An antibiofilm activity of maggots excretions/secretions against wound pathogens biofilms: the role of larval proteases

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ABSTRACT

An ability to form the biofilm is one of the most important virulent factors of bacteria present in chronic wound environment. Biofilm growth and its persistence within wounds have recently been suggested as contributing factors to impaired healing. Treatment of bacterial biofilm in the wound is complicated by the mechanisms underlying biofilm growth and it represents worldwide health care problem associated with high economical costs. Maggot debridement therapy (MDT), using larval stage of green bottle fly *Lucilia sericata,* is efficient, simple and low-cost therapy for the treatment of chronic wounds such as diabetic, pressure and venous ulcers, where bacterial biofilm is usually present. Indeed, the beneficial clinical effects of MDT are well documented, but particular bioactive compounds and mechanisms of healing cascade are not fully elucidated.

The goal of the study was to investigate the antibiofilm activity of maggot excretions/secretions (ES) against biofilm of wound isolates *Enterobacter cloacae* (*E. cloacae*) and *Staphylococcus aureus* (*S. aureus*). These bacteria are one of the most frequent strains isolated from chronic wounds.

In case of *E. cloacae*, the sufficient concentration of native ES able to significant reduce bacterial biofilm formation (40% inhibition) was 5 mg/ml, while active concentration 10 mg/ml of native ES nearly fully disrupted the established biofilm. ES at concentration of 10 mg/ml significantly decreased the number of viable cells within established biofilm. The antibiofilm activity of heat-treated fractions of ES was not detected in meaning of affecting the biofilm formation, biofilm degradation or cell viability of bacteria present in biofilm. This implies the antibiofilm activity of maggots ES against *E. cloacae* is due to heat-unstable components *e.g.* proteases.

In case of *S. aureus*, the sufficient concentration of native ES able to decrease bacterial biofilm formation and affect cell viability in biofilm was 5 and 10 mg/ml, respectively. In contrast to *E. cloacae*, heat-treatment of maggots ES did not affect its antibiofilm activity. This fact turns attention to bioactive role of termostabile defensin, lucifensin, active against Gram-positive bacteria.

It is known that L. sericata maggots secrete a mixture of proteolytic enzymes that degrade macromolecules and infected material. In order to identify possible larval proteases we constructed cDNA library from salivary glands of sterile maggots and identified five full-length and several incomplete cDNAs encoding proteases. We suggest that proteinases may play partial role in affecting the biofilm formation as well as degradation of established biofilm and cell viability within the established biofilm in bacterial pathogens, but further research is needed.

INTRODUCTION

An ability to form the biofilm is one of the most important virulent factors of bacteria present in chronic wound environment. Bacteria with biofilm phenotype are strongly adhesive to different kinds of abiotic surfaces used in medicinal practice, to protein and tissue structures present in the wound bed and also adhesive to other cell colonies. Biofilm-forming bacteria produce extracellular matrix which surrounds whole biofilm formation and serves as the adhesive structure, provide transport of the nutrition and cell to cell communication via quorum sensing. The extracellular matrix that surrounds the bacteria in biofilm consists of polysaccharides, proteins, fimbriae, mating pili, and extracellular DNA. This extracellular matrix is believed to offer protection against various factors including protozoan predation in environmental settings as well as host immune responses and antibiotic treatment in medical settings. Chronic wounds associated with biofilm colonization are worldwide problem joined with high economical costs, social and psychological deprivation and pain. Simply said, chronic wounds are prone to heal and one of the main causes of healing process failure is presence of bacterial its specific structure which is hardly permissible for medical treatment. biofilm with Maggot debridement therapy is efficient and cheap method of healing the non-healing or chronic wounds like diabetes, pressure and venous ulcers, where bacterial biofilm is usually present. This traditional method of debridement uses larval stage of green bottle fly *Lucilia sericata* for cleaning the wound bed from bacterial load, cell debris and necrotic tissue. Afterwards, the woundhealing process is able to progress from inflammatory to next phases. Maggots secrete a cocktail of heterogeneous substances with antibacterial, antibiofilm and immunomodulatory effect. The beneficial clinical effect of larval therapy is well documented, but particular bioactive compounds and mechanisms of healing cascade are not fully elucidated. We investigated antibiofilm activity of sterile maggot excretions and secretions (ES) against bacterial biofilm of gramm-negative *Enterobacter cloacae* and gramm-positive *Staphylococcus aureus*, both are clinical chronic wounds isolates. These strains are dominant in nonhealing wound bacterial flora, moreover *Enterobacter spp*. is frequently silver resistant. It is known that larvae secrete a mixture of proteolytic enzymes that degrade macromolecules and infected material, our findings also indicate degradation of gram-negative bacteria biofilm extracellular matrix. Until now, aspartyl, metalloproteinase, chymotrypsin and trypsin-like protease activities have been detected in larval secretions. In our study, we constructed cDNA library from salivary glands of sterile maggots and identified five full-length and several incomplete cDNAs encoding for proteases mainly belonging to serine protease families. They may also play crucial role in extracellular matrix degradation as well as affecting of formation and established biofilm of gramm-negative bacteria.

MATERIALS AND METHODS

Bacterial isolates: Bacterial isolates *S. aureus* 1141and *E. cloacae* 2383/10 from non-healing wounds were collected from the Department of Clinical Microbiology in Liptovsky Mikulas Hospital (Liptovsky Mikulas, Slovakia) and Prievidza Hospital (Bojnice, Slovakia). Isolates were transported to the Department of Microbiology, Faculty of Medicine, Slovak Medical University (Bratislava, Slovakia).

ES collection and preparation: Third instar nonsterile larvae were incubated in water for 60 min (50 larvae/200ul water). After incubation, the produced ES was collected, centrifuged and lyophilized. Dried ES was dissolved in Triptic Soy Broth medium (TSB) to obtain stock solution of 100 mg/ml and filtered using 0.22 µm microbial filter. Further dilutions of stock solution maggots ES were made to obtain concentrations of 5 mg/ml to100 mg/ml. Heat-treated ES were incubated for 10 min at 100°C, after incubation centrifuged and supernatant transferred into a new tube. For comparision of protease effect on biofilm we used proteinase K (1µg/ml).

Biofilm formation assays and bacterial growth: It was performed using a plastic 96-well tissue culture microtiter plate (Sarstedt, Germany). Briefly, a loopful of cells from a blood agar plate was transferred to a sterile polystyrene tube containing 4 ml of PBS. The cells were dispersed and suspension turbidity was adjusted to 10^8 CFU ml^{-1} and diluted with TSB broth to a final concentration of 10^6 CFU ml^{-1}. Ten µl aliquots of suspension were inoculated in each well of sterile 96-well polystyrene plates and supplemented with 90 µl of sterile medium or diluted/ heat-treated ES. Plates were incubated at 37°C for 24 hours, during incubation period, the bacterial growth was determined by monitoring the optical density at 570 nm every 60 min. for 10h, 22 h incubation was determined next day. Biofilm inhibition was examined after 24 h when the planktonic cells were removed and the wells were washed with sterile PBS. Subsequently, biofilms were exposed to a 2% (w/v) crystal violet solution for 8 min, washed and re-solubilized with 100 µl of 33% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 570 nm using an automated microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Biofilm disruption assay: It was performed similarly, biofilms were cultured for 24 h in a microtiter plate, then they were washed three times with sterile PBS and then treated with a particular concentrations of ES /heat-treated ES. Control biofilms were cultured in TSB broth alone. After an additional 24 h incubation, biofilms were quantified by CFU enumeration and crystal violet staining as described above. In the case of CFU enumeration, biofilms were rinsed three times and adherent bacteria were detached using a swabbing technique. A cotton-tipped swab was then transferred to 5 ml of PBS buffer and mixed by vortex agitating for 60 s. The bacterial suspensions were serially diluted and spread on blood agar plates. The plates were then incubated at 37°C for 24 h.

Determination of protease activity: It was performed using skim milk agar plates (Quiblier et al, 2011). ES concentrations and heat-treated ES (5 µl were added through a hole in the milk agar plates and incubated at 37°C during 24h and optically measured. LB medium and proteinase K were used as negative and positive controls.

Statistical analysis: Results are presented as the mean with standard error (SEM). All data were statistically analyzed from three independent experiments, where every experiment used new batch of ES. Differences between the values for ES-treated and non-treated bacteria were analyzed using a Student's t-test. The level of significance was set at $P < 0,05$. Analyses were performed using GraphPad Prism .

Bioinformatic analyse: Removal of 454 adapter sequences, trimming of the low quality sequence and assembly were done by Eurofins MWG using MIRA Assembler package. The resulting contigs were initially searched against GenBank (NCBI) Drosophila melanogaster refseq protein database through blastx. After initial database screening, cDNA sequences were de novo assembled, manually inspected and annotated by CLC Genomic Workbench and Vector NTI packages.

Fig. 1. The effect of ES on biofilm inhibition.

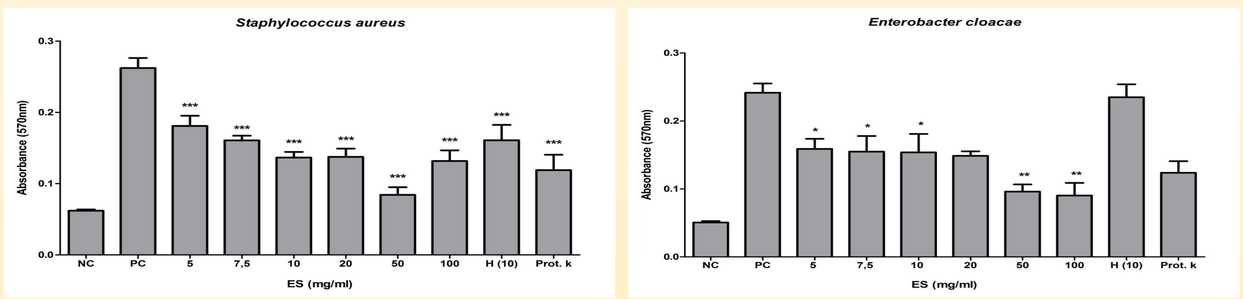


Fig. 2. The effect of ES on established biofilm disruption.

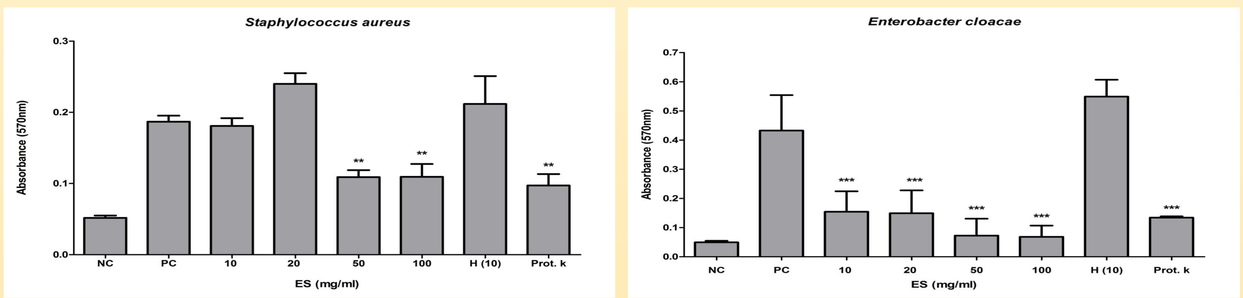
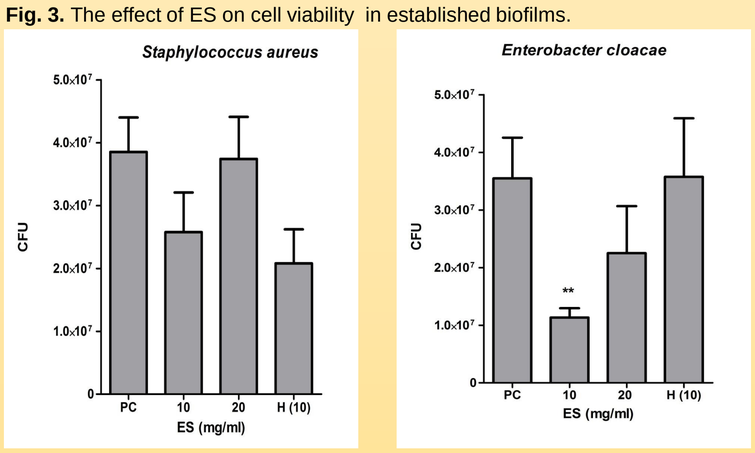


Fig. 3. The effect of ES on cell viability in established biofilms.



* P<0.05 was calculated vs. control
** P<0.01 was calculated vs. control
*** P<0.001 was calculated vs. control

NC – negative control
PC – positive control

Fig. 4. The effect of ES on bacterial growth.

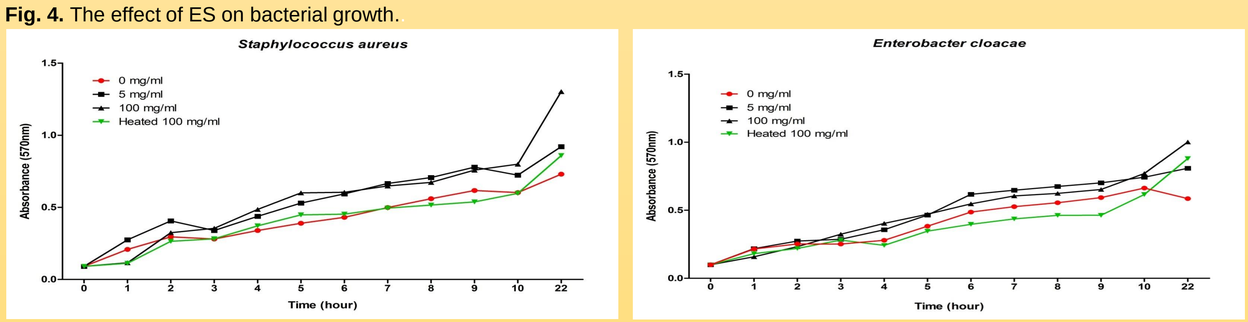
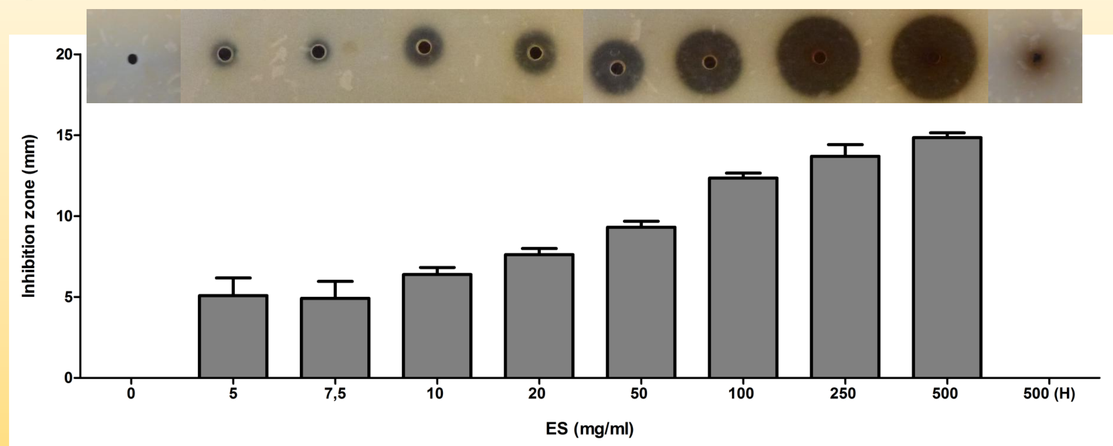


Fig. 5. The proteolytic activity of larval ES. H-heated



Tab. 1. Putative proteases identified in *L. sericata* salivary glands.

GenBank Acc. #	Best BLAST hits	E-value	Score	Amino acid identity
JN215468	AAA17382.1 serine proteinase [Lucilia cuprina]	6.32 e-50	511	101/108 (93%)
	AAA68986.1 chymotrypsinogen [Lucilia cuprina]	1.78 e-44	464	91/157 (57%)
JN215469	ADD18566.1 large serine protease [Glossina morsitans]	3.82 e-161	1474	274/424 (64%)
	ADD18568.1 salivary trypsin [Glossina morsitans]	3.46 e-154	1474	267/420 (63%)
JN215470	EDS29955.1 CAAX prenyl protease 1 [Culex quinquefasciatus]	2.79 e-153	1407	266/435 (61%)
	ABF18495.1 prenyl-dependent CAAX metalloprotease [Aedes aegypti]	1.06 e-152	1402	269/437 (61%)
JN215471	ADD19121.1 signal peptide protease [Glossina morsitans]	4.43 e-172	1568	301/355 (84%)
	AAF51486.1 signal peptide protease [Drosophila melanogaster]	2.52 e-167	1527	297/358 (82%)
JN215472	ADD20181.1 salivary serine protease [Glossina morsitans]	2.49 e-131	1217	226/381 (59%)
	ACN69171.1 salivary trypsin [Stomyx calcitrans]	2.61 e-80	777	151/264 (57%)

RESULTS

We examined the antibiofilm effect of maggots ES against *S. aureus* and *E. cloacae*, both dominant pathogens of chronic wounds (Fig.1, Fig.2). The concentration of 10 mg/ml statistically significant inhibited biofilm formation in *S. aureus* and *E. cloacae*. In case of disruption of established biofilm concentration 10 mg/m was sufficient in *E.cloacae*, while statistically significant ES concentration needed to disrupt *S. aureus* established biofilm was higher (50 mg/ml). Maggots ES shoved no significance, there was also need to examine the influence of maggots ES on bacterial growth, where we did not seen significance on bacterial growth curve.

Heat- treated ES showed values comparable with positive controls, only in case of *S. aureus* forming biofilm inhibition heat-treated ES showed significance.

This turned the attention to termolabile substances present in ES e.g. proteases, the proof of proteolytic activity brought fig. 5, proteolytic degradation generates concentration dependent zones in milk agar.

Afterwards we constructed cDNA library from salivary glands of third instar maggots and bioinformatic analyses brought 5 new proteases present (Tab.1).

CONCLUSIONS

This study showed that medicinal maggots used in larval debridement therapy are able to downregulate the biofilm of dominant pathogens present in chronic wound, that delays wound healing. We show attention to termolabile substances with proteolityc activity which are able to inhibit forming biofilm as well as disrupt established biofilm. These findings confirms that maggots play important role in MDT during debridement act, where the fundamental role may play substances like proteases, constantly secreted into a wound bed.

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