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Antimicrobial peptides expressed in the fat body of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in response to the microbial challenge

Peptídeos antimicrobianos expressos no corpo gorduroso de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) em resposta ao desafio microbiano

Péptidos antimicrobianos expresados en el cuerpo graso de Spodoptera frugiperda (Lepidoptera: Noctuidae) en respuesta al desafío microbiano

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Elisiane Pinto Feistler
ORCID: https://orcid.org/0000-0001-6012-525X
State University of Western Paraná, Brazil
E-mail: elisianeblog@gmail.com
Alexandre Maller
ORCID: https://orcid.org/0000-0002-8065-9184
State University of Western Paraná, Brazil
E-mail: alexandre.maller@unioeste.br
Rita de Cássia Garcia Simão
ORCID: https://orcid.org/0000-0003-2999-1218
State University of Western Paraná, Brazil
E-mail: rita.simao@unioeste.br
Marina Kimiko Kadowaki
ORCID: https://orcid.org/0000-0002-9174-5811
State University of Western Paraná, Brazil
E-mail: marinakk@unioeste.br
Luis Francisco Angeli Alves
ORCID: https://orcid.org/0000-0002-3102-764X
State University of Western Paraná, Brazil
E-mail: luis.alves@unioeste.br
José Luis da Conceição Silva
ORCID: https://orcid.org/0000-0002-5460-8797
State University of Western Paraná, Brazil
E-mail: jose.silva3@unioeste.br

Abstract
Spodoptera frugiperda (Lepidoptera: Noctuidae) is the key pest in maize crops and has a major impact on world agriculture. Several studies on the biological control of this species have been carried out, and these demonstrate resistance to biological control and pesticides. However, the humoral immune system of *S. frugiperda* is not yet completely known in relation to the innate immune response to biological control agents. The aim of this study was to evaluate the expression of *Gloverin*, *Sf-gallerimycin*, and *Attacin* genes in the fat body of larvae at the 6th instar of development. Our work confirmed the expression of *Sf-gallerimycin* in response to the bacterial challenge, 24 h post-inoculation, and revealed the expression of *Gloverin* and *Attacin* for the first time for both bacterial challenge and entomopathogenic fungus *Beauveria bassiana* challenge. *Gloverin* and *Attacin* genes were upregulated under the conditions analyzed. In addition, we revealed the presence of two probable antimicrobial peptides in the hemolymph, induced 24 h post challenge with the microorganisms. The 4.7 kDa band *b* is probably a *defensin-like* peptide, and the 6.1 kDa band *a* is a peptide not yet reported in *S. frugiperda.*
Keywords: Antimicrobial peptides; *Beauveria bassiana;* Fat body; Immune system; Gene expression.

Resumo
Spodoptera frugiperda (Lepidoptera: Noctuidae) é a principal praga da cultura do milho e tem grande impacto na agricultura mundial. Vários estudos sobre o controle biológico desta espécie têm sido realizados, e estes demonstram resistência ao controle biológico e agrotóxicos. No entanto, o sistema imune humoral de *S. frugiperda* ainda não é completamente conhecido em relação à resposta imune inata para agentes de controle biológico. O objetivo deste estudo foi avaliar a expressão dos genes *Gloverin*, *Sf-galerimycin* e *Attacin* no corpo gorduroso de larvas no 6º instar de desenvolvimento. Nosso trabalho confirmou a expressão de *Sf-galerimicina* em resposta ao desafio bacteriano, 24 h após

a inoculação, e revelou pela primeira vez a expressão de *Gloverin*a e *Attacina*, tanto com o desafio bacteriano quanto com o fungo entomopatogênico *Beauveria bassiana*. Os genes *Gloverina* e *Attacina* foram regulados positivamente nas condições analisadas. Além disso, revelamos a presença de dois prováveis peptídeos antimicrobianos na hemolinfa, induzidos 24 h após o desafio com os microrganismos. A banda b de 4,7 kDa é provavelmente um peptídeo do tipo defensina, e a banda a de 6,1 kDa é um peptídeo ainda não relatado em *S. frugiperda*.
Palavras-chave: Peptídeos antimicrobianos; Beauveria bassiana; Corpo gordo; Sistema imunológico; Expressão genética.

Resumen
Spodoptera frugiperda (Lepidoptera: Noctuidae) es la principal plaga del maíz y tiene un gran impacto en la agricultura mundial. Se han realizado varios estudios sobre el control biológico de esta especie, y estos demuestran resistencia al control biológico y plaguicidas. Sin embargo, el sistema inmune humoral de S. frugiperda aún no se comprende completamente en relación con la respuesta inmune innata a los agentes de control biológico. El objetivo de este trabajo fue evaluar la expresión de los genes *Gloverina*, *Sf-galerimicina* y *Attacina* en el cuerpo graso de larvas en el sexto estadio de desarrollo. Nuestro trabajo confirmó la expresión de *Sf-galerimicina* en respuesta al desafío bacteriano, 24 h después de la inoculación, y reveló por primera vez la expresión de *Gloverina* y *Attacina*, tanto con el desafío bacteriano como con el hongo entomopatógeno *Beauveria bassiana*. Los genes de *Gloverina* y *Attacina* aumentaron en las condiciones analizadas. Además, revelamos la presencia de dos probables péptidos antimicrobianos en hemolinfa, inducidos 24 h después del desafío con microorganismos. La banda b de 4,7 kDa es probablemente un péptido similar a la defensina, y la banda a de 6,1 kDa es un péptido aún no informado en *S. frugiperda*.
Palabras clave: Péptidos antimicrobianos; *Beauveria bassiana*; Cuerpo gordo; Sistema inmunitario; Expresion genica.

1. Introduction

Food cultivated in the world suffers attacks from pests, which cause approximately one third of food production losses during the process of growth, harvesting, and storage. Insects are a prominent group of organisms that attack crops, in addition to other small animals (Sarmento, *et al*., 2002). The attack of pests is intensified due to the biological imbalance caused by the elimination of natural enemies (Ribeiro, *et al*., 2016).

The corn culture (*Zea mays*) that has economic and social prominence (Melo, *et al.*, 2006) suffers attacks during almost the entire period of its development (Peterlini, *et al*., 2020), causing direct damage to plants (Wangen, *et al.*, 2015).

Although biological agents can help to control these insect pests, insecticides are now essential for effective and economical pest control on a large scale. The non-target effect of some pesticides is partly due to their effects on insect immunity, which is necessary for insect survival in natural environments (Casanova & Goodrich, 2013). In the defense against pathogens, insects depend mainly on their immune system (Viljakaínen, 2015).

In insects, the innate immune system is the first line of defense against invading pathogens. Although innate immune responses are nonspecific, they are widely distributed throughout the insect, allowing them to play a crucial role in maintaining homeostasis and preventing disease and infection (Sheehan, *et al.*, 2018). The immune response depends on several humoral and cellular factors, occurring both locally and systemically (Destoumieux, *et al.*, 2009).

Antimicrobial peptides (AMPs) are short proteins with antimicrobial activity (Pinto *et al.*, 2015), widely distributed among living organisms (Duvic *et al.*, 2012). Insect AMPs (humoral responses) play an important role in eliminating pathogens and parasites. These peptides are synthesized in specific tissues, such as the fat body and hemocytes; after their production, AMPs are rapidly excreted into the insect's hemolymph, performing an antimicrobial function (Bulet, *et al.*, 1999). They can potentially be applied in medicine and agriculture instead of traditional antibiotics, as they are easy to synthesize due to their relatively small size, have a fast and efficient action against pathogens, provide a wide range of antimicrobial activity, and have low toxicity to cells of vertebrates (Bang, *et al.*, 2012).

Most of these AMPs have been identified from insect hemolymphs using molecular and proteomic methods, such as mass spectrometry or cDNA cloning (Silva, *et al.*, 2010; Koehbach, 2017). Some researchers have used techniques to challenge insects in order to enhance the innate immune response (Charles & Killian, 2015).

The insect model of the study is the cartridge caterpillar, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), which is the main pest of the corn culture in Brazil. The *S. frugiperda* caterpillar has been studied using entomopathogenic fungi for biological control (Thomazoni, *et al.*, 2014); however, knowledge of the innate immune response of this species to entomopathogenic fungi is still scarce.

Recently the complete genome of this species has been elucidated (Kakumani, *et al.*, 2014), becoming a model organism for molecular studies, which allows the use of molecular techniques to analyze the expression of AMPs.

The aim of this work was to evaluate the expression of the genes of AMPs in *S. frugiperda*: *Gloverin*, *Sf-Gallerimycin* and *Attacin*. For the first time, the *Gloverin* and *Attacin* are revealed *in vivo* in corn borer larvae challenged by non-pathogenic bacteria and the fungus *Beauveria bassiana*. In addition, two peptides were revealed in the hemolymph in response to the septic challenge.

2. Methodology

2.1 Maintenance of cultivation, immune challenge and sample collection

Larvae of *S. frugiperda* were kept on an artificial diet at 27 ± 1 °C and 75 ± 5% relative humidity with a 16L:8D photoperiod. The entomopathogenic fungus *Beauveria bassiana* was maintained according to Thomazoni *et al.*, (2013), and the bacteria *Escherichia coli* ATCC 11229 and *Bacillus subtilis* ATCC 6623 were used in the challenge bacteria mix.

Sixth instar larvae were distributed in three groups: unchallenged (n=30), challenged with a mix (Gram-/Gram+) of bacteria (n=30), and challenged with *Beauveria bassiana* (n=30). The septic injury was performed by perforating the integument with a dental needle immersed in the pellets of the microorganisms. Samples were collected after 24 hours post-inoculation (24 h pi) at a controlled temperature of 25 ± 1 °C (Silva *et al.*, 2010).

Larvae were cleaned with 70% ethanol, and the hemolymphs were collected in microtubes containing phenylthiurea crystals (C_7HN_2S). After the material was centrifuged at 200 xg (1450 rpm) for 5 min at 4 °C, the supernatant was transferred to another tube, which was centrifuged at 20,000 xg (14,500 rpm) for 15 min at 4 °C, and the samples were kept at -20 °C until use. The fat body was dissected in 0.9% saline solution containing RNAse inhibitor and stored in microtubes with 250 µL of RNA latter® solution (Ambion) at 4 °C. The collected material was centrifuged at 400 xg (2,900 rpm) for 5 min at 4 °C. The supernatant was discarded, and the material was frozen at -20 °C.

2.2 Sample extraction, dosage and RT-PCR

The proteins from the hemolymph were extracted according to Silva *et al.*, (2010), and the dosage was performed according to the method of Bradford (Bradford, 1976).

TRIzol® reagent (Invitrogen®, EUA) was used to extract the total RNA from the fat body. The total RNA obtained was quantified by spectrophotometry at 260 nm, and the samples were then subjected to treatment with DNAse I, according to the manufacturer's protocol.

The cDNA synthesis was performed according to the instructions in the ImProm-II kit (Promega®). The primers for amplification of the PAM genes were synthesized by the company IDT DNA Technologies (Iowa/USA) by semi-quantitative RT-PCR and are shown in Table 1.

Table 1: Primers designed for the detection of AMPs in *S. frugiperda*.

Primers	Primer sequence	Amplicon pb
Attacin	F 5'GAC CAC CTG CCA TAC GAA CA 3' R 5'GGT TGT CGT TGT GGA ACA CG 3'	397 pb
Gloverin	F 5'GTA CTC CAA GTC AGT CCG CC3' R 5'AAG TTG CTG CTG TCT CCG TT3'	210 pb
Sf-Gallerimycin	F 5'AAG GTT TCA GTC ATG AAG GCT TGC3' R 5'TAC AAA CAT GGC AAG ATG GAG AGC3'	274 pb
Actin	F 5'ACT GCA GCG TGA CAT CAA GA3' R 5'CCG AAT ACT GGG TTG TGC CT3'	243 pb

F: Forward; R: Reverse; pb: base pairs. Source: Authors.

The *Sf-Gallerimycin* primer was used as described by Volkof *et al*., (2003), and the primer for Actin was used as a control of gene expression (Kakumani *et al*., 2014). The PCR reaction was performed with the Taq polymerase enzyme, and the PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

2.3 Analysis of samples by electrophoresis and quantification of expression

The visualization of the RT-PCR amplicons was performed on 1.5% agarose gel, and the electrophoresis of protein samples was performed in Tricine SDS-PAGE 16% (Schägger, 2006).

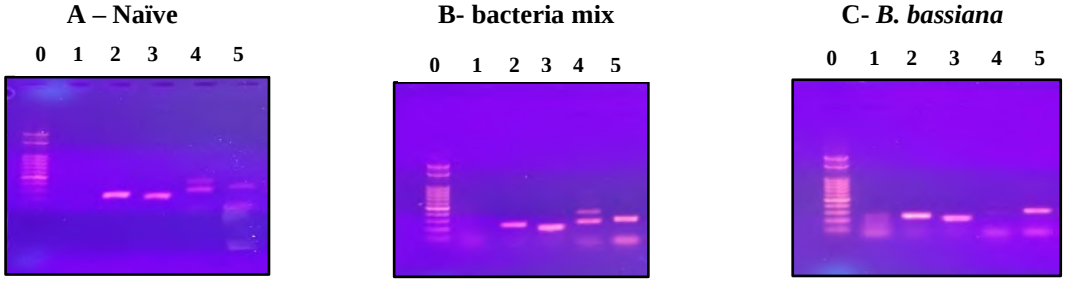
The ImageJ software (free software) was used for the densitometric quantification of the bands of differential expression in RT-PCR and the protein bands of the gels of Tricine SDS-PAGE. In the RT-PCR, we used the 100 bp Ludwig standard (0.1 µg/µL), using the 500 bp band (90 ng/5 µL) as a reference to determine the concentration of the AMP bands. In the Tricine SDS-PAGE, we used the area measurement of the bands to produce a correlation of the AMP expressions.

3. Results

3.1 AMPs expressed in the fat body of *S. frugiperda* challenged by septic injury

The fat body is the organ that produces AMPs in response to the septic challenge (Bulet *et al*., 1999). In our work, the study of PAM expression was carried out in sixth instar larvae challenged by microorganisms. The result of the entire process of extracting total RNA from the fat body and expression of the AMP genes by semi-quantitative RT-PCR can be seen in Figure 1.

Figure 1. Conventional RT-PCR of antimicrobial peptide gene expressed in the fat body. Lane 0 = Ladder 100 bp (Ludwig biotec). Lane 1 = Total RNA treated with DNAse and amplified by PCR with *Actin* primer; Lane 2 = *Actin* control gene fragment 243 bp; Lane 3 = *Gloverin* gene fragment 210 bp; Lane 4 = *sf-Gallerimycin* gene fragment 274 bp and Lane 5 = *Attacin gene* fragment 397 bp.



Source: Authors.

In lane 1 of all gels (A, B, and C), we have the total RNA treated with DNase I amplified by PCR with the actin primer to control contamination with genomic DNA. In lane 2, we have the amplicon from the RT-PCR of the actin gene (housekeeping). The *actin* gene expression was calculated in relation to the 500 bp band of the standard with the ImageJ software and served as a comparison in the analysis of the AMP genes.

In lane 3, we have the *Gloverin* gene with 210 bp amplicon, where the increased expression is seen in Figure 1-B compared to the actin control in Figure 1-B and 1-A. *Gloverin* expression was 1.4x or 40% upregulated compared to the actin gene in assay 1-B.

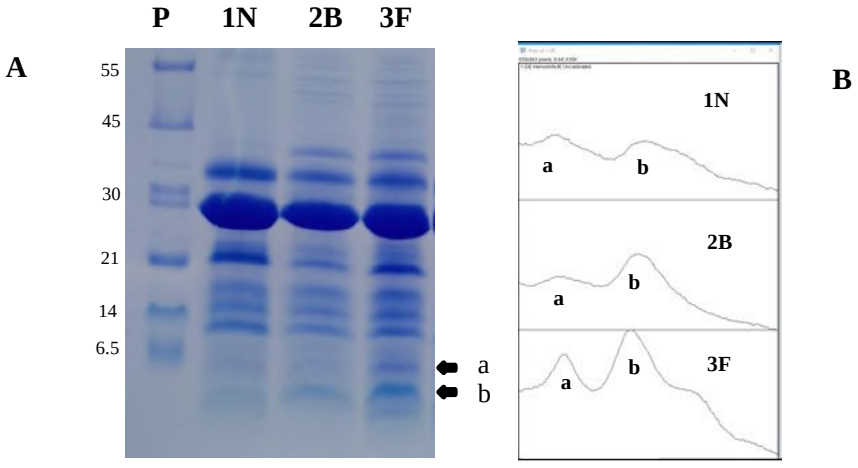
In lane 4 of the gels, we have the amplicon of the *Sf-Gallerimycin* gene (274 bp), where we can observe a nonspecific band, larger than the expected amplicon size; however, the gene was clearly induced (1.10x or 10.48% upregulated) in the challenge with the bacteria mix (1-B). In contrast, the *Sf-Gallerimycin* gene was downregulated in the challenge with the fungus *B. bassiana* (1-C).

In lane 5, we have the amplicon of the *Attacin* gene (397 bp), where we can see an increase in expression in 1-B and 1-C compared to 1-A (not challenged). In assay 1-B, the *Attacin* gene was 1.45x or 45% more expressed than the *Actin* (1-B control); in assay 1-C, it was 2.20x or 45.40% downregulated compared to the *Actin* (1-C control).

3.2 AMPs in the *S. frugiperda* hemolymph challenged by septic injury

We analyzed the presence of AMPs in the hemolymph, 24 h after the septic challenge. In Figure 2, the presence of AMPs was observed in samples 1N (native/unchallenged), 2B (challenged bacteria mix), and 3F (challenged by *B. bassiana*). In this assay, an increase in the concentration of AMPs was detected in the challenged samples compared to the native sample. The bands with molecular mass below 6.5 kDa are indicated by the arrow in Figure 2-A. The band *a* had a relative molecular mass of 6.1 kDa, and band *b* 4.7 kDa; both calculated through relative migration in the gel compared to the molecular mass standard.

Figure 2. 2-A. Electrophoresis in Tricine SDS-PAGE 16%. Lane P- Amersham Biosciences® molecular weight marker standard. Lane 1N: 50 µg of hemolymph from naive larvae; Lane 2B: 50 µg of hemolymph from challenged larvae with mix (Gram+/Gram-), Lane 3F: 50 µg of hemolymph from challenged larvae by *B. bassiana*. 2-B. ImageJ analysis of peptide *bands a* and *b* indicated by arrows in 2-A.



Source: Authors.

Figure 2-B shows the quantification with the ImageJ software, which shows an increase in the area measurement of bands *a* and *b*, mainly in the challenge with the fungus *B. bassiana*, in relation to the native sample. *B. bassiana* challenged the *band b* had an area of 14484.350 in comparation to the area (6539.945) of the native sample, representing 2.21x more expressed.

4. Discussion

One of the first lepidopterans to have its immune system studied was the moth *Hyalophora cecropia*. In *H. cecropia*, glycine rich AMPs were identified (Hultmark *et al*., 1983; Axén, *et al.*, 1997). From these initial findings, AMPs of this class were found in several other Lepidoptera, highlighting *Bombyx mori* (Tanaka *et al*., 2008).

In *Spodoptera exigua*, it was reported that *Gloverin* is important for resistance against *Bacillus thuringiensis*, and *Gloverin* expression was induced in a septic challenge with *Serratia marcescens* (Hwang & Kim, 2011; De Mandal, *et al.*, 2020).

In 2003, the presence of AMPs similar to the class of defensins in *S. frugiperda* were identified (Volkoff *et al.*, 2003), and Gloverin-3 expression was recently reported in *S. frugiperda* challenged by Ascovirus (Zaghloul, *et al.*, 2020). However, in the septic challenge of *S. frugiperda* by non-pathogenic bacteria and the entomopathogenic fungus *B. bassiana*, no *Gloverin* has been reported to date. In our work, although we have basal expression in native larvae, we have shown the induction of *Gloverin* expression with the septic challenge using the mix of bacteria and *B. bassiana.*

Attacins are mainly active against bacteria (Gram-) and have been described in *S. exigua* (Bang *et al*., 2012). The expression of *Attacin* and *Gloverin* were suppressed by infection with Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) in *S. exigua* (Choi *et al.*, 2012). In our work, we observed the induction of *Attacin* in the challenge with fungus and the mix of bacteria (Gram+/Gram-), indicating that this AMP is an important effector of the innate immune system. *Sf-Gallerimycin* was induced by the challenge of the mix of bacteria and suppressed in the challenge with *B. bassiana* after 24 h of inoculation. This AMP is active against bacteria and fungi, as reported by Volkof *et al*., (2003), and our work confirmed that induction by the fungus is a later response.

The peptides present in the hemolymph are products of the fat body and hemocytes, in response to the immune challenge. Our work revealed the presence of AMPs that were induced; however, they were not identified with the applied methodologies. The 4.7 kDa band *b* is probably a defensin-like peptide, which has a molecular mass of 4 kDa and is active against bacteria and fungi (Marshall & Arenas, 2003). Based on its molecular mass, the 6.1 kDa band *a* is a peptide not yet reported in *S. frugiperda*. These are PAM candidates that still need to be isolated and identified to understand their role in the immune response of *S. frugiperda*.

5. Conclusion

In our work, it was shown that microbial challenges induced the humoral immune response of the *S. frugiperda* larvae as observed in the RT-PCR assay for the analyzed genes. The induction of low molecular weight antimicrobial peptides was demonstrated in the hemolymph of challenged larvae as shown on SDS-PAGE. This study shows for the first time the induction of the *Attacin* and *Gloverin* genes in microbial challenge, in addition to pointing out possible antimicrobial peptides in the hemolymph of *S. frugiperda*.

However, the results of this article indicate that a research effort is needed in this area of insect immunology to understand its biology and then possibly we will have better perspectives for the control of insects and agricultural pests.

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