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Thermal stable and proteinase-K resistant insecticidal toxins produced by *Photorhabdus luminescens*

Ihsan Ullah* and Khalid M.S. Al-ghamdi

Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract

Photorhabdus is lives in a mutualistic association with nematodes from the family *Heterorhabditis*. Bacteria of the *Photorhabdus* can survive independently and cause toxicity in a larger variety of insects. In the present study, insecticidal activity of non-portentous heat-stable metabolites of *Photorhabdus luminescens* was evaluated against *Galleria mellonella*. For this purpose, the culture extract of *P. luminescens* was injected into the *G. mellonella* larvae, which killed almost 90% of larvae within 48 h. The extract showed 100% insecticidal activity after heat treatment of 70°C for 30 min and even 60% and 40% activity lasted at 80°C and 90°C respectively. The extract also showed a high degree of thermal stability and was 100% actives after 60 min at 70°C. In addition, insecticidal activity was preserved up to 100% after all proteinase-K treatments (0 µg/mL to 50 µg/mL). The results revealed that the extracts were non-portentous and showed high thermal resistance and stability.

Key words: *Photorhabdus*, insecticidal activity, toxins, heat stable non-proteinaceous

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**Corresponding Author:*
iullah@kau.edu.sa
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1. INTRODUCTION

Researchers have examined nematodes, parasitoids, entomopathogenic bacteria, and fungi in the laboratory against a wide variety of insects. *Beauveria bassiana* is an entomopathogenic fungus found in Europe that is used for pest control on cherry, grapevine, and strawberry. *Bacillus thuringiensis*, *Pseudomonas entomophila*, *Burkholderia* species, and *Photorhabdus* species have all been used for practical control of various Diptera species [1]. *Photorhabdus* bacteria are used as a bio-pesticide against a wide range of insect pests in various parts of the world [2]. It is highly entomopathogenic, especially in the larval stages, due to the presence of toxins [3]. The *Photorhabdus* gene pool contains genes that code for toxins like the toxin complex (Tc), *Photorhabdus* insect-related (Pir) [4]. *Photorhabdus* spp. has insecticidal activity that is not limited to proteinaceous toxins [5]. The majority of Photorhabdus genes are involved in the synthesis of non-portentous secondary metabolites that cause rapid insect toxicity [1,4]. According to Kim et al. [6], exposure to the photorhabdus toxin suppresses the immune system of the insect host. The insect immune response is suppressed by inhibiting phospholipase A2 (PLA2), a biochemical enzyme that catalyzes the release of eicosanoids. After non-self-recognition signals occur, eicosanoids suppress the immune system of several insect pathogens [5]. *Photorhabdus* bacteria produce highly insecticidal toxins as a biocontrol agent, but these have yet to be commercialized [7].

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Efficacy of *Photorhabdus* spp. against larvae of *Spodoptera litura, Manduca sexta*, *Plutella xylostella, Leptinotarsa decemlineata* and *Galleria mellonella* has been reported in several studies [8]. The discovery of Tc produced by *P. luminescens* has triggered a lot of interest in developing *P. luminescens* Tc alternatives to *Bacillus thuringiensis*. high molecular weight insecticidal protein produced by *Photorhabdus* caused toxicity in *L. decemlineata*, and *Bemisia tabaci* when the toxin was administered orally [9].

The study was designed to investigate the potential toxicity of extracellular thermostable toxins extracted from *P. luminescens*. Culture extracts were heated and treated with proteinase-K to remove potentially toxic compounds and investigate the non-toxic products of photorhabdus bacteria. The culture extract injected to five instar larva of *G. mellonella*.

2. MATERIALS AND METHODS

2.1 Antibiotics activities and growth conditions optimization

Photorhabdus luminescens was cultured in LB liquid broth and antibacterial activity of the *P. luminescens* was assessed against *Salmonella typhimurium Bacillus anthracis, Listeria monocytogenes Pantoea conspicua, Citrobacter youngae Bacillus aryabhattai* and *Enterobacter cowanii,* following Jang et al. [7]. Thirty microliter cultures of bacterial cultures were spread over the surfaces of LB agar medium, and *P. luminescens* and *Escherichia coli* DH5α colonies were stabbed on plants. Tetracycline (15.0 µg/mL) and *E. coli* DH5α were used as positive and negative controls respectively. The culture plates were then incubated at 30 ± 2°C for 48 h.

2.2 Insecticidal bio-assay against

Photorhabdus luminescens was cultured in 50 mL LB liquid broth *and incubated at* 30 ± 2°C for 36 h. The supernatant was separated after centrifugation at 10,000 × g for 10 min. Injection samples were made from the culture extract, which contains insecticidal metabolites in the supernatant.

Galleria mellonella larvae were cultured following Jang et al. [7] *and the* insecticidal test was performed on five instar *G. mellonella* larvae by injecting 5 µl of culture extract using a Hamilton syringe. E. coli DH5 culture extract, processed under the same conditions was used as control. After injecting the toxin into the hemocoel of larvae, the larvae were placed in a petri plate and incubated at 30 ± 2°C with 50 ± 5% relative humidity. mortality rate was examined and every 12 h of interval. Three replications with ten larvae per replication were used and the assay was repeated three times.

2.3 Heat treatment of extract

To determine the effect of temperature on the toxin, a culture extract was treated for 30 min at various temperatures ranging from 20°C to 100°C. Heat-treated samples of the toxin were injected into the hemocoel of larvae to test its insecticidal activity. Furthermore, the culture extract was heat treated at 70°C form a range of 0 min to 100 min to establish the thermal stability of the toxin. The toxin was tested for insecticidal capability by injecting heat-treated samples into the hemocoel of larvae. All of the samples were placed in a petri plate and incubated at 30 ± 2°C with 50 ± 5% relative humidity to examine the post-injection effects on larvae. The rate of mortality was monitored after every 12 h. Three replications with ten larvae per replication were used and the assay was repeated three times.

2.4 Proteinase-K treatments

In order to eliminate the effects of proteinaceous toxins of *P. luminescens,* the culture extract was subjected to proteinase-K treatment. Proteinase-K was added to the culture extract at different concentrations (10, 20, 30, 40, and 50 mg/L) and incubated at 37°C for 60 min and then injected to the hemocoel of larvae. All of the injected larvae were placed in a petri plate and incubated at 30 ± 2°C with 50 ± 5% relative humidity to examine the post-injection effects on larvae. The rate of mortality was monitored after every 12 h. Three replications with ten larvae per replication were used and the assay was repeated three times.

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2.5 Statistical analysis

Microsoft Excel 2007 (Redmond, WA, USA) was used to calculate the means and standard deviations. By using GraphPad online, the data was statistically examined for standard deviation. Student's *t*-tests were used to compare the mean values ($P < 0.05$).

3. RESULTS AND DISCUSSIONS

3.1 Antibacterial activity

Antibiotic synthesis by *P. luminescens* was confirmed using a simple experiment. Thirty microliter cultures of *S. typhimurium B. anthracis, L. monocytogenes P. conspicua, C. youngae B. aryabhattai* and *E. cowanii* were spread over LB agar medium and results (Table 1) indicated that antibiotics were produced by *P. luminescens*, which killed the bacteria on culture plates. The secretion of antibiotics functioned to form clear zones around the *P. luminescens* colonies. No clear zones formed around the *E. coli* DH5α colonies, which were included as a negative control.

Table 1. Antibacterial effects of extracts of *P. luminescens* against bacterial strains

S. No.	Name of Bacteria	Effects
1	*Salmonella typhimurium*	+Ve
2	*Bacillus anthracis*	+Ve
3	*Listeria monocytogenes*	+Ve
4	*Pantoea conspicua*	+Ve
5	*Citrobacter youngae*	+Ve
6	*Bacillus aryabhattai*	+Ve
7	*Enterobacter cowanii*	+Ve

A variety of secondary metabolites, including antibiotics, bacteriocins, stilbene, and carbapenem, are produced by *Photorhabdus* spp. The findings of our study were similar to those of a past study in which an antimicrobial compound produced by *Photorhabdus* spp. was found to have antibacterial activity against *Salmonella typhi, Staphylococcus aureus, Klebsiella pneumonia, Shigella flexneri* and *Pseudomonas aeruginosa* [9]. These substances produced by *Photorhabdus* spp. were considered to prevent the attack of other bacteria and hence prevent the putrefaction of infected insect carcasses over several weeks [10]. Culture extracts from *Photorhabdus* bacteria contain antimicrobial substances that can be used to combat a variety of bacteria that can cause problems in medical and agricultural settings [5].

3.2 Insecticidal bio-assay

To determine toxicity, *P. luminescens* was cultured in 250 mL of LB liquid broth media for 48 h at 30 ± 2°C, and the culture extract was bio-assayed against *G. mellonella* larvae. Intra-hemocoel injection resulted in 100% mortality in larvae within 36 h (Fig. 1). In addition, the larvae developed paralysis symptoms soon after being injected. Extracellular secondary metabolites are the primary source of those toxins. Extracellular metabolites i.e. phenylalanine-glycine, proline-tyrosine, benzylidene, oxindole, cis-cyclo-PY, and *p*-hydroxyphenyl propionic and indole acetic acid have been reported in *Photorhabdus* spp. [11]. Shrestha et al., reported PLA2 compound in *Photorhabdus* spp. which kills insects, by inhibiting the biosynthesis of eicosanoids [12].

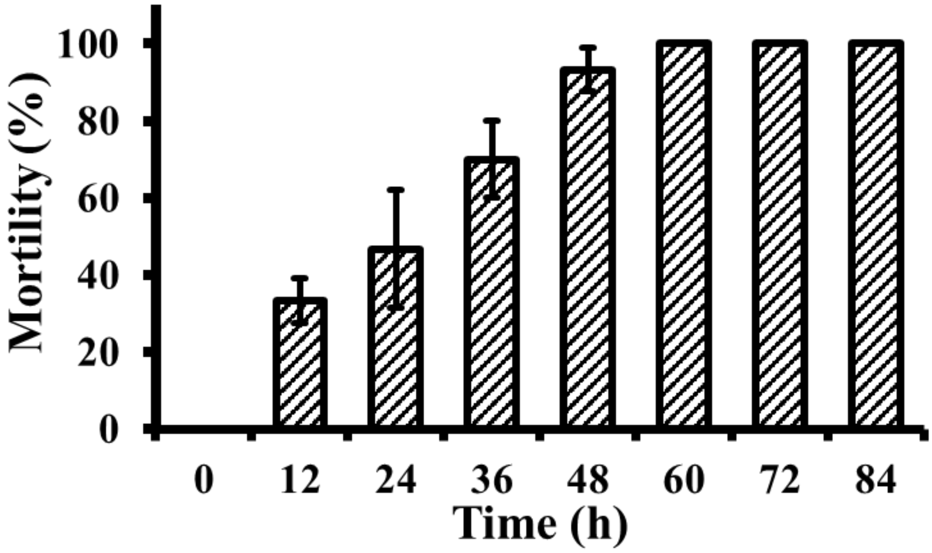

Fig. 1. The rate of mortality in *G. mellonella* caused by the extract of *P. luminescens*

3.3 Thermal resistance

We exposed extract of *P. luminescens* to 20°C to 100°C for 30 minutes to assess the heat resistance of the toxin. The results (Fig. 2) revealed that insecticidal activity was nearly 100 percent at 70°C. however, the activity was up to 65% and 40% when the temperature was elevated up to 80°C and 90°C, respectively.

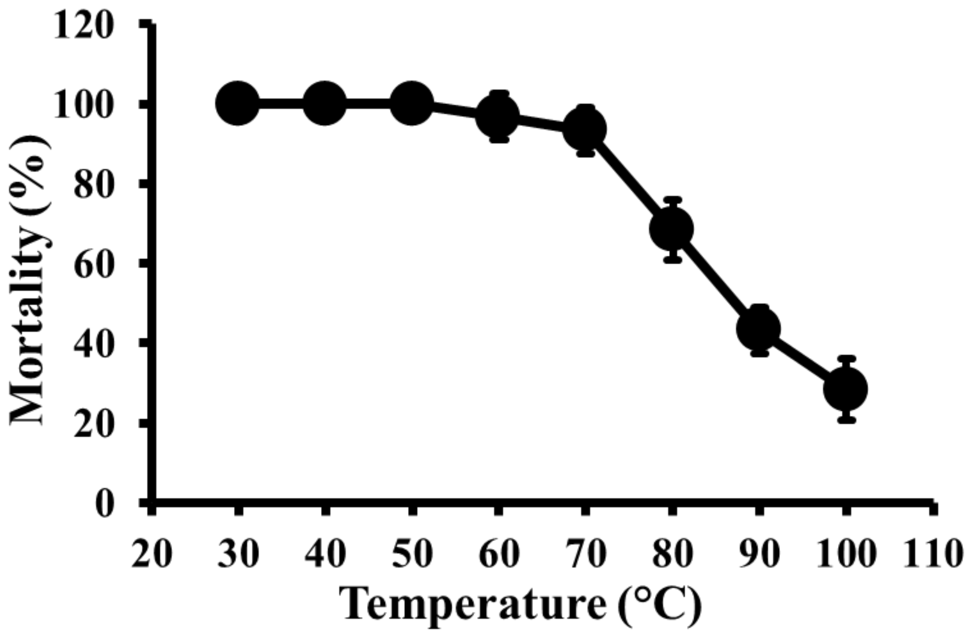

Fig. 2. Insecticidal activity of the extract of *P. luminescens* after heat treatment at different temperatures

Jang et al. [13] reported that Photorhabdus spp., produce non-proteinaceous toxins i.e. phenylethenyl-benzylidene, glycine-valine p-hydroxyphenyl. The metabolites kill insects, by inhibiting the biosynthesis of eicosanoids [14]. These non-proteinaceous toxins are heat resistant and can withstand temperatures as high as 100°C and can kill a variety of insects [15].

3.4 Thermal stability

The insecticidal activity of culture extract was further investigated for a period of 0 to 100 min at 70°C to evaluate the heat stability of toxin. The insecticidal activity was 90% after 60 min of heat treatment at 70°C and was reduced to 50% after 72 min. further, when the time duration was increased up to 84 min the activity was dropped to 30% (Fig. 3).

The extracellular metabolites produced by *Photorhabdus* bacteria have been shown to cause toxicity to a variety of insects according to [16]. Jang et al. [7] reported toxins produced by *P. luminescens* were heat-stable, however, heat treatment above 80°C or for extended periods steadily reduced their activity. *Photorhabdus* bacteria produce lipases, proteases, antibiotics, and lipopolysaccharides and other protein toxins which are heat liable [17].

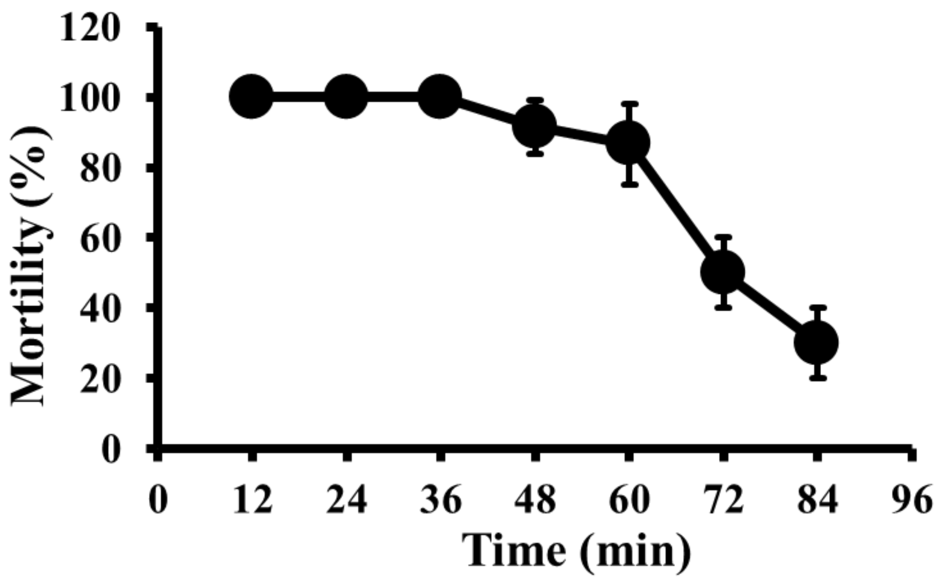

Fig. 3. Insecticidal activity of the heat stable extract of *P. luminescens*

However, Hu et al.[17] report that *Photorhabdus* spp. produce non-proteinaceous heat stable insecticidal toxins which were effective against *G. mellonella*, *L. decemlineata, S. litura,* and *M. sexta*. The toxins produced by the isolated *P. luminescens* was heat stable that might not be protein metabolites. Although toxins were non-protein metabolites, but prolonged heat treatment reduces their activity due to morphological and physiological changes [18]. This could be the reason that a declination in the toxicity was observed after longer time of heat treatment.

3.5 Proteinase-K resistance

Proteinase-K treatment at concentrations ranging from 10 to 50 µg/ml was used in addition to heat treatments, confirming the absence of protein in the toxin extracted from *P. luminescens*. Proteinase-K treatment had no effect on the toxicity of toxin, and 100% of insecticidal activity was maintained at all proteinase-K concentrations (Fig. 4).

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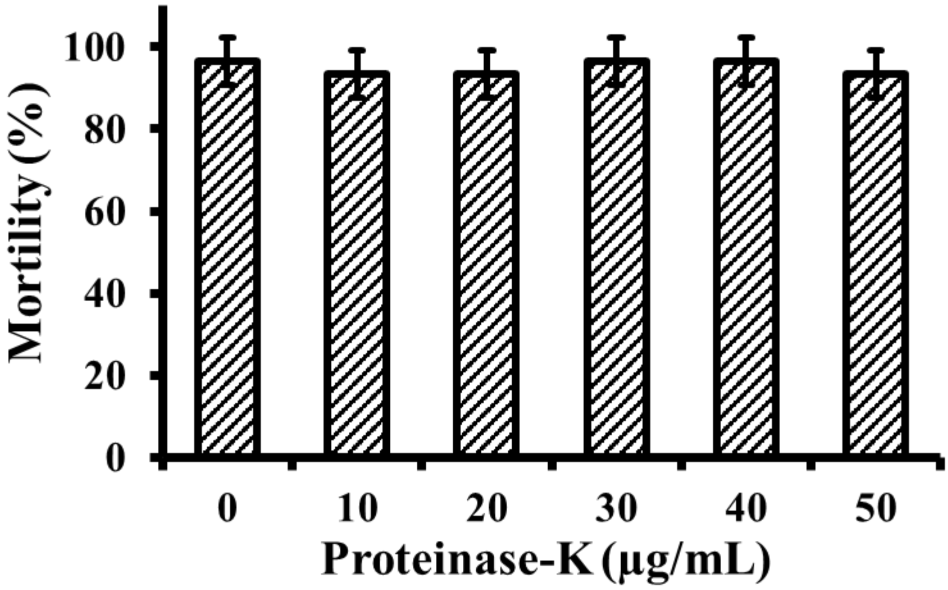

Fig. 4. Insecticidal activity of proteinase-K resistant extract of *P. luminescens*

In the light of these findings, *P. luminescens* toxins were proven to be non-proteinaceous. Several non-polar metabolites, including Ph1A hemolysin, isopropylstilbene, ethylstilbene, anthraquinone, and photobactin, had also been reported in previous studies which showed proteinase-K resistance [19 15 13 18]. Research on the mechanism of action of *P. luminescens* toxins and the factors(s) underlying them could be important given their potential to be utilized by the agriculture industry [18].

4. CONCLUSIONS

From the results it was concluded that extract of *P. luminescens* was highly toxic to the larvae of the *G. mellonella* when injected into larval hemocoel. Heat treatment of 70°C for 30 min resulted in 100% insecticidal activity, and 60 & 40% activity persisted at 80 & 90°C, respectively. The extract also showed a high level of thermal stability and was 100% active after 60 minutes at 70°C. Proteinase-K treatments (0 g/mL to 50 g/mL) did not show any effect of the toxicity of the extract, indicating non-pretentious characteristic of the extract.

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CONFLICT OF INTEREST

No conflict of interest has been found among the authors.

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