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# Induction of a gloverin-like antimicrobial polypeptide in the sugarcane borer *Diatraea saccharalis* challenged by septic injury

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## Abstract

*Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) is an important pest for Brazilian sugarcane. In the present study, we detected two distinct spots in hemolymph from septic injured larvae (HDs1 and HDs2), which are separated by 2DE gel electrophoresis. Both spots were subjected to in-gel tryptic digestion and MALDI-TOF/TOF analysis, which revealed the sequence VFGTLGSDDSGLFGK present in both HDs1 and HDs2. This sequence had homology and 80% identity with specific Lepidoptera antimicrobial peptides called gloverins. Analyses using the ImageMaster 2D software showed pI 8.94 of the HDs1 spot, which is similar to that described to *Hyalophora gloveri* gloverin (pI 8.5). Moreover, the 14-kDa molecular mass of the spot HDs1 is compatible to that of gloverins isolated from the hemolymph of *Trichoplusia ni*, *Helicoverpa armigera* and *H. gloveri*. Antimicrobial assays with partially purified fractions containing the HDs1 and HDs2 polypeptides demonstrated activity against *Escherichia coli*. This is the first report of antimicrobial polypeptides in *D. saccharalis*, and the identification of these peptides may help in the generation of new strategies to control this pest.

Key words: *Diatraea saccharalis*; Lepidoptera; Immune response; Gloverin; Sugarcane borer; Hemolymph

## Introduction

Insects are evolutionarily successful organisms that occupy almost all habitats in nature. They are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites and they are able to react with an efficient immune response against these invaders (1). Over the last decades, innate immunity has been extensively studied in insects, with emphasis on insect pest management in agriculture, the discovery of bioactive molecules and the revelation of the evolutionary roots of innate immunity (2). One of the most studied mechanisms of innate immunity in insects is the production of antimicrobial peptides (AMPs), which contain less than 150-200 amino acids (3).

Since the first report of the innate immune response of *Hyalophora cecropia* (4) (Lepidoptera: Saturniidae), many taxa of insects have been the subject of studies of innate immune response to septic injury. Insects can produce a variety of AMPs in response to microbial infection or body

injury, and most of these peptides are synthesized by the fat body, which is a major insect tissue involved in the immune response (5). AMPs are secreted into the hemolymph and are responsible for the growth arrest of the microbe at an early stage in the immune response (6).

The molecular mechanisms of the insect's innate immune system have been studied using both molecular and proteomic methods (7,8). *Drosophila melanogaster* is a model organism, and there is a vast amount of information available on its genetics as well as the molecular mechanisms of its immune response (9). In contrast, only a limited number of studies applying genomic and proteomic techniques are available for some lepidopteran species, which include mainly the domesticated silkworm, *Bombyx mori*, *Galleria mellonella* and *Manduca sexta* (10). The recent availability of the *B. mori* genome sequence has considerably increased the possibility of using proteomics

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to investigate insect innate immunity (11-14).

In Brazil, the sugarcane borer *Diatraea saccharalis* (Fabricius, 1794; Lepidoptera: Crambidae) is a pest with great economic impact on the sugarcane crop, causing considerable damage to the sugar and alcohol industries (15). Several studies intended to develop new strategies for biological control have been conducted to identify the physiological changes resulting from host-parasitoid associations (16). Micro-hymenopteran parasitoids were first used for the biological control of *D. saccharalis* in the 1980's and they have become one of the most valuable biopesticides currently used in commercial sugarcane crop management in Brazil (17). Although biological agents can control *D. saccharalis* infestation, further research on new approaches to insect management and the knowledge of *D. saccharalis* antimicrobial defenses should improve the biological control of this important pest (18).

In this study, we have detected two distinct peptides in the hemolymph from *D. saccharalis* 5th-instar larvae after septic injury. To identify these polypeptides we performed antimicrobial activity assay, electrophoretic analysis, in-gel tryptic digestion, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/TOF), and bioinformatic analysis.

## Material and Methods

### Experimental *D. saccharalis* challenge and sample preparation

Sugarcane borer larvae were reared on an artificial diet (19) under controlled environmental conditions (12-h light/dark, 25 ± 1°C, 70% humidity). For immune challenge, the 5th-instar larvae were pierced with a long 27G dental needle dipped into a pellet (1:1) of viable *Escherichia coli* (ATCC strain 11229) and *Bacillus subtilis* (ATCC strain 6623). Prior to hemolymph collection, the insects were chilled for 15 min at 4°C, and surface sterilized with a 70% (v/v) ethanol solution. Fifty larvae of each experimental group (naive and septic injured) were used in the experiments. The hemolymph (about 20 µL from each larva) was collected 14 h after the immune challenge for polypeptide extraction. The samples were obtained by cutting off an abdominal proleg with sterile scissors. The outflowing hemolymph was collected with a micropipette and immediately transferred to a sterile chilled Eppendorf tube containing a few crystals of phenylthiourea to prevent melanization. Hemocyte-free hemolymph was obtained by centrifugation at 300 g for 10 min to pellet hemocytes and the supernatant was subsequently centrifuged at 10,000 g for 10 min at 4°C to pellet any cell debris (20). The cell-free hemolymph was boiled for 10 min, centrifuged at 10,000 g for 20 min at 4°C and the resulting supernatant was subjected to electrophoretic analysis (21).

### Isoelectric focusing and 2DE analysis

Total protein in the sample was quantified by the method

of Bradford (22) and 5 µg total protein was loaded onto 16% Tricine SDS-PAGE. Alternatively, the samples were concentrated through filter systems YM-50 and YM-10 (Microcon Millipore, Ireland). Samples that displayed an induced immune response expression profile were then used for isoelectric focusing (IEF) and two-dimensional gel electrophoresis (2DE). For IEF, 50 µg concentrated protein was mixed with 250 µL rehydration buffer (GE Healthcare, Sweden) and loaded onto an IPG strip (13 cm, pH 3-10, linear; GE Healthcare, Sweden) following manufacturer instructions. Rehydration and IEF were carried out on the IPGphor platform (GE Healthcare, USA) at 20°C and 50 µA/strip, according to the following protocol: Step 1: 200 V-200 Vhr; Step 2: 500 V-500 Vhr; Step 3: Gradient 1000 V-800 Vhr; Step 4: Gradient 8000 V-11,300 Vhr; Step 5: 8000 V-12,000 Vhr; with a total of 24,800 Vhr accumulated in the IEF. After IEF separation, the gels were incubated for 30 min in equilibration buffer (50 mM Tris-base, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) containing 1% DTT, followed by the equilibration buffer containing 2.5% iodoacetamide for an additional 30 min. The gel strip was subjected to 15% SDS-PAGE sealed with 0.5% agarose. SDS-PAGE was performed at a constant power of 5 W/gel for 45 min and switched to 15 W/gel until the bromophenol blue frontier reached the bottom of the gel. The 2DE gels were visualized by Coomassie colloidal staining. The gels were scanned at an optical resolution of 300 dpi on a high-resolution image scanner (GE Healthcare, USA), and the images were analyzed using the ImageMaster Platinum 2D v6.0 software (GE Healthcare, USA). For both naive and challenged samples of larva hemolymph, three 2DE gels were prepared and analyzed in order to determine the reproducibility of the data.

### Mass spectrometry

The HDs1 and HDs2 spots detected in the induced hemolymph samples were excised from the 2DE gels and subjected to in-gel digestion with sequencing-grade trypsin (Promega, USA) as described by Shevchenko et al. (23) with minor modifications. After incubation at 37°C for 16 h, aliquots of each hydrolyzed sample were mixed with a saturated matrix solution of  $\alpha$ -cyano-4-hydroxycinnamic acid, spotted onto MALDI target plates and allowed to air dry. Mass spectra (MS) were acquired using a MALDI-TOF/TOF Autoflex II spectrometer (Bruker Daltonics, Germany) that was operated at a laser frequency of 50 Hz. MS analysis was performed in a positive ion reflection mode. The voltage parameters were set at IS1 19 kV, IS2 16.8 kV, lens 8 kV, reflector 1 20 kV, and reflector 2 9.54 kV. The delay time was 70 ns, and the acquisition mass range was 800-3200 Da. External calibration was performed using a peptide mixture containing ACTH (residues 1-24), ACTH (residues 18-39), Somatostatin, angiotensin I and angiotensin II (all from Sigma, USA). MS/MS analyses were performed in a positive ion LIFT reflection mode. The voltage parameters

were set at IS1 6 kV, IS2 5.3 kV, lens 3.15 kV, reflector 1 23.5 kV, reflector 2 9.7 kV, LIFT 1 19 kV, and LIFT 2 4 kV. The acquisition mass range was 40–2400 Da. Peak lists were created using the FlexAnalysis 3.0 software (Bruker Daltonics). The sophisticated numerical annotation procedure algorithm was used to detect the monoisotopic peak values, with a quality factor threshold of 30. The S/N threshold was set at 6 for MS analysis and at 3 for MS/MS analysis. Automated *de novo* sequencing was performed using the Peaks Studio software 4.5 Demo version (Bioinformatics Solutions, Canada). The parent ion mass tolerance was 50 ppm, carbamidomethylation of cysteines was set at fixed modification, fragment error was set at 0.3 Da, and all ion charge states were set as +1.

### Antimicrobial assay

Antimicrobial activity was monitored by a liquid growth inhibition assay against *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli* SBS363, and *Candida albicans* MDM8 using a procedure described by Silva Jr. et al. (24), and the microorganisms tested were obtained as previously described (24,25).

## Results and Discussion

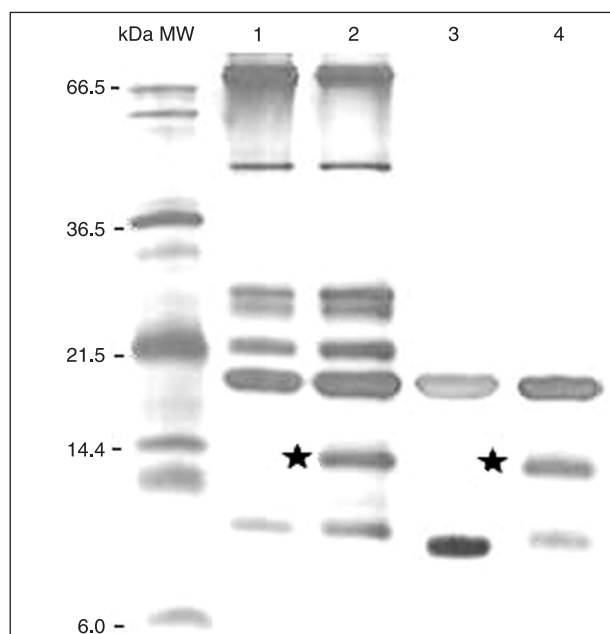
### Bacterial challenge induced a 14-kDa polypeptide in *D. saccharalis*

The 1DE analysis with hemolymph from bacterial-challenged larvae showed the presence of an approximately 14-kDa polypeptide that was not observed in naive larvae (Figure 1, lanes 1 and 2). After the pre-purification processes

with the filter systems YM-50 and YM-10 (Microcon Millipore; Figure 1, lanes 3 and 4), the polypeptide of approximately 14 kDa was detected in challenged larva hemolymph samples. When these samples were separated on 2DE gels (Figure 2A and B), two spots of different pIs at approximately 14 kDa were present only in hemolymph samples from challenged insects and were denoted HDs1 and HDs2 (Figure 2B). Both HDs1 and HDs2 were subjected to in-gel tryptic digestion and MALDI-TOF analysis, and the MS spectra were composed of 11 and 8 m/z monoisotopic peaks, respectively (Table 1). All peaks found in the HDs2 spectra were also found in the HDs1 spectra (molecular mass error <0.1 Da). Thus, the HDs1 and HDs2 fractions probably represent the same polypeptide and the differences observed in pI values suggest a putative post-translational modification. Recently, Mrinal and Nagaraju (26) reported that the evolutionary genetic mechanisms originated four isoforms of gloverins in *Bombyx mori*, *Bmg1v1*, *Bmg1v2*, *Bmg1v3*, and *Bmg1v4*. These different isoforms have a molecular mass of 19 kDa; however, no reports on biochemical aspects, such as the isoelectric point, are available. In contrast to being the result of post-translational modification, the two proteins described here could be the result of gene duplication events in which different alleles produce isoforms of gloverin-like proteins.

### The sequencing and antimicrobial assay showed a gloverin-like polypeptide

In order to identify the HDs1/HDs2 polypeptides, the tryptic peptides of HDs1 and HDs2 were subjected to MS/MS analysis (Table 1). No positive hits were observed on



**Figure 1.** One-dimensional gel electrophoretic (1DE) protein analysis of hemolymph from *Diatraea saccharalis* larva. Lane 1, Naive hemolymph; lane 2, induced hemolymph collected 14 h after septic injury; lanes 3 and 4, naive and induced hemolymph concentrated by Microcon YM-50 and YM-10 filters. Black stars indicate an approximately 14-kDa fraction, which is expressed in the septic injury-induced hemolymph. MW, molecular mass marker (Marker 12, Invitrogen, USA). 16% Tricine SDS-PAGE visualized by silver staining.



MS/MS spectra submitted to Mascot ion search using the NCBI database (12/03/2009). The MS/MS data were subjected to automated *de novo* sequencing using the Peaks 4.5 software. Only one peptide (m/z of 1499.682) generated an MS/MS spectrum that allowed *de novo* sequencing with confidence higher than 50%, yielding the sequence VFGTLGSDSGLFGK for both HDs1 and HDs2 (Table 1, Figure 3). These sequences were confirmed by manual inspection of the MS/MS data. A protein homology search of the NCBI database using the BLASTP 2.2.18+ algorithm adjusted to search for a short input sequence showed homology of the peptide with gloverins from diverse species of Lepidoptera. Gloverins are antibacterial proteins

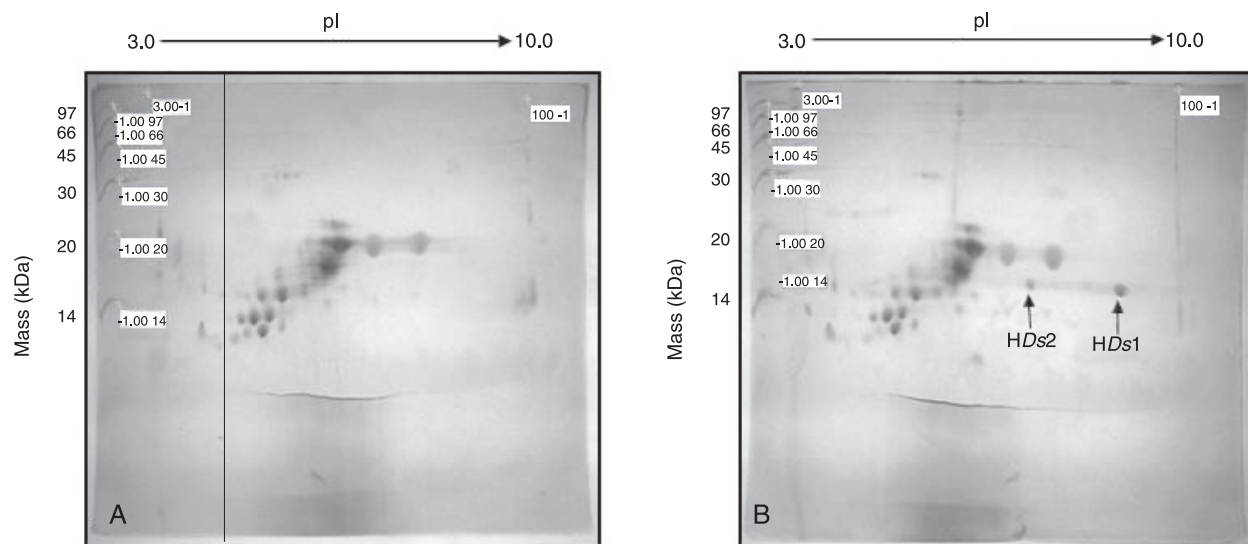
ranging in size from 8 to 30 kDa and along with other small peptides, such as cecropins and defensins, are components of the immune response in Lepidoptera (27). The approximately 14-kDa molecular mass of the HDs1 polypeptide is similar to that of gloverins isolated from the hemolymph of *Trichoplusia ni*, *Helicoverpa armigera* and *Hyalophora gloveri* (27-29). The pI of the HDs1 polypeptide (pI 8.94) is also similar to that described for *H. gloveri* gloverin (pI 8.5) (29). Gloverins are generally basic, heat-stable, glycine-rich (16-20%) proteins that act by inhibiting the synthesis of the outer membrane proteins, which would result in increased membrane permeability to Gram-negative bacteria (29).

Antimicrobial activity assays were carried out using

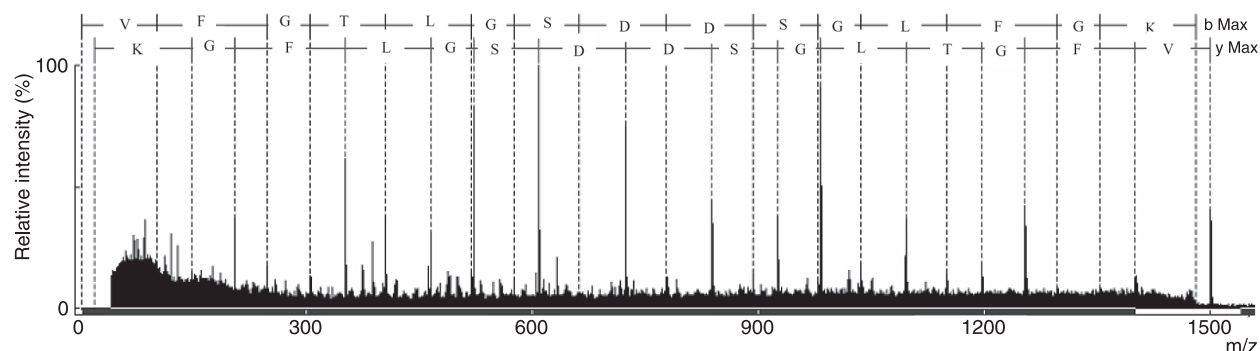
**Table 1.** Hemolymph polypeptides from induced *Diatraea saccharalis* larvae.

Spot	Mass (kDa)	pI	m/z of tryptic peptides in MS spectra <sup>a</sup>
HDs1	14	8.94	892.306; 979.422; 1302.687*; 1362.678*; 1427.661*; 1499.759* (VFGTLGSDSGLFGK) - 99%; 1513.769; 1521.741*; 1612.806; 2288.310; 3058.829
HDs2	14	7.27	892.256; 979.394; 1302.638*; 1362.620*; 1427.601*; 1499.682* (VFGTLGSDSGLFGK) - 77%; 1612.741; 3058.809

Molecular mass (kDa) was obtained by comparison of mobility with globular proteins (14 to 97 kDa low molecular weight calibration marker, GE Healthcare, USA) and pI by assuming IEF strips are linear (pI 3-10) - both with the ImageMaster Platinum 2D v6.0 software. <sup>a</sup>Monoisotopic m/z list was generated using FlexAnalysis 3.0 (Bruker Daltonics) but sequences were not obtained, except those shown in the Table. \*Peptides subjected to MALDI-TOF/TOF MS/MS analysis. The sequence of the peptide of m/z 1499 indicated in the table was obtained after MS/MS and automatic *de novo* sequencing using the Peaks Studio Demo version software (Bioinformatics Solutions, Canada). The number after the sequence indicates the percent confidence for that sequence being correct.



**Figure 2.** Two-dimensional gel electrophoretic (2DE) analysis of proteins from *Diatraea saccharalis* larva hemolymph. Partially purified samples, as shown in Figure 1, lanes 3 and 4, were resolved by 2DE gels. *A*, Sample from naive larva hemolymph; *B*, sample from septic injury-induced larva hemolymph. The polypeptide spots with molecular mass of approximately 14 kDa, HDs1 and HDs2, are indicated by arrows. The HDs1 and HDs2 spots were excised, submitted to tryptic digestion and analyzed by MALDI-TOF/TOF (Table 1). 15% Tris-glycine SDS-PAGE visualized by colloidal Coomassie staining; pI 3-10; 14 to 97 kDa low molecular weight calibration marker (GE Healthcare, USA).



**Figure 3.** MS/MS mass spectrum of the parental peptide 1499.682 (m/z) derived from trypsin digestion of spot HDs1. The mass spectrum and the *de novo* interpretation of the data were generated using the Peak 4.5 software. The y and b ion series shown confirm the VFGLGSDDSGFLFGK peptide sequence.

samples that were partially purified through the filter systems (Figure 1, lanes 3 and 4). The antimicrobial activity of the induced sample was efficient against *E. coli* (Gram-negative) growth. However, both the naive and induced samples were inactive against the Gram-positive bacterium *M. luteus* and the yeast *Candida albicans* (Table 2). Although in this study we could not obtain data on minimum inhibitory concentration, the activity against a Gram-negative bacterium, amino acid sequence and biochemical data (pI and molecular mass) support the classification of HDs1 as a gloverin-like polypeptide.

These results indicate that the polypeptide HDs1 may be involved in the innate immune response of *D. saccharalis*. Although several AMPs have been described in Lepidoptera, this is the first report of an immune polypeptide in this important sugarcane culture pest. Recent studies have shown that control strategies combined with fungi and nematodes are effective in controlling *D. saccharalis* (30,31). However, the action of these biological agents in the expression of antimicrobial peptides, as described for the Lepidoptera *Spodoptera exigua* when infected with *Xenorhabdus nematophila*, is unknown in *D. saccharalis* (32).

Therefore, a better understanding of the immune response of *D. saccharalis* will help with the development of new strategies to control this pest. Future RT-PCR experiments will be carried out using the sequence of the gloverin-like polypeptide in order to clone the gene. Thereafter, we will analyze gene expression in *D. saccharalis*

**Table 2.** Antimicrobial assay with *Diatraea saccharalis* polypeptides partially purified from naive and induced larvae hemolymph.

Microorganisms	Naive hemolymph	Induced hemolymph
<i>Escherichia coli</i> SBS363	+	-
<i>Micrococcus luteus</i>	+	+
<i>Bacillus subtilis</i>	+	+
<i>Candida albicans</i> MDM8	+	+

The hemolymph samples were concentrated through filter systems YM-50 and YM-10 (Microcon Millipore, Ireland) and lyophilized for the antimicrobial assay. A 10- $\mu$ L aliquot of the samples was dissolved in sterile Milli-Q water and applied to each well containing the microorganisms tested. + = no inhibition of growth; - = inhibition of growth.

and its possible applications in methods to control this sugarcane pest.

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