Mycopath (2016) 14(1&2): 29-35 **Research Article**

Partial purification and characterization of protease enzyme from soil-borne fungi

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Abstract

Soil borne fungi produce a broad variety of extracellular enzymes and protease is one of them. Alkaline protease extracted from fungal isolates (named as BY-1 and BY-2) was purified by precipitation with different concentration of ammonium sulfate. A purified enzyme extract obtained from isolates (BY-1 and BY-2) revealed increased protease activity as compared to the initial crude extract. Enzyme fraction obtained from isolate BY-1 after 70% saturation of $(NH_4)_2SO_4$ demonstrated higher protease activity (32.02 U mL^{-1}) as compared to its initial crude extract (31.84 U mL^{-1}) and it was named as enzyme fraction P1. Enzyme fraction obtained from isolate BY-2 after 80% saturation of $(NH_4)_2SO_4$ revealed increased protease activity (16.77 U mL^{-1}), when compared to the activity (9.15 U mL^{-1}) in its initial crude lysate and it was named as enzyme fraction P2. Partially purified enzyme was used for characterization study. Protease activity from both enzyme fractions (P1 and P2) was highest when the pH of the reaction mixture was adjusted to 8 and 9, indicates alkaline nature of protease. Optimum temperature for protease activity by both the enzyme fractions (P1 and P2) was found to be 50 ºC, indicates thermostability of enzyme. Ca^{2+} ion increased protease activity by 7.15% and 23.05% from fraction P1 and P2, respectively. Protease activity from fraction P1 and P2 was stimulated by Ca^{2+} and Mn^{2+}, respectively. *n*-butanol increased protease activity from enzyme fractions P1 and P2 by 5.70% and 35.71%, respectively, while methanol and acetone revealed inhibitory effect on protease activity.

Keywords: Enzyme characterization, partial purification, protease, temperature, pH, soil.

Introduction

Proteases, also known as proteinases or peptidases, are a broad category of enzymes that catalyze the hydrolysis of peptide bonds in polypeptide chains of protein. The proteases are classified based on the site of cleavage into two major groups: exopeptidases and endopeptidases. Proteases that cut peptide bonds at the C or N termini of polypeptide chains are known as exopeptidases and those that break internal peptide bonds within the polypeptide chain are called as endopeptidases (López-Otín and Bond, 2008). Proteases can be classified as glutamic, aspartic, threonine, cysteine and serine proteases based on the amino acids present in the catalytic site, or as metalloproteases if a metal ion is needed for biological activity (Sumantha *et al.,* 2006). Proteases are also classified into three main groups, i.e. acidic, neutral and alkaline proteases based on their acid-base nature.

Fungi are the main source of acidic proteases, plants are major source of neutral proteases and microorganisms are major source of alkaline proteases which work better at pH 8.0 or above (Sharma *et al.,* 2015a). Several microorganisms including bacteria (*Bacillus licheniformis, Bacillus firmus, Bacillus thuringiensis, Bacillus alcalo,* and *Bacillus subtilis*) and fungi (*Aspergillus flavus, Aspergillus miller, Aspergillus niger* and *Penicillium griseofulvin*) have been identified as protease producers (Sharma *et al.,* 2015b). The most important sources of fungal proteases are *Aspergillus* sp. and *Mucor* sp. (Chen *et al*., 1990; Ashour *et al*., 1996). Microbial proteases are extracellular in nature hence they are excreted directly into fermentation broth, thus simplifying purification of enzyme as compared to enzymes obtained from plants and animals (Sharma *et al.,* 2015c).

Proteases show broad applications in pharmaceutical preparations and various industries such as textile, detergent, leather processing, tanning, dairy etc. (Kataoka *et al*., 1987; Chen *et al*., 1990). Proteases can be obtained from plants and animals but microorganisms are the preferred

source of industrial production of proteases due to low cost, fast production, high productivity etc. (Laxman *et al.,* 2005). Today proteolytic enzymes represent one of the most valuable group of industrial enzymes and cover approximately 65% of the total worldwide enzyme market (Banerjee *et al*., 1999; Tari *et al*., 2006). It has been reported in many articles that global demand of proteases will increase dramatically (Godfrey and West, 1996; Tari *et al*., 2006). Various characteristic features of enzymes such as optimum pH, temperature, incubation time, stability in presence of various organic solvents and metal ions influence their use in various industrial processes (Zhang *et al.,* 2011). Therefore, the present study was undertaken to purify and characterize the extracellular protease enzyme from fungal isolates of soil.

Material and Methods

Protease production in submerged fermentation

One milliliter of spore suspension (containing 10^8 spores mL^{-1}) prepared from 7 days old slants of fungal isolates (BY-1 and BY-2) was transferred in 250 mL Erlenmeyer flask containing 100 mL of production medium containing (%, w/v): glucose 2.0; yeast extract 1.0; K_2HPO_4 0.1 g; $MgSO_4$ 0.02 g; KH_2PO_4 0.1 g; pH 7.0 (Keay and Wildi, 1970). The inoculated flasks were incubated in a shaker incubator at 28 °C, 120 rpm for 3 days. At the end of incubation, the contents of the flasks were filtered through Whatman filter paper No. 1 and then the filtrates were centrifuged at 8,000 rpm at 4 °C for 10 minutes. Pellet was discarded and clear supernatant was used as a source of protease enzyme. The crude enzyme supernatant was used for further studies (Josephine *et al.,* 2012).

Protease activity

Protease activity in the crude enzyme extract was determined according to the method of Cupp-Enyard (2008) using casein as substrate. The reaction mixture containing 1 mL of 0.65% casein solution (made in 50 mM potassium phosphate buffer, pH 7.5) and 0.2 mL of crude enzyme supernatant were incubated at 37 °C for 30 minutes and reaction was stopped by addition of 1 mL of trichloroacetic acid solution (110 mM). This mixture was left for 15 minutes and filtered using Whatmann's No 1 filter paper. 0.4 mL of filtrate was mixed with 1 mL of sodium carbonate solution (500 mM) and 0.2 mL of 2 fold diluted follin ciocalteus phenol reagent was added. The resulting mixture was placed in the dark at room temperature for 30 minutes for the development of blue colour. The concentration of liberated tyrosine was measured at 660 nm against a reagent blank using tyrosine standard. A standard curve was prepared using following concentration range of tyrosine: 27.5, 55, 110, 220 and 275 µM. One protease unit was defined as the amount of enzyme required to liberate 1 µM of tyrosine per minute per mL at 37 ºC, pH 7.5 (Mohapatra *et al.,* 2003). All the experiments were done in triplicates and mean values are presented.

The enzyme activity (U mL^{-1}) was calculated by following formula

$$\text{Protease activity (Units/mL)} = \frac{\text{µmole tyrosine equivalent releases} \times 2.2 \text{ (Total volume of assay)}}{\text{Volume of enzyme taken (0.2 mL)} \times \text{Incubation time (30)}}$$

2.2= Total volume of assay (mL).

0.2= Volume of enzyme used (mL).

Specific activity is the enzyme activity in one mg of total protein (expressed in µmol min^{-1} mg^{-1}).

The specific enzyme activity (U mg^{-1}) was calculated by following formula

$$\text{Specific enzyme activity (U mg}^{-1}\text{)} = \frac{\text{Enzyme activity (U mL}^{-1}\text{)}}{\text{Total protein content (mg mL}^{-1}\text{)}}$$

Protein assay

Total protein content in the crude enzyme extract of fungal isolates (BY-1 and BY-2) was determined by Folin- Lowry method using Bovine Serum Albumin (BSA) as standard (Lowry *et al.,* 1951).

Partial purification of protease

The supernatant obtained from fungal isolates (BY-1 and BY-2) was fractionated by precipitation with different concentration of $(NH_4)_2SO_4$ including 30%, 70% and 80% (Abirami *et al.,* 2011). Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 30% saturation and it was left for 3 hours at 4 °C. The precipitate was removed by centrifugation at 10,000 rpm at 4 ºC for 15 minutes. The pellet was reconstituted in minimum amount of potassium phosphate buffer (50 mM, pH 7.5) and protease activity in pellet was determined. Additional $(NH_4)_2SO_4$ was added to the supernatant to obtain 70% saturation and it was kept for 3 hrs at 4 °C. Again, it was centrifuged to obtain pellet. Protease activity was measured in pellet and additional $(NH_4)_2SO_4$ was added to the supernatant in order to obtain 80%

saturation and protease activity of 80% fraction was determined. Fold purification and percent yield were determined using following formulae.

$$\text{Fold purification} = \frac{\text{Specific protease activity of each fraction}}{\text{Specific protease activity of crude protein extract}}$$

$$\text{Percent yield} = \frac{\text{Protease activity of each fraction}}{\text{Protease activity of crude protein extrac}} \times 100$$

Characterization of partially purified protease

Partially purified protease enzyme fractions (P1 and P2) obtained from fungal isolates (BY-1 and BY-2) was further used for characterization study.

Effect of pH on protease activity

The effect of pH on the proteolytic activity of purified protease from fungal isolates (BY-1 and BY-2), was determined by assaying the protease activity at different pH values viz.: 4, 5, 7, 8 and 9 using potassium phosphate buffer (50 mM) at 37 ºC (Lakshmi *et al.,* 2014).

Effect of incubation temperature on protease activity

The impact of temperature on the activity of purified enzyme was studied by incubating the reaction mixture at different temperatures viz.: 40 ºC, 50 ºC, 60 ºC and 70 ºC (Lakshmi *et al.,* 2014).

Effect of metal ions on enzyme stability

The impact of various metal ions on the activity of the protease was studied by the addition of divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Cu^{2+}, Li^{2+} and Zn^{2+}) at a concentration of 5 mM. Protease fractions (P1 and P2) and the metal ions were mixed (1:1 v/v) and the resulting mixture was then incubated at 37 ºC for 2 h. The relative enzyme activity was determined with reference to the standard assay mixture, with no metal ion added (Ulker *et al.,* 2011).

$$\text{Relative enzyme activity (U mL}^{-1}) = \frac{\text{Enzyme activity of control (without metal ion)}}{\text{Enzyme activity with metal ion}}$$

The impact of various organic solvents viz.: methanol, ethanol, *n*-butanol and acetone at a concentration of 10% v/v was investigated on protease stability by pre-incubation of enzyme fractions (P1 and P2) and organic solvent (1:1 v/v) at 37 ºC for 2 h. Thereafter, protease assay was performed. Relative enzyme activity was determined by considering the activity of the enzyme without any organic solvents as 100% (Kumar *et al.,* 2012).

Results and Discussion

Partial purification of enzyme

Two fungal isolates (named as BY-1 and BY-2) obtained from soil sample were used for protease production in submerged fermentation. Protease from fermentation broth of both isolates was purified using ammonium sulfate and was then characterize for various parameters.

The protease activity in the crude enzyme supernatant of isolate BY-1 and BY-2 was 31.84 U mL^{-1} and 9.15 U mL^{-1}, respectively. Ammonium sulfate at a concentration of 70% totally precipitated protease from isolate BY-1 with 32.02 U mL^{-1} activity and 80% saturation of $(NH_4)_2SO_4$ precipitated protease from isolate BY-2 with 16.77 U/mL activity. Partially purified enzyme fraction obtained after 70% saturation of $(NH_4)_2SO_4$ from isolate BY-1 was named as P1. Partially purified enzyme fraction obtained after 80% saturation of $(NH_4)_2SO_4$ from isolate BY-2 was named as P2. Fold purification for P1 (at 70% saturation) and P2 (at 80% saturation) was found to be 3.04 and 9.60 (Table 1). Protease activity from enzyme fractions (P1 and P2) was found to be higher than the protease activity in their respective initial crude extract (Table 1).

Protease from *Bacillus* sp was purified by ammonium sulfate fractionation. It was purified by 1.32-fold as compared to crude extract (Josephine *et al.,* 2012). Kumar (2002) reported partial purification of alkaline protease by 80% saturation of $(NH_4)_2SO_4$, resulting in purification by 2-fold from initial crude extract. Abirami *et al.* (2011) reported purification of protease by 2 different concentration of $(NH_4)_2SO_4$ including 20% and 70%. Purification was increased by 1.3 fold.

Effect of pH on enzyme activity

Highest protease activity from enzyme fraction P1 and P2 was obtained at pH 9.0 of the reaction mixture. It was 6.64 ± 0.15 U mL^{-1} and 11.89 ± 0.31 U mL^{-1} for enzyme fractions P1 and P2, respectively. Enzyme fraction P1 and P2 was found to be stable at neutral (pH 7.0) and alkaline pH (8.0, 9.0) with increased protease activity. Protease activity from fractions (P1 and P2) was declined in acidic conditions (pH 4.0, 5.0). Alkaline nature of protease indicates it's

suitability in alkaline environment of various industries (Table 2).

Other investigators also reported optimum protease activity at different pH values such as 6.5 (Abirami *et al.*, 2011), 7.8 (Tsujibo *et al.*, 1990), 8.0 (Anwar and Saleemuddin, 1997) and 9.8 (Kumar *et al.*, 1999). Sevinc and Demirkan (2011) reported optimum pH 7.0 for maximum protease activity by *Bacillus* isolates.

Effect of temperature on enzyme activity

Protease activity from enzyme fractions P1 and P2 was highest when the incubation temperature of assay was 50 ºC. It was 11.65 ± 0.12 U mL^{-1} and 12.81 ± 0.23 U mL^{-1} for fraction P1 and P2, respectively. High protease activity at elevated incubation temperature indicates thermostability of enzymes. The protease activity from fraction P1 was found in following descending order: 50 ºC > 60 ºC > 40 ºC > 70 ºC. The protease activity from fraction P2 was affected by temperature in the descending order 50 ºC > 40 ºC > 60 ºC > 70 ºC (Table 3).

Similar to our results, Ammar *et al.* (2003) reported that, 55 ºC was the optimum temperature for thermostable purified protease enzyme. Sevinc and Demirkan (2011) reported optimum temperature 55 ºC for highest protease activity by *Bacillus* isolate. Josephine *et al.* (2012) reported 40 ºC as the optimum temperature for increased protease activity. Other investigators reported different optimum temperatures including 35 ºC (Gerze *et al.*, 2005), 40 ºC (Abirami *et al.*, 2011), 50 ºC (Anwar and Saleemuddin, 1997) and 70 ºC (Sookkheo *et al.*, 2000; Rahman *et al.*, 2006).

Effect of metal ions on enzyme stability

Highest protease activity from enzyme fractions (P1 and P2) was observed when they were pre-incubated with Ca^{2+} divalent metal ion. It was 31.17 ± 0.03 U mL^{-1} and 30.46 ± 0.32 U mL^{-1} for enzyme fraction P1 and P2, respectively. Calcium chloride increased protease activity by 7.15% and 23.05% from fraction P1 and P2, respectively as compared with control. Protease activity from fraction P1 was increased in presence of Ca^{2+} and Mn^{2+} while Mg^{2+}, Ba^{2+}, Cu^{2+}, Li^{2+} and Zn^{2+} decreased protease activity. Protease activity fraction P2 was increased in the presence of divalent metal ions such as Ca^{2+}, Mn^{2+} and Mg^{2+} and decreased in presence of Ba^{2+}, Cu^{2+}, Li^{2+} and Zn^{2+} (Table 4).

Similarly, some investigators have been reported that protease activity was increased by Ca^{2+} and Mn^{2+} (Nascimento and Martins, 2004; Jaswal and Kocher, 2007). Sevinc and Demirkan (2011) reported that protease activity from *Bacillus* sp. was increased by 28% and 26% in the presence of Mn^{2+} and Ca^{2+}, respectively. Metal ions such as Ba^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+} decreased protease activity.

Protease activity was decreased in the presence of Mn^{2+} and Ca^{2+} (Zambare *et al.*, 2007; Merheb-Dini *et al.*, 2009). Bhatiya and Jadeja (2010) reported inhibition of enzyme activity by Fe^{2+}, Li^{2+}, Hg^{2+}, Ba^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}. Rahman *et al.* (2006) reported increase in protease activity by 16% in the presence of Ca^{2+} at a concentration 10.0 mM.

Effect of organic solvents on enzyme stability

n-butanol increased protease activity from enzyme fractions P1 and P2 by 5.70% and 35.71%, respectively. It was 12.50 ± 0.06 U mL^{-1} and 11.59 ± 0.19 U mL^{-1} for fraction P1 and P2, respectively. Methanol, ethanol and acetone decreased protease activity. The protease activity from fraction P1 was affected by organic solvents in the descending order: *n*-butanol > ethanol > control > acetone > methanol. The protease activity from fraction P2 was found in following descending order: n-butanol > control > acetone > ethanol > methanol (Table 5).

Rahman *et al.* (2006) reported increase in protease activity by 85%, 83%, 78% and 59% in the presence of isooctane, hexadecane, dodecane and decane, respectively. Amid *et al.* (2014) reported that protease activity was not affected by the presence of acetate and ethanol at 10% v/v concentration while isopropanol and methanol decreased protease activity.

Conclusion

Extracellular protease from fungal isolates (BY-1 and BY-2) of soil was partially purified by different concentration of ammonium sulfate, resulting in a 3.04 fold increase in fold purification at 70% saturation of $(NH_4)_2SO_4$ for protease from isolate BY-1 and 9.6-fold increase in fold purification at 80% saturation of $(NH_4)_2SO_4$ for protease from isolate BY-2. Optimum pH and temperature for purified enzyme fractions (P1 and P2) was determined. Highest protease activity from fractions P1 and P2 was observed at pH 8-9, indicates alkaline nature of the enzyme, which is suitable for use in those industries where reactions are conducted in alkaline environment. The optimum temperature for protease activity from enzyme fractions (P1 and P2) was found to be 50 ºC, indicates superior thermal stability of enzyme. Metal ions and

organic solvents such as Ca^{2+}, Mn^{2+} and *n*-butanol increased protease activity up to 7.15%, 3.79% and 5.70% from fraction P1 and up to 23.05%, 18.25% and 35.71% from fraction P2, respectively as compared to the control. The purified protease from fungal isolates of soil can be used for various purposes in food, pharmaceutical and detergent industry.

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Table 1: Summary of partial purification of protease extracted from fungal isolates BY-1 and BY-2.

Percentage saturation of $(NH_4)_2SO_4$	Protease activity (U mL^{-1})		Protein content (U mL^{-1})		Specific activity (U mg^{-1})		Fold purification		Percentage yield	
	BY-1	**BY-2**	**BY-1**	**BY-2**	**BY-1**	**BY-2**	**BY-1**	**BY-2**	**BY-1**	**BY-2**
Crude enzyme extract	31.84	9.15	6.02	5.06	5.28	1.80	1	1	100	100
30%	7.35	4.57	1.76	1.09	4.17	4.19	0.78	2.32	23.08	49.94
70%	32.02	6.55	1.99	0.50	16.09	13.1	3.04	7.27	100.56	71.58
80%	6.86	16.77	1.31	0.97	5.23	17.28	0.99	9.6	21.54	183.27

Table 2: Effect of pH on the protease activity from partially purified enzyme fractions (P1 and P2).

pH	Concentration of liberated tyrosine (µM)		Protease activity (U mL^{-1})	
	P1	**P2**	**P1**	**P2**
4	14	12.66	2.56 ± 0.57	2.31 ± 0.46
5	26.66	30	4.88 ± 0.82	5.49 ± 0.28
7	28.33	31.66	5.18 ± 0.39	5.79 ± 0.05
8	33.33	36.66	6.1 ± 0.02	6.71 ± 0.13
9	36.33	65	6.64 ± 0.15	11.89 ± 0.31

Table 3: Effect of temperature on the protease activity from partially purified enzyme fractions (P1 and P2).

Temperature (°C)	Concentration of liberated tyrosine (µM)		Protease activity (U mL^{-1})	
	P1	**P2**	**P1**	**P2**
40	39.33	56	7.19 ± 0.24	10.24 ± 0.47
50	63.66	70	11.65 ± 0.12	12.81 ± 0.23
60	58.66	39.16	10.73 ± 0.87	7.16 ± 0.72
70	38.33	18.66	7.01 ± 0.63	3.41± 0.08

Table 4: Effect of various divalent metal ions on the stability of protease from partially purified enzyme fractions (P1 and P2).

Metal ions	Concentration of liberated tyrosine (µM)		Protease activity (U mL^{-1})		Percentage relative enzyme activity	
	P1	**P2**	**P1**	**P2**	**P1**	**P2**
Control	159	135.33	29.09 ± 0.22	24.76 ± 0.38	100	100
$CaCI_2$	170.33	166.50	31.17 ± 0.03	30.46 ± 0.32	107.15	123.05
$MgSO_4$	143.33	138.33	26.23 ± 0.18	25.31 ± 0.65	90.16	102.24
$MnCI_2$	1165	160	30.19 ± 0.47	29.28 ± 0.18	103.79	118.25
$BaCI_2$	141.66	129.5	25.92 ± 1.01	23.69 ± 0.27	89.11	95.71
$CuCI_2$	140	121.66	25.62 ± 0.26	22.26 ± 0.11	88.07	89.92
$LiSO_4$	146.66	135	26.84 ± 0.51	24.70 ± 0.77	92.26	99.77
$ZnSO_4$	126.66	112.66	23.18 ± 0.47	20.61 ± 0.20	79.68	83.27

Table 5: Effect of various organic solvents on the stability of protease from partially purified enzyme fractions (P1 and P2).

Organic solvents	Concentration of liberated tyrosine (µM)		Protease activity (U mL^{-1})		Percentage relative enzyme activity	
	P1	P2	P1	P2	P1	P2
Control	64.66	46.66	11.83 ± 0.61	8.54 ± 0.26	100	100
Methanol	41	17.33	7.50 ± 0.47	3.17 ± 0.34	63.42	37.14
Ethanol	64.66	35	11.83 ± 0.15	6.40 ± 0.54	100.03	75
n-butanol	68.33	68.33	12.50 ± 0.06	11.59 ± 0.19	105.70	135.71
Acetone	44.33	41.66	8.11 ± 0.39	7.62 ± 0.28	68.57	89.28

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