In vitro and *in vivo* expression of a metalloprotease from *Paenibacillus larvae* during infection of honeybee larvae

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INTRODUCTION

Paenibacillus larvae is the causative agent of American Foulbrood (AFB), the most severe bacterial disease that affects larvae of the honeybee *Apis mellifera*[1,2]. AFB presents a worldwide distribution, causing a significant decrease in honeybee populations and production[2].

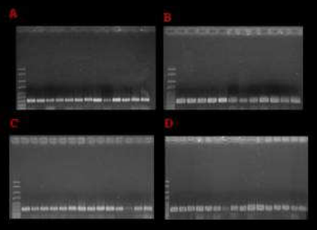
A. mellifera larvae become infected by swallowing food contaminated with spores. These spores germinate in the larval midgut, vegetative cells proliferate, move to the haemocoel and spread causing septicemia. As larvae die, their tissues decay and the consistency of the infected larval body changes to a brownish and viscous mass that then is dehydrated forming a scale [2,3]. Several authors reported that *P. larvae* secretes metalloproteases that are involved in the larval degradation[4,5,6].

The aim of the present work was to evaluate the distribution of a metalloprotease described for *P. larvae* among isolates from different geographic regions and to assess its expression *in vitro* and *in vivo*, during infection of honeybee larvae.

DISTRIBUTION OF *P. larvae* METALLOPROTEASE GENE

55 Uruguayan *P. larvae* isolates were used.

Primers Prot 1 and Prot 2 desgined to amplify a fragment of 280 pb of the *P. larvae* metaloprotease gene (Genbank access N° AF111421) were used.



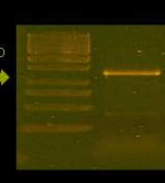
Name	Sequence
Prot.1	5'-GCAGCAAATCGTATTCAG-3'
prot.2	5'-GGTCCTTTGTAACGATTG-3'

P. larvae metalloprotease gene was presented in isolates from different geographic regions.

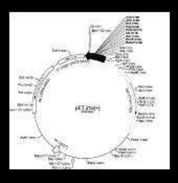
CLONING, EXPRESSION AND PURIFICATION OF METALLOPROTEASE

The gene that encodes for *P. larvae* metaloprotease was obtained by comparison of the partial published sequence with *P. larvae* genome (available at www.hgsc.bcm.tcm.edu). The encoding sequence was identified using Artemis program (www.sanger.ac.uk). Primers Met 5 and Met 6 were designed to amplify the complete gene.

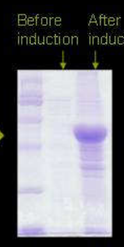
Name	Sequence
Met5	5´-GGAGGCATATGAATGAAGAAG-3´
Met6	5´-CCTTAAGCTTAGTGATGGTGATGGTGATGTTTGACTCCAACC



The complete gene encoding for *P. larvae* metaloprotease was succesfully amplified,



cloned into an expression vector

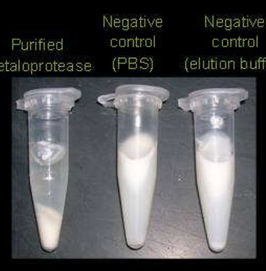


Pre-pro-enzyme (60 kDa)

transformed in *E. coli*, overexpressed *and purified.*

PROTEOLYTIC ACTIVITY

Proteolytic activity was evaluated using skim milk.

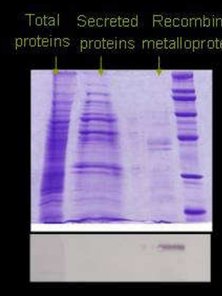


Mature recombinant enolase presented proteolytic activity

ANTISERUM ANTI-METALLOPROTEASE

Antiserum was raised in mouse using a recombinant metalloprotease for immunization.

Specificity was checked by SDS-PAGE and Western-blot.

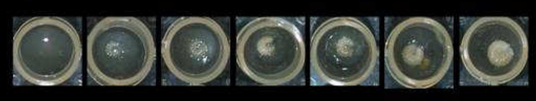


Antiserum against *P. larvae* metalloprotease resulted highly specific, recognizing only the protein used for immunization.

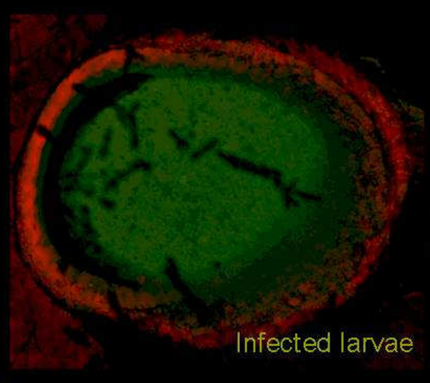
IN VIVO EXPRESSION OF METALLOPROTEASE

Exposure bioassay [7]

Young larvae (less than 24 hs old) were transferred to plastic microtiter trays filled with food. Larvae were fed with *P. larvae* spores (1000 esp/ul) within the food for 48 hs. After that, larvae were transferred daily to new microtiter trays with new food. Control larvae were fed with normal larval diet throughout the experiment.



Larvae were sacrificed five days after infection, fixed with PFA 4% washed with PBS, imbibed in a gelatin – albumin matrix and sectioned using a vibratome.

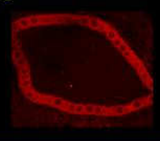


Infected larvae

FISH and immunofluorescence in larval midgut

Larval midgut cells were detected using the eukaryotic probe EUK516 5'-labelled with Cy3 (red) [3].

Metalloprotease expression was detected using antiserum against this protein and a secondary antibody anti-mouse conjugated to FITC (green).

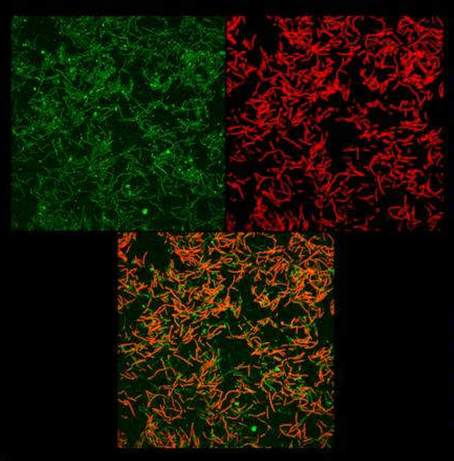


Control larvae

IN VITRO EXPRESSION OF METALLOPROTEASE

FISH and immunofluorescence on P. larvae vegetative cells and spores

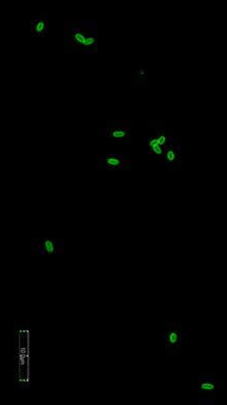
Metalloprotease expression was detected using antiserum against this protein and a secondary antibody anti-mouse conjugated to FITC (green).



P. larvae vegetative cells were visualized using a specific probe labeled with CY5 (red)[3].

Co-localization of *P. larvae* vegetative cells and metalloprotease (yellow) using confocal microscopy.

Metalloprotease expression by *P. larvae* spores.



CONCLUSSIONS

The *P. larvae* metalloprotease is wide distributed between isolates, suggesting that can be important for pathoegenesis.

The complete gene that encodes for this protein was succesfully cloned on an expression vector, overexpressed and the recombinant metalloprotease was purified. Preproenzyme undergoes autoproteolytic cleavage, obtaining the mature protein.

Mature recombinant metalloprotease presented proteolytic activity.

The metalloprotease is expressed by *P. larvae* vegetative cells and on the surface of *P. larvae* spores. It is also expressed *in vivo* during infection of honeybee larvae.

These results suggest and important role of the metalloprotease as a virulence factor involved in *P. larvae* pathogenicity.

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