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ORIGINAL ARTICLE

Partial Purification and Characterization of Protease from Local Isolate of *Beuveria bassiana*

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ABSTRACT

Objectives: Entomopathogenic fungi were identified as effective alternative to chemicals in insect pest control. Extra cellular proteases of these fungi are implicated as components of the insect cuticle degradation process. This study aimed to the purification and characterization of a protease from a local isolate of the entomopathogenic fungus *Beuveria bassiana* (*B. bassiana*).

Methods: The purification process of the protease from the culture filtrate of *B. bassiana* was carried out by dialysis, ethanol precipitation and DEAE-cellulose ion exchange chromatography. After that, the enzyme was characterized to establish the identity of the enzyme.

Results: The protease was partially purified by 2.3 folds with 16.3 yield. The results of enzyme characterization showed that the Km values were (4.43, 6.26 and 6.66) mg/ml using casein, gelatin and bovine serum albumin as substrates respectively. Furthermore, the Vmax values were (16.3, 16.66 and 16.66) mg/ml.min for the same substrates respectively. The optimum pH for the protease activity was 9 with heat stability range of 20-40 C°. The effects of protease inhibitors and activators showed that the enzyme was totally inhibited by PMSF at 1 and 5 mM. This indicated that the protease belonging to serine proteases. Conversely, calcium chloride and magnesium chloride showed stimulating effect on the enzyme activity.

Conclusion: : A protease was partially purified from an Iraqi isolate of *B. bassiana*. The enzyme can be used to degrade different types of insect cuticles as it showed a range of specificity toward different substrates.

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INTRODUCTION

The emergence of high tolerance to most chemical insecticides associated with environmental problems have limited their use. In recent decades, sustained efforts have made to develop Microbial Biocontrol Agents (MBCAs) as an effective alternative non-chemical strategy in the integrated control of insect pests[1].

Entomopathogenic fungi (EPF) are important components of MBCAs due to easy delivery, improving formulation, the availability of many pathogenic strains and the possibility of engineering and overexpression techniques. Moreover, EPF are important biological control agents

according to their broad host range and route of pathogenicity and/or virulence[2].

EPF invade their hosts via the insect integument (cuticle) which is a composite-complex structure of protein and chitin surrounded by lipid and wax layer or fatty acids. This structure reported to consist of 55-80% protein and 25-40% chitin[3]. EPF have a combination of enzymatic and mechanical mechanisms to penetrate the insect cuticle. The secretion of cuticle-degrading enzymes such as proteases, chitinases and lipases are important in the penetration process[4].

After the epicuticle degradation by lipases, the entomopathogenic fungi produce high quantities of proteases that break down the proteinaceous material. Subtilisin-like serine protease (designated as Pr1) and trypsin-like protease (designated as Pr2) are the most frequently investigated proteolytic enzymes in the literature[5]. Eleven isoforms are related to the Pr1 coding gene[6]. Multiple sequence alignment and structural analysis based on molecular modeling showed that Pr1 contains five cysteines forming two disulfide bridges and the residual cysteine is located near the active site that contains the substrate binding residues namely Asp39, His69 and Ser224. The enzymatic activities of Pr1 and Pr2 were identified in *Beuveria bassiana, Metarhizium anisopliae, Lecanicillium lecanni, Nomuraea rileyi* and *Metarhizium flavoviride*[7].

Beuveria bassiana is a well-understood member of the EPF that infect wide range of insect pests belonging to Lepidoptera, Coleoptera and Hymenoptera[8]. The fungus was used extensively in the integrated pest management (IPM)[9]. This study aimed to the purification and characterization of a protease from a local isolate of *B. bassiana*.

MATERIALS AND METHODS

A local isolate of *B. bassiana* obtained from department of Biology-Collage of Science and used in this study.

Preparation of seed culture:

Seed culture was prepared according to the method described by Dhar and Kaur[10] with minor modifications. The medium was inoculated with one disk from *B. bassiana* grown on Potato dextrose agar plate (PDA) (one disc contains approximately $2x10^7$ spores/ml). The inoculated medium was incubated at 30 ^{0}C in a shaking incubator for 72 hrs.

Preparation of production medium:

The production medium contains baker's yeast (1%), $MgSO_4.7H_2O$ (0.1%) and KH_2PO_4 (0.1%). The pH of the medium was adjusted to 6.5 prior to sterilization by autoclave. 50 ml of the production medium in250 ml conical flask was inoculated with seed inoculum (2%) and incubated at 30 ^{0}C under shaking for 3 days.

Protease activity assay:

Proteases activity was determined according to the method described by[11] with minor modifications. Briefly, 0.2 ml of the enzyme extract was mixed with 2 ml of the substrate (casein 2%) in Tris buffer (pH 8.5). The mixture was incubated in a water bath at 37^{0}C for 20 min., this reaction was stopped by adding 3 ml of Trichloroacetic acid (TCA) (10%). The mixture was centrifuged at 5000 rpm for 20 min to measure the released amino acids (due to the protease activity) in the supernatant spectrophotometrically. The absorbance was measured at 280 nm after calibration of the spectrophotometer. The activity unit is defined as the amount of enzyme that causes 0.001 increase in the absorbance at 280 nm.

Protein assay:

Protein concentration was determined according to the method described by[12] using Bovine serum albumin (BSA) as a standard protein.

Protease purification:

The enzyme was purified using the following steps:

- Dialysis: The crude extract of the protease produced by *B. bassiana* was dialyzed against Tris buffer (0.005 M, pH 8.0) three times at equal intervals of time.
- Ethanol precipitation: The dialyzed crude enzyme was precipitated by the addition of ethanol (70%). The mixture was centrifuged at 8000 rpm for 30 min at 4^{0}C. The precipitate was dissolved in Tris buffer.
- Ion exchange chromatography: further purification of protease was carried out using DEAE cellulose. The enzyme solution was loaded on DEAE cellulose column (1.8 x 18) cm. The column was washed with 0.005 M Tris buffer (pH 8.0) and eluted with increasing concentration gradient of NaCl (0.0 – 0.3) M. The eluted fractions were collected and analyzed for protease activity.

Characterization of protease:

- Identification of kinetic parameters: different concentrations of casein (0.05-2) % were used to evaluate the kinetic parameters of the purified protease namely Km and Vmax. These parameters were estimated according to the method described by[13].
- Substrate specificity of protease: Four substrates were used to study the specificity of the purified protease including casein, BSA, gelatin and ovalbumin using 2% concentration.
- Optimum pH of protease activity: Protease activity was determined by incubating the enzyme with casein prepared in buffers at different pH values ranging from 7-10.5.
- pH stability of protease: The purified protease was incubated at 37 ^{0}C for 30 min. with different buffers including acetate buffer at pH 5-6, phosphate buffer at pH 6.5 – 7.5 and Tris buffer at pH 8 - 10.5, after incubation the residual activity of the enzyme was determined.

Effect of temperature on protease stability:

The purified enzyme was incubated in a water bath for 30 min. at different temperatures ranging from 20 – 70 ^{0}C. The residual activity of the enzyme was determined to evaluate the enzyme stability.

Effect of some metal ions and chemicals on protease activity:

1-5 mM of some metal ions and chemicals were prepared to examine their effect on protease activity. These metals included $MnCl_2$, $HgCl_2$, $NiCl_2$, $MgCl_2$, $CaCl_2$, iodoacetamide, ethylene diamine tetra acetic acid (EDTA),

mercaptoethanol, boric acid and phenyl methyl sulphonyl fluoride (PMSF).

RESULTS AND DISCUSSION

All the purification steps were conducted under cooling conditions, these steps are listed in Table 1.

Table 1: **Purification parameters of protease from local isolate of *B. bassiana*.**

Purification step	Volume (ml)	Activity (unit/ml)	Protein concentration (mg/ml)	Specific activity (unit/mg)	Total activity (units)	Purification folds	Yield (%)
Crude enzyme	60	22	0.025	880	1320	1	100
Dialysis	61.8	19.5	0.020	975	1205.1	1.108	91.29
Ethanol precipitation (70%)	30	18.25	0.012	1520.83	547.5	1.728	41.47
Ion exchange with DEAE cellulose	41	5.25	0.0028	1875	215.25	2.13	16.3

Dialysis:

Dialysis of the crude enzyme were carried out against tris buffer (0.005 M, pH8) for 6 hours at 4°C, in this step the enzyme was partially purified with 1.108 purification folds and 91.29% enzymatic yield. Dialysis is one of the strategies that commonly used in the bio-separation of proteins depending on the osmotic difference between two solutions. After dialysis, small molecular size molecules can be removed in addition to concentrating the dialyzed sample[14].

Dialysis was used in the purification of proteases from different micro organisms such as *Aspergillus flavus* with 2.53 folds of purification and 3.73% enzymatic yield. Furthermore, this strategy was used during the purification of alkaline protease from *Penicillium expansum* with 801 folds of purification and 87.09% enzymatic yield. Dialysis was also used during the purification of protease from *Pleurotus sajor-caju* with 1.08 folds of purification and 73.33 enzymatic yield[15].

Ethanol precipitation:

B. bassiana protease was precipitated with Cold ethanol after dialysis. This purification step led to increased folds of purification 1.728 and enzymatic yield 73.33. Ethanol is a poor solvent for proteins as it is miscible with water and thus it is often used as a precipitation agent for these biomolecules. Moreover, ethanol increase protein-protein attraction forces as it has a lower dielectric point than water. The organic solvent is also able to form a solution with water which leave less water molecules to interact with the protein[16]. Ethanol was used as a first step in the purification of the alkaline serine protease from *myceliophthora* sp. With 106 purification folds and 79.5% enzymatic yield[17].

Ion exchange chromatography:

This was the last purification step of the protease from *B. bassiana*. The step was carried out using DEAE cellulose. After washing the resin, the bound proteins were eluted by NaCl gradient (0.0 – 0.3) M. several peaks were appeared Figure 1. Fractions 34-49 were pooled as they showed the highest protease activity. Specific activities of the pooled fractions were 1875 unit/mg protein. The purification parameters of this purification step were 2.13 folds and 16.3% recovery Table 1. Ion exchange chromatography was used in several studies for the purification of proteases from fungi. For example, DEAE cellulose was used in the purification of protease from *Beauveria* sp. with 10.02 and 38.6% folds of purification and enzymatic yield respectively[18]. Moreover, the same ion exchanger was used in the purification of protease from *Penicillium janthinellum* and *Neurospora crassa* with purification fold 3.1 and 9.3, and enzymatic yield 76% and 28%, respectively[17]. In this study, an Iraqi isolate of *B. bassiana* was collected and its protease was partially purified.

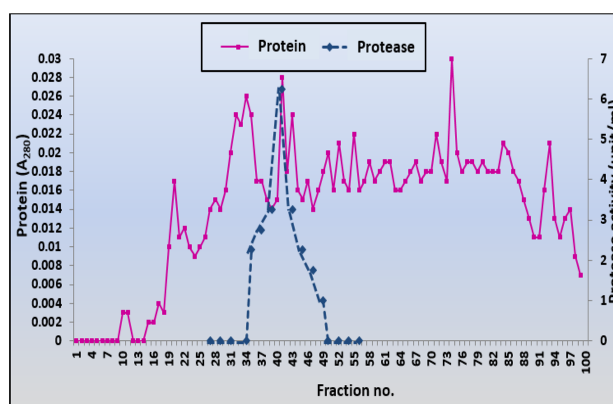

Figure 1. **Ion exchange chromatography of the protease from the entomopathogenic fungus *B. bassiana* using DEAE cellulose. The column (1.8x18 cm) was equilibrated with tris buffer (0.005 M and pH = 8). After loading the sample, the column was washed with the same buffer. After that, elution step was carried out using 0.0-0.3 M gradient of NaCl with 1 ml/min flow rate and 3ml fraction size.**

Characterization of *B. bassiana* protease:

Enzymatic characterization of the protease from the entomopathogenic fungus *B. bassiana* was carried out by identification of kinetic parameters (Km and Vmax), substrate specificity and the effect of pH, temperature and metal ions on the protease activity.

Measurement of kinetic parameters:

Lineweaver-Burk method was followed to estimate Km and Vmax of the protease using casein as substrate (Figure 2). The result showed that Km and Vmax were 4.34 mg/ml and 16.13 mg/ml.min respectively. It was

recorded that the Km and Vmax of the purified protease from *Beuveria* sp. were 5.1 mg/ml and 29.67 mg/ml.min respectively[18]. Moreover, the kinetic parameters of the purified protease from *Aspergillus oryzae* were 3 mg/ml and 715 mg/ml.min for Km and Vmax respectively using the same substrate namely casein[19].

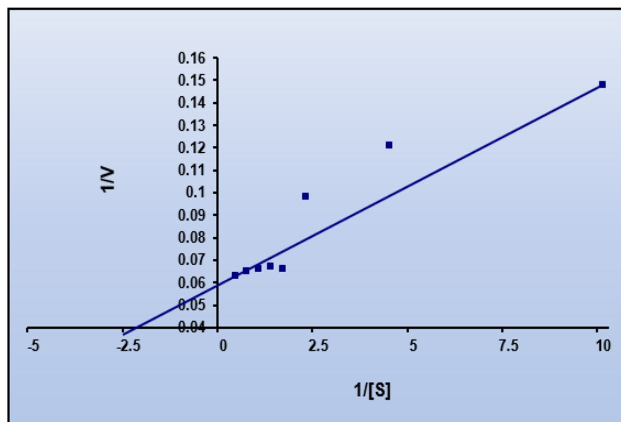

Figure 2. **Lineweaver-Burk double reciprocal plot to estimate the kinetic parameters (Km and Vmax) of the partially purified protease from *B. bassiana* using casein as substrate.**

Substrate specificity:
Alkaline Protease can specifically target a wide range of substrates including natural proteins and synthetic substrates. Figures 2, 3 and 4 show that the enzyme presented specificity for casein, gelatin and bovine serum albumin with Km values 4.34, 6.25 and 6.66 mg/ml respectively, whereas Vmax values for the same substrates were 16.3, 16.6 and 16.6 mg/ml.min respectively. The results revealed that casein is the optimum substrate for *B. bassiana* protease as compared to gelatin and bovine serum albumin. Km values can be highly varied from enzyme to another and among different substrates for the same enzyme[20]. These results are in agreement with data from kinetic study carried out using casein, hemoglobin and bovine serum albumin as substrates for the protease from *Beuveria* sp., Where the purified protease showed the highest substrate specificity toward casein[18].

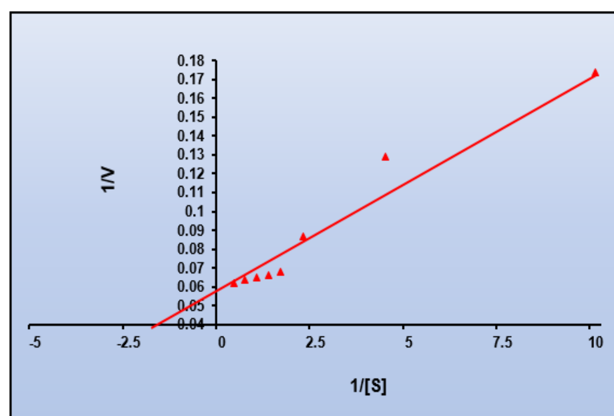

Figure 3. **Lineweaver-Burk double reciprocal plot to estimate the kinetic parameters (Km and Vmax) of the partially purified protease from *B. bassiana* using gelatin as substrate.**

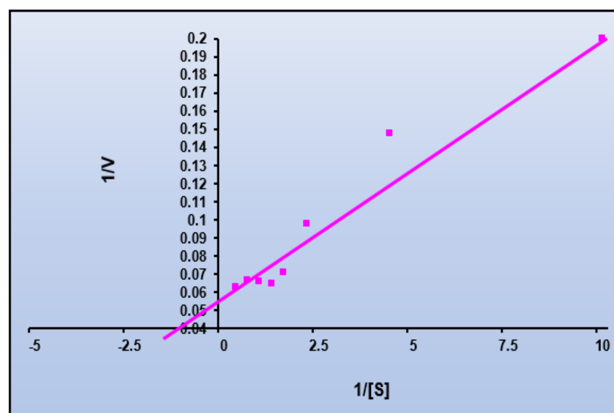

Figure 4. **Lineweaver-Burk double reciprocal plot to estimate the kinetic parameters (Km and Vmax) of the partially purified protease from *B. bassiana* using bovine serum albumin (BSA) as substrate.**

Effect of pH and temperature on protease activity:
The effect of pH and temperature on protease activity was investigated using casein as a specific substrate at different settings of pH and temperature. Figure 5 shows that the optimum pH for *B. bassiana* protease activity is 9. This emphasize that *B. bassiana* protease in this study can be considered an alkaline protease. However, further molecular and structural studies are required for verification. These results agree with other studies as the same optimum pH (namely 9) reported for the proteases purified from *Beuveria sp.*[18] and *Myceliophthora sp.*[17]. In other research, it was stated that the optimum pH of *B. brongiriaotii* purified protease was 8[21]. Temperature effect results showed that *B. bassiana* protease was stable at 20-40°C and maintained 90% of its activity at 45°C, whereas the protease was inactivated significantly at higher temperatures (50-55) and lost all of its activity at 70 °C (Figure 6).

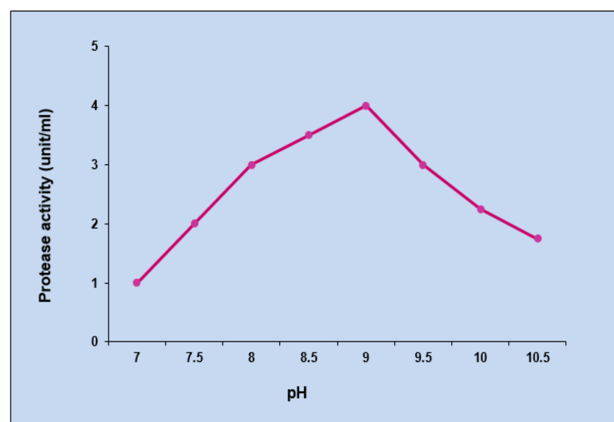

Figure 5. **Effect of pH on the proteolytic activity of the partially purified protease from *B. bassiana*.**

Effect of inhibitors and activators
The effect of various protease activators and inhibitors were assessed to establish the identity of the proteolytic enzyme (Table 2). Metal ions including Ca^{+2} and Mg^{+2} (5mM) induced *B. bassiana* protease activity to

174.67% and 140.83% respectively. This can be explained that these ions stabilize the enzyme structure. This can lead to protect the protease against thermal denaturation[22,23]. $HgCl_2$ decreased the proteolytic activity to 49.3% and 36.03% remaining activity using 1 and 5 mM respectively, possibly acting as denaturant where Hg ions can interact with thiol groups of cysteine, tryptophan and/or histidine residues in addition to the breakdown of the disulfide bridges[23]. 1and 5 mM of PMSF inhibited the protease activity entirely. This indicated the presence of serine-catalyzed protease activity where the amino acid in the active site is sulfonated by the inhibitor[24]. EDTA induced the activity to 144.13% and 163.75% using 1 and 5 mM respectively. This suggests that *B. bassiana* protease in this study is not a metalloprotease as no inhibition was observed with EDTA. Iodoacetamide (IAA) showed no enzyme inhibition as the protease activity increased to 114.63% using 5 mM. This result suggests the absence of thiol protease activity. Mercaptoethanol, the protective agent of sulfhydryl groups[25], showed some stabilizing effect on the extra cellular protease as the remaining activity increased from 54.59% using 1 mM to 67.25% using 5 mM. However, these sulfhydryl groups are not located in the active site as IAA did not inhibit *B. bassiana* protease activity.

Extra cellular proteases produced by *B. bassiana*[26] and other fungi[27] are implicated in the partial hydrolysis of a main component in insects cuticle[3] namely proteins. The hydrolysis process can be essential for fungal penetration as the pretreatment of insect cuticle with protease significantly increases chitin hydrolysis by chitinase[27].

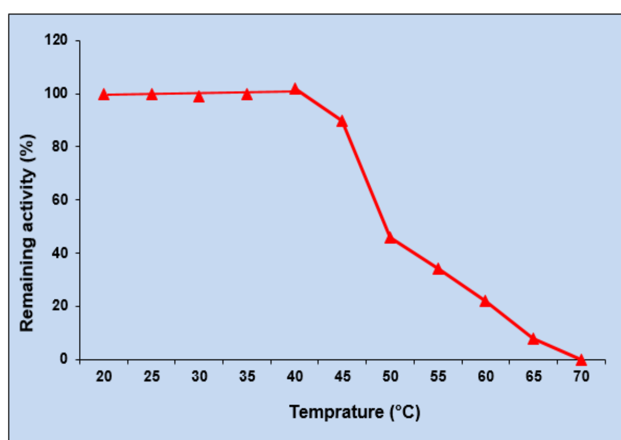

Figure 6. **Temperature stability of the protease from *B. bassiana*.**

Table 2. **Effect of activators and inhibitors on the protease activity of *B. bassiana*.**

Compound tested	Concentration (mM)	Protease activity (u/ml)	Remaining activity (%)
Untreated enzyme	-	4.58	100
$CaCl_2$	1	7.07	154.37
	5	8	174.67
$MgCl_2$	1	5. 2	113.54
	5	6.45	140.83
$HgCl_2$	1	2.25	49.13
	5	1.65	36.03
PMSF	1	0	0
	5	0	0
EDTA	1	7.5	163.75
	5	6. 83	144.13
Iodoacetamide (IAA)	1	4.5	98.25
	5	5.25	114.63
mercaptoethanol	1	2.5	54.59
	5	3.08	67.25

CONCLUSION

A protease was partially purified from an Iraqi isolate of the entomopathogenic fungus *B. bassiana*. The biochemical properties of the enzyme showed a range of specificity toward different substrates. In conclusion, this virulence factor can be used to degrade different types of insect integuments. Moreover, as the enzyme heat stability was limited it can be used in the biological control of insect pests during winter, autumn and spring rather than summer.

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