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Original Article

Purification and Characterization of Protease from *Rhizopus oligosporus*

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Abstract

In the present study, protease producing *Rhizopus oligosporus* fungal strain isolated from edible fruit stuff was purified and characterized. The crude enzyme obtained from culture filtrate was precipitated with ammonium sulphate and was purified by anion exchange chromatography on DEAE and gel filtration chromatography on Sephadex G_{75} with optimally active at the pH8 and at 50^0C. The molecular weight of the enzyme determined by SDS - PAGE was found to be 39kDa.
Keywords: *Rhizopus oligosporus*, Protease, Gel filtration, Precipitation, substrate.

Introduction

Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. They are also called protenase. Proteases of commercial importance are produced from microbial, animal and plant sources (Patel, 1985). A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes (Madan *et al*., 2002). Fungi are used in many industrial processes for the production enzymes and metabolites (Adrio *et al*., 2003).

Bulk production of enzymes has been reported in Aspergillus nigar, (Bosmann, 1973), *Aspergillus nidulans* (Sterns and sterns, 1980), *Aspergillums nidulans* (Stevens and Stevens, 1980), *Aspergillums clavatus* ESI (Hajj *et al*., 2007; 2008), *Aspergillus flaves* (Towhid Hossain *et al*., 2006), *Pencillium* spp (Sawada, 1963; Tomodo *et al*., 1964) and Rhizopus species (Kumar et al., 2005). Nutritional factor, physical factors such as inoculums concentration (Kaur *et al*., 1998), temperature, PH, (Tobe *et al*., 2005) and can also significantly effect protease production. Protease represents an important group of enzymes produced industrially and account for 60% of the worldwide scales values of the total industrial enzymes (Godfrey, 1996). Protease enzyme finds numerous application in the biotechnological production of detergents (pepsin) (Bailey *et al*., 1977), in dairy industries as milk-clothing agents (calf rennet composed mainly of chymosine and pepsin) (Fox, 1982) and as an agent for meat tenderization (Bernholdt, 1975), clinical and medical application (reduction of tissue inflammation) (Bailey *et al*., 1977; Nout *et al*., 1990). *Aspergillus oryzae* is used extensively to produce wide variety of industrial enzymes such proteases which have been produced either by solid-state (SSF) or by submerted fermentation (SMF) Sandhya *et al*., (2005). In our present study, purification and characterization of protease from Rhizopus oligosporus.

Materials and Method

Isolation and screening of fungi

The protease producing fungi was isolated from the contaminated edible fruit stuff. The organisms were streaked on sabouroud's dextrose agar containing 1% of cosein. The organisms were identified in microscope.

Protease Assay

Protease activity was assayed using Hayashi *et al*., (1967) modified method. The reaction mixture containing 1ml enzyme, 5ml of 1% casein (50mm Tris, p^H8) substrate were incubated for 1 hr at 37^0c. To the reaction mixture 5 ml of TCA (110mm) was added to stop the reaction and incubated for 30 min at 37^0C. The mixture solution was filtered. 2 ml of filtered solution was added to 5ml of 500mm sodium carbonate solution and 1ml of folin phenol reagent. This was incubated for 30 min at 37^0c and the colour intensity was observed at 660nm.

Protein content determination

Lowry's method (1951) was used in determining the protein content of the crude sample, ammonium sulphate precipitate and the fractions obtained from gel filtration with bovine serum albumin (BSA, as standard).

Optimization of enzyme production

Effect of substrate concentration of enzyme production

The organism was cultured in optimized various concentration of casein (0.5, 1.0, 1.5, 2.0,

2.5). Enzyme activity was assayed in standard assay condition.

Effect of temperature and pH on enzyme production

The organisam was inoculated in nutrient broth containing 1% casein and incubated is various temperatures. Such as 30, 40, 50, 60 and 70^{0}C. The standard growth broth was adjusted to various p^{H}3, 4, 5, 6, 7, 8, 9 and 10. At the end of the incubation period, the culture filtrate was obtained and it used for the enzyme assay.

Properties of proteases

Effect of temperature on enzyme stability

Effect of enzyme stability was studied by denaturing enzyme sample at 30, 40, 50, 60 and 70^{0}C for 30 min. The denatured sample was incubated with 1% casein for 1 hr. The enzyme activity was assayed in standard condition.

Effect of p^H on enzyme activity

The effect of PH was evaluated by measuring the enzyme activity of protease in the p^H range 3-10. The following buffers citric acid PG4, Trisbuffer PH7-10 were used.

Effect of metal ions on enzyme activity

Different types of 1m metal ions Ca^{2+}, $MnSo_4$, $Mgcl_2$, $Znso_4$, Nacl, $Hgcl_2$, $Cuso_4$, $Feso_4$ and NH_4cl were incubated with culture supernatant. The activity of enzyme was assayed in standard assay condition.

Partial purification of protease

Ammonium Sulphate precipitation:

Slowly, solid ammonium sulphate was added with the culture filtrate containing 1% casein and stirred slowly at various percentages 4^{0}c for 4hrs. The suspension was centrifuged 10000rpm for 15min. The pellet was dissolved in Tris buffer and subsequently dialyzed against the same buffer at 4^{0}C.

Anion exchange chromatography

Purification of protease enzyme, was carried out using DEAE cellulose anion exchange chromatography according to the method of Stempion, 1970. The dialysed sample was applied to a DEAE column. The column was washed with 50mm, Tris p^{H}8, and it was eluted with serially increasing concentration of Nacl (0.1M, 0.2). The eluted fractions were monitored by U.V. absorption spectroscopy at 280nm.

Gel filtration chromatography

The active fraction obtained from DEAE cellulose chromatography was loaded on the Sephadex G_{-75} column (1.8x110cm) of gel filtration column. The sample was eluted with the same buffer. The active fraction was assayed for protease activity.

Molecular mass determination

Molecular mass was estimated by SDS-polyacrylamide gel electrophoresis (Laemmili, 1970). Electrophoresis was performed in reducing and non - reducing conditions (with and without β - mercaptoethanol) using polyacrylamide gel. The molecular mass of proteases enzyme was estimated by comparing its mobility with that of following markers: Myosin (205kDa), Phosphorylase (97.4kDa), Bovine Serum albumin (66kDa), Ovalbumin (43), Carbonic anhydrase (29kDa). Gel was silver stained by the method of Blum *et al*.,(1987).

Results and Discussion

Microbial proteases are best classified by p^H optimum and inhibitor sensitivity rather than by most readily hydrolyzed substrate (Ong and Gaucher, 1973). Boer and Peralta (2000) reported the highest protease activity for Aspergillus tamarii from PH6 to 10 with optimum temperature at 40^{0}C. Protease production by *Aspergillus flavis* observed in the p^H range 7-9 (Sutar *et al*., 1992). In the present study, among the different p^H levels tested, p^{H}8 was found optimum for all the test isolates. Kalpana Devi *et al*., 2008 has reported that the Aspergillus niger was incubated at different temperatures ranging from 30^{0}C-60^{0}C and the enzyme activity was maximum at 45^{0}C. In the present study the optimum temperature for protease production was 50^{0}C.

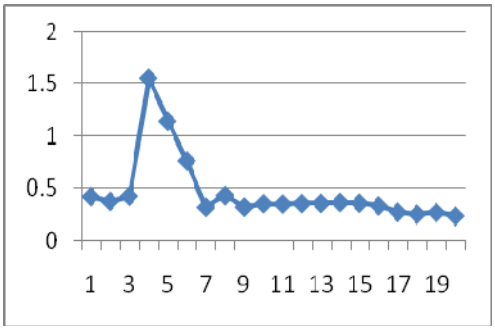

Fig.1: Gel filtration chromatography of Rhizopus oligosporus protease on Sepadex G_{75} column, absorbance at 280nm.

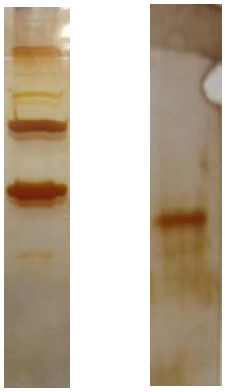

Fig. 2: SDS-polyacrylamide gel electrophoresis of the purified Rhizopus oligosporus protease in the presence of β-mercaptoethanol. Lane :1 : Standard non-denatured molecular weight marker protein, Lane : 2 : Purified protease.

Nehra *et al*., (2004) reported that Mg^{++} was found to be an activator for the alkaline protecise enzyme produced by Aspergillus Sp. In the present study the enzyme inhibited in Mn^{++} precipitation by ammonium sulphate is used in acedic and neutral $_{P}H$ solution (Aunstrup, 1980). When the ammonium sulphate protease was purified in anion exchange chromatography it gave the enzyme activity the fraction containing three bands. Therefore when protease active fraction was loaded on Sepadex G_{75} column single peak active protease can be observed as in Fig (1). SDS-PAGE of active protease gave single band with a molecular weight 39KDa as in Fig (2). The molecular weight of purified enzyme was determined by SDS-PAGE was found to be 38KDa. (Kalpana Devi et al., 2008).

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