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# CUTICLE-DEGRADING PROTEASES PRODUCED BY THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA* IN THE PRESENCE OF COFFEE BERRY BORER CUTICLE

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## ABSTRACT

A Brazilian isolate of *Beauveria bassiana* (CG425) that shows high virulence against the coffee berry borer (CBB) was examined for the production of subtilisin-like (Pr1) and trypsin-like (Pr2) cuticle-degrading proteases. Fungal growth was either in nitrate-medium or in CBB cuticle-containing medium under both buffered and unbuffered conditions. In unbuffered medium supplemented with cuticle, the pH of cultures dropped and Pr1 and Pr2 activities were detected in high amounts only at a pH of 5.5 or higher. In buffered cultures, Pr1 and Pr2 activities were higher in medium supplemented with cuticle compared to activities with nitrate-medium. The Pr1 and Pr2 activities detected were mostly in the culture supernatant. These data suggest that Pr1 and Pr2 proteases produced by strain CG425 are induced by components of CBB cuticle, and that the culture pH influences the expression of these proteases, indicating the occurrence of an efficient mechanism of protein secretion in this fungus. The results obtained in this study extend the knowledge about protease production in *B. bassiana* CG425, opening new avenues for studying the role of secreted proteases in virulence against the coffee berry borer during the infection process.

**Key-words:** subtilisin-like protease, trypsin-like protease, *Hypothenemus hampei*

## INTRODUCTION

The coffee berry borer (CBB) *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) is considered the most important coffee pest throughout the world. It is present in most coffee producing countries (19). The biology of CBB presents formidable challenges for the implementation of pest management programs. CBB lives the greatest part of its life cycle inside the coffee berry, which involves egg laying followed by the emergence of adult females from the berry. The control of CBB still depends largely on the application of synthetic insecticides which show limitations due to potential adverse effects to human health and the environment, and the development of pesticide resistance by the coffee berry borer to endosulfan (12). Due to its cryptic lifestyle, the use of biocontrol agents could be an effective alternative to chemical control.

The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin is the most prevalent fungus attacking CBB populations in African countries where this insect originated, as well as in those countries where the borer has spread, including Brazil (1,7,32). Currently, commercial products based on *B. bassiana* are available for CBB control (8,23). Recently, it was shown that the Brazilian strain CG425 of *B. bassiana* was the most virulent against the coffee berry borer among 60 strains tested (21). Furthermore, this strain showed compatibility with chemical insecticides used in the coffee crop, allowing the development of an integrated pest management program strategy for the crop with reduction in the amount of insecticides applied (21). This fungal strain also presented high mortality against the grasshopper *Rhammatocerus schistocercoides* (20) which is a serious pest of crops and native pastures in Central Brazil.

Entomopathogenic fungi exhibit many attributes that determine virulence toward their hosts, including the production

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of degradative enzymes. Fungal proteases are believed to play an important role in cuticle penetration (24). The best known determinant of fungal entomopathogenicity is based on subtilisin-like serine protease (designated Pr1) of *Metarhizium anisopliae*, where its role in host invasion has been clearly demonstrated (26). This enzyme is adapted to extensively degrade insect cuticular protein (25) and has been ultrastructurally located in the host cuticle during the early stages of penetration (13). A trypsin-like enzyme (Pr2) belonging to the serine protease group also occurs during the early stages of cuticle colonisation suggesting that it has some role in degrading extracellular proteins complementary to that of Pr1 (29).

A Pr1 protease from *B. bassiana* also appears to be a virulence factor given its ability to degrade insect cuticle (6) and considering that a protease-defective mutant was found to have decreased virulence against the migratory grasshopper *Melanoplus sanguinipes* (5). Gupta *et al*. (15) found that a high level of Pr1-like proteases produced by *B. bassiana* appeared to be related to early onset of mortality in the larvae of the wax moth (*G. mellonella*). According to these authors, trypsin-like proteases did not show a discernible trend. Furthermore, the Pr1 gene from *B. bassiana* which resembles the *M. anisopliae* Pr1 was cloned and sequenced (18), indicating that similar proteases may be widespread among entomopathogenic fungi.

The synthesis of extracellular *B. bassiana* Pr1 is controlled by a multiple regulatory circuit in which certain carbon sources together with a nitrogen source repress its synthesis. Its is also controlled by the levels of N-acetyl-D- glucosamine in the culture medium (3,4, respectively). Gupta *et al*. (14) showed that the production of Pr1 and Pr2 proteases is enhanced when *B. bassiana* is grown on insect cuticle. Campos *et al*. (9) described that tick cuticle also induces Pr1 production by *B. bassiana* and that the addition of the amino acid alanine to the culture medium repressed Pr1 secretion. Recently, Ito *et al*. (16) showed that a Brazilian strain of *B. bassiana* reactivated on CBB produced proteases in medium containing glucose and yeast extract. According to these authors *B. bassiana* produced 80% of the total of the protease activity in 48 h being the maximum activity on the 5$^{th}$ culture day.

In this study, we examined for the first time the production of Pr1 and Pr2 proteases by *B. bassiana* in the presence of *H. hampei* cuticle either in unbuffered or in buffered cultures, extending the knowledge about protease production by this fungus.

## MATERIALS AND METHODS

### Organism

*Beauveria bassiana* CG425 was obtained as a liquid nitrogen-stored stock culture from Cenargen/Embrapa-Brazil collection of entomopathogenic fungi, Brasília –DF.

### Enzyme secretion on solid media

Protease activity was determined by the ability to produce zones of clearing in agar mineral medium (MM)(22) lacking nitrogen source amended with either gelatin or casein at 0.2% (w/v) at pH 6.8 and 8.5. Spores were point-inoculated onto agar surfaces and incubated at 28ºC for up to 7 days. Activities were calculated as the ratio of diameter of the clear zone plus colony to that of the colony.

### Culture conditions

Conidia were obtained by harvesting sporulated cultures grown on agar plates composed of mineral medium (22) containing nitrate as nitrogen source. For enzyme production, conidia were added to 20 ml of liquid MM and MM+cuticle at a concentration of 1 x $10^7$ ml$^{-1}$ and grown in submerged culture (180 rpm) at 28ºC for up to 168 h. These media were left unbuffered (initial pH 6.8). The MM+cuticle medium corresponded to MM lacking nitrogen source amended with 0.5% (w/v) insect cuticle prepared from adult *Hypothenemus hampei* using an aqueous solution of 1% (w/v) potassium tetraborate. The cuticle extract was added to previously sterilised (121ºC for 15 min) MM lacking nitrogen source and autoclaved for 5 min at 115ºC.

In another set of induction experiments, both MM and MM+cuticle were buffered using a universal buffer (100 mmol l$^{-1}$ citric acid, 100 mmol l$^{-1}$ boric acid and 100 mmol l$^{-1}$ $KH_2PO_4$), pH 8.0.

All experiments were repeated three times, and the results represent mean values ± standard error of the mean (SD).

### Preparation of enzyme fractions

Following growth, mycelium was harvested by centrifugation at 8000 *g* for 15 min and washed in ice-cold 25 mmol l$^{-1}$ Tris-sodium phosphate buffer, pH 6.7. Weighed mycelium was ground to a fine powder in liquid nitrogen, resuspended in lysis buffer (1g ml$^{-1}$ of 25 mmol l$^{-1}$ Tris-phosphate, pH 6.7, 10% (v/v) glycerol and 1 mmol l$^{-1}$ EDTA) and centrifuged at 12000 *g* for 15 min. The supernatant recovered represented the intracellular (cell bound) soluble fraction. Culture filtrates were stored at -20ºC and used as the extracellular secreted fraction. Both fractions were assayed for protease activity.

### Enzyme assays

In this paper, subtilisin-like activity and trypsin-like activity of *B. bassiana* are referred to as Pr1 and Pr2, respectively. Pr1 and Pr2 activities were assayed using succinyl-$(alanine)_2$-proline-phenylalanine-*p*-nitroanilide and benzoyl-phenylalanine-valine-arginine-*p*-nitroanilide as substrates, respectively (25,33). Each assay consisted of 0.05 ml substrate (1 mmol l$^{-1}$), 0.85 ml 15 mmol l$^{-1}$ Tris-HCl buffer (pH 8.5) and 0.1 ml crude enzyme. The mixture was incubated for 1 h at 28ºC and the reaction was terminated by adding 0.25 ml of 30% acetic acid and left to stand for 15 min in ice, after which samples were centrifuged at 1250 *g* for 5 min at

4ºC. The supernatants were read at 410 nm. Activities were expressed as nanomoles nitroanilide (NA) released per milliliter per min (28). Assays were performed in duplicate for each sample.

## RESULTS

In this study, we determined the production of Pr1 and Pr2 proteases by *B. bassiana* CG425 in liquid culture, either in the presence or absence of coffee berry borer cuticle (*H. hampei*). In unbuffered medium containing nitrate as sole nitrogen source, the culture pH values ranged from 6.0 to 7.9 at different incubation times and the levels of Pr1 and Pr2 proteases were high at all incubation periods analysed (Tables 1 and 2). The pH of cultures dropped below 5.0 in unbuffered medium supplemented with cuticle, and Pr1 and Pr2 activities were detected in highest amounts in the late stage of growth when the culture pH reached 5.5 (Tables 1 and 2).

In buffered cultures (pH 8.0 in culture supernatants), Pr1-like proteases were also detected in the absence of *H. hampei* cuticle. However, in contrast to that observed in unbuffered cultures at its highest level, Pr1 activity was approximately 3-fold higher in mineral medium containing ground cuticle than in mineral medium containing nitrate as sole nitrogen source (Table1). In the former, protease production was high at 72 h and remained constant up to 168 h. Increased protease production was observed in nitrate-medium starting at 120 h. Similarly, Pr2-like activity was higher in medium supplemented with cuticle at all incubation times compared to activities on non-cuticular substrate (Table 2).

These data suggest that Pr1 and Pr2 proteases produced by strain CG425 are induced by protein components of coffee berry borer cuticle, and that the culture pH influences the expression of these protease types.

We also analysed protease activity on agar plates containing mineral medium lacking nitrogen source amended with either gelatin or casein. Protease activity in gelatin-containing medium was 3.56±0.22 and 3.25±1.08 in cultures at pH 6.8 and 8.5, respectively. In casein-containing medium, protease activity was 1.87±0.31 and 2.2±0.08 in cultures at 6.8 and 8.5, respectively. Unlike in liquid cultures, the plate clearing assay for protease determination did not reveal the effect of culture pH on protease activity.

As shown in Tables 1 and 2, Pr1 activities were higher than Pr2 activities in buffered media containing cuticle, although both types of proteases were detected after 48 h of growth, suggesting that these types of proteases are not coordinately expressed in this fungal strain.

We also analysed the distribution of Pr1 and Pr2 in both secreted and cell bound fractions after growth in unbuffered media, to increase our knowledge about protease secretion by this fungus. The Pr1 activities detected were mostly in the culture supernatant in either mineral medium or cuticle-containing medium. The percentage of Pr1 protease in the supernatant was 98-100% in mineral medium and 70-82% in cuticle-containing medium. The predominance of secreted Pr2 was also observed, even in nitrate-containing medium (up to 99%), suggesting the occurrence of an efficient mechanism of protein secretion by this fungus.

## DISCUSSION

Fungal pathogenesis is a complex and multi-factorial phenomenon, with particular virulence factors coming into play at various stages of infection and death. Like most fungal pathogens, *B. bassiana* might use a combination of enzymes to penetrate the cuticle and access the nutrient-rich host haemocoel.

The extracellular protease of *B. bassiana* has been implicated as a component of the insect infection process (5), and in this study we report on the regulation of Pr1 and Pr2 protease production by the isolate CG425 in liquid culture, as a function of nitrogen source and pH.

The detection of high levels of Pr1 and Pr2 proteases only in the late stages of growth in unbuffered medium containing *H. hampei* suggests that low culture pH had an effect on protease levels. In an earlier study, Bidochka and Khachatourians (3) described that an extracellular protease produced by *B. bassiana* in gelatin-containing medium was unstable at pH levels below 5. The decrease in pH in *B. bassiana* culture supernatants may have been due to the accumulation of metabolic acids such as oxalic acid in the medium as reported by Cordon and Schwartz (10). Our finding of a drop in pH only in medium supplemented with coffee berry borer cuticle needs further investigation, particularly as it relates to fungal utilisation of this substrate and to the process of insect infection.

In experiments where the pH of the culture supernatant was kept at 8.0, the addition of *H. hampei* cuticle to the medium had a positive effect on both types of protease (Pr1 and Pr2) activity. In the presence of cuticle, the production of these proteases seemed to be induced when the external pH is alkaline (Tables 1 and 2). These data suggest that both proteases are induced by specific components of the cuticle, and that their detection occurs at pH levels close to its pH optimum. *B. bassiana* Pr1 protease activity is maximum at pH 8.5 (2). St. Leger *et al.* (28) described that *M. anisopliae* produces extracellular proteases only at the pH at which they are active. According to these authors, there is evidence for a concerted action of pH and presence of cuticle on enzyme induction in *M. anisopliae*. In contrast, a subtilisin-type serine protease produced by *Aspergillus niger* is expressed at equally high levels at pH 3 and 8 (17). Recently Donatti *et al*. (11) described that the amino acid methionine seems to play a regulatory role in Pr1 secretion by *B. bassiana*, since both induction and repression seem to be dependent on the concentration of the amino acid present in

**Table 1.** Subtilisin-like (Pr1) activity in culture supernatants and in mycelium soluble fraction (intracellular) from *Beauveria bassiana* CG425 grown on unbuffered and buffered medium containing either nitrate or insect cuticle (0.5%) as nitrogen source.

| | Unbuffered medium | | | | | | | Buffered medium[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M M | | | MM+cuticle | | | | M M | | | MM+cuticle | | |
| Time (h) | pH[b] | Total activity[c] | secreted | intracellular | pH[b] | Total activity[c] | secreted | intracellular | Total activity[c] | secreted | intracellular | Total activity[c] | secreted | intracellular |
| 48 | 6.0 | 0.52±0.01 | 0.51±0.0 | 0.005±0.0 | 4.9 | 0 | 0 | | 0 | 0 | 0 | 0.04±0.03 | | 0 |
| 72 | 7.0 | 0.67±0.14 | 0.66±0.1 | 0.01±0.0 | 4.9 | 0 | 0 | | 0 | 0 | 0 | 0.99±0.15 | 0.98±0.15 | 0.01±0.0 |
| 96 | 7.9 | 0.88±0.17 | 0.88±0.1 | 0 | 4.9 | 0 | 0 | | 0 | 0 | 0 | 1.05±0.03 | 1.05±0.03 | |
| 120 | 7.9 | 0.82±0.13 | 0.82±0.1 | 0 | 5.5 | 0.40±0.23 | 0.39±0.1 | 0.01±0.0 | 0.32±0.16 | 0.31±0.1 | 0.01±0.0 | 1.01±0.06 | 1.01±0.06 | |
| 144 | 7.5 | 0.79±0.03 | 0.79±0.0 | 0 | 6.0 | 0.58±0.03 | 0.58±0.0 | 0.01±0.0 | 0.38±0.13 | 0.38±0.1 | 0 | 1.17±0.04 | 1.0±0.04 | 0.17±0.08 |
| 168 | 7.5 | 0.80±0.04 | 0.80±0.0 | 0 | 6.2 | 0.65±0.09 | 0.64±0.0 | 0.005±0.0 | 0.71±0.01 | 0.70±0.0 | 0.01±0.0 | 1.14±0.12 | 1.0±0.10 | 0.14±0.02 |

[a] pH 8.0 for culture supernatant, [b] pH of the culture supernatant; [c] Total activity is the sum of the activities determined in the two fractions. Pr1 activity expressed in nmol *p*-nitroanilide $ml^{-1}$ $min^{-1}$. Each result is the mean of three experiments ± standard error of the mean.

**Table 2.** Trypsin-like (Pr2) activity in culture supernatants and in mycelium soluble fraction (intracellular) from *Beauveria bassiana* CG425 grown on unbuffered and buffered medium containing either nitrate or insect cuticle (0.5%) as nitrogen source.

| | Unbuffered medium | | | | | | | Buffered medium[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M M | | | MM+cuticle | | | | M M | | | MM+cuticle | | |
| Time (h) | pH[b] | Total activity[c] | secreted | intracellular | pH[b] | Total activity[c] | secreted | intracellular | Total activity[c] | secreted | intracellular | Total activity[c] | secreted | intracellular |
| 48 | 6.0 | 0.64±0.05 | 0.63±0.0 | 0.005±0.0 | 4.9 | 0.01±0 | 0.01±0 | 0 | 0 | 0 | 0 | 0.08±0.06 | 0.08±0.06 | 0 |
| 72 | 7.0 | 0.70±0.04 | 0.69±0.1 | 0.01±0.0 | 4.9 | 0.04±0.03 | 0.04±0.0 | 0 | 0 | 0 | 0 | 0.23±0.22 | 0.19±0.1 | 0.03±0.0 |
| 96 | 7.9 | 0.69±0.02 | 0.68±0.1 | 0 | 4.9 | 0.01±0 | 0.01±0 | 0 | 0.06±0.03 | 0 | 0 | 0.71±0.05 | 0.60±0.03 | 0.10±0.06 |
| 120 | 7.9 | 0.70±0.01 | 0.70±0.0 | 0 | 5.5 | 0.30±0.28 | 0.22±0.1 | 0.08±0.0 | 0.22±0.12 | 0.19±0.1 | 0.03±0.0 | 0.64±0.04 | 0.55±0.04 | 0.09±0.02 |
| 144 | 7.5 | 0.68±0.10 | 0.43±0.1 | 0.15.±0.0 | 6.0 | 0.44±0.08 | 0.30±0.1 | 0.12±0.03 | 0.33±0.16 | 0.2±0.1 | 0.13±0.0 | 0.77±0.02 | 0.59±0.02 | 0.17±0.02 |
| 168 | 7.5 | 0.70±0.03 | 0.61.±00 | 0.092.±0.1 | 6.2 | 0.51±0.02 | 0.29±0.1 | 0.22±0.1 | 0.42±0.13 | 0.35±0.0 | 0.07±0.0 | 0.81±0.03 | 0.68±0.03 | 0.13±0.04 |

[a] pH 8.0 for culture supernatant, [b] pH of the culture supernatant; [c] Total activity is the sum of the activities determined in the two fractions. Pr1 activity expressed in nmol *p*-nitroanilide $ml^{-1}$ $min^{-1}$. Each result is the mean of three experiments ± standard error of the mean.

the culture medium. For *M. anisopliae* it has been suggested that alkalinization by ammonia production is adaptive by facilitating the utilization of proteinaceus substrates. Growing *M. anisopliae* in the presence of low levels of methionine resulted in high levels of ammonia formation (30).

Furthermore, the production of Pr1 and Pr2 by strain CG425 in cuticle-containing medium does not seem to be coordinately expressed, since both were detected after 48 h of growth, similar to that observed for *Metarhizium anisopliae* var. *acridum* (31).

In buffered cultures, both protease types were produced, albeit at lower levels, by carbon and nitrogen derepression alone indicating a substantial basal level of Pr1 and Pr2 (Tables 1 and 2). Similar results were obtained by Gupta *et al.* (14). In contrast, Bidochka and Khachatourians (3) reported that *B. bassiana* strain GK2016 did not produce protease in the absence of exogenous protein.

The main focus of most studies on cuticle-degrading enzymes produced by entomopathogenic fungi has been on extracellular activities. In this study, we analysed the protease activities in secreted and intracellular fractions. The high percentage of secreted proteases observed for both protease types (Pr1 and Pr2), compared to the intracellular activities, suggests the occurrence of an efficient mechanism of protein secretion in this fungus. Tiago *et al*. (31) also described a high percentage of secreted proteases from *M. anisopliae* var. *acridum* in cuticle-containing medium. Enzyme secretion by entomopathogenic fungi may be involved in the degradation of cuticular polymers during pathogenesis, assisting in the penetration of the insect exoskeleton and providing nutrients for fungal growth (13). However, there is evidence suggesting that certain *M. anisopliae* extracellular enzymes remain, in part, associated with the cell surface, which could be of benefit to the fungus as products of the enzyme action would be more readily absorbed (27).

The study of the regulation of virulence factors in entomopathogenic fungus is of particular importance because pathogenic specialisation may operate by way of regulatory controls that allow their expression. Furthermore, studies of the timing of the production of proteases and other factors in the presence of cuticular substrates could provide information about the role of the accumulated hydrolytic enzymes during pathogenesis. The results presented in this study increase the knowledge about protease production in *B. bassiana* CG425, opening new avenues for the study of the role of secreted proteases in virulence against the coffee berry borer during the infection process.

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## RESUMO

**Proteases degradadoras de cutícula produzidas pelo fungo entomopatogênico *Beauveria bassiana* em presença de cutícula de broca do café**

O isolado brasileiro de *Beauveria bassiana* (CG425) que apresenta alta virulência contra a broca do café (CBB) foi analisado quanto à produção de proteases degradadoras de cutícula, tipo-subtilisina (Pr1) e tipo-tripsina (Pr2). O crescimento fúngico foi realizado em meio contendo nitrato e em meio contendo cutícula da broca em condições de pH tamponado e não tamponado. Em meio não tamponado, suplementado com cutícula, o pH da cultura caiu e as atividades de Pr1 e Pr2 foram detectadas somente em valores de pH igual ou superior a 5,5. Em culturas tamponadas, as atividades Pr1 e Pr2 foram superiores em meio suplementado com cutícula, comparativamente as atividades em meio contendo nitrato. As atividades Pr1 e Pr2 ocorreram predominantemente no sobrenadante de cultivo. Os dados obtidos sugerem que Pr1 e Pr2 produzidas pelo isolado CG425 são induzidas por componentes da cutícula da broca do café (CBB), e que o pH da cultura influencia a expressão destas proteases, indicando a ocorrência de um mecanismo eficiente de secreção por este fungo. Os resultados obtidos neste estudo aumentam o conhecimento a respeito da produção de proteases por *B. bassiana* CG425, abrindo novos caminhos para o estudo do papel de proteases na virulência contra a broca do café durante o processo de infecção.

**Palavras chave**: protease tipo-subtilisina, protease tipo-tripsina, *Hypothenemus hampei*

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