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Isolation, purification and functional characterization of Serine protease from a biocontrol agent *Trichoderma harzianum*

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Abstract

Trichoderma harzianum is widely used as a biocontrol agent by virtue of its mycoparasitic abilities. An array of enzymatic proteins causing proteolysis of fungal cell wall make it competent parasite of numerous plant pathogenic fungi, protease is one such enzyme. In the present study *Trichoderma harzianum* producing extracellular protease was isolated from soil samples. The extracellular protease secreted by that *Trichoderma* isolate was purified to 41.13-fold and specific activity of the enzyme was calculated to be 145.42 U/mg. The purified enzyme was characterized for various optima. The study revealed higher stability levels of protease exhibiting upto 43% activity after an incubation of 30 min at 45°C and pH 10.0. The enzyme was found stable and highly active exclusively at strong alkaline conditions (pH=10) which showed its stability and suitability for various commercial applications. Moreover, the stability of enzyme illustrated its promising role in proficient mycoparastisic ability of *Trichoderma* isolate which can be employed as a biocontrol agent.

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Introduction

Proteolytic enzymes (proteases) catalyze the cleavage of peptide bonds in proteins. Proteases constitute 1-5% of the gene content in all organisms naturally. They are involved in a repertoire of physiological pathways at cellular as well as organ/organism levels, to produce surge systems such as homeostasis. They also have significant role for the processes which are complex like activities of the cell under abnormal pathophysiological conditions. Proteases of microbial origin are categorized best for industrial applications owing to higher stability and activity at alkaline pH and high temperature around 60°C (Vanitha *et al.*, 2014). Proteases on commercial scale are employed in leather processing, food supplementation, chemical industries, tanneries, waste treatment and systemic enzyme therapy (Vijay *et al.*, 2010). As proteases are physiologically needed for living organisms, they are ubiquitously found in a variety of sources such as microorganisms, animals and plants. Proteases play essential roles in all life forms like animals, fungi, prokaryotes and plants to survive on earth. Microbial sources are considered ideal for protease production as they are adapted to endure a variety of extreme conditions, have short life spans and can be easily cultured in flasks. Bacteria especially of genus *Bacillus* are being used in the production of the proteases commercially however; these proteases have low thermal stability and narrow pH range of 5 to 8 for active functioning (Rao *et al*, 1998). Consequently, potential fungal candidates are being explored as more preferential sources of protease and have advantages over other, microbial sources.

Trichoderma harzianum is a cosmopolitan soil borne, green-spored ascomycete that is commended as biocontrol agent against a variety of plant pathogenic fungi including members of ascomycota, basidiomycota and oomycota (Majid, 2012).

Genus *Trichoderma* is considered a blessing because of its enmity against other parasitic fungi (Migheli *et al.*, 2008). Several modes of action have been adduced explaining its biocontrol activity through mycoparasitism which involves sequential events including recognition, attack, subsequent penetration and killing of the host (Harman *et al.*, 2004). It is mainly due to the sequential expression of cell wall degrading enzymes mostly chitinases, glucanases and proteases. It has been shown that protease produced by *Trichoderma harzianum* inhibit spore germination and growth of pathogens in synergistic cooperation with chitinases, glucanases and antibiotics. It is well documented that *Trichoderma* can cease the growth of pathogenic fungi by digesting and deteriorating their hyphae, by coiling around and penetrating into them (Harman, 2006).

Species of *Trichoderma* genus are diverse in their use; naturally as decomposers, commercially as the producers of antibiotics, enzymes and also as biocontrol agent (Siddiquee *et al.*, 2012).

A number of species of *Trichoderma* are famous for their antagonism against fungal pathogens (Hjeljord and Tronsmo, 1998). *Trichoderma* mainly compete for space and nutrition with other fungi. Weindling (1932) was the first to observe its mycoparasitic and antibiosis activities. *Trichoderma* do so by using certain mechanisms like production of antifungal metabolites like chitinases, proteases and glucanases (Siddiquee *et al.*, 2009, 2010).

The enzymes which are involved in digesting fungal cell wall like chitinases, glucanases and proteases from mycoparasitic fungus *Trichoderma harzianum* have demonstrated high antifungal activity against a number of economically important plant pathogenic fungi (Besoaín *et al.*, 2007).

Trichoderma harzianum has been found effective against various plant pathogens like *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Phytophthora megasperma*, *Gaeumannomyces gramminis*, *Sclerotium rolfsii* and *Cylindrocladium scoparium* (Pérez *et al.*, 2007).

Objectives of this study are to characterize local *Trichoderma harzianum* isolate on morphological basis and isolation, purification and characterization of extracellular protease from that isolate.

Materials and methods

Microorganism

Preparation of PDA and Broth media

Potato starch was prepared by boiling 500 g of potato infusions in 500 ml of distilled water until the volume remained half. Then, it was filtered through muslin cloth to obtain liquid starch. In order to prepare 1000 ml of PDA, 20 g of agar and 20 g of sucrose were dissolved in an appropriate volume of distilled water and 250 ml of liquid starch was added. Total volume was made up to 1000 ml. Then, pH was adjusted to 5.5 following Ahmed, (2007). After autoclaving at a constant temperature 121°C and pressure 15 psi for 21 minutes, one ml of filter sterilized chloramphenicol (IM REXAPHENICOL) was added in order to check the bacterial growth. The protocol for PDB preparation is same just by excluding agar.

Production of Serine Protease

Mycelia of the isolate were inoculated in 500 ml of PDB containing casein as protein source in Erlenmeyer flask and placed in incubator (Irmeco GmbH, Germany) at 28°C for 72 hours in dark at 180 rpm for growth. After filtration through Whatmann standard filter paper grade 8 µm, the filtrate was centrifuged at the 15000 rpm for 10 min at 4°C in order to get cell free filtrate with maximum clarity. The supernatant (cell free filtrate) hereafter, referred to as crude enzyme extract was taken into new falcon tube and further employed for isolation and purification of protease.

Determination of Total Protein Contents

Total protein contents of the crude extract were quantified by adopting the Bradford method (Bradford, 1976) using Bovine Serum Albumin (BSA) as standard.

Isolation and Purification of protease

Step 1: Ammonium sulfate precipitation

For purification of enzyme, the crude enzyme extract was placed in ice and saturated by continuous addition of ammonium sulfate crystals up to 20%. Then, it was incubated overnight at 4°C. This was followed by centrifugation at 15,000 rpm for 15 minutes at 4°C. The pellet was separated and the supernatant was re-precipitated with 40, 60, 80, and finally with 100% ammonium sulfate by repeating the process of gradual dissolving, centrifugation and pellet separation (El-Safey and Rauf, 2004). Finally, the pellets (hereafter termed as crude enzyme) were saved at 4°C for further studies.

Step 2: Dialysis of crude enzyme

The pellets were resuspended in least volume of 0.2 M phosphate buffer (pH 7.2). For dialysis, a piece of dialysis membrane of proper pore size was cut, filled with resuspended crude enzyme and dialyzed against distilled water by employing four equal changes of water at an interval of 5 hrs to remove maximum ammonium sulfate.

Step 3: Gel Filtration Chromatography by Sephadex G-75 column

Partially purified enzyme was subjected to gel filtration chromatography using a glass column of internal diameter 2cm, packed with Sephadex G-75 (Sigma, USA) upto a height of 120cm and equilibrated with 0.2 M phosphate buffer (pH 7.2) (Sharma *et al.*, 2006). A total of 50 fractions (2 mL each) were collected by employing a flow rate of 0.5 mL/min. Total protein contents and protease activity was determined for each fraction.

Determination of Protease activity

The activity of purified protease was determined following McDonald and Chen (1965). The assay employed three tubes, one as a control (distilled water added instead of protease) and two as experimental (1 mL of protease was added). Two ml of 1% casein (as substrate) dissolved in Glycine-NaOH buffer (pH 10) was added into each tube and incubated at 30°C for one hour. Then, 3ml of 10% Trichloro Acetic Acid (TCA) was added in all the tubes and centrifuged for 3 min. One mL of the supernatant was taken into a new tube; 5mL of alkaline copper reagent was added and incubated for 15 min. Then, 0.5 ml of 50% Folin-Ciocalteau reagent was added into each tube and again incubated for 30 min. Optical density was measured at 700 nm using spectrophotometer.

One unit (U) of protease enzyme activity was defined as the amount of protease that releases 1 µg of tyrosine per mL per min under these conditions. Specific enzyme activity was expressed as units/mg of protein.

Tyrosine standard cure

Tyrosine solution of serial concentrations of 20, 40, 60, 80, 100 and 120 (µg/ml) were prepared in the HCl buffer (0.006N). Each concentration of tyrosine was incubated with 3 ml of TCA at 45°C for 15 min. Two tubes were employed as control (having distilled H_2O instead of the tyrosine solution). The measured absorbance was plotted against the concentrations and the resultant standard curve was further used to calculate the standard factor.

Determination of various optima for purified protease

Finally, the purified protease was assayed to evaluate its various optima by examining the effect of different pH values (6-10) using 0.2M phosphate buffer, effect of different incubation temperatures (00-60°C), effect of varying concentrations of substrate (0-4%), effect of varying concentrations of enzymes (0-3 mL) and incubation time (0-72 hours) on the activity of purified serine protease produced from *Trichoderma harzianum*. The enzyme activities for each case were determined under standard assay conditions using casein as substrate as described earlier.

Results

Production and purification of protease(s) from the Trichoderma harzianum

After 72 hours of incubation, when the growth of *Trichoderma harzianum* isolate was at its climax, 500 mL of the fungal broth culture was filtered as described earlier. The filtrate contained the total soluble extracellular proteins. This filtrate was then, centrifuged to get cell free filtrate which was then employed for protease purification.

Ammonium sulfate precipitation and dialysis

The soluble proteins present in cell free filtrate were then concentrated by the gradual addition of ammonium sulfate followed by centrifugation till the entire crude enzyme was retrieved.

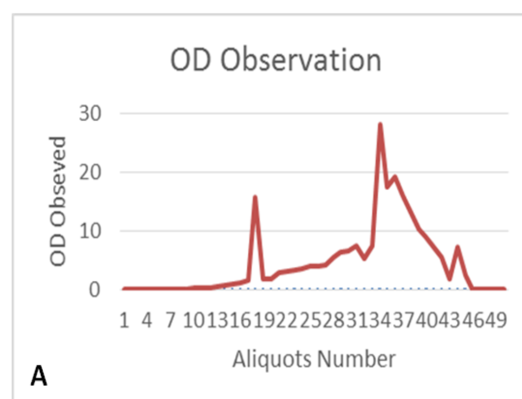
The crude enzyme was then subjected to dialysis. The process of dialysis was executed to remove the excessive salts (ammonium sulfate & others) present in the precipitated proteins because the excess amount of salts negatively affects the enzyme activity. The Dialysis was done against distilled water using dialysis membrane with several water changes at an interval of five hours in order to dialyze the proteins effectively.

Purification by Sephadex G-75 column chromatography

Sephadex column chromatogram was used to purify the partially purified product in order to increase the purification as much as possible. A total of 50 fractions (2 ml each) were collected in separate test tubes, numbered 1-50 and subjected to spectrophotometer for measuring optical density which resulted in the detection of proteins in fractions 26-41 while all other fractions had undetectable quantity of proteins. Among the fractions, 36th had the highest peak with a value exhibiting 28 U/mL (Fig.1-A).

Determination of specific enzyme activity

The enzyme undergoes harsh treatments during purification, concentration, dialysis and centrifugation which may severely affect the enzyme activity. Hence, the selected fractions were subjected to the evaluation of specific activity in order to find the activation rate against selected substrate (Casein) following optimized protocol (Chen *et al*., 2009). Then optical density was measured using spectrophotometer to quantify the released tyrosine amino acid by plotting against the tyrosine standard curve. The highest recorded activity was 242.400 U/ml (Fig.1-B).



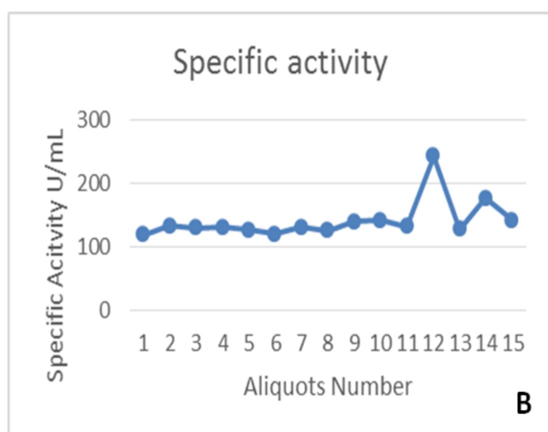


Fig. 1. Purification and determination of specific enzyme activity A, Measurement of optical densities of different aliquot fractions exhibiting presence of protein B, Determination of specific activity of different aliquot fractions.

Characterization of purified serine protease

Effect of different pH levels

The experiment was carried out to investigate the effect of different pH values on the activity of purified protease enzyme. The purified enzyme extract was incubated at different pH values ranging from 6-10 using 0.2M phosphate buffer.

Enzyme assay was performed after fifteen minutes of incubation using casein as substrate on spectrophotometer at the wavelength of 700 nm. Results of enzyme assay showed that the alkaline protease enzymes was completely stable in a large alkaline pH range (8-12) and presented an optimum activity for 275.4U/mL at a pH value of 10 (Fig. 2-A) whereas any further increase in pH up to 13 showed a decreasing trend in enzyme activity.

Effect of different enzyme concentrations

In order to determine the optimum protease concentration for highest enzyme activity, the purified enzyme was diluted eight fold and employed to the substrate solution of constant concentration. It was found that the activity appeared to almost steady from 1-3ml but a sudden increase was observed with further increase in enzyme quantity upto 4ml with a maximum activity of 217.37 U/ml. The best measured value is 217.37 U/ml (Fig. 2-B).

Effect of different substrate concentrations

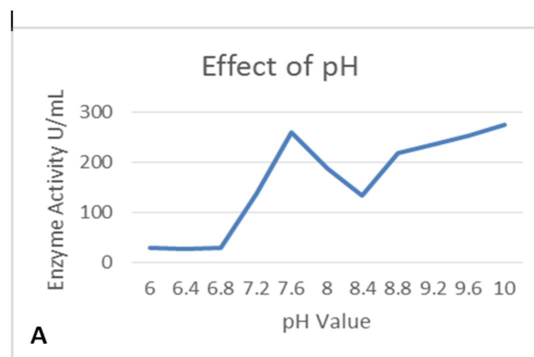
This study investigated the effect of various substrate concentrations on protease activity. At lower concentration of the substrate (0 to 2.0%) protease activity remained steady near 150 U/ml however further increase in substrate concentration upto 2.5% brought a significant increase in protease activity upto the highest mean value of 234.27 U/ml (Fig. 2-C).

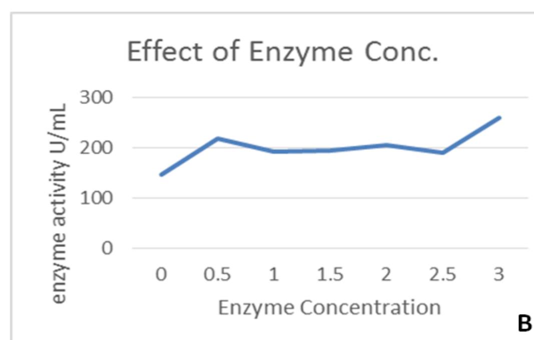
Effect of different temperature

The effect of various temperatures on enzyme activity was investigated in order to assess the temperature stability range of the isolated protease. The isolated enzyme was found to be stable at higher temperature up to 55ºC. However the enzyme worked best at 45ºC with a value of 71.16 U/ml (Fig. 2-D). The observed data revealed that the enzyme activity had increased with increasing temperature. But at higher temperature the enzyme showed low activity might be due to denaturation.

Effect of incubation time

It is obvious that the maximum protease activity was recorded at the incubation time of 18 hours which is 230.171 U/ml. The specific activity of the protease first deceases than increases and after 4% it started to decrease again as depicted in (Fig. 2-E).





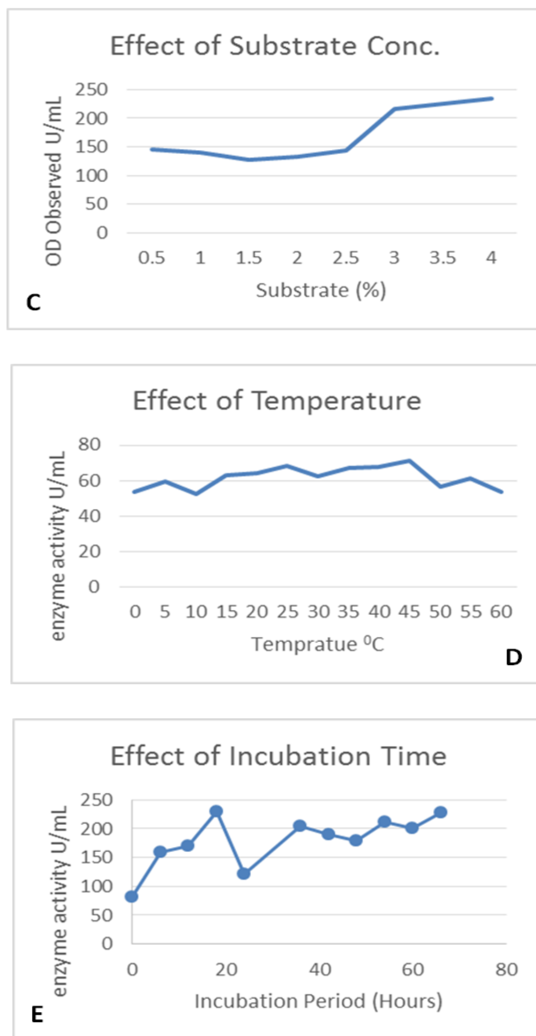


Fig. 2. Characterization of various optima of purified protease. A, Effect of pH on protease activity B, Effect of enzyme concentration on protease activity C, Effect of substrate concentration on protease activity D, Effect of temperature on protease activity E, Effect of incubation time on protease activity.

Discussion

Proteases interact synergistically with chitinases and glucanases to deteriorate disastrous pathogenic fungi. *Trichoderma harzianum* is a well-known source of proteases and has extensively been explored as biocontrol agent. Since protease producing ability is one of the core indication of the efficiency of biocontrol agent to control fungal parasites. Current studies were conducted to investigate protease production ability of local Trichoderma isolate. Further, isolated protease enzyme was characterized for various optima, in order to determine its affectivity.

The isolated enzyme appeared to have maximum specific activity (242.40 U/ml) against the substrate casein (1% w/v) at 28°C (Negi and Banerjee, 2010). As far as enzyme concentration is concerned, the best specific values were determined at 0.5 ml and 3ml. The enzyme specific values were 217.73 U/ml and 258.99 U/mL at 0.5 and 3.0 mL respectively. Enzyme activity was observed to be increased with increase in its concentration but at slower pace which can be attributed to higher activity of enzyme even at lower concentrations or least availability of substrate for higher doses of enzyme. Hence, we proved that the enzyme activity increases with increase in its concentration but upto certain threshold level (El-Safey and Abdul-Raouf, 2004). Dubey et al. (2010) and Niyonzima and More (2015) also reported similar behaviour of proteases isolated from *Aspergillus niger*.

Literature reports that protease isolated from *Trichoderma pseudokoningii* had highest activity at 60°C to 65°C and enzyme remains stable at 50°C whereas the best pH for enzyme activity is 8.5 though it remains functional at 10.6 (Chen et al., 2009). Our observations were a bit different from these as the isolated protease depicted maximum activity at 45°C and best pH for enzyme activity was observed to be 10. Similar range of temperature and pH, for maximum protease activity has also been reported by Janice and Felix, (2001). El-Safey and Abdul-Raouf (2004) were also of the view that optimum temperature and pH for the maximum activity of protease, isolated from *Bacillus subtillus* is 35°C and 7 respectively. They reported optimum incubation time for enzyme activity as 24 hrs contrary to ours which is 18 hrs. These differences in various optima, for enzyme activity may be owing to variable habitat, growth conditions and even substrate. Anyhow the enzyme, we isolated showed activity at desirable range of temperature and pH, hence may be promoted as a valuable industrial enzyme. Further, prevalence of active protease in the *Trichoderma* isolate confirms its potential as an effective biocontrol agent for the control of disastrous fungal pathogens.

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