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Research Article

Induction of *Manduca sexta* Larvae Caspases Expression in Midgut Cells by *Bacillus thuringiensis* Cry1Ab Toxin

Helena Porta, Carlos Muñoz-Minutti, Mario Soberón, and Alejandra Bravo

Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado postal 510-3, Cuernavaca 62250, Morelos, Mexico

Correspondence should be addressed to Helena Porta, helena@ibt.unam.mx

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Bacillus thuringiensis produces crystal toxins known as Cry that are highly selective against important agricultural and human health-related insect pests. Cry proteins are pore-forming toxins that interact with specific receptors in the midgut cell membrane of susceptible larvae making pores that cause osmotic shock, leading finally to insect death. In the case of pore-forming toxins that are specific to mammalian cells, death responses at low doses may induce apoptosis or pyroptosis, depending on the cell type. The death mechanism induced by Cry toxins in insect midgut cells is poorly understood. Here, we analyze the *caspases* expression by RT-PCR analysis, showing that the initial response of *Manduca sexta* midgut cells after low dose of Cry1Ab toxin administration involves a fast and transient accumulation of caspase-1 mRNA, suggesting that pyroptosis was activated by Cry1Ab toxin as an initial response but was repressed later. In contrast, *caspase-3* mRNA requires a longer period of time of toxin exposure to be activated but presents a sustained activation, suggesting that apoptosis may be a cell death mechanism induced also at low dose of toxin.

1. Introduction

Bacillus thuringiensis (*Bt*) are insecticidal Gram-positive bacteria that produce inclusion bodies composed by δ-endotoxin proteins, during sporulation [1]. The main components of these inclusions are the crystal (Cry) toxins, which possess highly selective toxicities against important agricultural and human health-related insect pests but are nontoxic to mammals or other organisms [2]. Cry toxins are classified as pore forming toxins (PFTs) based on its capacity to make pores in the midgut membrane of the larvae causing osmotic shock, rupture of the midgut cells, and finally the insect death [3].

In mammalian cells, PFTs are important virulence factors that kill eukaryotic cells by different mechanisms, depending on the dose and the cell type (for review, see [4]). At low doses, cell death occurs through two different mechanisms: (1) apoptosis, characterized by the activation of initiator-caspases (Cas) (Cas-2, -4, -8, -9, -10, and -12) that trigger effector-Cas (Cas-3, -6, and -7) to cleave cellular substrates. Apoptotic cells features include nuclear and cytoplasmic condensation and cellular fragmentation, Cas-3 activation, DNA damage, and formation of apoptotic bodies [5] and (2) pyroptosis characterized by the activation of inflammatory caspases, such as Cas-1, -5 and -11, that mediate cell death and inflammatory responses [6]. Apoptosis and pyroptosis are death processes confined to a discrete cell population that contends the insult and in some cases allows the organism to recover.

Some examples of PFT that induce apoptotic death response, when used at low doses are the *Staphylococcus aureus* Penton-Valentine leukocidin (PVL) that triggers Cas-9 and Cas-3 activity in neutrophils [7] and the *Clostridium perfringens* enterotoxin (CPE) induces activation of Cas-3 or Cas-7 in Caco-2 cells [8]. In endothelial cells, the *Streptococcus pneumoniae* pneumolysin (PLY) induces Cas-6, Cas-9, and Cas-3 [9], and in HeLa cells, the *Bt* parasoporin-1, activates Cas-3 [10]. Activation of Cas-1 and pyroptosis has been observed in macrophages treated with the streptolysin O (SLO) toxin from *Streptococcus* bacteria, the PLY, and the anthrax toxin [11–13]. It was shown that pore formation activity of these PFT is necessary to induce the apoptotic or the pyroptosis responses, since PFT mutants affected in pore formation, toxin-knockout mutants, or organisms in which the expression of PFT has been greatly reduced by antisense RNA were unable to induce these responses [7, 10, 14–16].

At high doses of PFT, cells respond starting a more severe cell death processes known as necrosis and oncosis, for example, high doses of CPE provokes oncosis in Caco-2 cells [8]. Oncosis is characterized by cellular and organelle swelling, membrane blebbing, and an increment in membrane permeability. Oncosis leads to depletion of the cellular energy stores and failure of the ionic pumps [17].

In the case of Cry toxins, there are no reports studying the induction of apoptosis or pyroptosis cell death processes in vivo in intoxicated Lepidopteran insects. The only report that analyzed the expression of *cas*-1 in third instar *Aedes caspius* larvae, a Dipteran insect, showed activation of *cas*-1 genes after treatment with two larvicidal bacteria, *Bt* and *B. sphaericus* [18]. The aim of our work was to explore if pyroptosis and apoptosis cell response processes, are activated *in vivo* in the midgut cells of *Manduca sexta* larvae in response to Cry1Ab intoxication. Here, we show that *M. sexta* larvae intoxicated with Cry1Ab induce a transient expression of *cas*-1, at low doses (LC_{25}) and short times. In contrast, the *cas-3* is induced after longer time of toxin exposure depending also in the toxin dose. Our results suggest that *M. sexta* midgut cells may induce first pyroptosis as an initial response to low doses of toxin, and then, apoptosis is triggered later with a more sustained pattern also at low toxin dose.

2. Materials and Methods

2.1. Extraction of the Crystal-Spore Suspension. Bt strains harboring pHT315-Cry1Ab plasmid [19] were grown at 30°C, in nutrient broth sporulation medium, supplemented with $10\,\mu g\,ml^{-1}$erythromycin until complete sporulation [20]. Crystal inclusions were observed under phase contrast microscopy and purified as previously described [21].

2.2. Manduca sexta larvae Intoxication. Cry1Ab was applied onto the diet surface in 24-well plates as described [20]. For clarity, we added thirty-five microliters of Cry1Ab crystal suspensions, containing two different toxin concentrations (1 and $2\,ng\,cm^{-2}$), corresponding to the lethal concentrations of LC_{25}, and LC_{50}, respectively, as previously described [20]. Once the surface was completely dry, a neonate first-instar *M. sexta* larvae was added per well, using 24 larvae per dose and time (from 0 min to 48 h) in order to analyze the initial response of the larvae to the toxin action. Two experiments were done with a total of 48 larvae per treatment. Feeding behavior was synchronized, since first instar larvae were quite voracious and immediately starting feeding when transferred to the artificial diet.

2.3. RT-PCR Assays. Total RNA isolated from midgut tissue from control *M. sexta* or with intoxicated larvae with LC_{25} and LC_{50} concentrations of Cry1Ab toxin at different times from 0 to 48 h was used for cDNA synthesis as previously reported [22]. Control larvae were feed with same diet without toxin addition at the same times. Briefly, 2 μg of total RNA and 500 μg of oligo(dT25)VN was used to synthesize cDNA with 1 μL (200 Units/μL) of Moloney murine leukemia virus Retroranscriptase (MoMLVRT) (Invitrogen, Carlsbad, Calif, USA). A volume of 2 μL of the first strand cDNA reaction was used in a 50-μL PCR reaction. Primers cas-1Forward (5′-CCA TTT ATT TTC AAT CAT GAA CAT T-TT G-3′) and cas-1Reverse (GGT TTA CCA GCA AGT GTG GGA-3′) or cas-3Forward (ACG AAG ATG TCG AAG CTC TGA AT-3′) and cas-3-Reverse (CAT TAA CCA GAT TTC GCG CTT C-3′) were used to amplify the *M. sexta cas-1* and *cas-3* cDNA, respectively. Taq DNA polymerase was used, and PCR reactions were performed as follows: (1) denaturing step, 1 min at 94°C, (2) extension step, 30 cycles of 30 sec at 94°C, 30 sec at 58°C, and 50 sec at 68°C, and (3) final extension, 5 min at 72°C. An aliquot of 5 μL of PCR product was analyzed by standard agarose gel electrophoresis and observed after ethidium bromide staining. As loading control a 268-bp RT-PCR product of *ribosomal protein S3* (*rpS3)* (Accession number U12708) from *M. sexta,* was also amplified by using rpS3-Forward, 5′-CCG-ATC GGA GAT CAT CAT CAT GGC C-3′ and rpS3-Reverse 5′-GCA ACC GCG CGC TTC AGA CTC C-3′ primers. The mRNA band intensity was quantified using the ImageJ software (http://rsb.info.nih.gov/ij/). The relative amount of mRNA for *cas-1* and *cas-3* were quantified using as reference the amount of the corresponding loading control (*rpS3*). The induction folds for *cas-1* and *cas-3* after Cry1Ab toxin administration was calculated in relation to the control larvae that were fed with the same diet and times without toxin addition. All data set were done two times, standard deviation of the data sets were calculated and error bars included in the figures.

2.4. Cloning and Sequencing. The PCR products obtained after *cas-1* and *cas-3* amplification reactions described above were cloned into the EcoR*V* restriction site of the pBCSK$^+$ plasmid (Invitrogen, Carlsbad, Calif, USA) and sequenced in the Instituto de Biotecnologia, UNAM facility using Taq FS Dye Terminator Cycle Sequencing Fluorescence-Based Sequencing in a Perkin Elmer/Applied Biosystems Model 3730 sequencer (ABI Prism; AppliedBiosystems, Carlsbad, Calif, USA).

2.5. Phylogenetic Analysis. The virtual translations of *cas-1* and *cas-3* gene sequences from *M. sexta* were aligned using Muscle 3.7 alignment [23]. The alignment includes Cas-1 and Cas-3 proteins from different Lepidoptera and Dipteran insects as well as some Cas-6, Cas-7, and Cas-8 that were identified in Dipteran insects. A maximum likelihood tree was constructed and drawn using PhyML version 3.0 [24] with a bootstrap of 500 replicates.

3. Results and Discussion

3.1. Isolation of M. sexta caspase-1 and caspase-3 Genes. To obtain the *M. sexta cas-1* and *cas-3* gene sequences, the *Bombix mori* gene sequences of these *cas* genes were used as a template to search for homologues sequences in an EST library from *M. sexta* deposited in the http://www.agripest-base.org/.

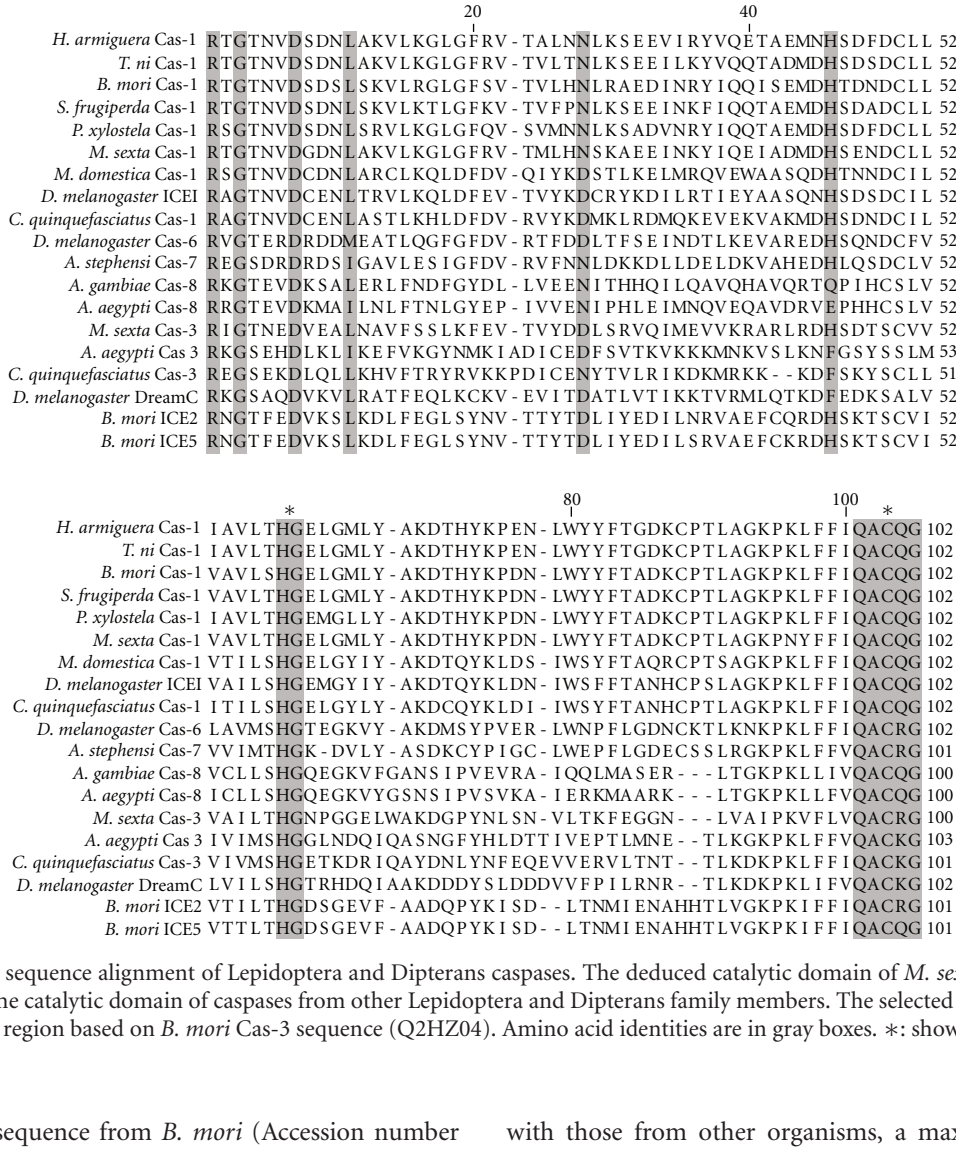

Figure 1: Multiple sequence alignment of Lepidoptera and Dipterans caspases. The deduced catalytic domain of *M. sexta* Cas-1 and Cas-3 is compared with the catalytic domain of caspases from other Lepidoptera and Dipterans family members. The selected sequence fragments include R^{73} to G^{173} region based on *B. mori* Cas-3 sequence (Q2HZ04). Amino acid identities are in gray boxes. ∗: shows the catalytic dyad.

When *cas-1* sequence from *B. mori* (Accession number AF448494) was used as query, a DNA contig containing a complete hypothetical *cas-1* of *M. sexta,* with 80% of identity, with *B. mori cas-1* query was obtained (ctg7180001044351). On the other hand, using the *B. mori cas-3* sequence as a template (Accession number AAW79564), a DNA contig from *M. sexta* (ctg7180001055914) with 40% identity to *B. mori cas-3*, was identified.

The deduced open reading frame from *M. sexta cas-1* codify for 289 amino acid residues and the partial *cas-3* gene for 154 residues. Alignment of several Lepidoptera and Diptera Caspase protein-fragments that include the catalytic domain showed that the catalytic dyad, characteristic of the Caspase family, is conserved. In *M. sexta* Cas-1, the catalytic dyad corresponded to His^{119} and Cys^{174} residues. The H and C catalytic residues in *M. sexta* Cas-3 partial protein sequence are also conserved (Figure 1).

3.2. The Cas-1 and Cas-3 Proteins from M. sexta Larvae belong to Two Independent Phylogenetic Groups Conserved between Lepidotera and Diptera. To obtain a better picture of the relationships between the *M. sexta* Cas-1 and Cas-3 proteins with those from other organisms, a maximum likelihood phylogenetic tree was constructed. The tree included representative Cas-1 and Cas-3 proteins from different Lepidoptera and Diptera organisms as well as some Cas-6, Cas-7 and Cas-8 archetypes from Dipteran insects (Figure 2). The Cas-1 and Cas-3 sequences are clustered in two independent branches of the phylogenetic tree, indicating that the *M. sexta* sequences used in this work were correctly identified. Each of the Cas-1 and Cas-3 clades, in turn, are separated in two groups: one containing the Lepidotera proteins and the other those belonging to the Diptera proteins. The Cas-1 group of Lepidopteran proteins contains *B. mori* (Q8I9V7), *Spodoptera frugiperda* (P89116), *Plutella xylostella* (D9IVD4), *Trichoplusia ni* (B6EEC1), and *Helicoverpa armigera* (A7L9Z3), and the Dipteran group includes *Drosophila melanogaster* (O01382), *Culex quinquefasciatus* (B0W0K2), and *Musca domestica* (B5AK94) Cas-1 proteins related to activation of cytokines during inflammation. The Cas-3 group of Dipteran sequences includes sequences from *Aedes aegypti* (Q178B6), *C. quinquefasciatus* (B0WZJ4), and *D. melanogaster* (Q7KHK9), while the Lepidopteran group contains two Cas-3 sequences from *B. mori* (Q2HZ04 and

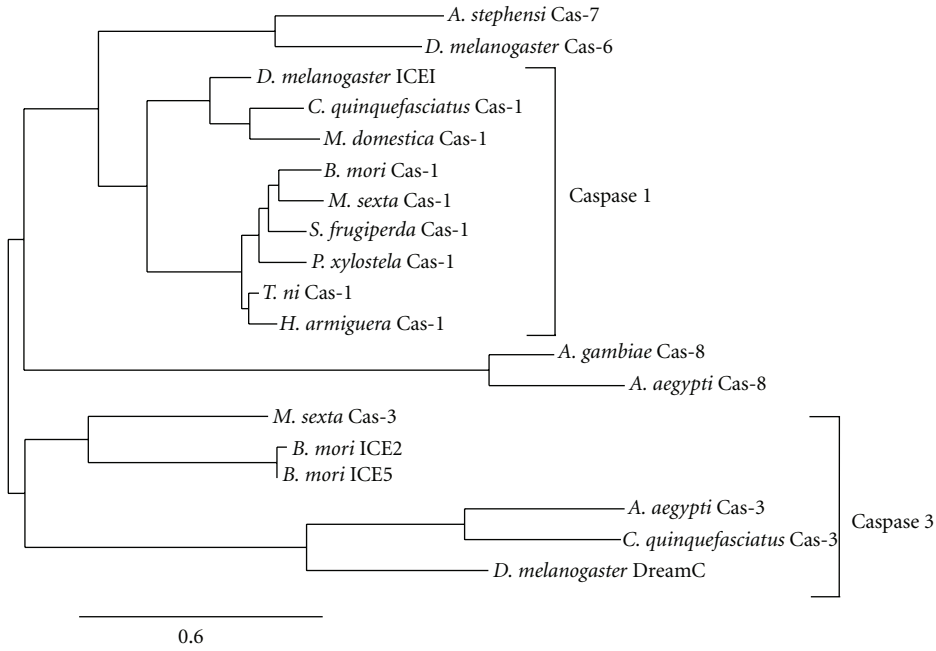

Figure 2: Phylogenetic tree of Cas-1 and Cas-3 proteins from *M. sexta*. A phylogenetic tree was constructed using the following sequences: Cas-1 from *Bombyx mori* (Q8I9V7), *Spodoptera frugiperda* (P89116), *Plutella xylostella* (D9IVD4), *Trichoplusia ni* (B6EEC1) *Helicoverpa armigera* (A7L9Z3), *Drosophila melanogaster* (O01382), *Culex quinquefasciatus* (B0W0K2), *Musca domestica* (B5AK94), and *Manduca sexta* (ctg7180001044351). Cas-3 from *Aedes aegypti* (Q178B6), *C. quinquefasciatus* (B0WZJ4), *D. melanogaster* (Q7KHK9), *B. mori* (Q2HZ04 and Q2HZ05), and *M. sexta* (ctg7180001055914). Cas-6 from *D. melanogaster* (Q9NHF9). Cas-7 from *Anopheles stephensi* (Q86FL0). Cas-8 from *An. gambiae* (Q5TMK1) and *Ae. Aegypti* (Q16H55).

Q2HZ05) that are related to apoptosis [25]. We also add some other caspase proteins to this analysis, such as the Cas-6 from *D. melanogaster* (Q9NHF9), Cas-7 from *Anopheles stephensi* (Q86FL0), and two initiator Cas-8 sequences, one from *An. gambiae* (Q5TMK1) and other from *Ae. Aegypti* (Q16H55), which are clustered in different branches from Cas-3 and Cas-1 (Figure 2).

3.3. Cry1Ab Intoxication Induces cas-1 and cas-3 Expression in M. sexta Larvae Midgut. To get new insights into the responses of *M. sexta* midgut cells to Cry1Ab intoxication, we analyzed the expression pattern of *cas-1* and *cas-3* as characteristic features of pyroptosis and apoptosis, respectively. *M. sexta* larvae were treated with two different doses of Cry1Ab toxin. The 25% lethal concentration and the medium lethal concentration were calculated in first instar *M. sexta* larvae feed 7 days with different concentrations of toxin by Probit statistical analysis. We then used a low dose, 1 ng cm^{-2} (corresponding to LC_{25}) and medium dose, 2 ng cm^{-2} (corresponding to LC_{50}) to study the initial response of *M sexta* midgut cells after ingestion of the toxin. *M. sexta* larvae feed with the same diet without contamination with Cry1Ab toxin, were used as control in each time analyzed. A RT-PCR approach was used to monitor *cas-1* and *cas-3* gene expression and the internal loading control *rpS3* was also analyzed. The predicted *cas-1* and *cas-3* cDNA sizes were 440 nt and 243 nt, respectively. The cDNA products obtained by RT-PCR, were cloned, and sequenced to corroborate their identity. Figure 3 shows representative RT-PCR electrophoretic patterns of *cas-1* and *cas-3* obtained in the control larvae and after treatment with the different toxin dose. This figure also shows the *rpS3* loading control. The numbers on the agarose gel represent the number of pixels observed in each mRNA band as determine by scanning optical density of the bands.

RT-PCR was used to analyze the expression of *cas-1* and *cas-3* in response to Cry1Ab intoxication. In panel A of Figure 4, we show the relative expression levels of *cas-1* and *cas-3* in control and toxin intoxicated larvae at the different times by analyzing the band intensity of *cas-1* or *cas-3* RT-PCR data over the band intensity of the constitutive *rpS3* control at the corresponding time and toxin dose. RT-PCR expression analysis showed that both *cas-1* and *cas-3* expression was slightly induced in the control larvae during the time frame of the analysis, where both *cas-1* and *cas-3* showed a twofold induction after 48 h of feeding in normal diet (Figure 4(a)).

In relation to the Cry1Ab intoxicated larvae, the results showed that *cas-1* was only induced after low dose of Cry1Ab administration, showing higher levels of *cas*-1 mRNA than the control feed larvae after 30 min and 2 h of feeding *M. sexta* with a LC_{25} Cry1Ab surface contaminated diet (Figure 4(a), upper panel). The *cas-1* mRNA expression was then repressed at longer times of incubation at this toxin dose and at all times after intoxication with higher toxin dose, LC_{50} (Figure 4(a) upper panel). It is noticeable that larvae looked healthy during the course of the experiment. In relation to the *cas*-3 mRNA expression, we found that this mRNA was also induced at low dose of toxin ingestion

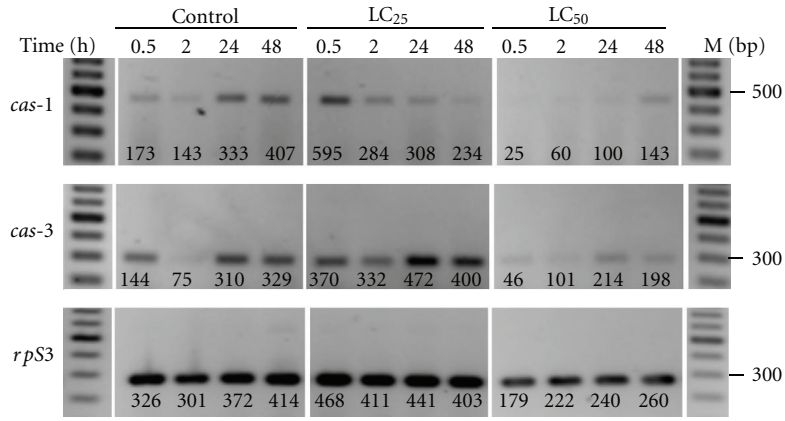

Figure 3: Representative electrophoretic patterns of RT-PCR amplifications of *cas-1*, *cas-3*, and *rpS3* genes from *M. sexta* larvae. RT-PCR analysis of the expression of *cas-1*, *cas-3*, and *rpS3* genes at different times were performed with control larvae or with Cry1Ab intoxicated *M. sexta* larvae with two different concentrations of Cry1Ab toxin. The numbers on the agarose gel represent the number of pixels observed in each mRNA band as determine by scanning optical density of the bands.

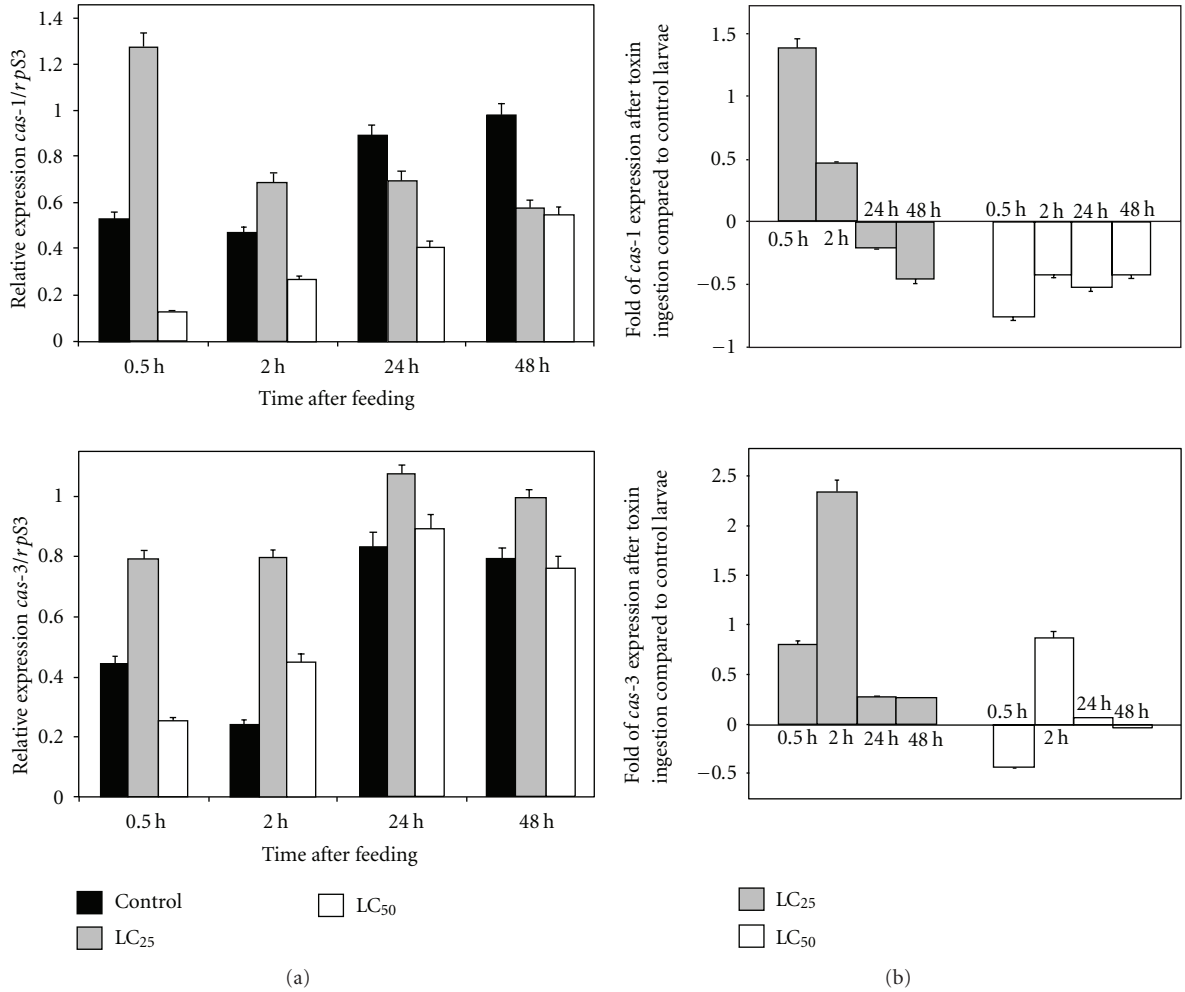

Figure 4: Accumulation of *cas-1* mRNA in response to LC_{25} and LC_{50} doses of Cry1Ab in *M. sexta* midgets. (a) shows analysis the relative expression of *cas-1* or *cas-3* over *rsp3* constitutive gene induced in control larvae (black bars) or after larvae intoxication with LC_{25} toxin dose (gray bars) or LC_{50} toxin dose (white bars). (b) shows the changes in fold expression of *cas-1* or *cas-3* after Cry1Ab intoxication compared to their expression in control larvae, fed with normal diet. Gray bars, LC_{25} toxin dose and white bars, LC_{50} toxin dose.

but requires longer times of toxin administration to be fully activated (Figure 4(a), lower panel). In LC_{50} toxin dose, the *cas-3* mRNA was only induced at 2 h after feeding with the toxin. It is interesting that this gene did not show a clear repression at longer times of intoxication with both toxin doses even at 48 h. The induction of *cas-3* mRNA was dependent on toxin dose, being higher with a LC_{25} than with a LC_{50} toxin dose (Figure 4(a), lower panel). Larvae looked paler than control larvae at 48 h feeding with LC_{50} toxin dose, but no death larvae were observed as expected, since LC_{50} value corresponds to the dose that kills 50% of the larvae after 7 days of intoxication.

In Panel B of Figure 4, we show the analysis of the changes in fold expression of *cas-1* and *cas-3* mRNA after Cry1Ab ingestion relative to control larvae fed with a noncontaminated diet. In this figure, it is clear that the highest expression of *cas-1* in response to Cry1Ab intoxication was observed after 30 min intoxication with a LC_{25} showing 1.5 fold induction, and then, the expression of this *cas-1* gene was repressed after 24 h of toxin ingestion as well as with higher LC_{50} dose of toxin administration, where it was repressed at all tested times, when compared with the control larvae fed with a normal diet (Figure 4(b), upper Panel). In contrast, the expression of *cas-3* mRNA showed an induction of 2.3 fold over the control larvae at 2 h after intoxication with LC_{25} toxin dose (Figure 4(b), lower Panel) and one fold induction after 2 h intoxication with LC_{50} toxin dose. The expression of *cas-3* mRNA did not show a clear repression when compared with the control larvae, during the time frame of this study (Figure 4(b), lower Panel).

In conclusion, two different toxin doses of Cry1Ab, LC_{25} and LC_{50}, were used in this study to analyze the initial response of *M. sexta* larvae to toxin intoxication. In this work, we identify the putative *cas-1* and *cas-3* genes of *M. sexta* larvae and analyzed their expression *in vivo* in the midgut tissue of the insect during intoxication with Cry1Ab toxin. As mentioned above, the inflammatory Cas-1 and the effector Cas-3 have been implicated in activation of different programmed cell death responses, named pyroptosis or apoptosis, respectively. In mosquito larvae, it has been described that Cas-3 protease is activated after invasion of midgut cells with the malaria parasites *Plasmodium gallinaceum* and *P. berghei,* suggesting that midgut cell death during penetration of the malaria ookinete is related to an apoptotic process [26]. In other reports, it was shown that the modulation of Cas-1 and Cas3 in midgut tissue of *Heliothis virescens* and in *Periplaneta americana* are implicated in the activation of programmed cell death during metamorphosis or during starvation, respectively [27, 28].

Our results suggest that *M. sexta* larvae trigger pyroptosis as an initial response to intoxication with a low dose of Cry1Ab, suggesting that this process may participate as a defense mechanism under this condition, and this response is inhibited at longer periods of toxin ingestion or at higher dose of toxin (Figure 4). In the case of other pore forming toxins, such as PLY, SLO, and lethal toxin from *B. anthracis,* it was shown that these toxins activate Cas-1 in macrophages promoting cell death though pyroptosis [11–13]. Cas-1-deficient macrophages were more resistant to cell-death induced by the SLO toxin [12]. In contrast, the expression of *cas-3* in *M. sexta* intoxicated larvae suggests that apoptosis may play a role latter, after longer times of Cry1Ab toxin administration and may be present at higher toxin doses showing a more sustained activation than Cas-1. These results are similar to the PVL toxin or the α-hemolysin produced by *Escherichia coli* that activate Cas-3 expression, inducing apoptosis in neutrophils, monocyes and macrophages when used at sublytic concentrations [7, 15], or to CPE from *C. perfringens* that also induces typical apoptotic cell death at low dose in mammalian Caco-2 cells by activating Cas-3 [15].

Regarding to higher doses of Cry1Ab intoxication, it was reported that cell death was produced by oncosis in *Trichoplusia ni* H5 ovarian cell line expressing the *M. sexta* Cry1Ab cadherin receptor. These authors showed that the broad-spectrum Cas inhibitor z-VAD-fmk did not suppress cell death in these cells, suggesting that Cas dependent responses such as pyroptosis or apoptosis were not activated with high doses Cry1Ab toxin in this cell line [29].

Further research is needed to analyze the role of Cas-1 activity in activating immune responses to low-dose administration of Cry1Ab toxin in *M. sexta* larvae. Similarly, the role of Cas-3 in activating apoptotic responses at low doses remains to be analyzed. Also, it will be desirable to analyze the effect of specific caspase inhibitors on the toxicity of Cry1Ab to determine the potential role of these programmed cell death responses on survival to Cry toxin intoxication.

Acknowledgments

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References

[1] E. Schnepf, N. Crickmore, J. Van Rie et al., "*Bacillus thuringiensis* and its pesticidal crystal proteins," *Microbiology and Molecular Biology Reviews*, vol. 62, no. 3, pp. 775–806, 1998.

[2] R. A. De Maagd, A. Bravo, C. Berry, N. Crickmore, and H. E. Schnepf, "Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria," *Annual Review of Genetics*, vol. 37, pp. 409–433, 2003.

[3] M. Soberón, S. S. Gill, and A. Bravo, "Signaling versus punching hole: how do *Bacillus thuringiensis* toxins kill insect midgut cells?" *Cellular and Molecular Life Sciences*, vol. 66, no. 8, pp. 1337–1349, 2009.

[4] A. Cancino-Rodezno, H. Porta, M. Soberón, and A. Bravo, "Defense and death responses to pore forming toxins," *Biotechnology and Genetic Engineering Reviews*, vol. 26, pp. 65–82, 2009.

[5] A. Samali, B. Zhivotovsky, D. Jones, S. Nagata, and S. Orrenius, "Apoptosis: cell death defined by caspase activation," *Cell Death and Differentiation*, vol. 6, no. 6, pp. 495–496, 1999.

[6] E. M. Creagh, H. Conroy, and S. J. Martin, "Caspase-activation pathways in apoptosis and immunity," *Immunological Reviews*, vol. 193, pp. 10–21, 2003.

[7] A. L. Genestier, M. C. Michallet, G. Prévost et al., "*Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils," *Journal of Clinical Investigation*, vol. 115, no. 11, pp. 3117–3127, 2005.

[8] G. Chakrabarti and B. A. McClane, "The importance of calcium influx, calpain and calmodulin for the activation of Caco-2 cell death pathways by *Clostridium perfringens* enterotoxin," *Cellular Microbiology*, vol. 7, no. 1, pp. 129–146, 2005.

[9] P. D. Guessan, B. Schmeck, A. Ayim et al., "The oligomeric state of *Bacillus thuringiensis* Cry toxins in solution," *Thrombosis and Haemostasis*, vol. 94, pp. 295–303, 2005.

[10] H. Katayama, Y. Kusaka, H. Yokota et al., "Parasporin-1, a novel cytotoxic protein from *Bacillus thuringiensis*, induces Ca^{2+} influx and a sustained elevation of the cytoplasmic Ca^{2+} concentration in toxin-sensitive cells," *Journal of Biological Chemistry*, vol. 282, no. 10, pp. 7742–7752, 2007.

[11] A. M. Timmer, J. C. Timmer, M. A. Pence et al., "Streptolysin O promotes group A streptococcus immune evasion by accelerated macrophage apoptosis," *Journal of Biological Chemistry*, vol. 284, no. 2, pp. 862–871, 2009.

[12] S. Shoma, K. Tsuchiya, I. Kawamura et al., "Critical involvement of pneumolysin in production of interleukin-1α and caspase-1-dependent cytokines in infection with *Streptococcus pneumoniae* in vitro: a novel function of pneumolysin in caspase-1 activation," *Infection and Immunity*, vol. 76, no. 4, pp. 1547–1557, 2008.

[13] S. L. Fink, T. Bergsbaken, and B. T. Cookson, "Anthrax lethal toxin and Salmonella elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 11, pp. 4312–4317, 2008.

[14] B. A. McClane and G. Chakrabarti, "New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin," *Anaerobe*, vol. 10, no. 2, pp. 107–114, 2004.

[15] T. J. Wiles, B. K. Dhakal, D. S. Eto, and M. A. Mulvey, "Inactivation of host Akt/protein kinase B signaling by bacterial pore-forming toxins," *Molecular Biology of the Cell*, vol. 19, no. 4, pp. 1427–1438, 2008.

[16] B. E. Menzies and I. Kourteva, "*Staphylococcus aureus* α-toxin induces apoptosis in endothelial cells," *FEMS Immunology and Medical Microbiology*, vol. 29, no. 1, pp. 39–45, 2000.

[17] G. Majno and I. Joris, "Apoptosis, oncosis, and necrosis: an overview of cell death," *American Journal of Pathology*, vol. 146, no. 1, pp. 3–15, 1995.

[18] A. A. Al-Roba, M. A. M. Aboul-Soud, A. M. Ahmed, and A. A. Al-Khedhairy, "The gene expression of caspasses is up-regulated during the signaling response of *Aedes caspius* against larvicidal bacteria," *African Journal of Biotechnology*, vol. 10, no. 2, pp. 225–233, 2011.

[19] R. Meza, M. E. Nuñez-Valdez, J. Sanchez, and A. Bravo, "Isolation of Cry1Ab protein mutants of *Bacillus thuringiensis* by a highly efficient PCR site-directed mutagenesis system," *FEMS Microbiology Letters*, vol. 145, no. 3, pp. 333–339, 1996.

[20] C. Muñóz-Garay, L. Portugal, L. Pardo-López et al., "Characterization of the mechanism of action of the genetically modified Cry1AbMod toxin that is active against Cry1Ab-resistant insects," *Biochimica et Biophysica Acta*, vol. 1788, no. 10, pp. 2229–2237, 2009.

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

[22] L. Castillo-Olamendi, A. Bravo-Garcìa, J. Morán, M. Rocha-Sosa, and H. Porta, "AtMCP1b, a chloroplast-localised metacaspase, is induced in vascular tissue after wounding or pathogen infection," *Functional Plant Biology*, vol. 34, no. 12, pp. 1061–1071, 2007.

[23] R. C. Edgar, "MUSCLE: multiple sequence alignment with high accuracy and high throughput," *Nucleic Acids Research*, vol. 32, no. 5, pp. 1792–1797, 2004.

[24] S. Guindon, J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, and O. Gascuel, "New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0," *Systematic Biology*, vol. 59, no. 3, pp. 307–321, 2010.

[25] Y. Sun, W. Wang, B. Li, Y. Wu, H. Wu, and W. Shen, "Synchronized expression of two caspase family genes, ice-2 and ice-5, in hydrogen peroxide-induced cells of the silkworm, *Bombyx mori*," *Journal of Insect Science*, vol. 10, Article ID 43, 10 pages, 2010.

[26] H. Zieler and J. A. Dvorak, "Invasion in vitro of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 21, pp. 11516–11521, 2000.

[27] R. Parthasarathy and S. R. Palli, "Developmental and hormonal regulation of midgut remodeling in a lepidopteran insect, Heliothis virescens," *Mechanisms of Development*, vol. 124, no. 1, pp. 23–34, 2007.

[28] M. S. Park, P. Park, and M. Takeda, "Starvation induces apoptosis in the midgut nidi of *Periplaneta americana*: a histochemical and ultrastructural study," *Cell and Tissue Research*, vol. 335, no. 3, pp. 631–638, 2009.

[29] X. Zhang, M. Candas, N. B. Griko, R. Taussig, and L. A. Bulla, "A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 26, pp. 9897–9902, 2006.