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# Molecular characterization of a defensin in the IZD-MB-0503 cell line derived from immunocytes of the insect *Mamestra brassicae* (Lepidoptera)

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**Abstract**

The induction of anti-microbial peptides against Gram positive and negative bacteria in the IZD-MB-0503 cell line from the lepidopteran *Mamestra brassicae* is demonstrated, while no anti-fungal activity is detected. The identification of a defensin-like molecule active against Gram positive bacteria is described for the first time in Lepidoptera. This molecule shows between 43% and 59% homology with group A defensins from other dipteran and hymenopteran species.



*Keywords:* IZD-MB-0503 cell line; *Mamestra brassicae*; Lepidoptera; Defensin gene; Immune response

## 1. Introduction

The literature on the presence of anti-bacterial peptides and polypeptides in fighting micro-organisms from invertebrates, in particular, insects, is extensive. These humoral components, known as attacins, cecropins, defensins, drosomicin, thanatin, etc., differ in their specific activities (Hetru et al., 1998).

The main production site of insect anti-bacterial peptides is the fat body, a functional equivalent of the mammalian liver (Fehlbaum et al., 1994). However, in response to bacteria and fungi, immunocytes are activated, resulting in the synthesis of a wide array of anti-bacterial peptides (Hultmark, 1993; Hoffmann, 1992), in addition to nodulation and encapsulation responses (Salt, 1970; Vass and Nappi, 2001).

Defensins are 4 kDa cationic peptides with a characteristic six cysteine/three disulfide bridge pattern and three domains, a flexible amino-terminal loop, a central α-helix and a carboxy-terminal anti-parallel β-sheet (Hanzawa et al., 1990; Bonmatin et al., 1992; Bulet et al., 1999).

Various papers have reported the isolation and description of anti-microbial defensins in different insects, but not in Lepidoptera (Chalk et al., 1995; Lowenberger et al., 1995; Cho et al., 1996, 1997; Miyanoshita et al., 1996; Richman et al., 1996; Dimopoulos et al., 1997; Gao et al., 1999; Muller et al., 1999; Eggleston et al., 2000). The only data available on defensin in Lepidoptera concern *Heliothis virescens*, in which the inducible molecule, heliomicin, has been found. Heliomicin exhibits anti-fungal activity and shows similarities with anti-bacterial insect defensins (Lamberty et al., 1999).

In the present paper, we describe, for the first time, a "classical", inducible defensin molecule active against Gram positive bacteria in the lepidopteran species, *Mamestra brassicae*.

## 2. Results

The anti-bacterial tests were performed using the conditioned medium obtained by exposing *M. brassicae* IZD-MB-0503 cell line to the Gram positive *S. aureus*. The medium completely inhibited the growth of *S. aureus* and this anti-bacterial effect was maintained for 72 h (Fig. 1A). When diluted (1:2), the conditioned medium inhibited bacterial growth for 7 h (Fig. 1A). The same result was obtained using the conditioned medium obtained from cells exposed to the Gram negative *E. coli* (Fig. 1B). Incubating *S. aureus* or

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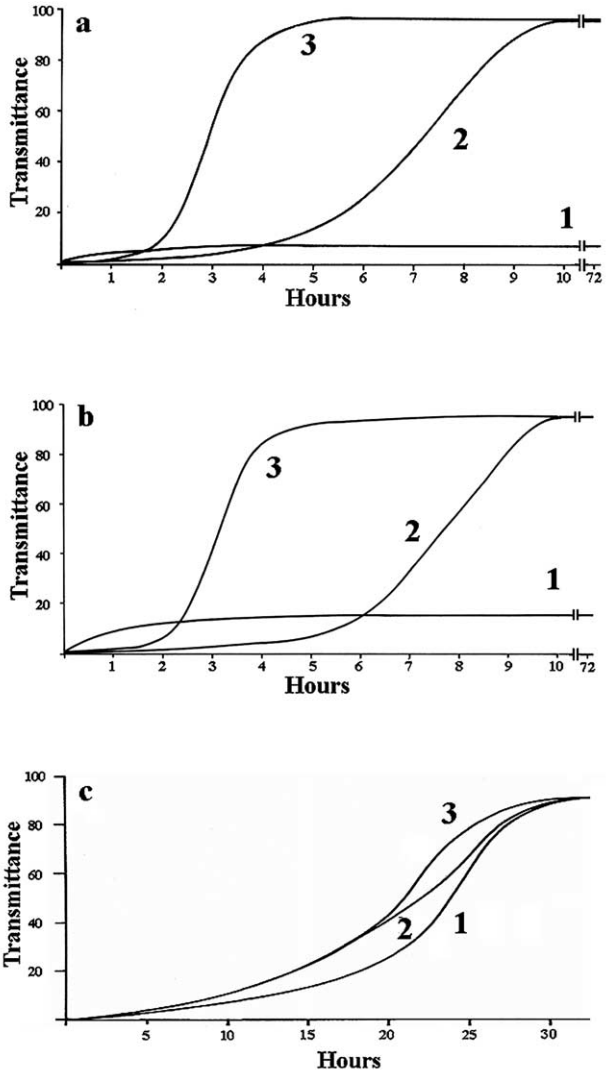


Fig. 1. (A) *S. aureus* growth in the presence of undiluted (1) and diluted (1:2) (2) conditioned medium. Bacterial growth in Ex-Cell 405 medium (control) (3). (B) *E. coli* growth in the presence of undiluted (1) and diluted (1:2) (2) conditioned medium. Bacterial growth in Ex-Cell 405 medium (control) (3). (C) *S. cerevisiae* growth in the presence of undiluted (1) and diluted (1:2) (2) conditioned medium. Fungal growth in Ex-Cell 405 medium (control) (3).

*E. coli* with a conditioned medium obtained from cells exposed to *E. coli* or *S. aureus*, respectively, gave the same picture. In both the cases, anti-bacterial activity was observed (data not shown). The conditioned medium obtained from insect cells exposed to *S. aureus* was devoid of any anti-fungal activity against both *C. albicans* (data not shown) and *S. cerevisiae* (Fig. 1C).

PCR amplification of an *M. brassicae* anti-microbial peptide gene evidenced a fragment of 280 bp, corresponding to a central portion of the gene encoding a defensin molecule. The gene sequence was completed by inverse PCR showing that the *M. brassicae* defensin gene consists of 294 bp (Fig. 2). Moreover, the absence of untranscribed regions was confirmed by RACE.

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atgctgtgcctcgctgacattcgtatcgtggcttcgtgttccgcc
M  L  C  L  A  D  I  R  I  V  A  S  C  S  A
gccattaagagtggatacggacagcaaccgtggctggcccacgtt
A  I  K  S  G  Y  G  Q  Q  P  W  L  A  H  V
gcaggcccttatgccaactctctattcgatgatgtgcccgcggat
A  G  P  Y  A  N  S  L  F  D  D  V  P  A  D
agctatcacgcggccgtcgagtacttgcgcctgatacccgccagt
S  Y  H  A  A  V  E  Y  L  R  L  I  P  A  S
tgttacctgctagacggatacgccgccggtcgtgatgactgtagg
C  Y  L  L  D  G  Y  A  A  G  R  D  D  C  R
gctcattgcatagccccacgcaaccgccgactatactgtgcctcg
A  H  C  I  A  P  R  N  R  R  L  Y  C  A  S
tatcaggtctgcgtctgtcgatattga
Y  Q  V  C  V  C  R  Y  *
```

Fig. 2. Complete sequence of *M. brassicae* defensin gene. The upper lines show the nucleotide sequence, while the putative amino acidic sequence is indicated by the lower lines.

The amplified fragments were cloned and sequenced. The alignment of *M. brassicae* defensin with homologous sequences from the defensin A genes of *Phormia terranovae*, *Drosophila melanogaster*, *Apis mellifera*, *Aedes aegypti* and *Anopheles gambiae* revealed homology ranging from 43% to 59% (Fig. 3).

The analysis of the putative protein indicated the presence of 98 amino acids, including eight cysteine residues. The evolutionary conserved localization of cysteine residues 3–8 suggests their involvement in the formation of three disulfide bridges.

Northern blotting experiments showed a constitutive expression of the defensin gene in *M. brassicae* cells. Moreover, its expression was induced by Gram positive, but not Gram negative bacteria (Fig. 4). Defensin induction was greater using live bacteria rather than heat-killed specimens (Fig. 4). No defensin gene induction was observed in the presence of *C. albicans* and *S. cerevisiae* (Fig. 4).

## 3. Discussion

Our findings suggest that the *M. brassicae* cell line IZD-MB-0503 is able to synthesize anti-microbial peptides active against Gram positive and negative bacteria, but inactive against fungi. The description of the presence of defensins in insect cell lines is not new (Fallon and Sun, 2000). However, the novelty of our findings is that we show, for the first time, the presence of a “classical”, inducible defensin A-like molecule in a lepidopteran species.

The molecular characterization of *M. brassicae* defensin gene shows between 43% and 59% homology with defensins of group A described in species belonging to Diptera and Hymenoptera (Hetru et al., 1998; Hoffmann and Hetru, 1992). *M. brassicae* defensin shows eight cysteine residues, suggesting the involvement of six cysteine residues in the formation of three disulfide bridges (Lee et al., 1998).

Recently, an inducible cysteine-rich molecule has been found in the lepidopteran *H. virescens* (Lamberty et al., 1999). This molecule, named heliomicin, shows the typical


Fig. 3. Alignment of the defensin peptide from *M. brassicae* (AF465486) (A), *Aedes aegypti* (AF156093) (B), *Apis mellifera* (D17670) (C), *Phormia terranovae* (AF182164) (D), *Drosophila melanogaster* (AC007414) (E) and *Anopheles gambiae* (AF063402) (F). Highly conserved nucleotides are boxed.

cysteine arrangement of insect defensins, but a peculiar and exclusive anti-fungal activity.

Our data, together with the results from Lamberty et al. (1999), seem to confirm that, as reported by Bulet et al. (1999), defensins form a family of anti-bacterial peptides that is widely distributed in insects, and Lepidoptera are no exception.

## 4. Materials and methods

### *4.1. Samples*

The IZD-MB-0503 cell line from the insect *M. brassicae* (Lepidoptera) (ATCC number: CRL-8003) was used. The cells were cultured in Ex-Cell 405 medium (JRH Biosciences, KS, USA) at 26 °C.

### *4.2. Micro-organisms*

The bacterial strains *Staphylococcus aureus* (ATCC 6538) (Gram positive) and *Escherichia coli* (Gram negative) were grown in nutrient broth for 48 h at 37 °C, while the fungal strains, *Candida albicans* and *Saccharomyces cerevisiae*, were grown in Sabouraud broth for 48 h at 25 °C.

### *4.3. Induction of anti-bacterial substances*

Cultures of *S. aureus* ($10^9$ cells/ml) and *E. coli* ($10^9$ cells/ml) were heat-killed at 65 °C for 45 min. The micro-organisms were then centrifuged (4000 rpm for 10 min), washed in phosphate-buffered saline (PBS) and re-suspended in Ex-Cell 405 medium in order to obtain a final concentration of heat-killed bacteria of $4 \times 10^8$ $ml^{-1}$. Both bacteria, of 4.5 ml suspensions, were added to IZD-MB-0503 cells at a final concentration of $1.8–2 \times 10^6$ $ml^{-1}$ and incubated for 24 h at 26 °C. After incubation, the suspensions were centrifuged and the supernatants were filtered. The medium obtained from insect cells exposed to *S. aureus*

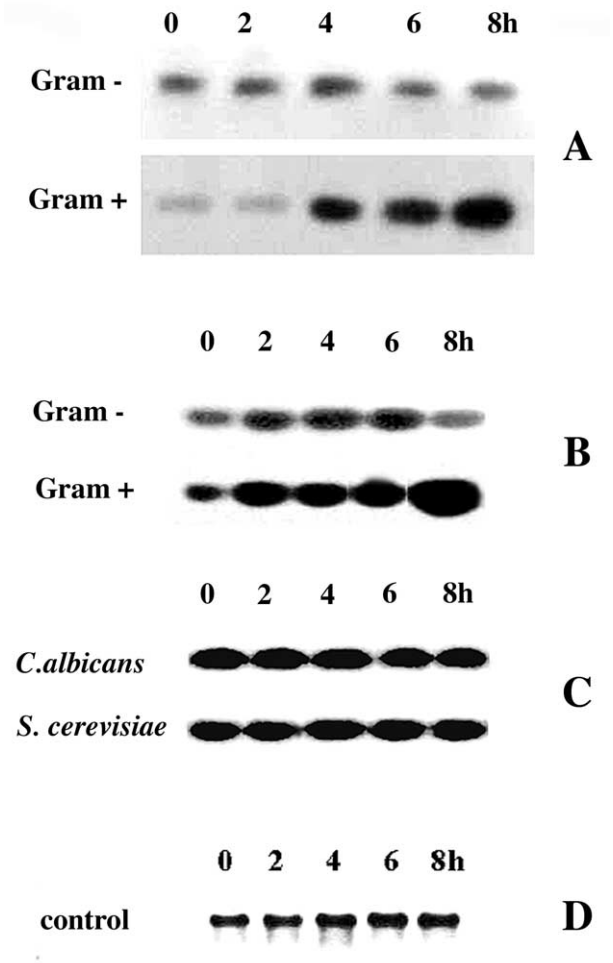


Fig. 4. Northern blotting with the defensin probe on RNA samples extracted at different times from *M. brassicae* cells after induction with heat-killed (A) and live bacteria (B) evidences a single band. Moreover, northern blotting indicates that defensin expression is increased by Gram positive bacteria, in particular, by live specimens, but not by *C. albicans* and *S. cerevisiae* (C). In order to verify that only defensin genes are induced and not the entire transcriptome, the expression of β-tubulin was verified without revealing any induction (D).

was tested undiluted, and diluted (1:2) against *S. aureus* or *E. coli*. The same procedure was followed using the medium from insect cells exposed to *E. coli*. The medium obtained from insect cells exposed to *S. aureus* was also tested undiluted and diluted (1:2) against *C. albicans* and *S. cerevisiae*. Both the anti-bacterial and anti-fungal activities of the conditioned medium were registered by Biophotometer recorder (Biophotometer-Bonet-Mauri, Isa Biologie, France) for 72 h. Micro-organism growth controls were carried out in Ex-Cell 405 medium.

### *4.4. PCR in the IZD-MB-0503 cell line*

Genomic DNA extraction from the IZD-MB-0503 cell line, electrophoresis and transfer of the DNA from agarose gel to nylon membrane were performed following the method of Mandrioli (2002). Quantitative assays were evaluated using the "Matrix" software (Quanta Vision, Madison, WI, USA).

PCR amplification of a portion of the defensin gene was carried out using two primers: F (5'-CCAAATGCCTCG-TCATCT) and R (5'-ATTAGAGTCAAGCTCAAAAGGG). These primers were selected depending on the insect defensin gene sequences available in GenBank. The amplification mix contained 100 ng of genomic DNA, 1 mm of each primer, 200 mm dNTPs and 2 U of DyNAZyme II polymerase (Finnzymes Oy, Finland). Amplification was carried out with a thermocycler performing 30 cycles at a denaturating temperature of 94 °C, an annealing temperature of 55 °C for 30 s and extension at 72 °C for 45 s. The *M. brassicae* defensin gene sequence was completed by inverse PCR following the method of Chowers et al. (1995). Rapid amplification of cDNA ends (RACE) was performed following the method of Frohman (1990).

The amplified fragments were cloned with the "pGEM T-easy cloning kit" following the Promega protocols (Madison, WI, USA). Sequencing was performed at the "Centro di sequenziamento ENEA Casaccia" and the sequence analysis was carried out using GCG Software (Madison, WI, USA).

The *M. brassicae* defensin gene sequence can be retrieved from GenBank under the accession number AF465486.

### *4.5. Analysis of defensin gene expression by northern blotting following bacterial and fungal induction*

Bacterial inductions were performed as previously described, but in the present experiment, both heat-killed and live bacteria were used. Moreover, the induction of antifungal peptides from both *C. albicans* and *S. cerevisiae* was performed by incubating insect cells with fungi for 24 h at 25 °C. RNA extraction was carried out 0, 2, 4, 6 and 8 h after induction using the "SV Total RNA Isolation kit" (Promega) following the manufacturer's protocols. RNA was successively electrophoresed on formaldehyde gel, transferred to a nylon membrane by capillary transfer and blotted with the digoxygenin (DIG)-labeled defensin probe following standard techniques. Northern blotting was carried out following the method of Mandrioli (2002). Pheromone binding protein genes (PBP) were amplified using the primers F (5'-ACTAGCATAGCAATTCGG) and R (5'-GCCTTGACCAT-TACGGACG). PBP amplification was used as a control of the RNA samples, since these genes are not expressed in the *M. brassicae* IZD-MB-0503 cell line and are therefore useful markers of DNA contaminants.

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## References

Bonmatin, J.M., Bonnat, J.L., Gallet, X., Vovelle, F., Ptak, M., Reichhart, J.M., Hoffmann, J.A., Ke Legrain, M., Achstetter, T., 1992. Two-dimensional 1H-NMR study of recombinant insect defensin A in water: resonance assignments, secondary structure and global folding. J. Biomol. NMR 2, 235–256.

Bulet, P., Hetru, C., Dimarcq, J.L., Hoffmann, D., 1999. Antimicrobial peptides in insects; structure and function. Dev. Comp. Immunol 23, 329–344.

Chalk, R., Albuquerque, C.M.R., Ham, P.J., Townson, H., 1995. Full sequence and characterization of two insect defensins: immune peptides from mosquito *Aedes aegypti*. Proc. R. Soc. Lond. B 261, 217–221.

Cho, W.L., Fu, Y.C., Chen, C.C., Ho, C.M., 1996. Cloning and characterization of cDNAs encoding the antibacterial peptide, defensin A, from the mosquito, *Aedes aegypti*. Insect Biochem. Mol. Biol 26, 395–402.

Cho, W.L., Fu, T.F., Chiou, J.Y., Chen, C.C., 1997. Molecular characterization of a defensin gene from the mosquito, *Aedes aegypti*. Insect Biochem. Mol. Biol 27, 351–358.

Chowers, Y., Holtmeier, E., Morzycka-Wroblewska, E., Kagnoff, M.F., 1995. Inverse amplification of rare T cell receptor messages from mucosal biopsy specimens. J. Immunol. Methods 179, 261–263.

Dimopoulos, G., Richman, A., Muller, H.M., Kafatos, F.C., 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. Proc. Natl. Acad. Sci. USA 94, 11508–11513.

Eggleston, P., Lu, W., Zhao, Y., 2000. Genomic organization and immune regulation of the defensin gene from the mosquito, *Anopheles gambiae*. Insect. Mol. Biol. 9, 481–490.

Fallon, A.M., Sun, D., 2000. Exploration of mosquito immunity using cells in culture. Insect Biochem. Mol. Biol 31, 263–278.

Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W.F., Hetru, C., Hoffmann, J.A., 1994. Septic injury of *Drosophila* induced the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J. Biol. Chem 269, 33159–33163.

Frohman, M.A., 1990. RACE: rapid amplification of cDNA ends. In: nis, D.A., Gelfand, D.H., Snisky, J.J. (Eds.), PCR Protocols. Academic Press Inc., San Diego, CA, USA, pp. 28–38.

Gao, Y., Hernandez, V.P., Fallon, A.M., 1999. Immunity proteins from mosquito cell lines include three defensin A isoforms from *Aedes aegypti* and a defensin D from *Aedes albopictus*. Insect Mol. Biol 8, 311–318.

Hanzawa, H., Shimada, I., Kuzuhara, T., Komano, H., Kohda, D., Inagaki, F., Natori, S., Arata, Y., 1990. 1H nuclear magnetic resonance study of the solution conformation of an antibacterial protein, sapecin. FEBS Lett 269, 413–420.

Hetru, C., Hoffman, J.A., Bulet, P., 1998. Antimicrobial peptides from insects. In: Brey, P.T., Hultmark, D. (Eds.), Molecular Mechanisms of Immune Response in Insects. Chapman & Hall, London, pp. 40–66.

Hoffmann, J.A., Hetru, C., 1992. Insect defensins: inducible antibacterial peptides. Immunol. Today 13, 411–415.

Hultmark, D., 1993. Immune reactions in *Drosophila* and other insects: a model for innate immunity. Trends Genet 9, 178–183.

Lamberty, M., Ades, S., Uttenweiler-Joseph, S., Brookhart, G., Bushey, D., Hoffmann, J.A., Bulet, P., 1999. Isolation from the lepidopteran *Heliothis virescens* of a novel insect defensin with potent antifungal activity. J. Biol. Chem 274, 9320–9326.

Lee, K.H., Hong, S.Y., Oh, J.E., 1998. Synthesis and structure–function study about tenacin 1, an antibacterial protein from larvae of *Tenebrio moltitor*. FEBS Lett 439, 41–45.

Lowenberger, C., Bulet, P., Charlet, M., Hetru, C., Hodgeman, B., Christensen, B.M., Hoffmann, J.A., 1995. Insect immunity: isolation of three novel inducible antibacterial defensins from the vector mosquito, *Aedes aegypti*. Insect Biochem. Mol. Biol 25, 867–873.

Mandrioli, M., 2002. Cytogenetic characterization of telomeres in the holocentric chromosomes of the lepidopteran *Mamestra brassicae*. Chromosome Res 9, 279–286.

Miyanoshita, A., Hara, S., Sugiyama, M., Asaoka, A., Tamiai, K., Yukuhiro, F., Yamakawa, M., 1996. Isolation and characterization of a new member of the insect defensin family from a beetle, *Allomyrina dichotoma*. Biochem. Biophys. Res. Commun 220, 526–531.

Muller, H.M., Dimopoulos, G., Blass, C., Kafatos, F.C., 1999. A hemocyte-like cell line established from the malaria vector *Anopheles gambiae* expresses six prophenoloxidase genes. J. Biol. Chem 274, 11727–11735.

Richman, A.M., Bulet, P., Hetru, C., Barillas-Mury, C., Hoffmann, J.A., Kafatos, F.C., 1996. Inducible immune factors of the vector mosquito *Anopheles gambiae*: biochemical purification of a defensin antibacterial peptide and molecular cloning of a preprodefensin cDNA. Insect Mol. Biol 5, 203–210.

Salt, G., 1970. The Cellular Defence Reactions of Insects. Cambridge. Cambridge University Press.

Vass, E., Nappi, A.J., 2001. Fruit fly immunity. Bioscience 51, 529–535.