Preliminary evaluation of glucose oxidase and its products in vitro antimicrobial activities on Paenibacillus larvae ATCC9545 vegetative form

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Abstract

Paenibacillus larvae is the pathogen responsible for American foulbrood disease in honeybees. Today, no useful tool has been identified to contrast its spreading due to the spores diffusion, except for hives burning. The effect of glucose oxidase and its reaction products, hydrogen peroxide and gluconic acid, was evaluated against P. larvae ATCC9545. Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by microdilution method. MIC values were employed to evaluate the survival growth rates of P. larvae after each treatment in a macromethod test. Results showed an inhibitory effect of glucose oxidase, gluconic acid and hydrogen peroxide at a minimum concentration of 0.28 µg/mL, 3.71 µg/mL and 0.74 µg/mL, respectively. For hydrogen peroxide MIC value corresponded to MBC value, while P. larvae survival growth rates at observed MIC was 98.3% and 91.6% for glucose oxidase and gluconic acid, respectively. Glucose oxidase MBC value (74.20 µg/mL) was remarkably higher than its MIC value. Even thought, the present work focused on the in vitro inhibitory effect of glucose oxidase and its reaction products only on P. larvae ATCC9545 vegetative form, the obtained results were promising and repeatable.

Key words: Apis mellifera, Paenibacillus larvae, glucose oxidase, American foulbrood disease.

Introduction

As all living organisms, Apis mellifera L. is subjected to microorganisms’ attack, such as deforming wing virus (Mazzei et al., 2015), Paenibacillus larvae (Ashiralieva and Genersch, 2006), Nosema ceranae (Higes et al., 2006); thus beekeepers are constantly looking for new tools to protect honeybees from these pathogens.

P. larvae is the etiological agent of American foulbrood disease (AFB), the most devastating bacterial disease affecting honey bee brood worldwide (Hansen and Brodsgaard, 1999). P. larvae is a gram-positive, spore-forming, facultative anaerobic microorganism (Genersch et al., 2005), which is transmitted by spore containing honey being fed to newly hatched larvae (Neuendorf et al., 2004); moreover adult bees also carry spores (Lindström et al., 2008a; 2008b). Spores, which represent the only infectious form, infect only larvae, while adult bees do not become infected upon ingestion of P. larvae spores (Genersch, 2010). Spores germinate and proliferate in the midgut lumen (Yue et al., 2008). Moreover, it has been shown that larvae can display a different susceptibility, according to their age (Brodsgaard et al., 1998) and the infecting P. larvae genotype (Genersch et al., 2005; Djkic et al., 2014).

It is demonstrated that vertical and horizontal transmission of AFB are both possible in daughter swarms (Fries et al., 2006) and among apiaries (Lindström et al., 2008a). Transmission is possible also by contaminated honey (Lindström et al., 2008b). Since the honey bee is a social organism, its health depends not only on the individual immune system, but also on the social immune system (Cremer et al., 2007). Spivak and Reuter (2001) showed that colonies selected for their hygienic behaviors were more resistant to AFB, indeed Oliver (2010) states that the honey-bee social immune system takes advantage of hygienic behaviour. The social immune system could also take advantage of several hive products, mainly honey (Gherman et al., 2014; Erler et al., 2014), but also royal jelly proteins and glucose oxidase, which showed antimicrobial activity (White, 1966; Fujiiwara et al., 1990; Bilkova et al., 2001; Fontana et al., 2004, Scarselli et al., 2005). Glucose oxidase is secreted by the hypopharyngeal gland (Takenaka et al., 1990; Ohashi et al., 1999); it has been found in honey (Schepartz and Subers, 1964) and in royal jelly (Furusawa et al., 2008). It has been shown that honey glucose oxidase is only active in diluted honey (Carina et al., 2014). Glucose oxidase catalyses the oxidation of D-glucose to D-gluconolactone and hydrogen peroxide (Keilin and Hartree, 1948; 1952; Schepartz, 1965). D-gluconolactone in presence of water produces gluconic acid spontaneously (Takahashi and Mitsumoto, 1963). The main function of glucose oxidase is to act as an antibacterial and antifungal agent (Wong et al., 2008). Both products of the reaction, gluconic acid and hydrogen peroxide, contribute to the disinfection of the colony, the prevention of the contamination by pathogens and the limitation of diseases diffusion (Alaux et al., 2010).

Most authorities consider the destruction of infected colonies, by burning, as the only effective control measure (Williams, 2000). Antibiotics can only mitigate the disease effects, but do not lead to complete sanification (Reybroeck et al., 2012), moreover, they are not allowed in EU apiculture (council regulation EEC n. 2377/90). In order to find alternative measures of control, the antibacterial activity of propolis components,
honey and essential oils were also tested (Albo et al., 2003; Roussenova, 2011; Mihai et al., 2012; Bilikova, 2013; Erler et al., 2014). Fuselli et al. (2006) tested essential oil of cinnamon and thyme. As well, they tested thymol compound and the mixture (62.5% of thyme, 12.5% of cinnamon and 25% of thymol) between them. They observed the following results: for cinnamon a minimal inhibitory concentration (MIC) value of 50-100 µg/mL and a minimum bactericidal concentration (MBC) value of 100-125 µg/mL; for thyme a MIC value of 150-250 µg/mL and a MBC value of 200-300 µg/mL; for thymol a MIC value of 100-125 µg/mL; for thyme a MIC value of 50-100 µg/mL and 125-250 µg/mL, respectively. Roussenova (2011), by testing eleven essential oils against P. larvae, demonstrated a high antimicrobial activity: MIC values recorded for cinnamon, thyme, clove, peppermint, lemongrass, sage and oregano essential oils were ≤ 0.06-0.015% v/v, while marjoram and tea tree oils exhibited a variable activity. Moreover, some reports on the sporicidal effect of natural substances (essential oil and plant extracts) are present in literature (Lawrence and Palombo, 2009; Lau and Rukayadi, 2015) concerning spore forming bacteria other than P. larvae.

The aim of this study was to evaluate the in vitro effect of glucose oxidase and its products on P. larvae growth to establish if they could be used as a fighting measure against AFB.

Materials and methods

Bacterial strain

The type strain ATCC9545 of P. larvae was obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium). It was grown in brain heart infusion broth (BHI) (Oxoid, Milano, Italy) at 37 °C for 24 h in aerobic condition or on tryptone soy agar (TSA) (Oxoid, Milano, Italy) at 37 °C for 48-72 h in aerobic condition.

Determination of MIC

Susceptibility of P. larvae against glucose oxidase and its products (gluconic acid and hydrogen peroxide) was evaluated by broth microdilution method. Glucose oxidase from Aspergillus niger (powder, 10 KU) was purchased from Sigma-Aldrich (Milano, Italy); gluconic acid (50% w/v aqueous solution) from Merck KGaA (Darmstadt, Deutschland) and hydrogen peroxide (40% w/v aqueous solution) from Carlo Erba (Milano, Italy).

MIC values were determined for each potential inhibitor, according to Flesar et al. (2010) with modifications. Glucose oxidase was solubilised in a solvent [2 M D (+) glucose (Panreac, Barcelona, Spain) and 100 mM HEPES buffer pH 7]. The obtained solution was thoroughly mixed for 1 hour. Gluconic acid (50% w/v) and hydrogen peroxide (40% w/v) solutions were diluted with sterile water. For each inhibitor, a two-fold serial dilution in BHI broth was performed using microtiter plates. No inhibitory activity against P. larvae was detected for these solvents. Tested concentrations ranged from 19000 to 0.035 µg/mL for glucose oxidase, from 118 to 0.000226 µg/mL for gluconic acid and from 95 to 0.000118 µg/mL for hydrogen peroxide. Microtiter plates containing different potential inhibitors were then inoculated with a P. larvae culture at a final concentration of 6 × 10⁸ CFU/mL, corresponding to point 2 of the MacFarland turbidity scale. For each microplate, a positive control (containing inoculated BHI broth) and negative control (containing sterile BHI broth) were included. After 24 h of incubation at 37 °C in aerobic conditions and a humid, dark chamber, MIC values for each inhibitor were determined visually as the lowest concentrations at which no growth was observed.

Determination of MBC

After determination of MIC values for each compound, samples contained in the microplate wells corresponding to concentrations equal or higher to the MIC were streaked on TSA plates and incubated at 37 °C for 48 h in aerobic condition to detect cellular proliferation and avoid spores germination. MBC was determined as the lowest concentration which allowed no colonies growth on TSA plates.

Determination of survival growth rate to MIC values

Observed MIC values were employed to evaluate P. larvae survival growth rate. A macromethod test in which BHI broth containing inhibitors in a concentration corresponding to the observed MIC values was used. P. larvae standard suspension (approximately 6 × 10⁸ CFU/mL) was inoculated in each tube. A control sample consisting of BHI broth inoculated with P. larvae standard suspension was also prepared. After incubation at 37 °C for 24 h, each sample was serially diluted in sterile saline solution and pour plated (1 mL) on TSA. After incubation at 37 °C for 48-72 h enumeration of the colonies was performed. Results obtained for each treatment were compared to those of the control sample.

Survival rate of honeybees to gluconic acid

Honey bees have been collected from the experimental apiary of Veterinary Sciences Department of Pisa University. Five concentrations (50, 25, 12.5, 6.25, 3.125% w/v sterile water solution) of gluconic acid and sterile water (control) have been tested. Each concentration has been tested in triplicate on 10 honey bees for replicate. Honey bees were placed in a Petri dish plate with a hole on the cap closed by a netting and fitted with a syringe with honey. An amount of 50 µl of gluconic acid solution was dripped on the thorax of each bee. The survival rate was recorded for 4 days.

Statistical analysis

MIC, MBC values, survival growth rate at the MIC values and survival rate of honeybees to gluconic acid were analyzed by ANOVA and to evaluate possible differences between inhibitors, mean values were tested by Tukey-Kramer HSD post-hoc comparisons.
Results

Table 1 shows MIC and MBC mean values of glucose oxidase and its products determined for *P. larvae* ATCC9545 vegetative form. Moreover, survival growth rates at the MIC values are shown.

Both glucose oxidase, in presence of its substrate, and its products showed an inhibitory effect against *P. larvae* although at different concentrations. Hydrogen peroxide exhibited an inhibitory effect with a MIC value of 0.74 µg/mL, corresponding also to the MBC value. Hydrogen peroxide MBC value resulted the lowest compared to the other MBC values observed (p < 0.001). Although both glucose oxidase and gluconic acid showed a high percentage of survival growth rate at the MIC values (98.3 and 91.6%, respectively), a bactericidal effect was observed at reasonably low concentrations, 73.20 µg/mL and 7.42 µg/mL, respectively. In particular, glucose oxidase MIC value resulted significantly lower (p < 0.001) than those of other tested inhibitors.

In order to investigate the honeybees’ mortality caused by gluconic acid an experiment was carried out. Results (table 2) showed that the survival rate was not significant different in honeybees treated with different gluconic acid concentrations compared to control group.

Discussion and conclusions

Our results not surprisingly showed a marked inhibitory activity of hydrogen peroxide on the vegetative form of *P. larvae* ATCC9545, but the practical application of hydrogen peroxide in the field could pose some problems due to its chemical instability and the possible toxic effect on the bees and oxygen. The novelty of this investigation is the use of glucose oxidase from *A. niger* and gluconic acid to inhibit *P. larvae* vegetative form in vitro. The choice of glucose oxidase from *A. niger* is due to its easy commercial availability and because its antibacterial effectiveness has been demonstrated by Zia et al. (2013). The pure enzyme glucose oxidase from *A. mellifera* is not available yet. Although they showed a less marked inhibitory effect, the use of glucose oxidase and gluconic acid could be more practical since they are compatible with the well-being of bees and bee larvae. In fact, glucose oxidase is normally secreted by honeybees on royal jelly and honey (Furusawa et al., 2008; Bucekova et al., 2014) and gluconic acid is a natural component of honey (Pulcini et al., 2004). In addition, glucose oxidase and gluconic acid showed a bactericidal effect at low concentrations. Moreover, since the hive microbial community contributes significantly to the social immunity, it would be important to investigate the effects of glucose oxidase and its products on symbiotic and non symbiotic hive microorganisms (Anderson et al., 2011; 2013), in particular those with an inhibitory activity against *P. larvae* (Alippi and Reynaldi, 2006). The main beneficial effect of the employment of glucose oxidase would be a constant production of hydrogen peroxide and gluconic acid, which would then contribute together to the eventual inhibitory activity.

Bucekova et al. (2014) showed that glucose oxidase is a regular component of natural honeys and its content varies significantly among honeys. Mato et al. (1997) analyzing 20 Spanish honey samples, have shown a gluconic acid content ranging from 3.91 to 11.71 g/kg; later Mato et al. (2006), carrying out a research on the determination of honey organic acids content, have highlighted a gluconic acid range from 3.7 up to 14.4 g/kg in 10 Spanish honey samples of different botanical origin. Pulcini et al. (2004) analyzed the total gluconic acid content in Italian unifloral honeys and found variable concentrations varying from 2 to 12.3 g/kg, values hundreds of times higher than the MBC value we observed. Moreover, it has been observed that stingless bee honey has a similar gluconic acid content (7.7-11.8 g/kg) (Persano Oddo et al., 2008). Erler et al. (2014) have already tested honeys with different hydrogen peroxide content against *P. larvae*. Therefore, it would be interesting to

Table 1. MIC, MBC mean values and *P. larvae* survival growth rate at the MIC values observed in 3 replicates of glucose oxidase, gluconic acid and hydrogen peroxide. Different letters into the columns show a statistically significant difference (p < 0.001).

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>MIC values</th>
<th>MBC values</th>
<th>Survival growth rate at the MIC values (% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose oxidase</td>
<td>0.28± ± 0.00 µg/mL</td>
<td>74.20± ± 0.00 µg/mL</td>
<td>98.3± ± 0.3</td>
</tr>
<tr>
<td>Gluconic acid</td>
<td>3.71± ± 0.00 µg/mL</td>
<td>7.42± ± 0.00 µg/mL</td>
<td>91.6± ± 0.2</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.74± ± 0.00 µg/mL</td>
<td>0.74± ± 0.00 µg/mL</td>
<td>0.0± ± 0.0</td>
</tr>
</tbody>
</table>

Table 2. Survival rate % of honey bees reared in vitro with honey and treated with different gluconic acid concentrations. No significant differences at p < 0.05.

<table>
<thead>
<tr>
<th>Survival rate, mean (SE)</th>
<th>Control</th>
<th>3.125% w/v gluconic acid</th>
<th>6.25% w/v gluconic acid</th>
<th>12.5% w/v gluconic acid</th>
<th>25% w/v gluconic acid</th>
<th>50% w/v gluconic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>83 (12)</td>
<td>80 (12)</td>
<td>100 (0)</td>
<td>100 (0)</td>
<td>97 (3)</td>
<td>100 (0)</td>
<td></td>
</tr>
</tbody>
</table>
investigate the inhibitory activity against P. larvae of honeys containing different gluconic acid and glucose oxidase concentrations, using diluted honeys (Carina et al., 2014) so that glucose oxidase would be able to perform its enzymatic activity.

Moreover, the evaluation of P. larvae wild strains resistance against glucose oxidase and gluconic acid would be needed in order to understand the effective antimicrobial potential.

Although we studied the inhibitory effect of glucose oxidase and its reaction products only on P. larve ATCC9545 vegetative form, and not on the spore, nonetheless we obtained promising and repeatable results in vitro.

Moreover, Dingman (2011) showed that hydrogen peroxide/peroxyacetic acid biocide is able to inactivate P. larvae endospores and thus could be employed as a sanitizing agent of beekeeping equipment. It would be then interesting to investigate the effects of glucose oxidase and its products on P. larve endospores.

This work represents a preliminary investigation; however, the employment of glucose oxidase/gluconic acid could be a new alternative tool against P. larve, compatible with honeybee well-being and beekeepers economy.

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