ISOLATION AND PURIFICATION OF CUTICLE DEGRADING EXTRA CELLULAR PROTEASES FROM ENTOMOPATHOGENIC FUNGAL SPECIES OF *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE*

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ABSTRACT : Enhanced Pr1 and Pr2 activities were observed in entomopathogenic fungi *Beauveria bassiana (*UB9) and *Metarhizium anisopliae* (UM4) supplemented with casein. Highest activity was observed at 72 hours of incubation when compared to minimal medium (MM) and colloidal chitin amended media. The extra cellular proteases (Pr1 and Pr2) thus isolated were purified through dialysis and their activities were found to be increased by two-fold. Pr1 and Pr2 were subjected to SDS - PAGE analysis and activity gel electrophoresis. The molecular weight of protease produced by *Beauveria bassiana (*UB9) is 19 KD and that of *Metarhizium anisopliae* (UM4) is 21 KD.
Key words: Extracellular proteases, Pr1, Pr2, *Beauveria bassiana*, *Metarhizium anisopliae.*

INTRODUCTION

Utilization of pesticides in recent years has lead to environmental problems such as soil and groundwater contamination with pesticide residues. The problems are not only associated with the environment but the pests itself. Random use of pesticides causes the insect pests to gain resistance against insecticides. Strong impetus to find an effective alternative to chemical pesticides focused on a more environment friendly sustainable approach that exploits natural predators of insect pests: the entomopathogenic fungi. The concern for the development of hyphomycete fungi as sustainable biocontrol agents for insect pests leads to the isolation of various entomopathogenic fungi. Among them the two well studied entomopathogenic fungi are *Beauveria bassiana* and *Metarhizium anisopliae* which indeed can be implemented as a sustainable approach in Integrated Pest Management (IPM).

Beauveria bassiana and *Metarhizium anisopliae* are natural soil borne fungi which infects a variety of insects and have the potential for insect pest control (Ferron 1985). These fungi generally infect the host insect by the passage through the cuticle (Pekrul and Grula 1979).

Germ tubes produced from conidia penetrate insect cuticle and it is also presumed that they orient towards the cuticle in the penetration by mechanical means accompanied by enzymatic action (Zacharuk 1970). After crossing the insect integument, fungi grow within aqueous haemolymph where they produce toxins which kill the host. Protease plays a prominent role in cuticle degradation as it is composed of 80% protein and 20% chitin. (Nerville 1975). Fungal entomopathogenicity is based on subtilisin-like serine protease Pr1 and trypsin-like enzyme Pr2 belonging to the serine protease group which is prominent during the early stages of cuticle colonization. Pr1 has broad substrate specificity but Pr2 has a much narrower host range. Broad-spectrum subtilisins are the main proteins produced by *M. anisopliae* and other entomopathogens during infection and degradation of insect cuticle. (St.Leger 1995) and several studies reported partial purification of extracellular proteases from *B. bassiana* and *M. anisopliae* (Gabriel 1968, Kucera 1971 & Samsinakova 1966). To elucidate the precise role of proteases in insect infection process, it is essential first to characterize the enzymes. This article reports the purification and characterization of proteases secreted *in vitro* by entomopathogenic fungal strains of *Beauveria bassiana* UB9 and *Metarhizium anisopliae* UM4.

MATERIALS AND METHODS

Culture and Growth conditions

Conidia of UB9 (USDA-ARS 2033) and UM4 USDA-ARS 2424) were inoculated into 250ml of three different media viz., Minimal medium (KH_2PO_4 -0.1gm, $MgSO_4$ - 0.05gm, NaCl - 0.25gm), MM + 1gm casein, MM + colloidal chitin (1%). The cultures were incubated at 3 different time intervals (48, 72 and 96 hours) on an orbital shaker at 180 rpm and 28^{0}C. The cultures were centrifuged at 10,000 rpm for 10min and the supernatant was used as crude enzyme.

Purification of protease

The supernatants were submitted to Ammonium sulphate precipitation by adding increasing amounts of salt and collecting different fractions of precipitated proteins. The fractions from 30% to 90% saturation were dialyzed for 24 hours at 4^{0}C against three changes of two liters of low salt buffer (0.01M Tris HCl and pH 8.0).

Proteolytic activity

Assay of general proteolytic activity was performed with casein as a substrate. Casein 1gm of casein was dissolved in 10ml of 0.1N NaOH and 0.01M Tris HCl at pH 8.0. To the 0.2ml crude as well as purified enzyme, 0.4ml of casein substrate and 0.2ml of 0.01M Tris HCl at pH 8.0 were added and incubated for 10 min at 37^{0}C. Later 1ml of 1.2M TCA was added to terminate the reaction. The contents were centrifuged at 8000 rpm for 5 min and the resulting supernatants were measured at 280nm.

Pr1and Pr2 activities

Pr1 and Pr2 activities can be measured with chromogenic synthetic substrates like N-Succinyl Alanine Prophepanilide (NA) and N-α-Benzoyl-DL-Arginine p-nitroanilide (BAPNA) respectively by dissolving them in DMSO and made to final volume of 100ml with 0.2M Glycine-NaOH Buffer (pH 8.5). These buffered substrates were added to 0.1ml of enzyme solutions and incubated on a rotary shaker at 20^0C for 1 hour and then absorbance was read at 520nm. Subtilisin activity of Pr1 was defined in nitroanilide units and that of trypsin like amidase activity for Pr2 was defined in BAPNA units.

Total protein determination

Total protein was measured by Bradford's method (1970) with Bovine Serum Albumin (BSA) as standard.

SDS-PAGE

SDS - PAGE was carried out to assess the number of proteolytic enzymes in the samples. The gel was polymerized from the mixture of 30% Acrylamide, 0.8% Methylene Bisacrylamide, 10% SDS, 1.5M Tris HCl (pH 8.8), distilled water, TEMED and Ammonium Per Sulphate. Electrophoresis was performed at room temperature with 0.05M Tris Glycine buffer at pH 8.3. Low molecular weight standards (19-97 KD) were ran in parallel and the gel was silver stained with 0.25% of 0.5gms of silver nitrate in 0.015% formaldehyde.

Activity gel Electrophoresis:

SDS gel was polymerized as described above along with 1% gelatin. Electrophoresis was performed at room temperature with 0.05M Tris - glycine solution as tank buffer. The gel was incubated in Triton - X for 1hour and in 0.01M Tris HCl for 6 hours to remove SDS. The gel was then placed in staining solution (0.2% Coomassie blue R-250 in methanol: acetic acid: water 3:2:1 respectively) at 60^0C for 45min and destained in the same solution.

RESULTS

Proteolytic activity appeared rapidly when UB9 and UM4 were grown in media supplemented with different substrates. After 46hours of incubation there was a gradual increase in the activities of Pr1 and Pr2 secreted by UB9 and UM4 in 1% casein supplemented medium than MM and MM + colloidal chitin (Table 1 & 2). After 72 hours the activities of Pr1 and Pr2 were found to be maximum in the medium with 1% casein. For *Beauveria bassiana* UB9, maximum Pr1 and Pr2 activities were 28.53 (NA units/ml) and 4.26 (BAPNA units/ml), while specific activities were 1.43 and 0.21 Tyr units/mg respectively (Table 1 & 2).

For *Metarhizium anisopliae* UM4 maximum Pr1 and Pr2 activities were 5.12 (NA units/ml) and 2.80 (BAPNA units/ml), while specific activities were 1.43 & 0.21 Tyr units/mg respectively. After 96 hours the activities of Pr1 and Pr2 were found to be gradually decreasing (Table 1 & 2), hence growth conditions for enhanced production of proteases for both strains were in the medium supplemented with 1% casein after an incubation of 72 hours. Total protein was also found to be higher in both UB9 as well as UM4 in casein amended medium when compared to other two media. The concentration of protein was maximum at 48 hours of incubation and it declined as the incubation time increased (Table 2 & 3). Ammonium Sulphate precipitation of centrifuged culture supernatant showed that protease was fractioned at 90% saturation. These fractions were further dialyzed against a low salt buffer. After dialyzing the protease samples, in a low salt buffer, the specific activities of Pr1 and Pr2 of both the strains were found to be increased by two-fold. Table 3 shows a summary of protease activities before and after dialysis. SDS-PAGE analysis revealed two single major bands (Figure 1) indicating that the molecular weight of UB9 protease as 21KD and that of UM4 as 19KD. A zymogram was used to assess the number of extracellular proteases produced. The activity gel electrophoresis (Figure 2) showed two colorless bands of dissolved gelatin for both the strains of UB9 and UM4.

Table 1: Pr1 &Pr2 activities of *Beauveria bassiana* isolate UB9

Medium	Pr1 (NA units/ml)	Pr2 (BAPNA units/ml)	Total protein (mg)	Specific Activity of Pr1 (TU/mg)	Specific Activity of Pr2 (TU/mg)	Proteolytic activity (TU/mg)
Incubation for 48 hours						
MM	3.86±0.02	3.13±0.08	50.85±0.02	0.07±0.00	0.06±0.00	1.29±0.03
MM+C	4.51±0.01	3.75±0.03	106.45±0.06	0.04±0.00	0.04±0.00	1.48±0.01
MM+CC	0.08±0.01	2.96±0.01	11.50±0.09	0.07±0.00	0.25±0.01	1.22±0.04
Incubation for 72 hours						
MM	3.65±0.04	3.04±0.02	12.27±0.03	0.29±0.00	0.21±0.00	1.31±0.02
MM+C	28.5±0.01	4.26±0.02	19.82±0.07	1.43±0.03	0.24±0.08	2.56±0.03
MM+CC	3.65±0.03	4.02±0.07	3.510±0.04	1.03±0.02	1.14±0.04	1.33±0.01
Incubation for 96 hours						
MM	2.80±0.02	2.19±0.06	7.80±0.08	0.35±0.01	0.28±0.01	1.24±0.08
MM+C	3.17±0.01	3.65±0.07	13.05±0.05	0.24±0.02	0.27±0.03	1.12±0.02
MM+CC	2.90±0.02	4.63±0.04	7.89±0.02	0.36±0.03	0.58±0.05	1.58±0.01

MM – Minimal medium
MM + C – Minimal medium amended with casein
MM + CC – Minimal medium amended with colloidal chitin

Figure 1: Gel showing different bands of proteins in the sample after dialysis

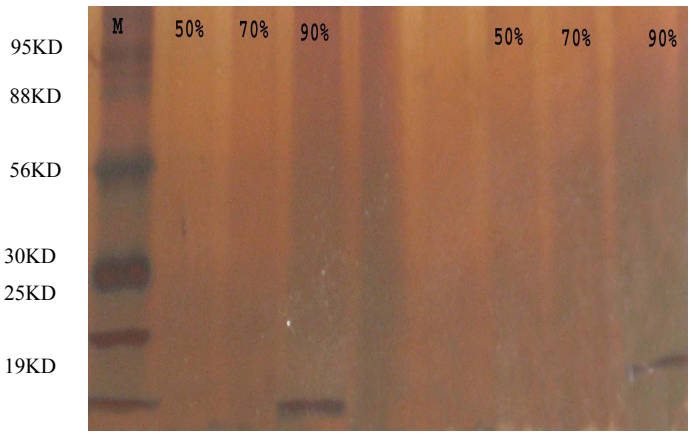

Table 2: Pr1 & Pr2 activities of *Metarhizium anisopliae* UM4

Medium	Pr1 (NA units/ml)	Pr2 (BAPNA units/ml)	Total protein (mg)	Specific Activity of Pr1 (TU/mg)	Specific Activity of Pr2 (TU/mg)	Proteolytic activity (TU/mg)
Incubation for 48 hours						
MM	4.08±0.07	3.95±0.05	48.09±0.04	0.08±0.00	0.08±0.00	1.06±0.04
MM+C	5.02±0.03	3.78±0.02	104.45±0.08	0.04±0.00	0.03±0.00	1.17±0.02
MM+CC	3.02±0.01	0.12±0.01	10.50±0.04	0.20±0.00	0.01±0.00	0.09±0.01
Incubation for 72 hours						
MM	3.98±0.02	3.24±0.02	11.30±0.09	0.35±0.00	0.28±0.04	1.58±0.09
MM+C	30.08±0.01	16.36±0.01	17.82±0.02	1.68±0.02	0.91±0.03	3.18±0.06
MM+CC	3.88±0.01	2.24±0.01	3.02±0.00	1.31±0.01	0.74±0.02	1.88±0.02
Incubation for 96 hours						
MM	3.02±0.04	2.08±0.04	7.25±0.00	0.41±0.00	0.28±0.05	0.90±0.01
MM+C	3.25±0.03	4.12±0.04	12.98±0.02	0.25±0.00	0.31±0.01	2.98±0.04
MM+CC	3.12±0.05	5.08±0.01	7.03±0.02	0.44±0.03	0.72±0.4	2.40±0.09

MM – Minimal medium
MM + C – Minimal medium amended with casein
MM + CC – Minimal medium amended with colloidal chitin

Figure 2: Activity gel containing 1% gelatin as substrate, showing protease bands

UB9 **UM4**

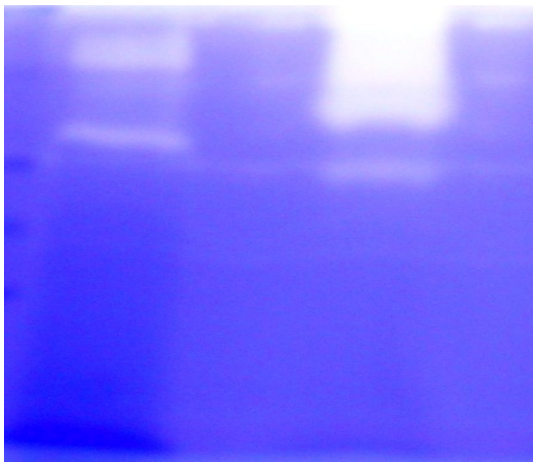

Table.3: Specific activity of protease before and after dialysis

Saturation	*B.*bassiana TU units/mg	*M.anasoplia e* TU units/mg
Before dialysis		
30%	1.23	1.57
50%	1.34	1.36
70%	1.90	1.93
90%	15.3	17.4
After dialysis		
30%	1.37	1.77
50%	1.45	1.43
70%	2.31	2.06
90%	31.2	32.4

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* and *M. anisopliae* use a combination of enzymes to penetrate the cuticle and extracellular proteases are implicated as components of insect infection process. Polymorphic growth characteristics for *B. bassiana, M. anisopliae* in different culture conditions have been observed in earlier studies (Macleod 1954).

When *B. bassiana* was grown in culture containing insect cuticle as the sole carbon and nitrogen source, extracellular proteases appear first followed by chitinases (Bidochka et.al, 1988). Results indicate that the extracellular protease production was increased in medium containing casein as substrate and increased after 72 hours of incubation. Protease production was found to be decreased after 96hours of incubation. Extracellular protease activity which was evident in medium with GlcNAc concentrations of <0.5mg/ml was related to the amount of growth in the culture and the latter depends on initial GIcNAc concentration in the medium (Bidochka et.al, 1988).High and low molecular weight proteases secreted by a strain of *B. bassiana* were observed which was in contrast to our findings (Kucera., 1971). With different nitrogen sources several proteases from these fungi may be produced (Leopold and Samsinakova 1970) however; *B. bassiana* UB9 and *M. anisopliae* UM4 produced a single protease when grown in medium containing casein as the sole carbon and nitrogen sources. Single major protein bands were detected in SDS-PAGE after purification procedures in oth the strains. Enzyme secretion by entomopathogenic fungi may be involved in the degradation of cuticular polymers during pathogenesis, assisting in penetration of insect exoskeleton and providing nutrients for fungal growth. High percentage of secreted proteases observed for both protease types Pr1 and Pr2, compared to the intracellular activities, The findings of our investigation suggest the occurrence of an efficient mechanism of protease secretion in both the fungi studied.

REFERENCES

1. M J Bidochka and G G. Khachatourians (1988) Applied and Environmental Microbiology: Vol. 54(11), 2699-2704.
2. M M Bradford (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein- dye binding. Anal. Biochem: 72: 248-254.
3. P. Ferron (1985). Fungal control: Vol.4, p.314-316. In G.A. Kerkut and L.I. Gilbert (ed.) Comprehensive insect physiology, biochemistry and pharmacology, Pergamon Press, Inc., Elmsford, N.Y
4. B P Gabriel (1968). Journal of Invertebrate Pathology: Vol.11, 82-89
5. M Kucera (1971). Journal of Invertebrate Pathology: Vol. 17, 211-215.
6. J Leopold and A Samsinakova (1970). Journal of Invertebrate Pathology: Vol. 15, 34-42.
7. R J St. Leger (1995). Canadian Journal of Botany: Vol. 73, 1119-1125.
8. D M Macleod (1954). Canadian Journal of Botany: Vol.32, 818-890.
9. A C Neville (1975). Biology of arthropod cuticle: 71-123.
10. S Pekrul and E A. Grula (1979). Journal of Invertebrate Pathology: Vol. 34, 238 -247.
11. A Samsinakova (1966). Journal of Invertebrate Pathology: Vol. 8, 395-400
12. R Y Zacharuk (1970). Journal of Invertebratae Pathology: Vol. 15, 372-396

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