Characterization of secreted proteases of Paenibacillus larvae, potential virulence factors involved in honeybee larval infection

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Abstract

Paenibacillus larvae is the causative agent of American Foulbrood (AFB), the most severe bacterial disease that affects honeybee larvae. AFB causes a significant decrease in the honeybee population affecting the beekeeping industry and agricultural production. After infection of larvae, P. larvae secretes proteases that could be involved in the pathogenicity. In the present article, we present the secretion of different proteases by P. larvae. Inhibition assays confirmed the presence of metalloproteases. Two different proteases patterns (PP1 and PP2) were identified in a collection of P. larvae isolates from different geographic origin. Forty nine percent of P. larvae isolates showed pattern PP1 while 51% exhibited pattern PP2. Most isolates belonging to genotype ERIC I – BOX A presented PP2, most isolates belonging to ERIC I – BOX C presented PP1 although relations were not significant. Isolates belonging to genotypes ERIC II and ERIC III presented PP2. No correlation was observed between the secreted proteases patterns and geographic distribution, since both patterns are widely distributed in Uruguay. According to exposure bioassays, isolates showing PP2 are more virulent than those showing PP1, suggesting that difference in pathogenicity could be related to the secretion of proteases.

1. Introduction

Paenibacillus larvae is the causative agent of American Foulbrood (AFB), the most severe bacterial disease that affects larvae of the honeybee Apis mellifera (Genersch et al., 2006; Hansen and Brodsgaard, 1999). AFB presents a worldwide distribution, causing a significant decrease in honeybee populations and production (honey, pollen, propolis, royal jelly and beeswax) (Hansen and Brodsgaard, 1999). Besides their importance for the beekeeping industry, honeybees play an essential role in the ecology of different environments throughout pollination, being essential for the production of agricultural systems and conservation of natural ecosystems.

Apis mellifera larvae become infected by swallowing food contaminated with spores. These spores germinate in the larval midgut, vegetative cells proliferate, move to the haemocoeel 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. Sporulation of bacterial vegetative cells occurs during the whole infection process (Yue et al., 2008; Hansen and Brodsgaard, 1999).

AFB infected ropes and scales are highly proteolytic, a feature that is often used for a quick diagnosis of the disease. Inoculation of infected material in milk produces protein coagulation in a few hours. These proteases are produced by P. larvae during the growth and sporulation processes (Hrabak and Martinek, 2007; De Graaf et al., 2006; Holst and Sturtevant, 1940) and are probably involved in the degradation of antibacterial peptides and in the degradation of larval tissues (Glinski and Jarosz, 1998; Casteels et al., 1989; Katzenelson and Lochhead, 1947).

Dancer and Chantawannakul (1997) reported the production by P. larvae of three different extracellular zinc-dependent metalloproteases, that belong to an atypical type of multimeric proteases, which vary according to the geographic origin of different isolates (Dancer and Chantawannakul, 1997). The aim of the present work was to characterize the proteases secreted by P. larvae isolates from different geographic regions and to evaluate their potential role in pathogenicity.

2. Methods

2.1. Bacterial strains and culture media

Fifty P. larvae isolates were randomly selected from the collection of the Department of Microbiology, Instituto de Investigaciones Biológicas Clemente Estable. These isolates were obtained
from worker bees, larvae and honey of different provinces of Uruguay between 1999 and 2002. Four isolates of *P. larvae* from Argentina were also used. *P. larvae* isolates corresponded to the different genotypes observed in previous works (Table 1, Antúnez et al., 2007). Isolates were routinely grown on J agar (Horntzky and Nicholls, 1993).

### 2.2. Protease production and relation with cellular growth

A *P. larvae* bacterial suspension (from a fresh culture of isolate 44) of 10⁶ colony forming unit (cfu)/ml (Mac Farland scale number 2.2. Protease production and relation with cellular growth

<table>
<thead>
<tr>
<th>Origin</th>
<th>Name</th>
<th>Genotype</th>
<th>Protease pattern</th>
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</thead>
<tbody>
<tr>
<td>Paysandí (Uruguay)</td>
<td>Ab1</td>
<td>ERIC I – BOX A</td>
<td>PP2</td>
</tr>
<tr>
<td>Paysandí (Uruguay)</td>
<td>Ab4</td>
<td>ERIC I – BOX A</td>
<td>PP2</td>
</tr>
<tr>
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<td>ERIC I – BOX A</td>
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</tr>
<tr>
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<td>ERIC I – BOX A</td>
<td>PP1</td>
</tr>
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<td>PP2</td>
</tr>
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<td>Salto (Uruguay)</td>
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<td>ERIC I – BOX A</td>
<td>PP2</td>
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<td>ERIC I – BOX A</td>
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<td>NRRL B-14152⁺</td>
<td>PLP</td>
<td>ERIC III – BOX PLP</td>
<td>PP2</td>
</tr>
</tbody>
</table>

PP1: Secreted proteases pattern 1.
PP2: Secreted proteases pattern 2.
NRRL = National Regional Research Laboratory (National Centre for Agricultural Utilization Research, USDA, USA).
* From Antúnez et al., 2007.

2.3. SDS–PAGE

Twenty microliters of *P. larvae* secreted proteins (approximately 40 µg) were analyzed by polyacrylamide gel electrophoresis under denaturing conditions, as described by Laemmli (1970) and stained with 0.1% (v/v) Coomasie brilliant blue. Wide Range protein molecular weight marker (BioRad, 6.500–20.0000 kDa) was used.

2.4. Zymograms

Twenty microliter of secreted proteins (approximately 40 µg) were electrophoresed in polyacrylamide gels under native conditions (Laemmli, 1970). Gelatin (0.1% in distilled water) was added to the resolving gel. After the analysis, the gel was washed with 1% Triton X-100 for 1 h and distilled water for 5 min. The gel was incubated at 37 °C during 12 h in PBS and stained with Coomassie brilliant blue. Proteolytic activity was assumed when translucent bands appeared in the gel.

2.5. Analysis of *P. larvae* secreted protease patterns

The secreted proteases patterns of the whole *P. larvae* isolates collection was evaluated by the method described above. Since maximum protease production was detected after 72 h of culture incubation, this period was chosen to compare between isolates. Secreted proteins from each isolate were analyzed at least twice in order to ensure reproducibility of results. Statistical analyses were performed to evaluate the correlation between secreted proteases patterns and genotypes. Data was summarized in a contingency table and analyzed using Chi square and Yates corrected Chi square test.

2.6. Proteases inhibition assays

Protease inhibition assays were performed using *P. larvae* isolates representing different secreted proteases patterns (isolates 44 and 29). Proteins were analyzed in native polyacrylamide gels supplemented with gelatin and electrophoresed in native conditions. Gels were incubated in PBS supplemented with different proteases inhibitors (Dunn, 1989): phenylmethylsulphonylfluoride (PMSF, specific for serine proteases, used at 1 and 5 mM), iodoacetamide (IA, specific for cysteine proteases, used at 0.05, 0.1 and 0.5 mM), Ethylenediaminetetraacetic (EDTA, specific for metalloproteases, used at 1, 5 and 10 mM), and 1,10 phenanthroline (specific for Zn²⁺ containing metalloproteases 0.5, 1 and 5 mM). Gels were incubated at 37 °C overnight. Controls were performed incubating the gel in PBS only. Experiments were carried out by duplicate.
2.7. Exposure bioassays

Worker larvae from the progeny of a single wild-mated honey bee queen (Apis mellifera ligustica) maintained in a disease-free apiary at the USDA Bee Research Lab (Beltsville, MD, USA) were used for this study. Larvae were collected and transferred to plastic microtiter trays (96 wells) for rearing, as described by Evans (2004). Larvae were fed ad libitum with an excess of a liquid diet consisting of 66% (vol/vol) royal jelly, 6% (wt/vol) glucose, 6% (wt/vol) fructose and 1% (wt/vol) yeast extract in sterile double distilled water. Larval food was supplemented with P. larvae at a final concentration of 500 spores per microliter. P. larvae isolates showing different protease patterns were used (85, NP2, 44, PL63 and PLP).

Groups of twelve larvae were used in each case. Larvae were fed with contaminated larval diet for the first 48 h after grafting and thereafter, normal larval diet was used for feeding. Control larvae were fed with normal larval diet throughout the experiment. Plates were incubated at 34.5 °C with high humidity for 6 days. Each day, larvae were taken out from the incubator and examined. Larvae were classified as dead when they lost their body elasticity or displayed a color change to brownish. The number of dead larvae was recorded, and surviving larvae were transferred to new wells filled with fresh food.

3. Results

3.1. P. larvae secreted proteins at different growth stages

P. larvae secreted proteins from isolate 44 were analyzed by SDS–PAGE and at least 10 different bands ranging between 6.5 and 200 kDa were observed (Fig. 1). The same protein pattern was observed at different times of culture incubation. Zymograms corroborated the presence of proteases among P. larvae secreted proteins. A well defined band with proteolytic activity was detected in the supernatants of bacterial suspensions from 24 to 96 hours of incubation. Growth curve indicates the predominance of vegetative cells during this culture period (Fig. 1).

3.2. P. larvae secreted proteases patterns

Fifty five P. larvae isolates from different geographic origins and belonging to different genotypes were analyzed in order to detect the presence of different secreted protease patterns and evaluate their use as an epidemiologic marker. Two different proteases patterns could be defined in the entire collection, pattern 1 (PP1), composed of only one proteolytic band and pattern 2 (PP2), that showed four proteolytic bands (Fig. 2A). Both protease patterns were highly reproducible.

Forty nine percent of P. larvae isolates showed pattern PP1 while 51% exhibited pattern PP2. Most isolates belonging to genotype ERIC I – BOX A showed PP2 (59%), while most isolates belonging to ERIC I – BOX C showed PP1 (62%) although statistical analysis demonstrated that relations were not significant (p > 0.05) (Table 1). Isolates belonging to genotypes ERIC II and ERIC III presented PP2, although it is important to notice than only one isolate from each genotype was analyzed.

No correlation was observed between the secreted proteases and geographic distribution, since both patterns are widely distributed in Uruguay.

3.3. Proteases inhibition assays

P. larvae secreted proteins (belonging to different proteolytic band patterns) were incubated with different proteases inhibitors in order to elucidate the nature of the bacterial proteases. Inhibition assays resulted reproducible. Proteases from pattern PP1 were not inhibited by IA nor by PMSF at any of the concentrations used, while EDTA and 1,10-phenantroline showed an inhibiting effect, indicating the presence of metalloproteases (Fig. 2B–E). Proteases from pattern PP2 gave similar results, and all assays were consistent for the two replicates (Fig. 2F–I).
3.4. Secreted protease patterns and relationship with bacterial virulence

Exposure bioassays were carried out in order to evaluate if the secretion of different proteases patterns is related to P. larvae virulence. Variation on virulence was observed on P. larvae isolates showing different secreted protease patterns (Fig. 3). When isolates of different genotypes and showing different secreted protease patterns were compared, a higher virulence was detected on isolates showing PP2 (isolates PL63 and PLP, 85) than those showing PP1 (44, NP2). Even more, when isolates belonging to the same genotype (isolates 44 and 85, belonging to ERIC I – BOX C) were compared, isolate 85, showing PP2, was more virulent.

4. Discussion

Proteases have been described as important virulence factors in a great variety of microorganisms (Miyoshi and Shinoda, 2000) and so far, a few studies have been carried out to characterize P. larvae proteases (Dancer and Chantawannakul, 1997; Hrabak and Martinek, 2007). Although we can not discard the production of proteases during sporulation stage, we showed that P. larvae produces and secretes different proteins with proteolytic activity during the replication of vegetative cells. Also, these proteases can be classified as metalloproteases according to the results of inhibition assays.

Dancer and Chantawannakul (1997) reported different secreted proteases patterns in P. larvae isolates from different locations which could be used for strain classification and epidemiological studies. In the present work we also found different secreted proteases patterns (PP1 and PP2), which were highly reproducible. No significant relationship was detected between genotype and secreted protease pattern. Correlation between the secreted proteases patterns and geographic distribution was neither observed.

To further characterize P. larvae secreted proteases and to evaluate their relation with virulence we used exposure bioassays using A. mellifera larvae. Variation on virulence have been previously reported on P. larvae isolates belonging to different genotypes (Genersch et al., 2005). P. larvae isolates belonging to genotype ERIC II resulted more virulent than those belonging to genotype ERIC I. Similar results were obtained in the present work. When analyzing the relation of virulence with secreted protease patterns, we found that isolates showing a PP2 are more virulent than those showing PP1, independently from their genotype, and even comparing strains from the same genotype, the isolate presenting PP2 resulted more virulent than the isolate presenting PP1. These results suggest that the proteases could be important in the P. larvae pathogenicity.

The existence of more or less virulent strains of P. larvae influences disease pathogenesis and transmission, so the analysis of secreted protease patterns can be a useful technique for epidemiological studies.

This work may contribute to the knowledge related to basic aspects of pathogenicity of a bacterium of great economic significance, to characterize P. larvae potential virulence factors and to elucidate the mechanism of insect-pathogen interaction.

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References


