit the same susceptibility to proteolytic digestion with trypsin as the wild-type. We cannot rule out the possibility that the base substitutions introduced into block 5 (Fig. 1) result in instability of the *cryIVA* mRNA, causing poor production of the mutant CryIVA proteins. However, 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. Since block 5 of CryIVA is supposed to be in the core of the 130 kDa protein molecule, based on the crystalline structure of the CryIIIA protein molecule [5], it is not surprising that changes in the amino acid sequence of block 5 cause critical conformational alterations. According to the microscopic observation, the mutant inclusions, the production of which was poor, were smaller than the wild-type inclusions, and also their shapes were altered. These results also suggest the following possibility; the mutations in block 5 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. As a consequence, the inclusions become small and immature in their shapes. Alternatively, some amino

acid residues in block 5 may directly involve the inter-molecular association among the 130 kDa proteins. That is, the mutations cause a conformational change in the 130 kDa protein molecules, so that it is difficult for them to aggregate into inclusions that are highly resistant to proteolytic degradation; and the 130 kDa protein molecules that fail to aggregate are degraded upon proteolytic attack in the *B. thuringiensis* subsp. *israelensis* cells. Thus, we may conclude that block 5 of CryIVA is one of the major elements that in some way determine the structural stability of the toxin protein molecule.

The  $\delta$ -endotoxins, to which the lepidopteran larvae are specifically susceptible, are proteolytically processed to produce 60–70 kDa activated toxins in the midguts of target insect larvae [2]. In contrast, Angsuthanasombat et al. obtained a 48 kDa protein from the 130 kDa protein of CryIVA through treatment with gut extracts of *Aedes aegypti*, *Aedes gambiae*, and *Culex quinquefasciatus*, and demonstrated that the 48 kDa protein is active against *A. gambiae* and *C. quinquefasciatus* cells [6]. Now we have shown that, upon treatment with the gut extracts of *C. pipiens pallens*, a 45 kDa protein is produced from the 130 kDa protein of the dipteran-specific CryIVA toxin (Fig. 3). The 45 kDa protein may be an active toxin because its size is in good agreement with that of the 48 kDa of the activated toxin of CryIVA that was shown by Angsuthanasombat et al. [6]. According to the result of the thermolysin digestion, the 45 kDa protein is considerably more stable than the 130 kDa protein. Possibly, the 45 kDa protein acts as a very stable activated form of the toxin in insect midgut.

In the case of CryIA(b), upon proteolytic digestion, the protease-resistant 60 kDa fragment cannot be produced from the altered protoxin carrying the mutation at Arg<sup>601</sup>, which correspond to Lys<sup>671</sup> of block 5 of CryIVA [23]. However, upon treatment with trypsin or gut extracts, the 45 kDa fragment can be produced from the 130 kDa protein of the mutant CryIVA-Lys671Ala (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 exhibits the same thermal stability as the wild-type (Fig. 3). 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 (Table 1), we may conclude that the mutations in block 5 of CryIVA have no direct effect on the insecticidal activity. Nevertheless, the two mutants CryIVA-Asp660Asn, and -Lys671Ala exhibit 2- to 3-fold increases in the LC<sub>50</sub> values as compared with the wild-type. These reductions in the insecticidal activity may be meaningful, and remain to be examined further.

Table 1

Insecticidal activity against *C. pipiens pallens* larvae of mutant inclusions

Mutant CryIVA <sup>a</sup>	Insecticidal activity (LC <sub>50</sub> ) <sup>b</sup>
Wild-type	55 (41–72)
Asp670His	95 (42–272)
Asp670Tyr	76 (52–100)
Asp670Asn	57 (39–81)
Asp670Leu	37 (26–52)
Asp670Pro	62 (17–198)
Asp660Asn	112 (80–162)
Asp684Tyr	62 (42–90)
Asp684His	54 (17–161)
Val667Ala	87 (65–119)
Leu668Ala	88 (66–118)
Ile669Ala	80 (62–105)
Asp670Ala	71 (27–188)
Lys671Ala	136 (107–176)
Leu672Ala	75 (34–176)
Glu673Ala	77 (58–104)
Phe674Ala	86 (61–123)
Leu675Ala	46 (11–152)
Pro676Ala	74 (54–103)

<sup>a</sup> The amino acid replacements are indicated.

<sup>b</sup> LC<sub>50</sub> values represent the concentrations (ng/ml) of inclusions giving 50% mortalities after 24 h. The 95% confidence intervals are shown in parentheses.

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