Research Article

Variable induction of cuticle-degrading enzymes of *Beauveria bassiana* isolates in the presence of different insect cuticles

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**Abstract:** In the present study, the cuticle-degrading enzymes production potential of five native *Beauveria bassiana* (TV, OZ, UN, DV and DE) isolates was investigated in the presence of cuticles from *Eurygaster integriceps*, *Ephestia kuehniella* and *Zophobas morio*. Furthermore, histopathology of infected insects by *B. bassiana* was studied. The level of cuticle degrading enzymes was the highest and lowest for TV (as the most virulent isolate) and DE (as the weakest isolate), respectively. *E. integriceps* nymphs as the most sensitive host produced the highest level of cuticile degrading enzymes (Pr2, exochitinase, and lipase) while *Z. morio* as the most resistant host, produced the lowest level of hydrolytic enzymes. According to histopathological study, the fungal isolate could not penetrate into *Z. morio* cuticle, as no mycelia or hyphae were observed in its tissues after inoculation, while fungal bodies were detected in microscopic slides of the other two insects. Overall, the chemical and topographical structure of insect cuticle had a substantial effect on the virulence of entomopathogenic fungus. Production of enzymes including proteases (especially Pr2), chitinase (*N*-acetyl-glucosaminidase), and lipases was positively related to virulence of fungus isolates. It can be concluded that not only the hydrolytic activity of *B. bassiana* isolates, but also host cuticle composition determine the pathogenesis and virulence cascade in fungus-insect interactions.

**Keywords:** *Beauveria bassiana*, cuticle-degrading enzymes, virulence, histopathology

**Introduction**

Fungal entomopathogens are important biological control agents all over the world and have been studied intensely for more than 100 years (Vega *et al.*, 2012). Among them, *Beauveria bassiana* can affect a wide range of arthropod pests such as coleopterans, hemipterans and mites, but its distinct isolates differ in their host range and specificity (Lacey *et al.*, 1999; Mayoral *et al.*, 2006; Talaei-Hassanlou et al., 2007).

Entomopathogenic fungi can infect their hosts by direct penetration through the insect cuticle, which is composed of chitin embedded in a matrix with cuticular proteins, lipids, and other compounds (Richard *et al.*, 2010). Fungal penetration to the insect cuticle can be mediated by both mechanical processes and enzymatic attack (Vega *et al.*, 2012). Therefore, the physical and chemical properties of cuticle could affect the virulence of entomopathogenic fungi. Enzyme production by entomopathogenic fungi may be involved in many biological processes,
including the degradation of cuticular polymers during infection, assisting penetration through the insect exoskeleton, and additionally, providing nutrients for fungal growth (Goettel et al., 1989). Proteases, chitinases, and lipases break down the cuticle, those are considered as the most important enzymes in the infection process (Mustafa and Kaur, 2009). Understanding the mode of relationship between enzyme activities and virulence could be useful in developing screening methods to identify new efficient fungal isolates (Gupta et al., 1994).

Correlation between the virulence and the enzyme activity of fungal isolates have been previously reported in different articles (Pinto et al., 2002; Dhar and Kaur, 2010; Zare et al., 2014). A comprehensive study regarding the effects of three enzymes (Proteases, chitinases, and lipases) has been conducted by Pelizza et al. (2012). They indicated that the highest protease, chitinase, and lipase activities were recorded in the most virulent B. bassiana isolate on Schistocerca cancellata Serv (Orthoptera: Acrididae). Similarly, a positive correlation of the virulence with the protease and chitinase activities of B. bassiana isolates was demonstrated against Spodoptera litura Fabricius and Helicoverpa armigera Hübner (Padmini-Palem and Padmaja, 2013). Moreover, studies by Khan et al. (2012) on hydrolytic activities of some isolates of B. bassiana and Verticillium lecanii Zimm revealed that lipase participated more in total virulence to Myzus persicae Sulzer in comparison with protease and chitinase.

In this research, we studied the protease (Pr1 and Pr2), chitinase (N-acetyl glucosaminidase), and lipase activities of different B. bassiana isolates, in response to cuticles from Eurygaster integriceps (Hem: Scutelleridae), Ephemia kuhihniella (Lep: Pyralidae) and Zophobas morio (Col: Tenebrionidae) to illuminate the effect of cuticles from disparate insect orders as substrate and their interaction with production of B. bassiana hydrolytic enzymes. Furthermore, the possible correlation between the virulence and the enzyme activities of fungal isolates and histopathology of infected insect cuticles by B. bassiana has been studied.

Materials and Methods

Insects rearing

Sunn pest, E. integriceps, adults were collected from wheat fields of Varamin, Tehran province, Iran and reared in plastic boxes (30×30×50 cm) on wet wheat seeds (Triticum aestivum var. Pishtaz), a piece of cotton soaked with water was used as a water source. Folded strips of paper were hung in containers as oviposition substrates. Newly emerged nymphs were transferred to plastic shelves with pots of wheat and wet wheat seeds. Fifth instar nymphs (identified by rudiments of hind wings and developmental periods) were used in experiments, as they are physiologically more stable than previous instars and their developmental period is long enough for successful penetration of the fungus. The flour moth colony was prepared from the Biological Control Laboratory, College of Agriculture and Natural Resources, University of Tehran and bred in plastic containers containing flour and yeast (10 g yeast per kg of flour), then fourth instar larvae identified by mean head capsule wide and moltings (Yazdanian et al., 2005) were used in experiments. Initial colony of Z. morio beetle was obtained from a pet food store and reared in plastic containers (20×15×10 cm) containing wheat bran and pieces of potato. The last instar larvae of the new generation were used in experiments. Rearing condition was 25 ± 1 °C, 70 ± 5% R. H. and 16:8 (L: D) h photoperiod for all insects.

Beauveria bassiana isolates

Five B. bassiana isolates encoded TV, OZ, UN, DV and DE (soil origin, Seyyedalebi et al., 2017) were grown on Sabouraud Dextrose Agar (SDA) plates and maintained at 25 ± 1 °C, 70 ± 5 % RH, and a photoperiod of 16:8 (L: D) h for 14 days. Cultures were scrapped after sporulation and conidia were obtained (Goettel and Inglis 1997). Virulence of the isolates has been previously studied on these insects, as TV and DE were the most and the least virulent.
isolates, while the others had moderate virulence on *E. kuehniella* larvae and *E. integriceps* nymphs. None of the isolates significantly affected the survival of *Z. morio* larvae (Seyed talebi et al., 2018).

**Cuticle preparation**

For cuticle preparation, larvae of *E. kuehniella* and *Z. morio* and nymphs of *E. integriceps* were dissected and their internal organs were removed. Cuticles were rinsed several times with saline solution (6 mol/L NaCl), dried and ground to be used in liquid culture media (Ramzi and Zibae, 2014).

**Enzymes assays**

- **Total protease activity**

Total protease activity was assessed using the method described by Erlacher et al. (2006). The substrate was 250 μl of 2% (w/v) azocasein solution in 20 mM Tris HCl buffer (pH 8.0). The reaction was initiated by loading 150 μl of sample to the substrate. The mixture was incubated at 37 °C for 30 min, then the reaction was stopped by addition of 1.2 ml of 10% (w/v) trichloroacetic acid (TCA). Thereafter, the samples were centrifuged (Universal 32R) at 8000 rpm for 4 min and 600 μl of the clear supernatant were transferred to new microtube containing 700 μl of 1.0 M NaOH and vortexed. The absorbance was read at 440 nm with a Microplate reader (Bio TeK, USA). The experiment was conducted in three replicates.

- **Subtilisin (Pr1) and Trypsin (Pr2) activity**

Pr1 and Pr2 activities were assayed using the described method by St. Leger et al. (1987) and specific synthetic substrates, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and Benzoylphenylalanine -valine-arginine-p-nitroanilide (BAPNA), respectively. Each substrate (50 μl of 0.1 Mm) was mixed with 0.85 ml of 20 mM Tris–HCl buffer (pH 8.5) and 100 μl of citrate buffer. The reaction mixture was incubated for 30 min at 28 °C, then terminated using 0.25 ml of 30% acetic acid. Absorbance was observed at 405 nm using Microplate reader (Bio TeK, USA) and the concentration of para-nitro aniline was determined. One unit of protease activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitroaniline per min under the above conditions. The experiment was conducted in three replicates.

- **Chitinase activity**

N-acetyl-β-D-glucosaminidase (NAGase) activity was assessed according to the method of St. Leger et al. (1998). The reaction mixture contained 80 μl of 0.1 M citrate buffer (pH 5.0), 10 μl of 10 mM *p*-nitrophenol-N-acetyl-β-D-glucosaminide, and 10 μl of culture supernatant. Reaction was carried out at 37 °C for 30 min, then terminated by the addition of 100 μl of 0.5 M NaHCO3–Na2CO3 buffer. The release of *p*-nitrophenyl (pNP) was determined at 405 nm (Microplate reader, Bio-Tek, USA). Activities were expressed as 1 μmol of *p*-nitrophenol released per min. The experiment was conducted in three replicates.

- **Lipase activity**

The enzyme assay was carried out as described by Tsujita et al. (1989). Twenty μl of the enzyme, 100 μl of 20 mM Tris–HCl buffer (pH 7) and 50 μl of 27 Mm *p*-nitrophenyl butyrate, were incorporated, thoroughly mixed and incubated at 37 °C. After 15 min of inoculation, 100 μl of NaOH (1 N) was added to each tube and absorbance was read at 405 nm. One unit of enzyme releases 1 μmol of *p*-nitrophenol per min. The experiment was conducted in three replicates.

The protein concentration was determined by the method described by Bradford (1967), using BSA as standard for all experiments.

- **Paraffin embedded histopathological sections**

TV (as the most virulent) isolate was selected for this experiment. The individuals of sunn pest nymphs, the flour moth and king meal worm larva were dipped into 20 ml of the fungal suspension (5 × 10⁷ conidia/mL) for 10 s, distilled water containing 0.05% Tween-80 solution was used as control, based on our previous experiments (Seyed talebi et al., 2018). There were 20 insects in each treatment. After dryness, the insects were transferred to a plastic dish containing relevant food and incubated under 25 ± 1 °C, 70 ± 5% R.H. and 16:8 (L: D) h photoperiod conditions. At 24 h intervals, 3 - 4 larvae were picked out randomly for paraffin sectioning.
The larvae were fixed with Formaldehyde 10% for 24 h, and dehydrated in increasing graded (30, 50 and 75%) ethanol for 1 h. The fixed samples were embedded in paraffin wax and cut into sections of 5 - 6 μm. The sections were stained with hematoxylin and eosin then slide preparations were assessed under a Zeiss microscope and pictures were taken with Dino-Lite digital lens Dinocap®.

**Statistical analysis**
All experimental data were subjected to analysis of variance (ANOVA). Pooled data of two-time repeats of the whole assays were analyzed to determine possible significant differences among the treatments via F-LSD test post-significant ANOVA. A possible correlation between enzyme activity and virulence of isolates was analyzed through the Pearson correlation coefficient (SAS Institute, 2002).

**Results**

**Proteolytic activity**
The proteolytic activity was significantly different among isolates \((F_{4, 15} = 183.08, p < 0.001)\) and insects \((F_{2, 15} = 1377.70, p < 0.001)\). The lowest enzyme activities were recorded on *Z. morio* larvae cuticle (Fig. 1). Among the evaluated isolates, maximum proteolytic activity was observed for TV isolate. There was a positive correlation between the protease activity and the virulence of isolates for two of the insects, *E. kuehniella* larvae \((r = 0.92, p < 0.001)\) and *E. integriceps* \((r = 0.97, p < 0.001)\).

**Pr1 and Pr2 activity**
The Pr1 activity was statistically different among fungus isolates \((F_{4, 15} = 30.09, p < 0.001)\) and insect cuticles \((F_{2, 15} = 211, p < 0.001)\). In the same way, the Pr2 was variably produced by different *B. bassiana* isolates \((F_{4, 15} = 10.12, p < 0.005)\) and on three distinct insect cuticles \((F_{2, 15} = 72, p < 0.001)\). The highest activity of Pr1 and Pr2 was recorded for isolate TV in presence of *E. kuehniella* and *E. integriceps* cuticle, respectively (Figs. 2 and 3). There was a positive correlation between the Pr1 and Pr2 activity and the virulence of isolates on *E. kuehniella* larvae \((r = 0.92, p < 0.001. r = 0.78, p < 0.007)\) and *E. integriceps* \((r = 0.90, p < 0.0003. r = 0.77, p < 0.009)\).

![Figure 1](attachment:image.png)  
**Figure 1** Mean activities of proteases (± SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, P < 0.05).
Figure 2 Mean activities of Pr1 enzyme (± SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, P < 0.05).

**Chitinase activity**
There were significant differences between isolates ($F_{4, 15} = 19.12$, $p < 0.001$) and insect cuticles ($F_{2, 15} = 276.72$, $p < 0.001$) in chitinolytic activity. TV isolate had the highest enzymes activity (Fig. 4). There was a positive correlation between the chitinase activity and the virulence of isolates on *E. kuehniella* ($r = 0.94$, $p < 0.001$) and *E. integriceps* ($r = 0.83$, $p < 0.003$).
Lipase assay
Lipase activity was significantly different among isolates ($F_{4, 15} = 0.19, p < 0.003$) and insects ($F_{2, 15} = 6.71, p < 0.001$). The highest and lowest enzyme activities were observed for *E. integriceps* nymphs and the *Z. morio* larvae, respectively (Fig. 5). There was a positive correlation between the lipase activity and the virulence of isolates for *E. integriceps* ($r = 0.81, p < 0.004$) unlike *E. kuehniella* ($r = 0.61, p > 0.6$).

**Figure 4** Mean chitinase activity ($\pm$ SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, $P < 0.05$).

**Figure 5** Mean lipase activity ($\pm$ SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, $P < 0.05$).
Histopathological study
Microscopic slides indicated different morphological characteristics in the cuticles of the studied insects. The cuticle of the flour moth larvae had numerous folding on the surface (Fig. 6A), Sunn pest cuticle was characterized with many microscopic thistles in different parts (Fig. 6B) and in the case of king meal worm larvae it had a flat surface, it was also thicker (Fig. 6C). Histological examinations, after 96 h exposure to B. bassiana, revealed the growth of fungal hyphae in the tissues of the infected E. kuehniella (Fig. 7A) and E. integriceps (Fig. 7B). In the exposed Z. morio larvae, fungal growth was not detected (Fig. 7C).

Figure 6 The cuticle (× 1000) of flour moth larvae, Ephestia kuehniella (left), Sunn pest, Eurygaster integriceps (middle) and king meal worm, Zophobas morio (right). (cu: cuticle, ep: periderm, h: tissue and b: thistle).

Figure 7 Fungal hyphae of Beauveria bassiana in the body tissues of the infected Ephestia kuehniella (left) and Eurygaster integriceps (middle), with no fungal growth in the body tissues of Zophobas morio (right).

Discussion
Our results revealed that the enzymatic activity of B. bassiana was significantly different based on both fungus isolates and insect cuticles. Although some pre-penetration aspects related to conidial size, vigor, germination speed, and attachment affect the host-fungus interaction (Altre et al., 1999; Safavi et al., 2007; Safavi, 2011; Faria et al., 2015), penetration through the insect cuticle is carried out via a combination of mechanical pressure and hydrolytic enzymes (Lu and St. Leger, 2016). Degradation of cuticular hydrocarbons starts with the reaction of enzyme complexes (Barra et al., 2015). These cuticle-degrading enzymes are important in infection process, as they hydrolyze the major components of insect cuticle including polymer protein, chitin and lipid complexes (Petrisor and Stoian, 2017).

Different induction of hydrolytic enzymes on three insect cuticles in our experiments revealed the crucial role of the host cuticle in the infection process. The composition of the components of host cuticle may induce or inhibit fungal development. This depends on a variety of different factors involved in the specificity of the interaction between the insect cuticles and entomopathogenic fungi (Crespo et al., 2002). The most sensitive and resistant hosts to B. bassiana were E. integriceps nymphs and Z. morio larvae, respectively.
Cuticle degrading enzymes of Beauveria bassiana

Seydtaeabi et al., (2018). Also, in most cases the maximum enzymatic activity was observed in the presence of E. integriceps cuticle, unlike Z. morio cuticle which induced minimum levels of enzymatic activity. Regulation of degradative enzymes is in response to differences in the composition of insect cuticle which varies from species to species (Gupta et al., 1994). Experiments by El-Sayed et al. (1993) revealed that expression of hydrolytic enzymes was different when cuticles of Trichoplusia ni Hübner and Helicoverpa zea Boddie were used as substrates.

Based on our findings, TV isolate, as the most virulent isolate (Seydtaeabi et al., 2018), had the highest proteolytic activity. The entomopathogenic fungus can produce diverse enzymes as virulence factors in response to different insect cuticles. Comparisons among isolates for virulence and production of enzymes indicate the great variability within a fungal species for numerous factors, many of which may influence cuticle-degrading enzyme activity (Petrisor and Stoian, 2017). Extracellular enzymes such as proteases, chitinases and lipases are in some way related to virulence of entomopathogenic fungi (Fang et al., 2005; Kaur and Padmaja, 2009; Safavi, 2012; Dhawan and Joshi, 2017). In the same way, our data indicated that there was a positive correlation between protease, chitinase, and lipase activities and fungal isolate virulence. A study by Gupta et al. (1994) supports the notion that cuticle degrading enzymes may determine not only specific virulence, but also host specificity of fungal isolates. However, other studies showed no correlation between the virulence of fungus and enzyme activities (Rosato et al., 1981).

Insect cuticle is essentially composed of proteins and chitin associated with lipids and phenolic compounds, but protein content of this complex and composite structure is higher than the others. There are different types of proteins in insect cuticle, which vary among different insects. Thus, main focus has been directed on the role of proteases in penetration process (Andersen et al., 1995; Dombrovsky et al., 2003; Charnley, 2003). Although proteases initiate degradation, they act synergistically with chitinases in solubilization of the insect cuticle (Smith et al., 1981; St. Leger et al., 1986). Subtilisin-like serine protease (Pr1) and trypsin-like enzyme (Pr2) are synthesized in the early stages of cuticle colonization, suggesting their critical role in degrading proteins (St Leger et al., 1988; Mohanty et al., 2008). The significantly variable protease activities that was evident in our experiments are in line with other studies indicating degrees of variation in production of cuticle degrading proteases in different isolates of entomopathogenic fungi (Clarkson and Charnley, 1996; Pinto et al., 2002; Boldo et al., 2009; Dhar and Kaur, 2010; Revathi et al., 2011). Also, different insect cuticles induced diverse levels of protease activities. The maximum levels of total protease and Pr1 activity was observed when E. kuehniella cuticle was used as substrate, while Pr2 activity was mostly stimulated with E. integriceps cuticle. Moreover, as there was a positive correlation between proteolytic activities and fungal isolate virulence, a direct relationship of B. bassiana and activity of cuticle degrading enzymes is proposed (Chui-Chai et al., 2012). Besides, positive correlation of proteolytic enzymes and virulence has been proved previously (St. Leger et al., 1996; Feng, 1998; Gillespie et al., 1998; Zare et al., 2014).

Our results showed a crucial role of chitinolytic enzyme in virulence, because more virulent fungal isolates secreted more chitinase. The highest enzyme level was produced by TV isolate in the presence of E. integriceps and E. kuehniella cuticles. The hydrolysis of chitin by chitinolytic enzymes occurs through the endochitinases, and the exochitinases. N-acetylglucosaminidase is an exochitinase that cleaves chitin from its non-reducing end, releasing dimers (GlcNAc2) (Duo-Chuan, 2006; van Aalten et al., 2011). Studies have shown that chitinase is secreted later into the cuticle, after the protease, and plays a secondary role in infection process (St. Leger, 1993; St. Leger et al., 1996). Production of the exochitinases in entomopathogenic fungi was studied previously
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References


Cuticle degrading enzymes of Beauveria bassiana


St. Leger, R. J., Joshi, L. and Roberts, D. W. 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Applied and Environmental Microbiology, 64(2): 709-713.


القای متغیر آنژیم‌های تجزیه کننده کوتیکول در حشرات مختلف

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چکیده: در تحقیق حاضر تولید آنژیم‌های تجزیه کننده کوتیکول در پنج جدایی از Beauveria bassiana در حضور کوتیکول حشرات مختلف و نسبت حساسیت این جدایی به کوتیکول باید باید با توجه به تأثیر تیمار در حشرات مختلف پژوهش خواهد شد. 

Beauveria bassiana در حضور کوتیکول Eurygaster integriceps و Zophobas morio باعث تولید آنژیم‌های تجزیه کننده کوتیکول بود و در حضور کوتیکول می‌توانند قدرت بیشتری در حضور کوتیکول را نشان دهند. در این مطالعه، استفاده از این کوکقابل‌کونی در دو حشره دیگر دیده شد. به‌طور کلی، تحقیقات فیزیکی و شیمیایی کوتیکول حشرات در حشرات مختلف و چسب‌گیری به‌طور مستقیم دارد. سطوح فلختی آنژیم‌های موجود در این گونه به‌طور کلی باید تشخیص قوی‌ترین جدایی‌ها را نشان دهد.

واژگان کلیدی: آنژیم‌های تجزیه کننده کوتیکول، حشره‌ها، Zophobas morio