Ovicidal effect of chitinase and protease enzymes produced by soil fungi on the camel tick *Hyalomma dromedarii* eggs (Acari:Ixodidae)

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Abstract The aim of this study was to evaluate the effect of chitinase and protease enzymes produced by environmentally safe soil Fungi; *Aspergillus* sp. NRC 4/5H; *Mucor* sp. NRC 5; *Trichoderma* sp. NRC 4/56; *Aspergillus sojae*; *Mucor ranosisinus*; *Aspergillus oryzae* on embryo development of the camel tick *Hyalomma dromedarii* eggs. The experiment was carried out on the 7 day aged eggs. Concentrations of the chitinase and protease crude enzymes [1:1, 1:2, 1:3, 1:4 and 1:5 (v/v)] were prepared from six stock solutions whose concentrations were 5 units/ml each. The prepared chitinase and protease enzymes produced by soil fungi were tested in vitro study for eradication of ticks. Ovicidal effect of the chitin concentrations of extracellular lytic enzymes (chitinase) produced by *Aspergillus* sp NRC 4/5H, *Mucor* sp. NRC 5, *Trichoderma* sp. NRC 4/56 were tested on *H. dromedarii* eggs. The results showed that the ovicidal effect increased with increasing the chitin concentration in case of *Mucor* sp. NRC 5. The maximum inhibitory effect which ranged from 95.3 to 100 % was at concentrations ranging from 1:3 to 1:5 ml/ml, respectively. The results of protease enzymes produced by *A. sojae*, *M. ranosisinus* and *A. oryzae* revealed that, it is highly effective in all concentrations on *H. dromedarii* eggs. It is indicated that the chitinase and protease enzymes produced by fungal species could be used for biological control of the camel tick infestation to avoid the use of carcinogenic chemical.

Keywords Chitinase · Protease · Fungi · Tick · *Hyalomma dromedarii* · Eggs

Introduction

The arthropod parasites (ectoparasites) are a major cause of production losses in livestock throughout the world and many arthropod species act as vectors of diseases for both animals and humans (George et al. 2004; Kusiluka and Kambarage 2006). The tick *Hyalomma dromedarii* is an obligate parasite, principally on camels. This species is known as the most important obstacle to camel production in several areas of the Middle East. In an integrated tick control program covering different methods, the control of this parasite is performed mainly through chemical acaricides. However, continuous use of acaricides has led to the problem of resistance in these arthropods. The most widely used method for controlling tick populations are based on the application of synthetic acaricides both in the environment and to animals. However, the indiscriminate use of these substances inevitably leads to resistance and potentially can harm the environment. A study of the literature shows that promising results have been achieved using fungi that are pathogenic for arthropods (Sewify and Habib 2001; Kaaya and Hassan 2001; Broglio-Micheletti et al. 2012; Lonc et al. 2014).

Therefore, the search for alternatives to chemical pesticides gained the attention of scientists. Insect killing fungi have high potential in controlling agriculturally harmful pests, but their slow progress and high variation in killing insects are major impediments to their successful industrialization (Kim and Je 2010). There is a great demand to introduce this technology in veterinary practices as well as overcome its manufacturing problems for controlling

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animal external parasites. Fungi are found frequently in the environment, particularly in nesting places and soil. Animal faeces fertilize the soil in such a way as to give the fungus competitive advantage over other soil microorganisms. A diverse group of fungi is associated with arthropods, the former constitute the largest class of eukaryotic species on Earth, and play a role in controlling the populations of the latter, in particular of insects (Cizek et al. 2012). The most well known insect-associated fungi are entomopathogens, which are necrotrophic fungi that actively penetrate the host exoskeleton and proliferate in the hemocoel until all internal tissues have been degraded. The infection process of entomopathogenic fungi depends on the secretion of a plethora of enzymes and toxins, which serve to penetrate and kill the host, as well as to provide nutrients through the action of biopolymer-degrading enzymes (Hasan et al. 2013; Barreto et al. 2004). The best-characterized example of a relation between an entomopathogenic fungus and its hosts is the genus *Metarhizium* (Sewify and Habib 2001; Kaaya and Hassan 2001). Chitinases from various sources have been attracting interest for biotechnological applications in the chemical and pharmaceutical industry because they can convert chitinous material from natural sources (such as crab shells) into usable components. Recombinant insect-derived chitinases may serve as powerful enzymes in such catalytic systems. In addition, chitinases and their inhibitors possess high potential as fungicides for the treatment of mycoses in animal and humans, therapeutic compounds against parasites, and biopesticides for the control of insect pests (Merzendorfer 2013). This study focuses on Fungi-derived chitinases and protease and the ovicidal effects of them on *H. dromedarii* eggs.

Materials and methods

Fungi

Isolation

Samples were collected from soil. The samples were diluted in sterile saline solution (0.89 % w/v). The diluted samples were plated onto sterile starch nitrate plates (pH 7) containing (g/l) Starch 20; KNO₃ 1; K₂HPO₄ 0.5; MgSO₄ 7H₂O 0.5; NaCl 0.5; FeSO₄ 0.01; and agar 20 and incubated at 30 °C (Kuester and Williams 1964). After 7 days, the isolated fungi colonies were subcultured in fresh plates and then the single uniform colonies were transferred into slants of the same medium and preserved in the refrigerator at 4 °C until use.

Chitinase production

LB medium is a common medium used for identifying fungi, and selective medium used below is for the sake of obtaining chitinase-producing fungi. The strain will show red colony when grown on medium containing beef extract or peptone (Ghafil 2013). Selective medium contained 1 % colloidal chitin (w/v), 0.5 % (NH₄)₂SO₄ (w/v), 0.05 % MgSO₄ 7H₂O (w/v), 0.24 % KH₂PO₄ (w/v), 0.06 % K₂HPO₄ (w/v), pH 7.0. (Pranav and Mukund 1989). Solidified selective medium was obtained by adding 1.5 % agar (w/v) into the selective medium. LB medium contained 1 % beef extract (w/v), 1 % peptone (w/v), 0.5 % NaCl (w/v), pH 7.0. Solidified LB medium was from adding 1.5 % of agar (w/v) into LB medium. The isolated strain was identified by biochemical tests in National Research Centre (Microbial Chemistry Dept.).

Protease production

Two ml of spore suspension was inoculated on 48 ml liquid medium (medium containing % soy bean, 30; dextrin, 15; CaCO₃, 10; MgSO₄, 1.0 pH 7.0) in 250 ml Erlenmeyer flasks. There after, the flasks were incubated for 6 days at 30 °C in a shaker incubator (200 rpm). The culture medium was centrifuged at 6000 rpm for 10 min and the supernatant was assayed for protease activity and protein content.

Screening of fungi for production of chitinolytic and proteolytic activities

Chitinase activity

The basal medium used for the optimization studies contained (g/l): KH₂PO₄, 3.0; K₂HPO₄, 1.0; MgSO₄, 0.7; (NH₄)₂SO₄, 1.4; NaCl, 0.5; CaCl₂, 0.5; yeast extract, 0.5; bacto-peptone, 0.5 and chitin, 5.0. The pH of the medium after autoclaving was between 5.0 and 5.5 for fungi, all the experiments were carried out in shake flasks (100 ml medium/500-ml Erlenmeyer flask) incubated at 28 °C with shaking (150 rpm) for 7 days. For further studies, medium optimized for enzyme production was used.

In all the experiments, spore inoculums (10⁷/flask) from 7-day-old slants was used.

Protease activity

The culture was inoculated in 250 ml Erlenmeyer flasks containing 100 ml of production medium, consisting of: Glucose 150 mg, K₂HPO₄ 20 mg, KH₂PO₄ 20 mg, MgSO₄

10 mg, CaCl₂ 10 mg, casein 200 mg, NaNO₃ 100 mg pH 8.5 and put on an environmental shaker at 150 rpm at 28 °C for 5 days and checked for enzyme activity. The supernatant was collected after centrifugation at 15,000 rpm, at 4 °C temperature for 15 min and was used as crude enzyme.

Preparation of dead enzyme

The crude culture filtrate was autoclaved at 121 °C, 1.5 atm for 5 min.

Quantitative assay of protease

Protease activity in the culture filtrate was assayed by method of Tsuchida et al. (1986) by using casein as the substrate. One protease unit is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per min under the above assay conditions.

Determination of chitinase activity

Chitinase activity was determined by measuring the amount of the reducing end group, degraded from colloidal chitin, as described in the previous by Bergmeyer et al. (1983). One unit of activity is defined as the amount of enzyme that liberated 1 µmol of reducing sugar per min.

Protein concentration

The protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a

Ovicidal effect of chitinase and protease enzymes

Test was carried out on the egg aged 7 day. Concentrations of the chitinase and protease enzymes derived from *Aspergillus* sp NRC 4/5H; *Mucor* sp. NRC 5; *Trichoderma* sp. NRC 4/56; *A. sojae*; *M. ranosisinus*; *A. oryzae* were prepared by using distilled water. The test included five concentration; 1:1, 1:2, 1:3, 1:4, 1:5 [crude enzyme:distilled water(v/v)]. The concentrations of the six stock solutions from which these dilutions were prepared had enzyme concentration of 5 units/ml. Distilled water was used alone as a control treatment for each concentration. Each concentration or control treatment was replicated 3 times, each replicate included 50 healthy eggs. Treatment was applied by dipping eggs for 50 s in 200 µl from each concentration dilution or distilled water in control treatment and left to dry and then incubated at room temperature until hatchability occurred. Calculated mortality percentage of eggs were based on eggs with brown–black color and abnormal shape and corrected by Abbott's formula (Abbott 1925 and Habeeb et al. 2007). Normal eggs were normal shape (oval) and colors (shiny brown) were left to develop until hatching occurred.

Inhibitory activity

Inhibitory activity of tested fungi enzymes on *H. dromedarii* eggs were determined at the end of the incubation period. The rate of eggs hatching in both exposed and control of eggs were evaluated according to Hegazi et al. 2007. The reduction percentage of *H. dromedarii* egg hatching was estimated using the following formula:

$$\text{Inhibitory activity} = \frac{\text{Hatching \% of control eggs} - \text{Hatching \% of exposed eggs}}{\text{Hatching \% of control eggs}} \times 100.$$

standard.

Ticks

Engorged females of *H. dromedarii* were collected from the ground of camel market in Burkash village, Giza governorate, Egypt. Identification of females was confirmed in the laboratory according to Hoogstraal 1956 and Estrada-Peña et al. 2004. The females were incubated at a constant temperature of 24 ± 2 °C with a relative humidity of 75 ± 5 % in permanent darkness to obtain eggs as previously described by Patrick and Hair (1975).

Statistical analysis

Data were subjected to statistical analysis including the calculation of mean and standard deviation (SD). Differences between treated groups were tested for significance using a two-way analysis of variance followed by Duncan's multiple range test. Differences were considered significant at *P* < 0.01 level, Snedecor and Corchran 1989, using SPSS version 10 computer program download from <http://www.SPSS.com>.

Results

Screening of chitinase and protease produced by the isolation fungi

Primary screening for chitinase and protease producing fungi were done to study the growth in the culture medium suggested that maximum growth in all isolates occurred, data in Tables 1 and 2.

Ovicidal effect of chitinase and protease enzymes

Five concentrations of enzymes chitin and protein ranged from 1:1 to 1:5 ml/ml were tested at room temperature on *H. dromedarii* eggs and the results were obtained on the 7 day after the treatment (after hatching) (Table 3). Ovicidal effect of the chitin concentrations of extra cellular lytic enzymes (chitinase) produced by *Aspergillus* sp NRC 4/5H, *Mucor* sp. NRC 5, *Trichoderma* sp. NRC 4/56 were

Table 1 In-vitro growth on medium containing Casein for protease or chitin for chitinase

Strain	Chitinase	Protease
<i>Aspergillus sojae</i>	+	++++
<i>Mucor ranosisinus</i>	–	++++
<i>Aspergillus oryzae</i>	–	+++++
<i>Aspergillus</i> sp NRC 4/5H	+++++	–
<i>Trichoderma</i> sp. NRC 4/56	++++	+
<i>Mucor</i> sp. NRC 5	+++++	–
– No growth		
+ Weak growth		
++ Moderate growth		
+++ Heavy growth		
++++ Vigorous growth		

Table 2 Production of chitinase and protease by fungi

Strain	Chitinase activity (units/mg protein)	Protease activity (units/mg protein)
<i>Aspergillus sojae</i>	–	889.93 ± 0.91
<i>Mucor ranosisinus</i>	–	3136.33 ± 22.3
<i>Aspergillus oryzae</i>	–	1200.39 ± 1.79
<i>Aspergillus</i> sp NRC 4/5H	5605 ± 7.0	–
<i>Mucor</i> sp. NRC 5	4420.67 ± 0.58	–
<i>Trichoderma</i> sp. NRC 4/56	3458.67 ± 8.39	–

tested on *H. dromedarii* eggs. The results showed that ovicidal effect increased with increasing the chitin concentration in case of *Mucor* sp. NRC 5. The maximum inhibitory effect which ranged from 95.3 to 100 % was at concentrations which ranged from 1:3 to 1:5 ml/ml (v/v), respectively. In *Aspergillus* sp NRC 4/5H chitin, the ovicidal effect showed that, marked increase in concentration 1:5 ml/ml (100 %) and marked decrease in concentration 1:4 ml/ml (85.3 %). Ovicidal effect of *Trichoderma* sp. NRC 4/56 chitin showed fluctuations between the concentrations. The protein concentrations of extracellular lytic enzymes (protease) produced by *A. sojae*, *M. ranosisinus* and *A. oryzae* were tested on *H. dromedarii* eggs. The protease enzymes produced by *A. sojae*, *M. ranosisinus* and *A. oryzae* revealed high effective inhibition in all concentrations on *H. dromedarii* eggs. Ovicidal effect of the protease concentrations, produced by *A. sojae* showed, marked effect in concentration 1:2 ml/ml (100 %). The ovicidal effect of 1:3, 1:5 ml/ml protease concentrations produced by *M. ranosisinus* on *H. dromedarii* eggs was 100 %.

Inhibitory activity

In chitinase enzymes produced by *Aspergillus* sp NRC 4/56, the highest inhibitory activity on egg development was observed in all concentrations. At concentrations 1/1, 1/2 and 1/4 of chitinase produced by *Mucor* sp. NRC 5 and *Trichoderma* sp. NRC 4/56, the high inhibitory activity on *H. dromedarii* egg development was 100 %. The highest inhibitory activity on egg development treated with protease enzymes was 100 % at concentrations 1/1 and 1/5 in *A. oryzae*, *M. ranosisinus* and *A. sojae* respectively.

Discussion

Among the major benefits of incorporating biopesticides into agricultural systems is that they are generally more environmentally friendly and do not damage the soil, water supply or wildlife-including the beneficial insects. Their safety to beneficial organisms is one area where biopesticides are definitely having a fit.

Hassan et al. (2015) reported that, Chitinase enzyme was produced and extracted from cell walls of *Candida albicans* and *Aspergillus fumigatus* isolates that were recovered from soil and assayed for control of cattle tick's infestation, *Boophilus microplus* in comparison with one of the most commonly used arsenical pesticide. These fungi showed the ability to eradicate 90 and 80 % of active ticks, respectively. The mean time effect per day gives an indication on the efficacy of pesticide, the shorter the time, the better is the efficacy of the product. Their results proved

Table 3 Effect of protease and chitinase enzymes of *Aspergillus sojae*, *Mucor ranosisinus*, *Aspergillus oryzae*, *Aspergillus* sp. NRC 4/5H, *Mucor* sp. NRC 5 and *Trichoderma* sp. NRC 4/56, fungi on *H. dromedarii* eggs

Concentration	Tested protease			Tested chitinase			Marginal mean
	<i>Aspergillus sojae</i>	<i>Mucor ranosisinus</i>	<i>Aspergillus oryzae</i>	<i>Aspergillus</i> sp. NRC 4/5H	<i>Mucor</i> sp. NRC 5	<i>Trichoderma</i> sp. NRC 4/56	
1/1	96.0 ± 5.3	99.3 ± 1.2	100.0 ± 0.0	96.7 ± 4.2	100.0 ± 0.0	97.3 ± 4.6	98.22 ± 1.4 ^a
1/2	100.0 ± 0.0	96.3 ± 4.7	96.0 ± 6.9	92.0 ± 9.2	85.3 ± 13.3	95.3 ± 4.2	94.2 ± 1.3 ^{bc}
1/3	98.0 ± 3.5	100.0 ± 0.0	95.3 ± 8.1	98.0 ± 5.3	96.6 ± 5.8	96.7 ± 3.1	97.4 ± 1.3 ^{ab}
1/4	99.3 ± 1.2	87.3 ± 15.5	100.0 ± 0.0	85.3 ± 5.0	94.0 ± 2.0	94.7 ± 1.2	93.4 ± 1.3 ^c
1/5	96.6 ± 5.8	100.0 ± 0.0	96.3 ± 6.5	100.0 ± 0.0	96.7 ± 3.1	98.0 ± 3.5	97.9 ± 1.3 ^{ab}
Marginal mean	98.0 ± 1.4	96.6 ± 1.4	97.5 ± 1.4	94.4 ± 1.4	94.5 ± 1.4	96.4 ± 1.4	

F value between treatment is 1.096 (insignificant)

F value between concentration is 2.992 (significant with *P* value 0.026)

Small letters represent significant differences between concentrations

that these times were 4.3, 6.85, 1.5 days for, *C. albicans*, *A. fumigates* and *Arsenicals* respectively. In our study, the test was carried out on the egg aged 7 day. In fact, our study illustrated that the extracellular lytic enzymes (chitinase) produced by *Aspergillus* sp NRC 4/5H, *Mucor* sp. NRC 5, *Trichoderma* sp. NRC 4/56 were having significant inhibitory effect on camel tick *H. dromedarii* eggs, our results are supported by Merzendorfer (2013) who mentioned that the genome of the entomopathogenic fungus *Metarhizium anisopliae* encodes several chitinases. One of these chitinases, chitinase 2 (Chi2), is involved in the pathogenicity of this fungus. Strains that overexpress Chi2 showed higher efficiency to kill its host.

Recent advances toward understanding the roles of chitinases in fungal biology, as well as substrate-binding properties and new mechanistic insights in different chitinases should include biotechnological implications of these findings. In aggressive fungi and parasites, e.g. mycoparasites and ectoparasites of invertebrates, chitinases are involved in the attack of other fungi and insects. Overexpression of single chitinases in mycoparasitic, insects and entomopathogenic fungi and/or addition of a chitin-binding domain to these chitinases was shown to improve the mycoparasitic potential and insect virulence, respectively, of these fungi Boldo et al. (2009).

Several extracellular enzymes from entomopathogenic fungi, including protease (especially the subtilisin family of serine proteases), chitinase and collagenase involved in the infection of arthropod, have been identified, cloned, and homologously or heterologously expressed, respectively. They are at least partly responsible for fungal penetration of the insect cuticle and/or digestion of the internal tissues of the host, Wojda et al. (2012). Inhibitory effect of the protease concentrations, produced by *A. sojae*, *M.*

ranosisinus and *A. oryzae* revealed that, they are highly effective in all concentrations on *H. dromedarii* eggs. Our results are supported by Wojda et al. (2012).

Conclusion

Our results concluded that the protease enzymes produced by *A. sojae*, *M. ranosisinus* and *A. oryzae* revealed that, they are highly effective in all concentrations on *H. dromedarii* eggs. Chitin concentrations of extracellular lytic enzymes (chitinase) produced by *Aspergillus* sp NRC 4/5H, *Mucor* sp. NRC 5, *Trichoderma* sp. NRC 4/56 showed that the ovicidal effect increased with increasing the chitin concentration in case of *Mucor* sp. NRC 5. In *Aspergillus* sp NRC 4/5H chitin, the ovicidal effect showed marked increase in concentration 1:5 ml/ml (100 %). Inhibitory effect of *Trichoderma* sp. NRC 4/56 chitin showed fluctuations between the concentrations.

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