Cry10Aa Protein is Highly Toxic to Anthonomus grandis Boheman (Coleoptera: Curculionidae), an Important Insect Pest in Brazilian Cotton Crop Fields

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Research Report

Introduction

Bacillus thuringiensis (Bt) is a naturally occurring, Gram positive, spore forming soil bacterium. The entomocidal properties of Bt are mediated by Cry proteins (δ endotoxins), which form parasporal crystal inclusions during the bacterial stationary growth phase (Monnerat and Bravo, 2000; Schnepf et al., 1998). These crystal inclusions are produced by one or more insecticidal proteins, which can exhibit toxicity and specificity toward a select group of Lepidopteran, Coleopteran and Dipteran insect species (Schnepf et al., 1998). Different Cry toxins showed great potential for the control of several economically devastating insect-pests when bioengineered into crop plants (Betz et al., 2000; Chattopadhyay et al., 2004).

Cotton is one of the most important crops cultivated worldwide. The cotton productive chain is one of the most important in Brazil and in the world, as it generates thousands of direct and indirect jobs and annually only the Brazilian cotton industry generates around US $ 1.5 billion (Martins et al., 2007). The cotton boll weevil, Anthonomus grandis (Coleoptera: Curculionidae), is a devastating cotton pest responsible for more than 50% of insecticide costs in Brazilian cotton crop fields. Moreover, A. grandis larvae resides inside floral buds and results in the destruction of fiber quality and hampers chemical control, causing considerable yield losses (Martins et al., 2007). However, due to A. grandis economic importance, the search for Cry toxins specific to this species is as essential as the knowledge on the toxic processes involved.

Abstract

The cry10Aa gene from the Brazilian Bacillus thuringiensis subsp israelensis S1804 strain was introduced into the genome of the baculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) in order to evaluate its expression in insect cells and its toxicity to A. grandis. The recombinant virus (vSyncry10Aa) was amplified in Spodoptera frugiperda larvae. Total extracts from S. frugiperda infected with the recombinant viruses were analyzed by SDS-PAGE and showed the presence of a polypeptide around 85 kD. Cuboid-shaped protein crystals were observed in insect extracts by light and scanning electron microscopy. Bioassays, using the recombinant virus infected-insect extracts, showed high toxicity to A. grandis larvae, with a LC50 of 7.12 µg/mL. Furthermore, a competition binding assay with the recombinant biotin-labeled Cry10A protein and brush border membrane vesicles (BBMV) from A. grandis indicated that the toxin binds specifically to BBMVs. Therefore, the Cry10A protein has a potential to be used in transgenic cotton plants for the control of this important insect pest.

Keywords Bacillus thuringiensis; Toxicity; Recombinant protein

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performed with another Bti strain, have shown that Bti was also toxic to A. grandis and this toxicity was not due to Cry4A, Cry4B, Cry11 and Cyt proteins individually or their combination (Martins et al., 2007). So, in order to find out which protein was responsible for the high level of toxicity we investigated the action of Cry10Aa towards A. grandis, since Bti strains also have a cry10A gene.

In the present study, we have introduced the cry10Aa gene isolated from the Brazilian S1804 Bti strain, into the genome of a baculovirus and analyzed the recombinant protein expression in cultured insect cells and insect larvae. We have shown that the recombinant Cry10A protein might be responsible for the toxicity of this Bti strain to A. grandis larvae, since it was shown to be toxic to this insect and bound to A. grandis BBMVs.

1 Results and Analysis
1.1 Amplification, cloning and sequencing of a cry10Aa gene from B. thuringiensis subsp. israelensis S1804 strain
The cry10Aa gene of B. thuringiensis S1804 strain, was amplified by PCR using Cry10Aa-specific oligonucleotides, which were designed from the published cry10Aa gene sequence (GenBank accession number: M12662) (Thorne et al., 1986), and cloned into the pGEM®-T Easy vector (data not shown), resulting in the recombinant plasmid pGemcry10Aa. The DNA fragment was sequenced and the sequence analysis revealed that the entire sequence of the cry10A gene was present but an error in the design of the reverse oligo removed one nucleotide (T) before the stop codon of the gene. Blast analysis showed that, besides the missing base at the 3-end, the sequence is practically identical to the cry10Aa gene described by (Thorne et al., 1986) (Genebank accession number: M12662), with only two nucleotide differences at positions +1779 and +1885, respectively (change of A to G and C to G). These two nucleotide changes generate two amino acid changes in the Cry10Aa protein (T589A and T624S).

1.2 Construction of recombinant baculoviruses
The DNA fragment containing the cry10Aa without a stop codon was removed from the pGemcry10Aa plasmid and cloned into the transfer vector psSynXIV+X3 generating the plasmid pSyncry10Aa (Figure 1). When cloned into this plasmid, a new stop codon was generated after 152 base pairs downstream of the gene which resulted in a new ORF of 723 amino acids (not shown). The pSyncry10Aa was then used in a co-transfection of insect cells with the DNA of a linearized occluded negative virus (occ-) vSynVTgal and the recombinant vSyncry10Aa was purified. The vSyncry10Aa virus, besides having a cry gene, has the AcMNPV polyhedrin gene, which makes easy the purification of the recombinant by the presence of viral occlusion bodies (OBs) inside the nucleus of infected cells. In order to confirm the insertion of the heterologous gene into the recombinant virus genome, PCR reactions were carried out with cry-specific oligonucleotides (data not shown).

![Figure 1 Diagram showing the different plasmids and viruses used in this work](image-url)

Note: A: Plasmid pSynXIVVI+X3 containing the polyhedrin promoter (Ppol), a mutated promoter derived from polyhedrin promoter (PXIV), a synthetic promoter (pSyn) and the polyhedrin gene (Pol); B: Plasmid pSyncry10Aa, showing the cry10Aa gene inserted at the EcoR I site of the pSynXIVVI+X3 plasmid; C: region of the vSynVI-gal virus genome where heterologous genes are inserted after homologous recombination with transfer vectors based on the pSynXIVVI+X3 plasmid; D: Region of the recombinant vSyncry10Aa virus genome where the cry10Aa gene was inserted. The cry10Aa was cloned into the plasmid pSynXIVVI+X3 generating the pSyncry10Aa plasmid that was co-transfected with vSynVI-gal DNA into insect cells, generating the recombinant virus vSyncry10Aa by homologous recombination.

1.3 Structural and ultra-structural analysis of insect cells and insects infected with the recombinant viruses
Six-well plates (TPP, Techno Plastic Products AG, Switzerland) were seeded with BTI-TN5B1-4 cells...
(10^6/well) and infected with wild-type AcMNPV and different recombinant viruses (10 pfu/cell). After 1 h (zero times p.i.), virus inoculum was removed and the plates incubated at 27°C. Ninety-six h.p.i, cells were photographed (Figure 2), collected by centrifugation (1,000×g, 5 min) and stored at -80°C. T. ni cells infected with vSyncry10Aa produced possible Cry10Aa protein crystals inside the cells’ cytoplasm as seen by light microscopy (Figure 2D). Sucrose gradient purified samples from extracts of larvae infected with recombinant vSyncry10Aa viruses showed the presence of large cuboidal crystals and baculovirus occlusion bodies (Figure 2E). The cuboidal shape of these crystals are different from the spherical shape of crystals found in B. thuringiensis subsp. israelensis strains toxic to insects of Lepidoptera, Coleoptera and Diptera orders (Praça et al., 2004).

1.5 Binding assay with isolated BBMV of Antonomus grandis

Since we showed that the Cry10Aa recombinant protein was toxic to A. grandis we decided to analyze the binding of the toxin to the midgut of A. grandis larvae. The binding assay showed that the toxin binds to A. grandis BBMVs and that the binding was specific since no binding was detected in the presence of a 100 fold excess of unlabelled Cry10Aa toxin (Figure 4).

Figure 2 Structural and ultra-structural analysis of putative Cry10Aa crystals produced in insect cells and larvae

Note: A: Light microscopy of BTI-Tn5B1-4 cells not infected; B: BTI-Tn5B1-4 cells infected with vSynVI-gal at 96 h.p.i, (C) BTI-Tn5B1-4 cells infected with AcMNPV at 96 h.p.i. (D), BTI-Tn5B1-4 cells infected with vSyncry10Aa at 96 h.p.i. and (E) Scanning electron microscopy of purified viral occlusion bodies (OBs) and crystals (Cry) from S. frugiperda larvae infected with the recombinant vSyncry10Aa. In each photograph was added an inset which show a cell in detail. Long arrows show viral occlusion bodies (OBs) short arrows show putative Cry10a large cuboidal crystals. Scale bar in all photographs = 20 µm, except in E

1.4 Heterologous protein expression analysis

Extracts of wild type and different recombinant viruses-infected insects were then analyzed by SDS-PAGE (Figure 3). The heterologous protein was expressed in insect since we have detected a protein band of around 85 kD by SDS-PAGE in vSyncry10Aa-infected insect extracts that was not present in other insect cells extracts infected with wild type AcMNPV and other recombinant baculovirus (Figure 3).

Figure 3 Recombinant Cry10Aa protein expression: 12% SDS-PAGE of extracts from wild type and recombinant infected insect extracts (120 h.p.i.)

Note: Lane 1: Molecular marker High-Range (GE); Lane 2: AcMNPV-infected insect extract; Lane 3: vSynVI-gal-infected insect extract; Lane 4: vSyncry10Aa-infected insect extract. The arrows indicate the viral occlusion body protein polyhedrin (lower) and the recombinant Cry10A protein (upper), respectively

Figure 4 Binding assays on BBMV isolated from A. grandis.

Note: Biotinylated trypsin-activated Cry toxins were incubated with the BBMV in the absence or in the presence of a 100-fold excess of unlabelled toxin. After 1 h post incubation, unbound toxins were removed, and vesicles containing bound toxins were run in an SDS-PAGE gel and blotted onto a nitrocellulose membrane. Labeled proteins were visualized by incubating the membrane with streptavidin-peroxidase conjugate and Luminal. Lane 1: biotinylated protein marker (Biorad), Lane 2 and Lane 3: biotinylated.
1.6 Bioassays

The heterologous Cry10Aa proteins present in the recombinant virus infected-insect extracts were shown to be highly toxic to neonate *A. grandis* larvae with a LC$_{50}$ of 7.12 µg/mL (Table 1). No toxic activity was detected for second instar *A. gemmatalis* and *S. frugiperda* larvae when incubated with the heterologous Cry10Aa protein (data not shown). Bioassays performed with IPS82 and S1804 strains of *B. thuringiensis israelesiss* showed LC$_{50}$ of 740 and 300 µg/mL, respectively (Table 1).

Table 1 LC$_{50}$ values of recombinant Cry10Aa protein against *Anthonomus grandis*

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Slope (± SE)</th>
<th>LC$_{50}$ (FL) (µg/mL)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry10Aa protein</td>
<td>25</td>
<td>1.64 (± 0.26)</td>
<td>7.12 (5.27-9.8)</td>
</tr>
<tr>
<td>IPS82 Bt strain</td>
<td>25</td>
<td>2.64 (± 0.33)</td>
<td>740 (610-910)</td>
</tr>
<tr>
<td>S1804 Bt stain</td>
<td>25</td>
<td>3.23 (± 0.45)</td>
<td>300 (250-360)</td>
</tr>
</tbody>
</table>

Note: The results of three different bioassays with their respective mean LC$_{50}$ values are shown. N: number of insects used; Bt: Bacillus thuringiensis; $^a$Lc$_{50}$ (LC: lethal concentration and FL: Finducial Limites) calculated by Probit analysis.

2 Discussion

The expression of *cry* genes in insect cells using baculovirus expression vectors (BEVs) is an alternative method for the study of single Cry proteins. Some Cry proteins have already been expressed in insect cells using BEVs, such as Cry1I Cry1C, Cry2A (Aguiar et al., 2006; Martens et al., 1990; Martins et al., 2008; Lima et al., 2008), showing that the recombinant proteins were biologically similar to their native counterparts, with toxicity towards different insects.

Previous studies have shown that the *B. thuringiensis subsp. israelesiss* strain, was also toxic to *A. grandis* and this toxicity was not due to Cry4A, Cry4B, Cry11 and Cyt proteins individually or their combination (Martins et al., 2007). Since *Bti* strains also have a cry10A gene, we wanted to test the hypothesis that the toxicity of this strain towards *A. grandis* was in part due to the Cry10A protein.

Currently various Coleopteran insects were shown to be susceptible to the *B. thuringiensis* Cry proteins, such as: *Chrysomela scripta* (Coleoptera: Chrysomelidae) and *A. grandis* (Martins et al., 2007; Martins et al. 2005; Tailor et al., 1992), *Epilachna varivestis* (Coleoptera: Chrysomelidae) (Tamez-Guerra et al., 1999), *Hypothemenus hampeii* (Coleoptera: Scolytidae) (Arrieta et al., 2004; Bradley et al., 1995), *Xanthogaleruca luteola* (Coleoptera: Chrysomellidae) (Arbab et al., 2001). Moreover, *A. grandis* have different susceptibility to Cry proteins. For example, Cry1Aa have shown a LC$_{50}$ of 21.5 µg/mL to *A. grandis neonate* larvae (Martins et al., 2007), while, the Cry1Ba a LC$_{50}$ of 305.32 µg/mL (Martins et al., 2010).

The recombinant Cry10Aa is equally or even more toxic to *A. grandis* than the recombinant Cry1Ba and Cry1B proteins also expressed in insect cells, with a LC$_{50}$ of 7.12 µg/mL (Martins et al., 2008). Furthermore, the recombinant Cry10Aa protein had the capacity of crystallization into cuboidal crystals in insect cells. Since the cry10Aa gene is under transcriptional control of two promoters in tandem, pSyn and pXIV (Wang et al., 1991), the high expression level of the Cry10Aa protein may have contributed to the crystallization of the protein. SDS-PAGE of purified crystals produced by vSyncry10Aa recombinant viruses showed the presence of a protein of molecular mass of approximately 85 kD (Figure 3).

This difference in shape from the spherical form, present in S1804 strain to a cuboidal form in insect cells, probably could be associated with the presence and association of others proteins during the formation and assembly of the crystal or the presence of the extra amino acids present in the C-terminal of the recombinant protein. Cry proteins have been expressed in high amounts in insect cells and some formed larger crystals than when expressed in *Bti*, suggesting that the size of the crystal produced in *Bti* is limited by the size of the bacteria (Aguiar et al., 2006; Ribeiro and Crook, 1993). The formation of crystals by Cry proteins in insect cells infected with recombinant baculoviruses has been reported for the Cry1Ab, Cry1Ac, Cry11Aa, Cry1Ac, Cry1L, and Cry1Ca proteins (Aguiar et al., 2006; Lima, 2008; Lu and Miller, 1997; Martins et al., 2008; Ribeiro and Crook, 1993).

The transcription analysis of the cry10Aa gene by RT-PCR demonstrated that a cry10Aa gene transcript was detected at 96 h.p.i. indicating that transcript was present during the late phase of infection in
vSyncry10Aa-infected insect cells (data not shown). Consistent with the transcriptional analysis we have detected the Cry10Aa protein of vSyncry10Aa by SDS-PAGE in vSyncry10Aa-infected insect cells extracts in the very late phase of the infection.

Several Bt toxins have already been identified and their toxic activity described for several insects. However, new toxins are still being discovered, as current diversity in pesticidal activity of crystal proteins does not yet approach the diversity reported for naturally occurring Bt isolates (Frankenhuyzen, 2009). The recombinant Cry10Aa was able to bind specifically to BBMVs from A. grandis larvae and previous research have shown that Cry proteins, after activation by proteases localized in the midgut of the target insect, bind to specific receptors localized in the apical microvilli of the midgut cells of susceptible insects from Lepidoptera (Hofmann et al., 1988), Coleoptera (Bravo et al., 1992) and Diptera (Hofte and Whiteley, 1989) orders. Recently, (Martins et al., 2010) showed the binding of an activated Cry1B toxin to BBMVs from A. grandis. Two GPI-anchored proteins with alkaline phosphatase (ALP) activity and around 65 kD and 62 kD, respectively were shown to bind the Cry1B toxin.

The study of the Cry toxins interactions to receptors present on BBMVs of different insect is an important tool for the understanding of molecular basis of toxin-receptor interaction and will be useful for the development of new recombinant Cry toxins with novel specificities and improved toxic activities, contributing to the management of insect resistance in the field and development of new plant cultivars resistant to A. grandis. In recent studies, some Cry toxin receptors have been characterized (Pigott and Ellar, 2007). The best characterized among them are the APN receptor (Pigott and Ellar, 2007) and the cadherin-like receptor (Gahan et al., 2001; Likitvivatanavong et al., 2011; Pacheco et al., 2009; Valaitis et al., 2001) identified in lepidopterans. Other putative receptors include ALP (Fernández et al., 2006; Jurat-Fuentes and Adang, 2004; Jurat-Fuentes and Adang, 2006), a 270 kD glycoconjugate (Valaitis et al., 2001), and a 252 kD protein (Hossain et al., 2004). There is no transgenic plant resistant to boll weevil so far, although this Coleopteran insect-pest is economically important in cotton crop in different producer countries, being the most devastating cotton insect pest in Brazil. The commercially available Bt transgenic cotton expresses Cry1Ac/Cry2Ab toxins that confers mild resistance against Spodoptera frugiperda (Lepidoptera: Noctuidae) (Hamilton et al., 2004), and no resistance towards A. grandis. In this work a recombinant version of the Cry10Aa from B. thuringiensis was shown to be highly toxic to the cotton boll weevil and its gene a promising candidate to be inserted into cotton plants for the control of this devastating cotton pest.

3 Materials and Methods

3.1 Cells, viruses and bacteria

Trichoplusia ni (BTI-Tn5B1-4) cells were maintained at 27°C in TC-100 medium supplemented with 10% fetal bovine serum (Gibco-BRL) and served as host for the wild type virus AcMNPV, the recombinant viruses vSynVI-gal (Wang et al., 1991), and vSyncry10Aa (constructed in this work). Bacillus thuringiensis subsp. israelensis S1804 and IPS82 strains were obtained from the Bacillus subsp. Bank of the Embrapa Recursos Genéticos Biotecnologia (Brasilia, Brazil).

3.2 Recombinant virus construction

The cry10Aa gene of B. thuringiensis S1804 strain, was amplified by PCR using the oligonucleotides F1 (5’-GGATATCCTCCGGAGGAAATGATATGAAATC-3’) and R1 (5’-ATAGTGAATGATTTATTTGTAA GGATCC-3’), which were designed from the published cry10Aa gene sequence (GenBank accession number: M12662) (Thorne et al., 1986). The PCR reaction consisted of 100 pg of total DNA from B. thuringiensis S1804, 0.4 µmol/L of each oligonucleotide primer, 10 µmol/L of each dNTP, 2.5 µL of Taq DNA polymerase buffer (10×), 2 mmol/L MgCl2 and 5 U of Taq DNA polymerase (Invitrogen) in a total volume of 50 µL. Amplification steps were: 94°C/30 s, followed by 35 cycles at 95°C/30 s, 52°C/30 s, 68°C/4 min and a final extension of 68°C/8 min. BamHI restriction sites were introduced into the oligonucleotides F1 and R1 (letters in bold). The amplified fragment was cloned into the plasmid pGEM-T® easy (Promega®) following the manufacturer’s instructions and sequenced (MEGA
BACE 1000, Amersham Bioscience). DNA from the recombinant plasmid (pGemcry10Aa) was digested with EcoRI and separated by electrophoresis in a 0.8% agarose gel following standard protocols (Sambrook et al., 2001). The 2,077 bp fragment was gel-purified using the GFX Kit (GE) and cloned into the transfer vector pSynXIIVI+X3 (Ribeiro and Crook, 1993) previously digested with EcoRI. One µg of the recombinant plasmid (pSyncry10Aa) DNA and 0.5 µg of vSynVI-gal DNA, previously linearised with the restriction enzyme Bsu36I, were co-transfected into a monolayer of BTI-TN5B1-4 cells (10⁶ cells) in a 60-mm plate, using liposomes (Cellfectin®, Invitrogen®) following the manufacturer’s instructions. The plate was incubated one week at 27 °C until the appearance of viral occlusion bodies (OBs), when the supernatant was collected and used to purify the recombinant virus through end-point dilution in 96 well plates (O’Reilly et al., 1992). The single Bsu36I site of vSynVI-gal is located inside the β-galactosidase gene, and linearization makes it non-infective facilitating recombinant virus purification (O’Reilly et al., 1992). Furthermore, the pSyncry10Aa plasmid possesses, besides the cry10Aa gene, the polyhedrin gene (disrupted in vSynVI-gal). Upon homologous recombination of plasmid and viral DNA during co-transfection, vSynVI-gal regains expression of polyhedrin, which is made evident by the formation of OBs by the new recombinant virus (vSyncry10Aa). The latter was purified by three rounds of serial dilution in 96 well plates (O’Reilly et al., 1992; Jarvis, 1997).

3.3 Recombinant protein expression
BTI-Tn5B1-4 (10⁶ cells/plate) cells were infected with recombinant and wild-type AcMNPV viruses (10 pfu/cell) and observed by light microscopy. At 120 h.p.i., cells were collected by centrifugation (5,000 g/10 min) and stored at -80°C. Third instar S. frugiperda larvae were infected with injection of 10 µL of viral stocks (10⁶ pfu/mL for vSyncry10Aa and 10⁷ pfu/mL for AcMNPV) into the hemolymph. Extracts of virus-infected larvae (120 h.p.i.) were analysed in a 12% SDS-PAGE (Mini-protean II —Biorad®) following the manufacturer’s instruction. A band around 85 kD on SDS-PAGE was quantified by densitometry using the program Image Phoretix 2D (Pharmacia). The purification of protein crystals was carried out by ultracentrifugation of virus-infected S. frugiperda extracts on a discontinuous sucrose gradient as described elsewhere (Chang et al., 1993). The purified crystals from vSyncry10Aa infected larvae were solubilized for 1 h at 4°C with 0.1 mol/L NaOH. The pH of the solution containing the solubilized protoxin was decreased to pH 9 by addition of same volume of 1 mol/L Tris-HCl, pH 8.0, the protoxin was then activated with trypsin (1:50 w/w) for 2 h at 37°C, and the reaction stopped by adding trypsin inhibitor (Sigma). The molecular mass and integrity of the recombinant protein were determined by SDS-PAGE.

3.4 Structural and ultra-structural analysis of putative Cry10Aa crystals produced in insect cells and larvae
Insect cells were infected with AcMNPV and vSyncry10Aa as described above and at 96 h.p.i., analyzed in a light microscope (Axiovert 100, Zeiss), and photographed. One hundred third-instar S. frugiperda larvae were infected with the recombinant vSyncry10Aa by injection of virus as described above, and at 120 h.p.i., OBs and protein crystals were purified following the protocol described by (Praça et al. 2004). The purified OBs and crystals were processed for scanning electron microscopy and analyzed under a Zeiss DSM962 scanning electron microscope at 10 kV or 20 kV.

3.5 Preparation of BBMV of Antonomus grandis
BBMVs were prepared from dissected midguts of fourth instar larvae of A. grandis by differential precipitation using MgCl₂, as previously reported (Wolfersberger et al., 1987), and stored at -80°C until use. The purity of a preparation was checked by evaluating the enrichment of the brush border membrane marker enzymes aminopeptidase N and alkaline phosphatase. Aminopeptidase N (EC 3.4.11.2) and alkaline phosphatase (EC 3.1.3.1) activities in the homogenate and in the BBMV preparation were determined as described previously (Giordana et al., 1982). Protein concentrations in the BBMV preparations were determined by the method of Bradford, using BSA as the standard (Bradford, 1976).

3.6 Protein binding assays
Recombinant solubilized, trypsin-activated Cry10Aa
toxin was biotinylated using biotinyl-N-hydroxysuccinimide ester (GE Helthcare) following the manufacturer's instructions. Binding of 10 nmol/L labelled toxin to 10 µg of A. grandis BBMV total protein was carried out in 100 µL binding buffer (1 × PBS, 0.1% BSA, 0.1% Tween 20, and pH 7.6). After 1 h at 25°C, unbound toxin was removed by centrifugation (10 min at 14,000×g). The pellet containing the BBMV with bound toxin was washed twice with 100 µL of the same buffer and finally suspended in 1X PBS, pH 7.6. An equal volume of 2X sample loading buffer (0.125 mol/L Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue) was added, samples were boiled for 3 min, the proteins separated in a SDS-PAGE gel (10%), transferred to a PVDF membrane, and the BBMV total protein in 100 µL binding buffer.

3.7 Bioassays

Five doses of recombinant Cry10Aa crystals purified by centrifugation in sucrose gradients (100 µg/mL, 50 µg/mL, 20 µg/mL, 10 µg/mL and 5 µg/mL), IPS82 strain (1.5 µg/mL, 1.0 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.1 µg/mL) and S1804 strain (1.5 µg/mL, 1.0 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.1 µg/mL) were separately added to A. grandis artificial diet as described by (Martins et al., 2010). Each protein dose was added to 5 mL of the artificial diet before it was poured out into six well cell culture plates (TPP, Techno Plastic Products AG, Switzerland). Five holes were punched in each well and each hole received a A. grandis neonate larva. The bioassay was kept in an incubator with photoperiod of 14 h/10 h (light/dark) at 27°C. Mortality was recorded seven days later, and the LC₅₀ obtained by Probit analysis (Finney, 1971).

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