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Full Length Research Paper

Production of cuticle - degrading proteases by *Beauveria bassiana* and their induction in different media

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The concern for the development of hyphomycete fungi as suitable biocontrol agents of insect pests leads to the isolation of various insect pathogenic fungi. Amongst them, one of the most studied entomopathogenic fungus is *Beauveria bassiana*. The conidia of mitosporic fungi adhere to the host cuticle and germinate to produce an infectious propagule, and produce a sequential release of extra cellular enzymes to breach the insect cuticle. Protease is one of the most important and earliest enzymes involved in the host invasion. Extracellular protease production by seventeen *B. bassiana* isolates was investigated in the present study. High protease activity was observed during four to six days of culture incubation. Induction mechanism of subtillisin type Pr1 and trypsin type Pr2 activity were investigated utilizing different media. Minimal medium supplemented with casein (1%) showed high protease production and minimal medium supplemented with colloidal chitin (2%) was also able to induce Pr1 activity. The pH, ammonia and oxalic acid production in *in vitro* conditions was also investigated and the alteration in pH for protease production was not significant irrespective of the medium used. The protease activity gel was also studied and a common 66 kDa protease was observed in all the seven isolates studied.

Key words: Ammonia, *Beauveria bassiana*, oxalic acid, subtillisin type Pr1, trypsin type Pr2, protease, pH.

INTRODUCTION

The entomopathogenic fungi are natural soil borne insect pathogens and are currently being reviewed as an effecttive alternative to chemical pesticides. Amongst them, *Beauveria bassiana*-based mycoinsecticides have been developed and registered worldwide for control of agricultural pests (Hajek et al., 2001; St. Leger et al., 1992). These fungi are unique in their pathogenesis, where the infection of the host initiates by the attachment of the spores on the hydrophobic cuticle and subsequent development of the infectious structures called appresoria, which releases an array of cuticle solubilizing enzymes (Inglis et al., 2001). During the fungal pene-tration through the host cuticle, hydrolytic enzymes such as proteases, chitinases and lipases are produced and secreted and are important for the initiation of the infection process (St. Leger et al., 1986, 1996). The secretion of proteolytic enzymes is believed to be an im-portant pathogenic factor for fungal attachment on cuticle (St.Leger et al., 1987b). The best understood model of fungal entomopathogencity determinant is based on the subtilisin like endoprotease designated Pr1 (St.Leger et al., 1995). Several studies indicated that Pr1 is essential for cuticle penetration that results in pathogenicity (Shah et al., 2005; Wang et al., 2002). It is an important virulence determinant and is induced by insect cuticle, derepressed under starvation and repressed in the presence of excess nutrients although the typsin - like protease Pr2 is the first to appear during *in vitro* growth on the cuticle (Butt et al., 1998; Wang et al., 2002; Gillespie et al., 1998). The role of Pr2 in insect parasitism is not yet elucidated completely, although St. Leger et al. (1994) reported that Pr2 as well the other cuticle - degrading proteases may complement each other in the splitting of peptide bonds in the insect cuticle. Results ob-tained by Gillespie et al. (1998) are consistent with a role

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of Pr2 in the induction or activation of Pr1, since Pr2 was detected before Pr1 in a culture containing ground locust cuticle. St. Leger et al. (1988) observed the regulation mechanism of Pr1 and Pr2 in *M. anisopliae* and reported the catabolic repression of the enzymes. Bidochka and Khachatourians (1988) reported a high protease production from *Beauveria bassiana* in medium containing 1% gelatin as sole source of carbon and nitrogen. Induction of protease by protein is also reported in the fungus *Neurospora crassa* (Drucker, 1972). The caseinolytic activity of *M. anisopliae* in the culture filtrates is mainly due to the presence of two proteases, Pr1 and Pr2 both capable of hydrolyzing casein (St. Leger et al., 1987a). Two chymotrypsin like proteinases were produced when *B. bassiana* was grown on different carbon and nitrogen sources (Chrzanowska et al., 2001).

The relationship between gene regulation and pH sensibility by the organism is a well established fact. Regulation of gene expression by pH is observed in many fungi (Caddick et al., 1986; St Leger et al., 1998). Mutations in the pH regulated gene effecting the expression was also observed in *Y. lipolytica* (Otero and Gaillardin; 1996). In *M. anisopliae* regulation of gene expression by pH include the cuticle - degrading subtilisin proteases and other putative virulence factors (St. Leger et al., 1999). Insect cuticle infected with *M. anisopliae* demonstrated an increase in pH compared to unaffected cuticles (St. Leger et al., 1998). This suggests that *M. anisopliae* produces one or more basic compounds. Subtilisins like proteases from *M. anisopliae* are only produced under alkaline conditions (St. Leger et al., 1996). The production of cuticle degrading proteases is often subjected to the alteration of the pH by the production of ammonia in the media (St. Leger et al., 1999). Ammonia and oxalic acid production has also been studied by many workers but concluded that it does not affect the virulence (Bidochka and Khachatourians, 1993). Ammonia production by *M. anisopliae* is tightly regulated by amino acids which as protein degradation products could serve as signals for the presence of proteinaceous nutrients in the environment (St. Leger et al., 1999). Given that ammonium is linked with nitrogen catabolite repression there are presumably additional regulatory mechanisms that allow protease production in the presence of ammonia (Arst and Cove, 1969). Various protease isozymes were observed for *M. anisopliae* (St. Leger et al., 1998). A 35 kDa serine protease was produced by *B. bassiana* grown in a liquid medium containing gelatin as sole carbon nitrogen source (Bidochka and Khachatourians, 1987).

In this study the variation in the proteolytic activity of seventeen *B. bassiana* isolates was investigated for a period of ten days to study the effect of incubation time on the protease production. Subtilisin type Pr1 and trypsin like Pr2 activity of the high protease producing isolates was studied in media containing different carbon and nitrogen source to elucidate the induction of these two enzymes. At the same time studies on pH, ammonia and oxalic acid release into the media and their effect on protease production was also studied.

MATERIALS AND METHODS

Fungal isolates and culture conditions

A total of sixteen isolates were obtained from ARSEF (USDA-ARS Plant Protection Unit, Ithaca, NY) and one isolate was from India (Table 1). The isolates were routinely sub cultured on SDA (Sabouraud Dextrose Agar) slants at 28°C and maintained at 4°C. Seven day old SDA slants were used for the preparation of conidial suspension (1 x 10^6 conidia/ml) to inoculate SDY broth (4% Dextrose, 1% Peptone, and 1% Yeast extract) and incubated at 28°C and 180 rpm for 3 days. The harvested mycelium was washed twice with sterilized distilled water and inoculated into induction medium containing casein (1%) (Sigma) in basal salts medium (0.1% KH_2PO_4, 0.05% MgSO4, 0.05% NaCl) at 20% v/v based on the final volume (50 ml) of the culture for the determination of proteolytic activity. The pH of the culture media was adjusted to 8.0 and the cultures were incubated at 28°C and 180 rpm for a period of 10 days. The mycelia were harvested by centrifugation and the supernatant was assayed for proteolytic activity on alternate days of growth till tenth day of culture.

For the determination of subtilisin (Pr1) activity and trypsin (Pr2) activity three different media; minimal media, minimal media supplemented with casein (1%) and minimal media supplemented with colloidal chitin (2%) were used and the pH was maintained at 7.0. For the enzyme production, conidia at a concentration of 1 x 10^6 conidia/ml were inoculated into each media (three replicates were maintained) and incubated as shake cultures at 180 rpm, 28°C for 72 h. The mycelia were harvested by centrifugation and the supernatants assayed for enzyme activity (Pr1 and Pr2).

Enzyme assays and protein determination

Proteolytic activity

The protease assay was done by the method of Kunitz (1947). Casein substrate was prepared for enzyme assay by dissolving 2 gm of casein (Sigma) in 90 ml of 0.01 M Tris HCl (pH 8.0) containing 10 mM $CaCl_2$ (pH 8.0) and the volume made up to 100 ml with H_2O. A 400 µl of casein substrate was added to 200 µl of the culture supernatant in 0.01M Tris HCl pH 8, 10 mM $CaCl_2$. The reaction mixture was incubated at 35°C for 10 min and the reaction terminated by the addition of 1.2 M TCA. The reaction mixture was centrifuged at 4000 rpm for 5 min and the supernatant was taken and the absorbance at 280 nm (A_{280}) was observed against water as blank. One unit of protease activity was defined as the amount of enzyme that produced 1 mM of Tyrosine per minute under the above conditions. Tyrosine was taken as standard for all the enzyme assay calculations.

Subtilsin (Pr1) and Trypsin (Pr2) activity

The subtilisin (Pr1) activity and typsin (Pr2) activity was assayed by the method of St. Leger et al. (1987a). The Pr1 activity was assayed using specific synthetic substrate, N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide and Pr2 activity was assayed using specific synthetic substrate, Benzoyl-phenylalanine-valinearginine-*p*-nitroanilide (BAPNA). Each substrate (50 µl) was mixed with 0.85 ml of Tris-HCl buffer (pH 8.5) and 100 µl of culture supernatant. The reaction mixture was incubated for 1h at 28°C and the reaction was terminated using 0.25 ml of 30% acetic acid. The reaction mixture was left to stand for 15 min on ice prior to spectrophotometry. Absorbance at 410 nm (A_{410}) was observed and concentration of para-nitro aniline was determined. One unit of protease activity was defined as the amount of enzyme that produced 1 µM of para-nitro aniline per minute under the above conditions. Protein content was measured by the method of Bradford (1976) using Bovine Serum Albumin as a standard for proteolytic, Pr1 and Pr2 activity.

Table 1. Source of *B. bassiana* isolates.

Isolates	Code No./ Accession No. ARSEF	Host insect	Geographical location
UB1	1788	*H. virescens*	Spain
UB2	2041	*C. medinalis*	Philippines
UB3	5278	*B. tabaci*	U.S.A
UB4	2417	*H. puer*	India
UB5	2597	*S. litura*	India
UB6	6646	*C. septumpunctata*	India
UB7	4027	*C. septempunetata.*	Denmark
UB8	1166	*H. armigera.*	Spain
UB9	2033	*Coccinella* sp.	U.S.A
UB10	2034	*Coccinella* sp.	U.S.A
UB11	4018	*C. septempunetata*	Denmark
UB12	1886	*C. infuscatellus*	India
UB13	2412	*X. jamaicensis*	India
UB14	8250	*B. fulvicornis*	India
UB15	6650	*S. litura*	India
UB16	2660	*Adult Coleoptera*	India
AB1	Local	Unknown	India

Quantification of ammonia

Ammonia concentration was measured by the colorimetric method of Chaney and Marbach (1962). An uninoculated broth was used as a negative control. For this study two solutions were prepared, solution I (Phenol 10 gm/l, Sodium nitroprusside 0.05 gm/l) and solution II (NaOH 5 gm/l, NaCl 0.42 gm/l) and added in equal volumes to 1 ml of culture supernatant. Absorbance at 625 nm (A_{625}) was determined after incubating the solution at room temperature for 30 min.

Quantification of oxalic acid

Oxalic acid concentration was determined by the method of Yan et al. (2004). To the culture supernatant (1ml), 1 M H_2SO_4, 400 µl of 0.03 M $K_2Cr_2O_7$ and 8 ml of ddH_2O was added. After a gentle mix, 400 µl of 1×10^{4} M Victoria blue B was added and the total volume of the solution were made to 10 ml by ddH_2O. The solution was incubated for 9 min at 60°C. Mixture was cooled to quench the reaction with tap water for 2 min and absorbance at 610 nm (A_{610}) of the solution was determined.

Protease activity gel

Molecular weight of the native enzyme was observed using gelatin acrylamide gel electrophoresis. SDS-PAGE was performed on 12.5% gels containing 0.3% gelatin as a copolymerized substrate under non-reducing conditions. After electrophoresis, the gels were shaken gently at room temperature for 1 h in 2.5% Triton X -100 solution to remove SDS and denaturize the enzymes. Gels are then incubated in 0.01 M Tris-HCl (pH 8) – 10 mM $CaCl_2$, buffer at 37°C for 5 - 8 h. The gels were fixed and stained with Coomassie brilliant blue R - 250 for 3 h and then detained for 1 h. clear proteolytic zones in the gel were visible against dark blue background.

Statistical analysis

Statistical analyses were performed by SPSS software. Test of significance were carried out using Tukey's test.

RESULTS

Proteolytic activity

B. bassiana isolates incubated for ten days showed variation among different isolates studied and the enzymatic activity was maximum from day four to day eight (Table 2). On the second day of incubation, isolates UB8, UB10 and UB 14 showed a activity of 0.58, 0.60 and 0.52 U/ml respectively. On the fourth day, isolates UB 3, UB 9, UB13 produced maximum enzymatic activity of 0.76, 0.90 and 0.77 U/ml respectively. Isolates UB3 (0.75 U/ml), UB 8 (0.74 U/ml), UB 11 (0.76 U/ml) on sixth day and isolates UB11 and UB16 showed an activity of 0.89 and 0.84 U/ml respectively on 8^{th} day and with decreased protease activity on 10^{th} day. Isolates UB3, UB9 and UB13 showed high enzymatic activity on all days of study. High specific activity was observed on 10^{th} day in almost all the isolates with highest specific activity by isolates UB 3 (10.41 mU/mg) and UB 9 (14.58 mU/mg) respectively. High enzyme producing strains were screened based on the enzyme activity on 4^{th} day and further analysis of Pr1 and Pr2 activity was deter-mined along with pH, ammonia and oxalic acid release.

Table 2. Enzymatic activity and specific activity of *B. bassiana* isolates

Isolates	Enzyme and specific activity as on									
	Day 2		Day 4		Day 6		Day 8		Day 10	
	Enzymatic activity (U/ml)	Sp. activity (mU/mg)	Enzymatic activity (U/ml)	Sp. activity (mU/mg)	Enzymatic activity (U/ml)	Sp. activity (mU/mg)	Enzymatic activity (U/ml)	Sp. activity (mU/mg)	Enzymatic activity (mU/ml)	Sp. activity (mU/mg)
UB1	0.4247^{c}	1.26^{f}	0.5748^{d}	1.39^{g}	0.5718^{c}	2.37^{f}	0.5710^{d}	5.19^{c}	0.5705^{b}	0.87^{f}
UB2	0.4422^{c}	1.61^{f}	0.4367^{e}	2.46^{f}	0.3967^{de}	5.54^{c}	0.4010^{e}	7.65^{a}	0.4137^{d}	1.39^{e}
UB3	0.3538^{d}	2.01^{e}	0.7633^{b}	3.45^{e}	0.7466^{a}	3.37^{e}	0.6985^{c}	7.61^{a}	0.6088^{b}	10.41^{a}
UB4	0.0156^{h}	7.08^{a}	0.1109^{h}	4.32^{d}	0.1243^{g}	4.79^{d}	0.1279^{h}	4.38^{d}	0.1313^{f}	1.13^{f}
UB5	0.1757^{f}	1.57^{f}	0.3497^{f}	9.43^{a}	0.3467^{e}	1.59^{g}	0.3421^{f}	2.49^{f}	0.3401^{e}	3.71^{d}
UB6	0.1141^{g}	0.518^{g}	0.2081^{g}	7.43^{b}	0.2548^{f}	9.78^{a}	0.2842^{g}	1.65^{g}	0.3022^{e}	3.27^{d}
UB7	0.1423^{g}	1.17^{f}	0.1476^{h}	1.15^{g}	0.2355^{f}	1.75^{g}	0.3211^{f}	3.23^{e}	0.3371^{e}	3.39^{d}
UB8	0.5889^{a}	3.63^{c}	0.5679^{d}	2.87^{f}	0.7347^{a}	6.19^{b}	0.7582^{b}	5.30^{c}	0.6084^{b}	6.01^{b}
UB9	0.4336^{c}	3.25^{c}	0.9021^{a}	3.09^{e}	0.6216^{b}	3.89^{e}	0.5864^{d}	6.69^{b}	0.5211^{c}	14.58^{a}
UB10	0.6059^{a}	4.71^{b}	0.6536^{c}	6.74^{c}	0.4105^{d}	5.60^{c}	0.4526^{e}	6.24^{b}	0.4269^{d}	6.10^{b}
UB11	0.4204^{c}	3.43^{c}	0.4925^{e}	3.02^{e}	0.7583^{a}	5.03^{c}	0.8946^{a}	5.07^{c}	0.8277^{a}	5.05^{c}
UB12	0.1575^{g}	1.01^{f}	0.3723^{f}	1.42^{g}	0.4224^{d}	2.01^{f}	0.4014^{e}	4.31^{d}	0.3515^{e}	3.99^{d}
UB13	0.0524^{h}	0.546^{g}	0.7747^{b}	2.49^{f}	0.6706^{b}	2.96^{f}	0.6660^{c}	4.05^{d}	0.6454^{b}	9.22^{a}
UB14	0.5216^{b}	3.35^{c}	0.5531^{d}	3.53^{e}	0.5179^{c}	4.51^{d}	0.4745^{e}	4.26^{d}	0.4429^{d}	4.57^{c}
UB15	0.1755^{f}	1.92^{e}	0.2845^{g}	3.71^{e}	0.2059^{f}	2.50^{f}	0.1831^{h}	2.12^{f}	0.1819^{f}	2.06^{e}
UB16	0.2672^{e}	2.86^{d}	0.5222^{d}	3.23^{e}	0.5982^{c}	4.47^{d}	0.8405^{a}	5.43^{c}	0.8309^{a}	4.95^{c}
AB1	0.0360^{n}	0.070^{n}	0.0817^{i}	0.15^{n}	0.0804^{n}	0.16^{n}	0.1024^{n}	0.46^{n}	0.2031^{r}	1.71^{e}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test.

pH, ammonia, oxalic acid, Pr1 and Pr2 activity in minimal medium

High Pr1 activity was observed for isolate UB13 (4.44 U/ml) followed by isolate UB 3 (3.88 U/ml) (Table 3). High specific activity of Pr1 enzyme was observed for isolate UB 5 (3.89 U/mg) followed by isolate UB 3 (1.26 U/mg). Almost all the isolates showed same level of Pr2 activity except isolate UB 1 (2.03 U/ml). The pH of the medium increased from the initial (7.0) for all the isolates except UB 1 and a high Pr1 activity was observed at pH 7.02 and 7.05 respectively. The ammonium concentration was high for isolate UB 3 followed by UB 2. Oxalic acid concentration was least (26.0 µg/ml) for the isolate UB 13 that showed high Pr1 activity (4.44 U/ml).

pH, ammonia, oxalic acid, Pr1 and Pr2 activity in minimal medium supplemented with Casein (1%)

High Pr1 enzyme activity was observed for isolate UB 1 and UB 2 (4.77 U/ml) followed by isolate UB 9 (4.04 U/ml) (Table 4). Specific activity was observed to be extremely low compared to enzyme activity for both Pr1 and Pr2 enzymes. High Pr2 activity was observed for isolate UB 5 (0.75 U/mg) and the rest of the isolates showed similar enzymatic activity. The pH of the medium showed slight change in case of isolates UB 1 (7.17), UB 2 (7.08) and a pH of 7.06 for the isolate UB 9. The ammonia concentration was the least for the isolate UB 1 (0.015) which showed high Pr1 activity. The other two isolates which showed

Table 3. pH, ammonia, oxalic acid concentration, Pr1 and Pr2 activity of *B. bassiana* isolates in minimal medium.

Isolates	pH	Ammonia concentration (µg/ml)	Oxalic acid concentration (µg/ml)	Pr1		Pr2	
				Enzyme activity (U/ml)	Sp. activity (U/mg)	Enzyme activity (U/ml)	Sp. activity (U/mg)
UB1	6.99	0.022	33.5	0.76^{e}	0.17^{e}	2.03^{a}	0.45^{b}
UB2	7.01	0.065	43.0	1.49^{c}	0.38^{d}	0.15^{b}	0.04^{c}
UB3	7.05	0.068	60.5	3.88^{b}	1.26^{b}	0.16^{b}	0.05^{c}
UB5	7.02	0.054	85.5	0.35^{f}	3.89^{a}	0.16^{b}	1.78^{a}
UB9	7.01	0.059	75.5	1.24^{d}	0.67^{c}	0.17^{b}	0.09^{c}
UB10	7.03	0.055	94.5	0.28^{f}	0.12^{e}	0.17^{b}	0.07^{c}
UB13	7.02	0.052	26.0	4.44^{a}	0.33^{u}	0.16^{u}	0.01^{c}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test.

Table 4. pH, ammonia, oxalic acid concentration, Pr1 and Pr2activity of *B. bassiana* isolates in MM supplemented with casein (1%).

Isolates	pH	Ammonia concentration (µg/ml)	Oxalic acid concentration (µg/ml)	Pr1		Pr2	
				Enzyme activity (U/ml)	Sp. activity (U/mg)	Enzyme activity (U/ml)	Sp. activity (U/mg)
UB1	7.17	0.015	-	4.77^{a}	0.05^{a}	0.14^{b}	0.001^{a}
UB2	7.08	0.027	-	4.77^{a}	0.05^{a}	0.09^{b}	0.009^{a}
UB3	7.06	0.037	-	2.56^{d}	0.04^{a}	0.13^{b}	0.002^{a}
UB5	7.05	0.033	-	3.77^{c}	0.05^{a}	0.75^{a}	0.009^{a}
UB9	7.06	0.026	-	4.04^{b}	0.06^{a}	0.18^{b}	0.003^{a}
UB10	7.02	0.029	-	1.87^{e}	0.03^{a}	0.11^{b}	0.002^{a}
UB13	7.11	0.035	-	0.07^{f}	0.001^{u}	0.14^{u}	0.009^{a}

high Pr1 activity also showed less ammonia concentration when compared to other isolates. Interestingly, oxalic acid production was not observed in any of the isolates in this medium.

pH, ammonia, oxalic acid, Pr1 and Pr2 activity in minimal medium supplemented with Colloidal chitin (2%)

Pr1 activity was moderately high for all the *B. bassiana* isolates. Isolate UB 13 showed high Pr1 activity (4.36 U/ml) and UB 5 showed high specific activity for Pr1 (0.39 U/mg) (Table 5). High Pr2 activity was observed for the isolate UB 3 (0.18 U/ml). Pr2 specific activities were indistinguishable among the isolates. The pH of the medium showed slight changes, and the isolates showing high Pr1 activity also showed relatively high concentration of ammonia. Interestingly, even in this medium oxalic acid production was not observed.

Protease activity gel

Isolate UB 1 and UB 5 both showed same kind of zymograms with two bands corresponding to 97 and 66 kDa of native protease enzymes (Figure 1). A 97.4 kDa protease was observed in the zymogram for the isolates UB 2, UB 3 and UB 9. A 66 kDa native protease was observed in common in all the isolates. A 55 kDa protease was observed in isolate UB 3 which was absent in all other isolates studied.

DISCUSSION

Protein is one of the most primary constituent in insect cuticle after chitin. Protease is one of the most important and earliest enzymes involved in the invasion followed by chitinase after the eventual exposure of chitin in the host cuticle after the proteolytic degradation of cuticular proteins (Smith et al., 1981; St.Leger et al., 1986). Extracellular proteases were even found in insect haemolymph (Shimizuy et al., 1993). An immense variation in the protease activity was observed among the seventeen *B. bassiana* isolates. Variation in the time of maximum protease production was also observed with different isolates. The variation in protease production might be attributed to the geographical origin of the isolates as three among four high protease producing isolates viz. UB3, UB9, UB10 were from same geographical origin (USA), although variation cannot be directly correlated with the insect host. Most of the isolates showed

Table 5. pH, ammonia, oxalic acid concentration, Pr1 and Pr2 activity of *B. bassiana* isolates in MM supplemented with colloidal chitin (2%).

Isolates	pH	Ammonia concentration (µg/ml)	Oxalic acid concentration (µg/ml)	Pr1		Pr2	
				Enzyme activity (U/ml)	Sp. activity (U/mg)	Enzyme activity (U/ml)	Sp. activity (U/mg)
UB1	7.17	0.015	-	3.53^{c}	0.24^{b}	0.11^{bc}	0.01^{a}
UB2	7.08	0.027	-	3.59^{c}	0.34^{a}	0.09^{bc}	0.01^{a}
UB3	7.06	0.037	-	3.02^{d}	0.26^{b}	0.18^{a}	0.02^{a}
UB5	7.05	0.033	-	3.79^{b}	0.39^{a}	0.09^{bc}	0.01^{a}
UB9	7.06	0.026	-	2.66^{e}	0.21^{b}	0.10^{bc}	0.01^{a}
UB10	7.02	0.029	-	1.53^{f}	0.15^{c}	0.05^{c}	0.01^{a}
UB13	7.11	0.035	-	4.36^{a}	0.26^{b}	$0.09b^{c}$	0.01^{a}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test.

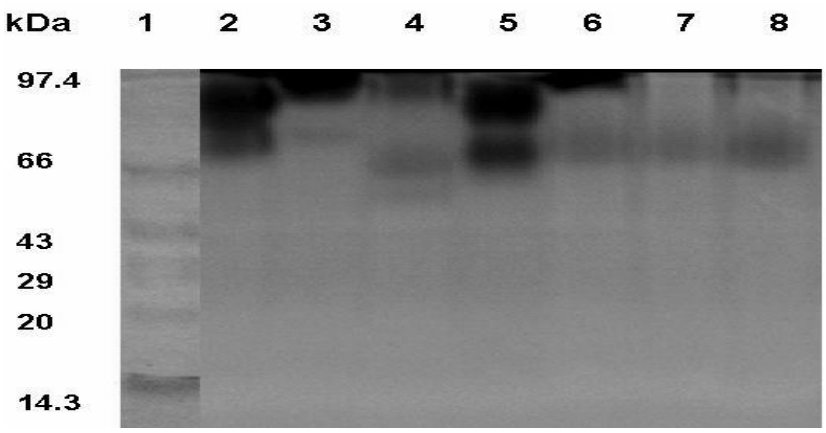

Figure 1a. Protease activity in gelatin polyacrylamide gel electrophoresis. Lane 1 corresponds to MW marker after complete destaining of the gel. Lane 2,3,4,5,6,7,8 corresponds to isolates UB 1, UB 2, UB 3, UB 5, UB 9, UB 10 and UB 13, respectively.

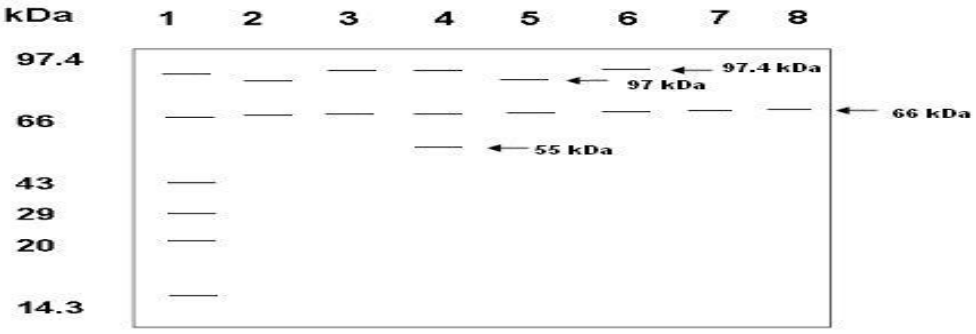

Figure 1b. Schematic representation of Figure 1a with the corresponding MW for each isozyme.

high protease production during four to six days of culture which is comparable with the observations by Ito et al. (2007), demonstrating high protease activity on fifth day of culture in *B. bassiana*. The time for maximum protease production does not necessarily depend upon the media constituents and Kucera (1971) observed high amounts of protease release 3 days post inoculation in the media with different nitrogen sources. Proteolytic activity of *B. bassiana* isolates decreased with increase in culture age probably due to nutrient limitation or autolysis of the

culture. Caseinolytic and elastolytic activity of an *M. anisopliae* isolate was observed to be high during four to six days of culture and then a steep decrease up to sixteenth day of culture probably due to increasing degree of autolysis of the aging culture (Braga et al., 1999).

The caseinolytic activity can be attributed to the presence of Pr1 and Pr2 activity in the culture filtrate (St. Leger, 1987a). The Pr1 and Pr2 activity in three media with different carbon and nitrogen sources was investigated for the selected seven isolates. The use of minimal medium for protease production could possibly emulate the enzyme activity in the initial stages of host infection when readily metabolizable compounds are extremely low. Almost all the isolates showed moderate to high Pr1 activity and low levels of Pr2 activity even in the minimal medium indicating that the enzyme production is constitutive. The Pr1 and Pr2 activity was observed to increase rapidly during carbon and nitrogen depression for entomopathogenic fungus *M. anisopliae* (St. Leger, 1988), although Bidochka and Khachatourians (1988) reported the lack of protease production from one *B .bassiana* isolate in the absence of exogenous protein. Constitutive production of protease was also found in *Aspergillus* sp. (Cohen and Drucker, 1977). In the present study, the Pr1 and Pr2 activity was observed to be high in the medium containing casein (1%) as a sole source of carbon and nitrogen. The reason perhaps is the presence of protein in the minimal medium acting as an inducer for both the enzymes. Similar results of protease induction by protein were observed by Drucker (1972) in *Neurospora crassa*. The Pr2 production could be enhanced by the addition of proteins like gelatin, BSA or casein (Paterson et al., 1993). The most notable observation was that the isolates that showed maximum Pr1 production in minimal medium viz., UB 3 (3.88 U/ml) and UB 13 (4.44 U/ml) showed decreased Pr1 activity in minimal medium supplemented with casein (1%), UB 3 (2.56 U/ml) and UB 13 (0.07 U/ml), which was found to be contrary in case of other five isolates that showed low Pr1 activity in minimal medium and high in minimal medium supplemented with casein (1%). These results indicate the presence of catabolite repression of Pr1 activity produced by different isolates. St. Leger et al. (1988) reported that modifying the concentrations of carbon and nitrogen independently could repress protease production even in the absence of other repressor molecule. All the isolates showed high Pr1 activity in minimal medium supplemented with colloidal chitin (2%).

The exploitation of colloidal chitin as sole source of carbon and nitrogen appeared to induce the Pr1 activity which is indeed comparable with the results of St. Leger et al. (1988) who observed that protease activity could be induced by using polymeric substances like cellulose and insect cuticle. Chitin, the main constituent of insect cuticle enhances the Pr1 activity either by inducing the protease or possibly that colloidal chitin was unable to produce catabolite repression. However the role of colloidal chitin to induce or repress Pr2 enzyme was unclear as very low levels of Pr2 activity was detected in this medium containing colloidal chitin (2%).

Genes that are involved in virulence and pathogenesis are often regulated by environmental signals like pH (De Bernardis et al., 1998). An increase in pH was observed on insect cuticle after 60 h growth of fungus which is consistent with the pH regulation of the protease production during growth by *M. anisopliae* in liquid culture containing cuticle. The pH of the culture media rises due to the release of ammonical by - products of cuticle protein degradation (St. Leger et al., 1998; St.Leger et.al., 1989). Protease production in *M. anisopliae* was also correlated with increased pH of culture medium caused by the production of ammonia; however oxalic acid producing mutants showed decreased activity (St Leger et al., 1999). However, in this investigation no such correlation was observed in relation to pH of the culture medium and subtilisin type protease production (Pr1). The pH of the unbuffered media changed diminutively from the initial pH 7 in the three different media. High ammonia production in minimal medium supplemented with casein (1%) could be correlated with the high Pr1 production, although high Pr1 production in minimal medium supplemented with colloidal chitin (2%) cannot be associated with low ammonia concentration. Dias et al. (2008) reported moderate production of Pr1 from *B. bassiana* in unbuffered minimal medium at pH 7 after 72 h of culture. High concentration of oxalic acid should not be directly correlated with the low protease production in nutrient deprived minimal medium. However oxalic acid was not detected in other two media suggesting a favorable condition for high Pr1 secretion by ammonia production alone. The native proteases from the seven *B. bassiana* isolates differed in their molecular weights.

Almost all the isolates showed a 66 kDa protease. St. Leger et al. (1996) observed two trypsin like proteases in *M. anisopliae* with 30 kDa and 27 kDa molecular weight. A 103 kDa and 12 kDa metalloprotease was reported from *M. anisopliae* conidia (St. Leger et al., 1998).

The results represented in this study would increase the knowledge about the degree of protease production in various *B. bassiana* isolates and the incubation time as well as the Pr1 and Pr2 production by *B. bassiana* isolates could lead to new course of understanding of the role of proteases in virulence of the entomopathogenic fungi.

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