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Production of extracellular proteolytic enzymes by *Beauveria bassiana*

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The production of proteolytic enzymes by two strains of *Beauveria bassiana* 278, *B. bassiana* 446 and one strain of *Ascosphera apis* 496 was analysed. It was demonstrated that the strain of *B. bassiana* 278 proved to be the best producer of basic and acid proteases. The influence of different environmental factors such as nitrogen and carbon sources on the production of extracellular hydrolytic enzymes was assessed. In addition the acid protease from *B. bassiana* was partially characterized.

Key words: proteolytic enzymes, *Beauveria bassiana*, acid protease, entomopathogenic fungi.

INTRODUCTION

Insect pathogenic fungi (entomopathogens) that include the strains of *Deuteromycetes* and *Ascomycetes* have been recognized as being very effective in producing an array of hydrolytic enzymes. Among them the most important are those active against components of insect cuticle, namely proteins, chitin and lipids (St Leger et al. 1986).

The insect cuticle is mainly composed of proteins 55–80% of which a significant part is complexed with chitin fibrils (Bidochka and Khachatourians 1992). The main proteins which occure in insect cuticle include resilin, an elastic tissue unique to invertebrates, and collagen (Bidochka and Khachatourians 1987). The insect cuticle proteins are very susceptible to proteolytic degradation, and indeed,

it was shown that proteolytic enzymes of entomopathogenic fungi, which are thought to be a significant factor of their virulence, attack the cuticle before the chitinolytic enzymes (chitinases and (β-N-acetylglucosaminidases) do (St Leger et al. 1986, St Leger et al. 1996).

Many of the proteases of entomopathogens are classified as collagenases (Hurien et al. 1977; Dean and Domnas 1983) or chymoelastases with an active site serine residue and show strong homology with the subtilisin family of proteases (St Leger et al. 1992).

Proteases and other entomopathogenic hydrolytic enzymes are promising agents for biological control of agricultural insect pests. Moreover, these proteases due to their broad specificity, may appear to be useful also in selective tenderizing of beef meat containing large quantity of connective tissue.

The aim of this work is to investigate the influence of different environmental factors on the production of extracellular proteases by three strains of entomopathogenic fungi. In addition the *Beauveria bassiana* 278 acid protease was described.

MATERIAL AND METHODS

Strains and growth media

Two strains of *Beauveria bassiana* (Bals) Vuill 278 and 446 and one strain of *Ascosphera apis* (Olive et Spiltaire) 496 were kindly provided by Prof. E. Lamer-Zarawska (Medical University of Wrocław). Fungi were maintained on yeast extract-peptone-glucose agar (Bidochka and Khachatourians 1988), grown at 25°C and stored at 10°C. They were cultivated as submerged cultures in Erlenmeyer flasks on a rotary shaker at 180 rpm at 25°C. The medium contained the following basal salts: NaCl, $MgSO_4 \times 7H_2O$ and K_2HPO_4 (each 0.3 g/l); pH 7.0. Variable carbon and nitrogen sources were added to the medium at different concentrations (Tabs 1 – 5). All the peptones used in the cultures, with the exception of bio-trypcase (from bioMerieux) were products of Difco. The conidial suspensions ($5-6 \times 10^6$/ml) were used to inoculate cultures. After 4 days of growth, the mycellium was removed by filtration followed by centrifugation at 3000 g for 20 min. The supernatant was used for enzymatic assays. The growth medium used for production of acid protease was composed of minerals at concentrations as mentioned above and glucose (10 g/l), gelatin (1 g/l) and ground hemolymph deprived larvae of *Apis mellifera* (5 g/l).

Determination of protease activities

Proteolytic activity was determined with different proteins as substrates: 4% hemoglobin (at pH 3.2), 2% casein (at pH 7.6 and 8.5). The final assay mixture (2ml) contained either 1 ml of 0.2 M Tris-HCl buffer, pH 7.6, or 8.5 or 1 ml of 0.2 M acetate-HCl buffer, pH 3.2, 0.5 ml of enzyme solution and 0.5 ml of substrate in appropiate buffer. This mixture was incubated at 37°C for 30 min. The reaction was stopped with 3 ml of 5% trichloroacetic acid and after 10 min. the mixture was centrifuged at 7000 g for 10 min. The absorbance of supernatant was determined at 280 nm. One proteolytic unit was defined as the amount of enzyme giving an increase in absorbance of 0.01 at 280 nm under the conditions described above.

The activity against hide powder azure (HPA) was determined in Britton--Robinson buffer at pH range 3.0–10.2 according to St Leger et al. (1986).

For the inhibition studies of the acid protease, the enzyme was pre-incubated for 10 min. at room temperature with 1 mM solution of inhibitors: phenylmethylsulfonyl fluoride (PMSF), EDTA, iodoacetate, pepstatin at 0.2 M acetate-HCl buffer, pH 3.2.

Aminopeptidase activity was assayed by colorimetric method using 10 mM leucine-p-nitroanilide (from Fluka) as substrate. The release of nitroaniline was determined by measuring the absorbance at 410 nm. The enzymatic reaction was performed by incubating a mixture of 0.4 ml 0.1 M Tris-HCl, pH 8.5, 0.5 ml of enzyme solution and 0.1 ml of substrate at 37°C for 30 min and stopped by adding 0.5 ml of 30% acetic acid. Aminopeptidase activity was expressed in units assuming that 1 unit corresponded to the increase of optical density by 0.01 under the conditions of the reaction.

Protein was determined by the microbiuret method of Goa (1953), or spectrophotometrically at 280 nm.

pH stability of *B. bassiana* 278 acid protease was examined by incubation of the enzyme solution in 0.2 M acetate buffers at pH 3.0–5.5 or in 0.2 M glycine – HCl buffer, pH 2.0.

RESULTS AND DISCUSSION

The results of extracellular proteolytic activity determinations of the three strains of entomopathogenic fungi *B. bassiana* 278 and 446, and *A. apis* 496 grown in media containing different nitrogen sources are presented in Table 1. It was found that the strain of *B. bassiana* 278 cultivated in the medium with swine blood plasma as nitrogen source appeared to be the best enzyme producer. After 4 days of cultivation, caseinolytic activity (at pH 7.6) of

this fungus reached the value of 26 units/ml. The same medium was the best for the enzyme production by two other strains of entomopathogens. However, cultivation of fungi in the medium containing collagen as nitrogen source no or very low activity was noticed.

For further investigations the strain of *B. bassiana* 278 was choosen, as being the most productive one. Different microorganisms including filamentous fungi like *Aspergillus niger* (Jarai and Buxton 1994) or yeasts, such as *Yarrowia lipolytica* (Glover et al. 1997) may secrete both alkaline and acid extracellular proteases. The expression of genes responsible for this was found to be regulated by environmental pH. The production of alkaline and acid proteases was also observed in *B. bassiana* 278 cultures. The cultivation of this fungus in the medium with the same composition of minerals and nitrogen source (blood plasma at different concentrations and gelatin) but with or without addition of glucose significantly influenced both the pH of the culture and proteolytic enzymes production (Tab. 2). The removal of glucose from the medium resulted in the increase of pH in the culture above 8.0, after 4 days of cultivation. Caseinolytic activity with blood plasma in medium increased to 182 u/ml (Tab. 2B) but activity was not detectable at pH 3.2. However, in the presence of glucose in medium,

Table 1

Proteolytic activity in cultures of *Beauveria bassiana* and *Ascosphera apis* grown in mineral organic medium with different N-sources

Strain	Proteolytic activity u/ml		
	0.1% gelatin	0.5% swine blood plasma	0.1% collagen
Beauveria bassiana 278	4.0	**26.0**	2.0
Beauveria bassiana 446	0.0	2.0	0.0
Ascosphera apis 496	2.0	8.0	0.0

Table 2

Proteolytic activity of *Beauveria bassiana* 278 broth grown in mineral-organic medium containing different nitrogen sources with glucose (A) at 1% concentration or without glucose (B)

N-source	Proteolytic activity u/ml (A)		Proteolytic activity u/ml (B)	
	pH 8.5	pH 3.2	pH 8.5	pH 3.2
0.1% gelatin	0.0	50	144	0.0
0.1% swine blood plasma	0.0	100	100	0.0
0.2% swine blood plasma	0.0	**130**	**182**	0.0
0.3% swine blood plasma	0.0	100	180	0.0

pH of cultures dropped below 3.0 and no protease active against casein at pH 8.5 was found (Tab. 2A). By contrast, the synthesis of an enzyme active under acidic conditions was very high (130 u/ml). Similar changes in pH and the protease activity of *B. bassiana* GK 2016 cultures, but only at pH 8.5 in the presence or absence of glucose in the medium was observed by Bidochka and Khachatourians (1988).

The decrease in pH in *B. bassiana* culture may be linked with H^+ being pumped out of the cells during glucose transport (Jennings 1974) or might be attributed to the accumulation of metabolic acids in the medium (Cordon and Schwartz 1962).

The addition of different peptones and casein, as a sole carbon and nitrogen sources, to the culture medium of *B. bassiana* resulted in increase of alkaline enzyme biosynthesis when compared to the gelatin medium (Tab. 3). Especially good medium constituents for enzyme production appeared to be peptones: proteose-pepton and tryptose, and soy-bean flour hydrolysate: bacto-soyton, but casein was much less effective. The synthesis of acid proteases was several times lower than that of alkaline enzymes regardless of the carbon and nitrogen source. This was somewhat surprising, since in the case of other fungi e.g. *Fusarium moniliforme* (Kołaczkowska et al. 1988) and *Penicillium camemberti* (Chrzanowska et al. 1992) casein proved to be the best stimulator of acid enzyme biosynthesis.

Table 3

Proteolytic activity of *Beauveria bassiana* 278 grown in medium with different nitrogen sources

N-source	Proteolytic activity u/ml	
	pH 8.5	pH 3.2
Casein	93.0	41.0
Casein hydrolysate	100.0	20.0
Proteose-pepton	**232.0**	80.0
Tryptose	**181.0**	50.0
Bio-trypcase	140.0	20.0
Bacto-soyton	**202.0**	**5.0**

In the medium containing gelatin as the major source of carbon and nitrogen *B. bassiana* GK 2016 produced one extracellular serine protease (Bidochka and Khachatourians 1987). The strain of *B. bassiana* used by Kucera and Samsinakova (1968) was found to produce more than one extracellular protease when growing on complex nitrogen sources such as corn steep liquor.

The influence of different carbon compounds on the enzyme synthesis by *B. bassiana* is presented in Table 4. In all the cases the swine blood plasma at 0.2% concentration was used as a nitrogen source. Further modification of medium composition including substitution of glucose by other sugars (10 g/l): monosaccharides, disaccharides and glycerol significantly affected the synthesis of enzymes studied. The proteolytic activity at pH 8.5 was below 5 u/ml and in cultures grown at pH 3.2 in the presence of disaccharides such as lactose, saccharose, maltose was also very low. On the other hand, the replacement of glucose by galactose or fructose had no effect on the level of protease production. However when glycerol was used as a carbon source a high increase of proteolytic activity at pH 3.2 was observed.

Table 4

Proteolytic activity of *Beauveria bassiana* 278 grown in medium with different carbon sources

C-source	Proteolytic activity u/ml	
	pH 8.5	pH 3.2
Galactose	tr	**125.0**
Fructose	tr	**130.0**
Glycerol	tr	**210.0**
Lactose	tr	20.0
Saccharose	tr	10.0
Maltose	tr	10.0

tr — traces of activity

In the case of other entomopathogenic fungi e.g. *Metarhizium anisopliae*, *B. bassiana* it was demonstrated that when the insect cuticle was used as a sole carbon and nitrogen sources the production of protease was stimulated (Leger et al. 1986; Cole et al. 1993; Joshi et al. 1995). In our work the supplementation of medium by ground larvae of *Apis mellifera* previously deprived of hemolymph which is known to contain high levels of protease inhibitors (Polanowski and Wilusz 1996), had also significant effect on extracellular protease production (Tab. 5). In the medium composed only of minerals and larvae, high caseinolytic and aminopeptidase activities were found at pH 8.5 (280 u/ml and 108 u/ml, respectively), whereas when the medium was additionally enriched by glucose, proteolytic activities both of exo- and endoproteases, at alkaline pH were not detectable. The proteolytic activity at pH 3.2 remained quite high (104 u/ml). The addition of gelatin to the medium containing minerals, ground larvae and glucose caused about 3 fold increase of proteolytic activity at pH 3.2, but aminopeptidase was very low (36 u/ml) and caseinolytic activity was below detection limit.

Table 5
Proteinase and aminopeptidase activities of *Beauveria bassiana* 278 grown in complex medium

Medium	Proteolytic activity u/ml		Aminopeptidase activity u/ml
	pH 3.2	pH 8.5	pH 8.5
G + M + L	**104.0**	0.0	0.0
G + Z + M + L	**312.0**	0.0	36.0
M + L	2.0	**280.0**	**108.0**

G – glucose, Z – gelatin, L – ground larvae corps of *Apis mellifera*, M – minerals

Taking this into consideration and the results obtained by other authors it may be concluded that *B. bassiana* can utilize for its growth, sporulation and synthesis of hydrolytic enzymes a wide variety of substrates including larvae of *Apis mellifera*, insect cuticles (Smith and Grula 1981; St Leger et al. 1986, 1996), various peptones (Barnes et al. 1975) and carbohydrates (Campbell et al. 1983). In particular the supplementation of *B. bassiana* growth media by carbohydrate affects the production of extracellular acid proteases.

Partial purification of *B. bassiana* acid protease

The extracellular acid proteolytic enzyme of *B. bassiana* was purified from the broth by batch adsorption on CM-Sephadex C-50 at pH 4.0 in 0.05 M acetate buffer. The bound active protease was eluted with 0.2 M NaCl in the same buffer. This step of purification procedure allowed to achieve a 30-fold increase of specific activity with 50% yield. The fraction with the highest specific activity were pooled, then concentrated in a stream of cold air and subjected to a Bio-Gel P-30 column, equilibrated with 0.05 M acetate buffer, pH 4.0. On gel filtration the protein resolved into 2 peaks of which the first one contained all proteolytic activities. This two step purification procedure increased the specific activity of protease by 40-fold with a 36% recovery (Tab. 6).

Table 6
Partial purification of *Beauveria bassiana* acid protease

Treatment	Protein (mg)	Activity		Purification factor	Yield (%)
		Total (units)	Specific/mg)		
Culture filtrate	337*	12600	37.3	1	100
CM-Sephadex C-50	5.88	6500	1105	29.6	51
Bio-Gel P-30	3.08	4530	1470	39.4	36

*determined by method of Goa

At 1 mM concentration pepstatin, a specific aspartyl protease inhibitor, reduced the proteolytic activity of the enzyme by half, but the other inhibitors used i.e. PMSF, EDTA, iodoacetate had no effect, which indicated that the protease produced by *B. bassiana* 278 was a member of aspartyl proteinase family. The activity of acid protease, stored at 4°C in the range of pH 3.0–5.5, was found to be stable for two months. However at pH 2.0 its activity decreased rapidly. The enzyme isolated from *B. bassiana* culture broth had optimum activity at pH 3.5 against hemoglobin and at 6.0 against HPA. The tenderizing effect of this protease on beef meat is under investigation.

From among entomopathogenic fungi this type of enzyme has been isolated so far only from *Metarhizium anisopliae* cultures by St Leger et al. (1987). The protease with a pH optimum of 5.0–5.5 did not degrade elastin and occurred as multiple isoenzymes. This enzyme was, however, not characterized in more details.

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Produkcja zewnątrzkomórkowych enzymów proteolitycznych przez *Beauveria bassiana*

Streszczenie

Zbadano produkcję enzymów proteolitycznych u dwóch entomopatogennych szczepów *Beauveria bassiana* 278 i 446 oraz u *Ascosphera apis*. W wyniku przeprowadzonych badań stwierdzono, że szczep *B. bassiana* 278 wykazywał najwyższą zdolność do biosyntezy kwaśnych i zasadowych proteaz. Określono wpływ różnych źródeł azotu i węgla na produkcję zewnątrzkomórkowych hydrolitycznych enzymów jak również częściowo scharakteryzowano kwaśną proteazę z *B. bassiana* 278.