

Purification and characterization of the cuticle-degrading proteases produced by an isolate of *Beauveria bassiana* using the cuticle of the predatory bug, *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae)

Hossein Firouzbakht¹, Arash Zibae^{1*}, Hassan Hoda², Mohammad Mehdi Sohani³

¹ Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, 41635-1314 Iran

² Biological Control Department, National Institute of Plant Protection, Amol, 91951-46191 Iran

³ Department of Agronomy and Plant Breeding, Faculty of Agricultural Sciences, University of Guilan, Rasht, 41635-1314 Iran

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Abstract: The entomopathogenic fungi-like *Beauveria bassiana* must penetrate via the integument of an insect to reach the hemocoel. Since proteins are the molecules responsible for integument strength in insects, the proteins must synthesise the cuticle degrading proteases which will then enable the proteases to penetrate. It is important to determine the biochemical properties of these proteases so that fungal virulence can be better understood. In the current study, a recently collected isolate of *B. bassiana*, namely AM-118, was inoculated in liquid media containing 0.5% of *Andrallus spinidens* Fabricius cuticle to obtain specific proteases. The crude samples were purified via a three step process using ammonium sulfate, Sepharyl G-100, and DEAE-Cellulose Fast Flow. The results revealed two proteases known as subtilisin-like (Pr1), and trypsin-like (Pr2), with the molecular weights of 105 and 103 kDa. The optimal pH and temperature values were found to be 8 and 35°C for Pr1 and 8 and 40°C for Pr2, respectively. Inhibitors like AEBSE, EDTA, TPCK, and phenanthroline significantly affected proteolytic activities. Here, we reported two fungal proteases by high molecular weight from an Iranian isolate of *B. bassiana*. These findings will help us to better understand fungal virulence against insects.

Key words: *Andrallus spinidens*, *Beauveria bassiana*, protease, purification

Introduction

Entomopathogenic fungi belong to various orders, such as Zygomycota, Ascomycota, and Deuteromycota (Samson *et al.* 1988). These fungi are important components in modern agriculture as they are used as biocontrol agents against a wide range of agricultural pests (Rodgers 1989; Andreev *et al.* 2008). Entomopathogenic fungi are able to infect their hosts by passing through the integument. A mechanical or biochemical process is involved (Pucheta *et al.* 2006; Liu *et al.* 2009). Efficient virulence of the entomopathogenic fungi depends on the interactions between the host and the cell walls of the fungi (Liu *et al.* 2006). This is because the integument of insects is the first barrier against the germ tube of the entomopathogenic fungi as it tries to reach the hemolymph (Gillespie *et al.* 1998). Entomopathogenic fungi utilise two major proteases known as subtilisin-like serine protease (Pr1) and trypsin-like protease (Pr2) (St. Lager *et al.* 1986; Braga *et al.* 1999; Dias *et al.* 2008) to overcome this barrier. Several entomopathogenic fungi, such as: *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicilium lecanii*, *Numuraea rileyi*, and *Metarhizium flavoviride* have demonstrated Pr1 and Pr2 activities (Bidochka and Meltzer 2000). During the initial stages of cuticle degradation, these proteases

are secreted via a signal transduction mechanism by activation of the protein kinase A (PKA) mediated by antimicrobial peptides (AMPs) (Fang *et al.* 2009). It is believed that Pr1 is the key component in the penetration process since it is a virulent indicator for the entomopathogenic fungi (Castellanos-Moguel *et al.* 2007). The enzyme contains eleven isoforms. The molecular structure of the enzyme has five cysteines forming two disulfide bridges but another cysteine is found near the catalytic triad formed of Asp39, His69, and Ser224 (Liu *et al.* 2007).

The predatory bug, *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae), is a promising biocontrol agent of the Pyralidae and Noctuidae rice pest families. This predatory bug also has biocontrol potential against *Chilo suppressalis* Walker (Lepidoptera: Crambidae) (Sorkhabi-Abdolmaleki *et al.* 2013). It is necessary to consider the possible interactions of the biocontrol agents when they are to be used synchronologically against a pest. Since *C. suppressalis* have shown resistance to the chemical insecticide, diazinon, in several areas of northern Iran, biocontrol agents such as *B. bassiana* and *A. spinidens* seem to be appropriate candidates to efficiently and safely control the pest. Ramzi and Zibae (2014) recently determined virulence of several entomopathogenic fungi, including

*Corresponding address:

arash.zibae@gmx.com; arash.zibae@guilan.ac.ir

B. bassiana, on the larvae of *C. suppressalis*. Understanding the potential adaptability of the two biocontrol agents is crucial to preventing conflicts in an agro-ecosystem. Therefore, we are interested in the virulence of two isolates of *B. bassiana* – BB2 and AM-118 – on *A. spinidens* (Safavi 2010; Chitgar *et al.* 2014; Firouzbakht *et al.* 2015). In this context, we have conducted the current study by extracting and purifying Pr1 and Pr2 proteases produced by isolate AM-118. The enzymes were purified via chromatography and their properties were investigated by considering pH, temperature, and specific inhibitors. This is the first time that such a study has been carried out using the cuticle of a biocontrol agent.

Materials and Methods

Beauveria bassiana culture

Isolate AM-118 of *B. bassiana* was cultured at 25±1°C on Potato Dextrose Agar (PDA) (pH 5.6) supplemented with 1% yeast extract. After 14 days, conidia of the fungus were washed off using a 0.01% aqueous solution of Tween 20. A concentration of 10⁷ spore · ml⁻¹ was prepared for the liquid culture medium.

Culture and growth conditions

The liquid culture medium used for protease production consisted of CaCl₂ (0.01%), KH₂PO₄ (0.02%), Na₂HPO₄ (0.02%), MgSO₄ (0.01%), and ZnCl₂ (0.01%). The medium was inoculated with 10⁷ spores · ml⁻¹ of isolate AM-118 and 5 g of *A. spinidens* cuticle. Incubation took place for 8 days on a rotatory shaker (120 rev · min⁻¹) at 25±1°C, in 300 ml of Erlenmeyer flasks with a working volume of 100 ml (Hajji *et al.* 2007). The flasks were sealed and shaken for the incubation period.

Sample preparation

Mycelia in the liquid media, were gathered by centrifugation at 8,000 × g for 15 min and ground to a fine powder. The powder was incubated in the lysis buffer (1 mg in 1 ml of 25 mM universal pH 8, 10% [v/v] glycerol and 1 mM EDTA) for 24 h and centrifuged at 12,000 × g for 15 min. The given sample was stored at -20°C and used for enzymatic assays (Dias *et al.* 2008).

Proteolytic assays

Subtilisin-like (Pr1) and trypsin-like (Pr2) activities in the isolate AM-118 were determined using succinyl-(alanine) 2-prolinephenylalanine-*p*-nitroanilide and benzoyl-phenylalanine-valinearginine-*p*-nitroanilide as substrates, respectively (Dias *et al.* 2008). Each assay consisted of 10 µl of the substrate (1 mM), 40 µl of 20 mM universal buffer (pH 8; and containing succinate, glycine, and 2-morpholinoethanesulfonic acid) (Frugoni 1957), and 5 µl of the enzyme. The mixture was incubated for 10 min at 25°C and the absorbance was read at 405 nm. Activities were expressed as micromoles nitroanilide (NA) released per ml per min (St. Leger *et al.* 1998).

Protease purification

Purification of the proteases was based on a three-step procedure described by Hajji *et al.* (2007). The samples were initially precipitated with a 70% concentration of ammonium sulfate. The precipitated fraction was collected by centrifugation at 15,000 g prior to adding the universal buffer. Finally, the sample solution was subjected to dialysis tubing (12,000 Da) for 20 h. Then, the dialysed sample was subjected to gel filtration on a Sephacryl G-100 column equilibrated with a universal buffer. Enzyme fractions at 1 ml were collected at a flow rate of 20 ml · h⁻¹ with the same buffer. Protein content and protease activities were measured separately, and the fractions showing the highest activities were pooled for the final step. The active fractions were applied to a DEAE-Cellulose Fast Flow equilibrated with universal buffer. After washing with the same buffer, bound proteins were eluted with a linear gradient of NaCl in the range of 0.1 to 0.5 M. The fractions showing the highest proteolytic activities were pooled and stored at -20°C for further analysis.

Protein determination

Protein concentrations were assayed according to the method described by Lowry *et al.* (1951). The method recruits reaction of Cu²⁺ produced by the oxidation of peptide bonds with Folin-Ciocalteu reagent. In the assay, 20 µl of the homogenised sample was added to 100 µl of reagent. Incubation was done for 30 min prior to reading the absorbance at 545 nm (as recommended by Ziest Chem. Co., Tehran-Iran).

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of the purity and molecular mass of the proteases, as described by Laemmli (1970), using a 5% (w/v) stacking gel and a 10% (w/v) separating gel. After loading the samples, electrophoresis was run at a voltage of 80 V. At the end, the gel was separated from the glasses, washed with distilled water and incubated in a staining solution containing: water : acetic acid : ethanol (4 : 2 : 1) and 0.2 g of Coomassie Brilliant Blue.

Measurement of kinetic parameters

Kinetic parameters of the purified proteases were evaluated using different concentrations of each substrate (1, 2, 3, 5, 7, and 10 mM). The reaction mixture was prepared using the above mentioned components and each substrate concentration. The given data was changed to specific activity. Finally the Michaelis constant (K_m) and the maximal velocity (V_{max}) were estimated using Sigmaplot software version 6.

Effect of pH and temperature on the activity of the enzyme

The effects of temperature and pH on Pr1 and Pr2 activities were studied using specific substrates. The optimal

pH was determined using universal buffer (20 mM) with the pH set at 5–11. The effect of temperature on protease activity was determined by incubating the reaction mixture at 20–50°C.

Effect of specific inhibitors on protease activity

The effects of specific inhibitors on protease activity were studied using 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 5 mM, as serine inhibitors), ethylenediaminetetraacetic acid (EDTA, 5 mM, as metalloprotease inhibitor), tosyllysine chloromethyl ketone hydrochloride (TLCK, 5 mM, as trypsin inhibitor), tosyl phenylalanyl chloromethyl ketone (TPCK, 5 mM, as chymotrypsin inhibitor), and phenanthroline (5 mM, as metalloprotease inhibitor). The purified enzyme was pre-incubated with the inhibitors for 10 min at 30°C.

Statistical analysis

All data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's student test when significant differences were found at $p < 0.05$ (SAS 1997).

Results and Discussion

Purification of proteases

The virulence of an entomopathogenic fungi demonstrated as the percentage of mortality on a host. The efficient virulence highly depends on ability of a fungus to penetrate via integument. The penetration is a multi-complex processes in which both mechanical forces and relevant enzymes regarding the chemical composition of integument are crucial to facilitate fungal penetration. It is believed that proteases and esterases are the enzymes present in the first 24 h of fungal infestation (St. Leger *et al.* 1986). Since 80% of insect integument consists of proteins, the proteases play a prominent roles in the degradation

process. As indicated earlier, penetration of entomopathogenic fungi via integument requires subtilisin-like serine protease Pr1 and trypsin-like enzyme Pr2. In the current study, the proteases produced by isolate AM-118 from *B. bassiana* were purified using the three-step procedure: ammonium sulfate precipitation, Sephacryl G-100, and DEAE-Cellulose Fast Flow chromatographies. After ammonium sulfate precipitation, the specific obtained activities of Pr1 and Pr2 were 13.3 and 22 U · mg⁻¹ protein; recovery (%) for Pr1 and Pr2 was found to be 37.67 and 94.42%; purification folds were 3.43 and 8.59 (Tables 1, 2). In Sephacryl G-100 chromatography, fractions 16–25 for Pr1 and 12–16 for Pr2 showed the highest proteolytic activities (Fig. 1).

Specific activities of the pooled fractions for the enzymes were found to be 17.72 and 32.27 U · mg⁻¹ protein (Tables 1, 2). Fractions 7–12 for Pr1 and 10–13 for Pr2 showed the highest activities (Fig. 1) in DEAE-Cellulose Fast Flow column. At the end of the purification process, Pr1 and Pr2 proteases showed specific activities of 72.30 and 114.61 U · mg⁻¹ protein, recovery of 26.62 and 63.94%, and a purification fold of 18.68 and 44.86, respectively (Tables 1, 2). Finally, SDS-PAGE of the samples showed a single band by 105 and 103 kDa of molecular weights for Pr1 and Pr2, respectively (Fig. 2). Biodochka and Khachatourians (1987) found a molecular weight of 35 kDa for a protease produced by *B. bassiana*. Pei *et al.* (2000) purified a protease by molecular weight of 27 kDa produced by *M. anisopliae*. Zibae and Bandani (2009) showed molecular weight of 47 kDa in a purified protease produced by *B. bassiana* in the presence of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). Lakshmi *et al.* (2010) reported the molecular weight of 19 and 21 kDa for proteases produced by *B. bassiana* and *M. anisopliae*. In the current study, we collected an Iranian isolate of *B. bassiana* and extracted its protease using an *A. spinidens* cuticle. Species, media of production, and level of the fungal interaction by its host may affect molecular weight, isoforms and biochemical properties of the en-

Table 1. Purification parameters of Pr1 from isolate AM-118 of *Beauveria bassiana*

Purification steps	Unit activity [U]	Specific activity [U · mg ⁻¹ protein]	Recovery [%]	Purification fold
Crude extract	3.53	3.87	100.00	1.00
Ammonium sulfate 70%	1.33	13.30	37.67	3.43
Sephacryl G-100	1.17	17.72	33.14	4.57
DEAE-Fast Flow	0.94	72.30	26.62	18.68

*all experiments have been carried out at 4°C

Table 2. Purification parameters of Pr2 from isolate AM-118 of *Beauveria bassiana*

Purification steps	Unit activity [U]	Specific activity [U · mg ⁻¹ protein]	Recovery [%]	Purification fold
Crude extract	2.33	2.56	100.00	1.00
Ammonium sulfate 70%	2.20	22.00	94.42	8.59
Sephacryl G-100	2.13	32.27	91.41	12.60
DEAE-Fast Flow	1.49	114.61	63.94	44.76

*all experiments have been carried out at 4°C

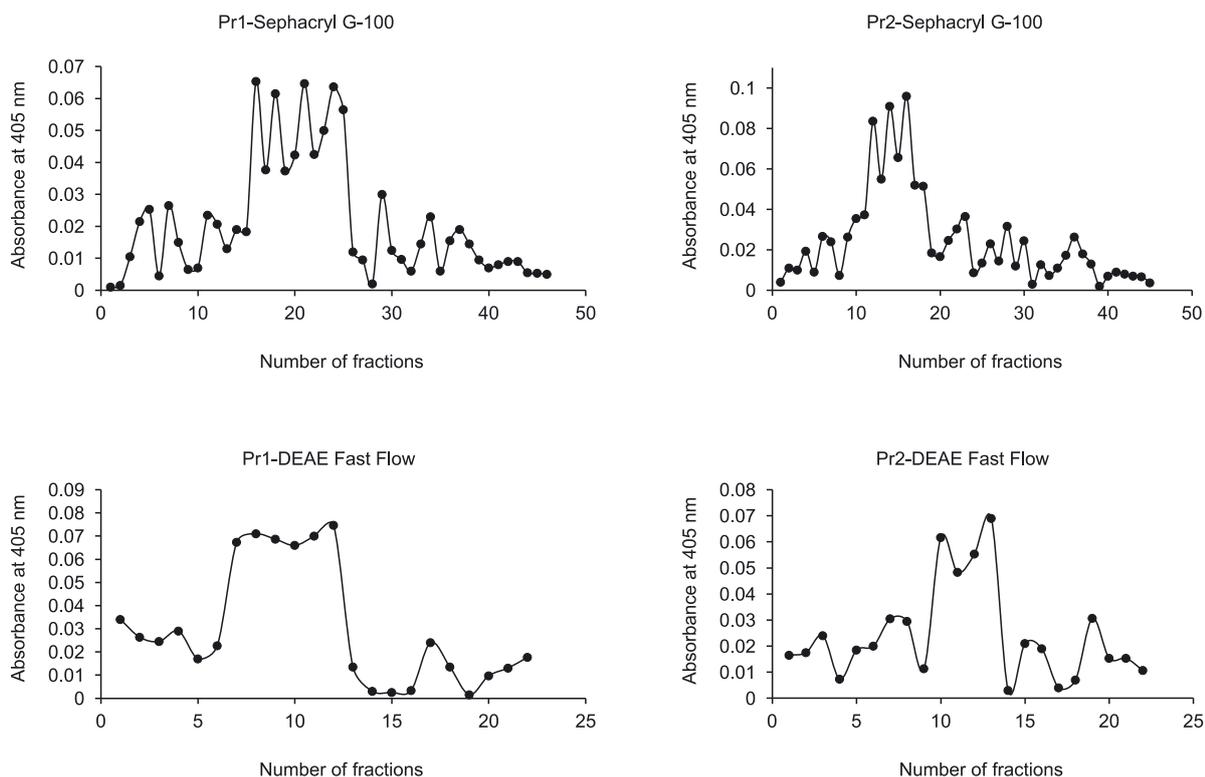


Fig. 1. Purification of Pr1 and Pr2 from isolate AM-118 of *Beauveria bassiana* via Sephacryl G-100 and DEAE-Fast Flow chromatographies

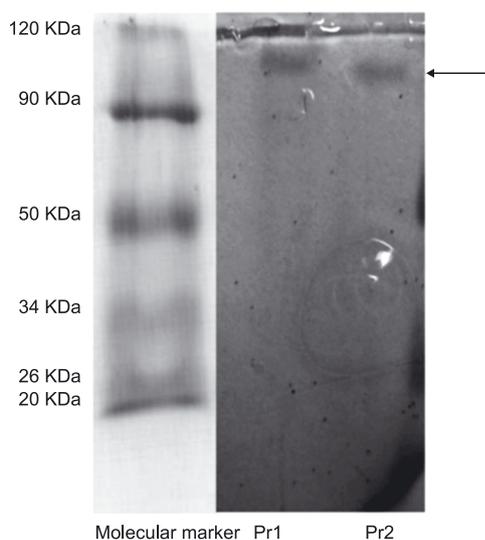


Fig. 2. SDS-PAGE showing purity and molecular weight of the proteases from isolate AM-118 of *Beauveria bassiana*

zymes produced by microorganisms. Since proteins may be in various structures, different isoforms of proteases were required to be digested. This diversity may cause some types of host-microbe interactions evolving enzyme secretions and their inhibitors.

Kinetic parameters of proteases

Kinetic parameters of the purified Pr1 and Pr2 were found as V_{max} values of 3.33 and 1.17 $U \cdot mg^{-1}$ of protein, and K_m values of 0.7184 and 0.216 mM, respectively (Fig. 3).

Since Pr1 has a higher maximal velocity than Pr2, it may be that Pr1 has a higher efficiency for degrading the host's cuticle. On the other hand, Pr2 could cause a more stable complex with its substrate. The K_m has an inverse relationship with the substrate concentration, meaning that saturating the active sites of the enzyme is required. A high K_m would indicate weak binding and a low K_m would indicate strong binding. Hence, it was clearly depicted that Pr1 has stronger activity in comparison with Pr2 due to having a higher V_{max} and lower K_m . Consequently, Pr1 is able to better degrade the insect cuticle to facilitate fungal penetration.

Effect of pH and temperature on protease activity

The effects of pH and temperature on Pr1 and Pr2 activities were determined using their specific substrates at various pH and temperature settings (Figs. 4, 5). An optimal pH of 8 was found for both proteases by specific activities of 149.08 and 305.19 $U \cdot mg^{-1}$ protein, respectively. There was a slight, significant difference between the pH of 7 and 8 in the case of Pr2 (Fig. 4). As concerns the activity of the proteases, the optimal temperature values for Pr1 and Pr2 were 35 and 40°C for Pr1 and Pr2, with specific activities of 492.12 and 94.36 $U \cdot mg^{-1}$ protein, respectively (Fig. 5). Bidochka and Khachatourians (1987) reported a pH value of 8.5 and a temperature of 37°C as the optimal values for proteolytic activity in *B. bassiana*. Moreover, Larcher *et al.* (1992) in *Aspergillus fumigatus*, Tunga *et al.* (2003) in *A. parasiticus*, Hajji *et al.* (2007) in *A. clavatus*, and Zibae and Bandani (2009) in *B. bassiana* – showed optimal pHs of 8–9.5 and temperatures of 30–45°C. Temperature could significantly affect the growth and sur-

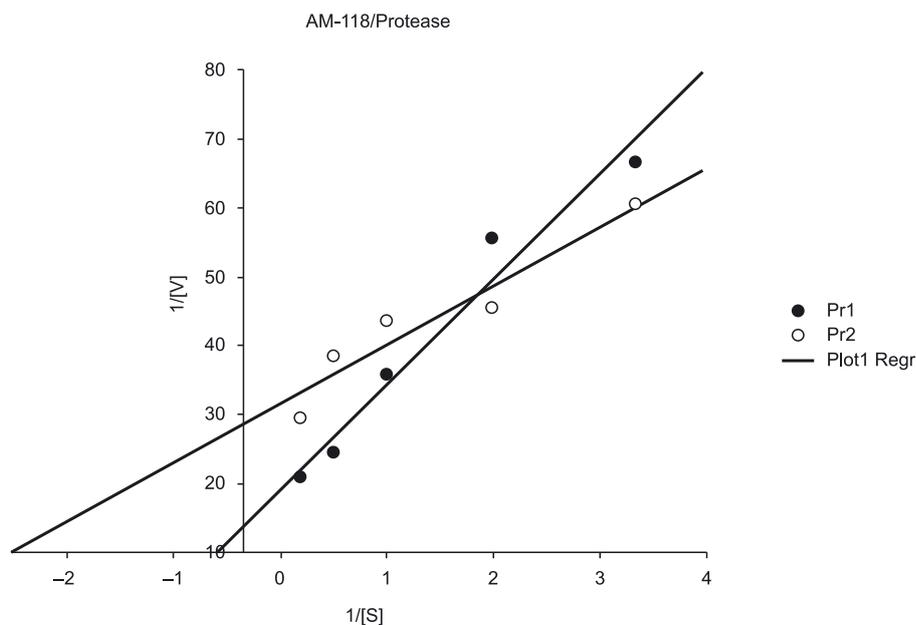


Fig. 3. Double reciprocal plot to show the kinetic parameters of the purified Pr1 and Pr2 from isolate AM-118 of *Beauveria bassiana* ($1/V_{max}$ – intercept on the $1/V_0$ ordinate; $-1/K_m$ – intercept on the negative side of the $1/[S]$ abscissa). R^2 for Pr1 is 0.85 and Pr2 is 0.91

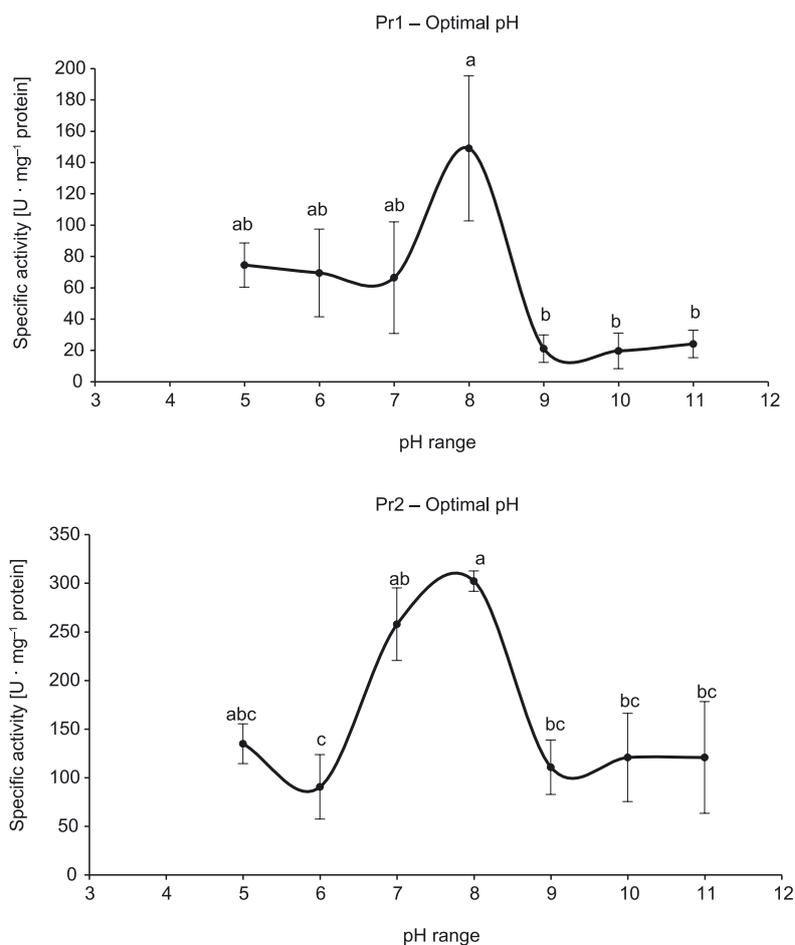


Fig. 4. Optimal pH determination of the purified Pr1 and Pr2 from isolate AM-118 of *Beauveria bassiana*. Statistical differences have been shown by various letters ($p \leq 0.05$; Tukey test)

vival of *B. bassiana*. Determining the temperature value in which the proteolytic activity of *B. bassiana* reaches its highest value is important when expecting pathogenicity on hosts. Moreover, the pH of growth media has been

shown to have significant effects on entomopathogenic fungi. Hence, the evaluation of this value regarding the pathogenicity of *B. bassiana* might make it clear whether the enzyme is efficient or not.

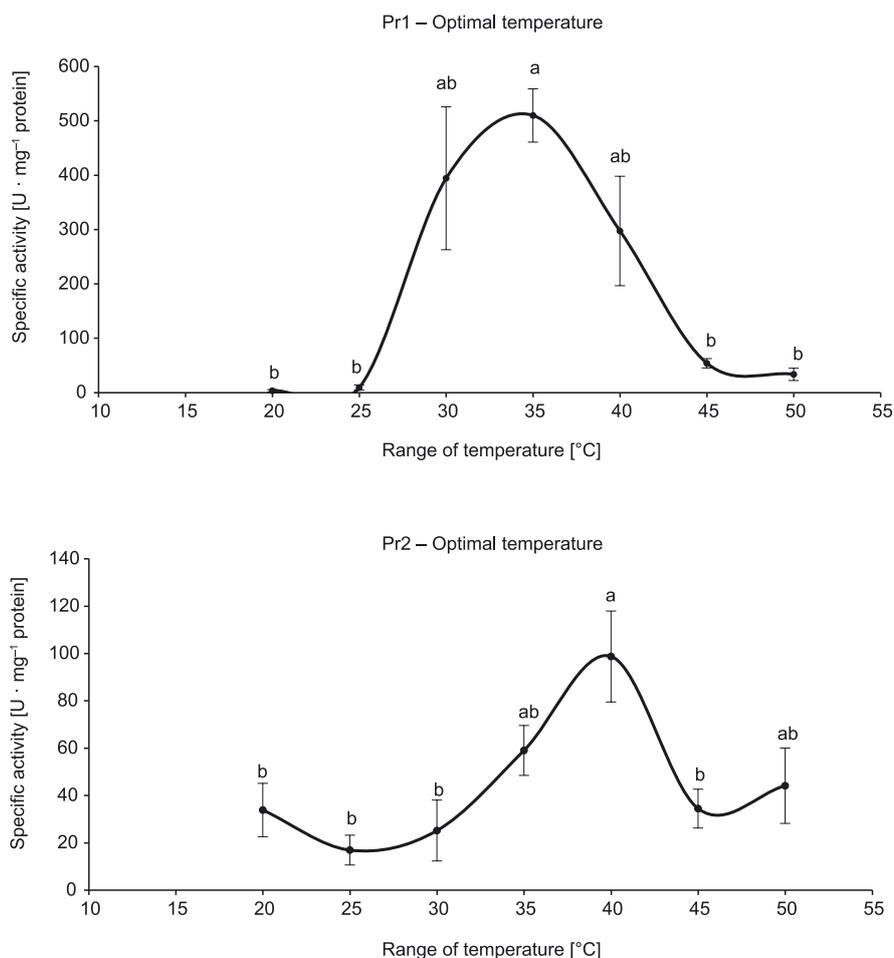


Fig. 5. Optimal temperature determination of the purified Pr1 and Pr2 from isolate AM-118 of *Beauveria bassiana*. Statistical differences have been shown by various letters ($p \leq 0.05$; Tukey test)

Table 3. Inhibition of the purified Pr1 and Pr2 by specific inhibitors

Inhibitors	Concentrations [mM]	Inhibition of Pr1 [%]	Inhibition of Pr2 [%]
The control	–	0 d*	0 d
AEBSF	5	69.44 a	81 ab
TLCK	5	40.26 b	90 a
TPCK	5	26.38 c	0 d
Phenanthroline	5	0 d	45 b
EDTA	5	0 d	46 c

AEBSF – 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, TLCK – tosyllysine chloromethyl ketone hydrochloride, TPCK – tosyl phenylalanyl chloromethyl ketone, EDTA – ethylenediaminetetraacetic acid

*statistical differences have been shown by different letters

Effect of enzyme inhibitors

Various inhibitors were evaluated for their effects on the purified Pr1 and Pr2 from AM-118. The protease inhibitors: AEBSF, TLCK, and TPCK had significant inhibition on Pr1, but EDTA and phenanthroline showed no effects (Table 3). The trypsin-like Pr2 was inhibited by TLCK, AEBSF, EDTA, and phenanthroline while TPCK showed no inhibition (Table 3). In contrast, Bidochka and Khachatourians (1987) reported no inhibition of *B. bassiana* proteases by metal-chelating agents, sulfhydryl reagents, trypsin inhibitor, and chymotrypsin inhibitor. Zibaee and Bandani

(2009) found significant inhibition of the enzyme by PMSF and EDTA. These findings revealed that Pr1 contains just amino acids in its active site; mainly serine, histidine, and aspartic acid, but Pr2 may contain metal ions since EDTA, and phenanthroline, inhibit the activity of Pr2.

Conclusions

We extracted and purified two proteases from an Iranian isolate of *B. bassiana*. The proteases were novel due to their high molecular weight and their response to specific inhibitors. Knowing the biochemical properties of the en-

zymes help improve our understanding about the natural degrading of insect integument. Improving the strain selection of entomopathogenic fungi for pest control can then be done. Determination of the enzymatic structure, and identification of the genes responsible for synthesizing these enzymes, are necessary for finding the enzymatic capability and interactions with different hosts.

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