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Antibacterial properties of lucifensin in *Lucilia sericata* maggots after septic injury

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated that the antimicrobial peptide lucifensin was not found in the digestive tract of the larvae of the fly *L. sericata* as was believed and that lucifesin was isolated to the haemolymph and found higher antibacterial activity of such haemolymph in comparison to non-stimulated larvae.
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ABSTRACT

Objective: To investigate the antibacterial properties of lucifensin in maggots of *Lucilia sericata* after septic injury.
Methods: In our preliminary study we have shown that injuring the maggots with a needle soaked in lipopolysaccharide solution induced within 24 h lucifensin expression in the fat body and in the grease coupler of the salivary glands. It is assumed that lucifensin is secreted solely from this tissue into the haemolymph (similar to other insect defensins) and not into secreted/excreted products. We used high-performance liquid chromatography fractionation and radial diffusion assay to investigate the antibacterial properties of haemolymph extracted from larvae after septic injury.
Results: After septic injury, production of lucifensin in the haemolymph is increased. This led to higher antibacterial activity of such haemolymph in comparison to non-stimulated larvae.
Coclusions: These results suggest that beside the previously demonstrated role of lucifensin in the debridement therapy, lucifensin is simultaneously important as a part of the systematic immune response.

KEYWORDS
Lucilia sericata, Wound bacteria, Defensin, Lucifensin, Immune-challenge

1. Introduction

Lucifensin is one of the well-characterised antibacterial substance from maggots of *Lucilia sericata* (*L. sericata*) involved in maggot therapy[1]. It is assumed that it plays a role in the inhibition of some wound pathogens since it has been found in excretion/secretion of maggots. Lucifensin was originally isolated from larval guts and was subsequently detected in salivary glands, the fat body and haemolymph[1]. Using *in situ* hybridisation, expression of lucifensin has been confirmed in the salivary glands of all larval stages. Expression has been also occasionally

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detected in a few cells of the fat body and in the grease coupler of salivary glands. Surprisingly, no expression of lucifensin has been detected in the gut although lucifensin was originally purified from this tissue. This could mean that, after secretion from salivary glands into the environment, lucifensin is ingested by maggots along with food and passes through the digestive tract[2].

The antibacterial activity often results in a constitutive expression of antibacterial factors (produced at a constant level) or in an inductive expression of antibacterial factors upon bacterial stimulation[3]. It has previously been described that the larval immune system might be activated to induce production of antibacterial substances to survive in an infectious environment[4,5]. Synthesis of antimicrobial peptides in the fat body (a functional equivalent of the mammalian liver) and their rapid release into the haemolymph is important and the best characterised aspect of the insect immune response. Using *in situ* hybridisation, it has been shown that an infectious environment could increase the expression of lucifensin in the fat body of *L. sericata* larvae[2]. Lucifensin should be secreted solely from this tissue into the haemolymph (similar to other insect defensins) and not into excretion/secretion products. Injuring sterile maggots with a sterile needle increased fourfold the antibacterial activity of haemolymph within 24 h. When infected needle was used the antibacterial activity of haemolymph increased sixteenfold[6].

The aim of this study was to investigate the antibacterial properties of haemolymph extracted from the larvae after septic injury.

2. Materials and methods

2.1. Rearing of L. sericata larvae

Colonies of the green bottle fly (*L. sericata*) were maintained at the Institute of Zoology, Slovak Academy of Sciences under constant conditions. Imagos were exposed to 12 h light/dark photocycles at (25±1) °C and a relative humidity of 40-50%. Larvae were fed on ground beef liver mixed with bran.

2.2. Preparation of whole body larval extracts

The whole body extract from 4-day old larvae in the middle of third instars (n=300) was prepared as previously described with some modifications[1]. Briefly, larvae collected from beef liver, were washed and homogenised in grinding mortar using a methanolic extraction buffer (methanol/water/acetic acid: 90/9/1). The larval extract was vortexed and centrifugated at 10 700 r/min for 30 min at 4 °C to remove particular material. The supernatant was collected and lyophilized, and the obtained pellet was dissolved in 1 mL of ultrapure water.

2.3. Purification and identification of larval antibacterial lucifensin

Whole body larval extract was used for isolation of antibacterial peptide-lucifensin. The purification was performed as previously described with some modifications[1]. Briefly, extract was loaded onto HiTrap CM Sepharose HP column (GE Healthcare, UK) and eluted fractions with antibacterial activity were pooled and concentrated. This material was submitted to fractionation under reverse phase-high performance liquid chromatography (RP-HPLC) with a C18 column (250 mm× 4.6 mm; 5 μm) (Grace, IL USA) at a flow rate 0.3 mL/min by using a gradient from 0 to 90% (v/v) acetonitrile [containing 0.1% (v/v) trifluoroacetic acid], during 70 min, after initial 5 min at 0% acetonitrile. After lyophilisation, the fractions were dissolved in 100 μL of ultrapure water and tested for antibacterial activity.

Mass spectra of antibacterial fraction were acquired in positive ion mode using electrospray ionization on a Apex-Qe Ultra Fourier transform mass spectrometry instrument equipped with a 9.4 T superconducting magnet (Bruker Daltonics, Billerica, MA, USA).

2.4. Immune-challenge of L. sericata maggots

Second instar larvae of *L. sericata* were punctuated dorsolaterally with a needle that was contaminated with an lipopolysaccharide (LPS) solution (10 mg/mL, crude preparation of *Escherichia coli* endotoxin 0111: B4, Cat. No.: L2630, Sigma, Taufkirchen, Germany) and subsequently, 24 h post immune-challenge animals were used for collection of haemolymph.

2.5. Collection of haemolymph

Approximately 50 pieces of feeding larvae or larvae after 24 h post immune-challenge were removed from liver, thoroughly washed, then placed into a 50 mL Erlenmeyer flask and cut by scissors into multiple pieces and kept 1 h at 4 °C. The released liquid was decanted and centrifuged at 11 000 r/min for 5 min at 4 °C to remove all the debris before further processing.

2.6. RP-HPLC of haemolymph extracts

Haemolymph extracts were fractionated by using the same

HPLC specification and conditions as mentioned previously, except that 200 μL was injected onto the column. Fractions were collected in 5 min intervals, lyophilized and solid resuspended in 200 μL of sterile ultrapure water.

2.7. Determination of antibacterial activity

Radial diffusion assay was used in order to evaluate antibacterial effects of haemolymph fractions. Briefly, one bacterial colony of *Micrococcus luteus* in overnight agar plate culture was suspended in phosphate buffer saline and the turbidity of suspension was adjusted to 10^8 CFU/mL. One-hundred microlitre aliquot of suspension was inoculated to 10 mL of melted Luria-Bertani broth containing 0.7% (w/v) agar pre-heated at 48 °C and poured into 90 mm Petri dishes. After solidification, 5 mm-diameter wells were punched into Luria-Bertani agar and 5 μL of the sample was added to each well. Antibacterial activity of examined samples was compared on the basis of radius of clear inhibition zone around well after 18-24 h incubation at 37 °C.

3. Results

3.1. Isolation and identification of lucifensin from larval extract

The whole larval body extract showed antibacterial activity against model bacterium *Micrococcus luteus*. The fraction with antibacterial activity after purification on a C18 RP-HPLC column (Figure 1) was subjected to mass spectrometry analysis. High resolution mass revealed that the most intensive signal was observed at m/z 4 114.8951 Da which corresponded to a recently published oxidized form of lucifensin (theoretical mass $[M+H_+]$=4 114.8932)[1].

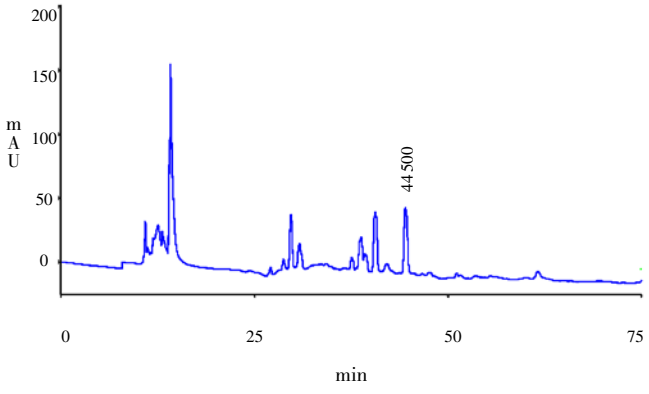

Figure 1. RP-HPLC of fraction eluted from CM Sepharose column and exhibiting antibacterial activity.
Peak at shown retention time (44.5 min) corresponds to the lucifensin as confirmed by MS chromatogram drawn at 280 nm.

3.2. Effect of immune-challenge on lucifensin expression and secretion into larval haemolymph

Maggots in the second instars were punctuated dorsolaterally with a needle that was contaminated with an LPS solution and after 24 h used for collection of haemolymph. The final larval haemolymph was used for examination the antibacterial activity. Haemolymph extracted from non-challenged larvae has been used as a control. Both haemolymph extracts were tested for antibacterial activity. We observed significant increase in the antibacterial activity of the haemolymph following the septic injury (data not shown). After this primary test, extracts have been subjected to fractionation using the same protocol as for the purification of lucifensin. The only fraction exhibiting antibacterial activity was collected at the retention time where lucifensin is supposed to be eluted (Figure 2). We could therefore conclude that increased antibacterial activity of haemolymph in LPS-stimulated larvae was solely caused by the increased expression of lucifensin.

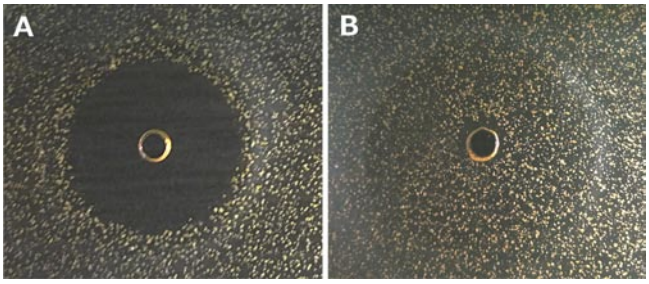

Figure 2. Antibacterial activity of haemolymph fractions from LPS-stimulated and normal larvae.
Injuring larvae with LPS caused significant increase in antibacterial activity of lucifensin containing haemolymph fraction (A) in comparision to the non-challenged one (B).

4. Discussion

In this study, peptide lucifensin with molecular mass of 4 113.89 Da was isolated and identified as an exclusive antibacterial compound in maggots. Lucifensin belongs to the insect defensins, small (~5 kDa), basic, cysteine-rich antibacterial peptides with efficacy against Gram-positive bacteria[7,8]. Most defensins almost immediately kill bacteria by permeabilization of their cytoplasmic membrane[9-11]. Insect defensins are either inducibly expressed in the fat body during systemic immune responses or alternatively might be constitutively expressed in tissues which are in continuous contact with potentially infectious environments, like salivary glands of *L. sericata*. Lucifensin expression has been detected in the salivary glands of all larval stages and occasionally in a few cells of the fat body and

in the grease coupler of salivary glands. Certain infectious environment could increase lucifensin expression in the fat body and the secretion into haemolymph[2]. Immune-challenging the larvae with LPS caused the same systemic response. Lucifensin production has been upregulated and haemolymph of such animal show increased antibacterial activity. Using liquid chromatography techniques we confirmed that the increased antibacterial activity corresponded solely with lucifensin fraction.

In conclusion, our results suggest, that beside the previously demonstrated role of lucifensin in the maggot debridement therapy, lucifensin is simultaneously important as a part of the systematic immune response.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The study of antimicrobial peptides is an important research field, and in the present work the authors focused on the determination of the antimicrobial activity of a member of these antimicrobial peptides, such as the lucifensina in maggot before and after a LPS challenge. Indeed found that these peptides are inducible.

Research frontiers

In this paper, the authors provide with the frontiers of knowledge to establish that the antimicrobial peptide lucifensina which was not found in the digestive tract of the larvae of the fly *L. sericata* as was believed, was considered as a component of the products of excretion/secretion.

Related reports

There are numerous publications on insects antimicrobial peptides as components of the innate immune system and its antibacterial activity.

Innovations and breakthroughs

This study establishes the antimicrobial property of peptide lucifensin, which was not found in the digestive tract of the larvae of the fly *L. sericata* as was believed.

Peer review

This is a valuable research work in which authors have demonstrated that the antimicrobial peptide lucifensin was not found in the digestive tract of the larvae of the fly *L. sericata* as was believed and that lucifesin was isolated to the haemolymph and found higher antibacterial activity of such haemolymph in comparison to non-stimulated larvae.

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