PURIFICATION AND SOME PROPERTIES OF ALKALINE PROTEASE FROM PENICILLIUM EXPANSUM

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SUMMARY: An alkaline protease was purified from culture broth Penicillium expansum by fractioning with acetone and column chromatography on Sephadex G-100 and DEAE-Sephadex A-50. The homogeneity of the final product was demonstrated on polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate which showed a single protein band. The purified enzyme had a molecular weight of about 20500 on polyacrylamide disc electrophoresis. The optimal pH and temperature for enzyme activity were found to be 10.5 and 35°C, respectively. The activity was found to be enhanced by addition of cysteine, mercaptoethanol and Co^{2+} whereas inhibition was observed in the presence of Hg^{2+} and Ag^{2+}. Addition of Ca^{2+}, Zn^{2+} and Mn^{2+} did not produce any significant effect on enzyme activity.

Key Words : Penicillium expansum, rice husk, alkaline protease, cysteine, mercaptoethanol, activation.

INTRODUCTION

A number of extra-cellular proteases from various strains of filamentous fungus have been characterized (2,18,22). However, the nature of proteolytic enzymes from fungal origin is less well known than that of animal and plant origin. Keay (11) and Fogarty (8) have classified the microbial proteases according to the nature of optimum pH of enzyme activity such as acidic, neutral and alkaline. The properties of alkaline proteases from fungi have not been described in detail but only a few reports have been published (2,10,15-17,19). In recent years, tremendous uses of proteases in industry have served to enhanced research in this area. The use of alkaline protease has been increased in the preparation of medicines, in food processing, leather and textile industries (8,11).

During a recent study it was observed that Penicillium expansum strain CMI 39761 is capable to produce greater amount of an extra-cellular alkaline protease when grown in rice husk mineral medium (6). This work was undertaken to purify and to elucidate some basic enzymatic properties of Penicillium expansum alkaline protease.

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MATERIALS AND METHODS

Strain

Penicillium expansum strain CMI 39761 was used which was o+btained from the department of botany, University of Glasgow. The stock culture was maintained at 27°C on agar slants, containing 2% bactoagar, 2% dextrose and 1% peptone.

Basal medium

Basal medium was used for the growth of Penicillium expansum as reported by Burrel *et. al.* (3).

Inoculum

A spore suspension of the fungus prepared as reported earlier (4).

Cultivation conditions

800 ml of basal medium without glucose supplemented with 8.0 g of rice husk fine powder (40 mesh) was taken in

2000 ml conical flask plugged with cotton wool and autoclaved at 1.5 kg/cm^2 for 20 minutes. The pH of the culture medium was adjusted to 6.0 before sterilization. The sterilized media cooled at room temperature was inoculated with 8.0 ml of inoculum containing 50x10^6 spores/ml. The flasks were incubated in a cooled orbital shaking incubator (Gallenkamp) at 220 rev m^{-1} at 35°C. After 48 hours, the cells were removed from from the culture medium by filtration through Whatman No. 1 filter paper and the filtrate was used as a crude enzyme for the enzyme purification.

Determination of protease activity

Protease activity was determined as reported earlier (5,6) with slight modification, Casein was dissolved in glycine-HCl buffer pH 9.0.

One unit of the protease activity was defined as the amount of enzyme that liberated 1μg of tyrosine under the standard assay conditions.

Enzyme purification

To the crude enzyme prepared, as described above, was added slowly 2 fold cold acetone with constant stirring. The mixture was allowed to stand for two hours at 4°C. The precipitates were collected by centrifugation at 500 x g for 20 minutes (Kubota refrigerated centrifuge at 4°C) and dissolved in small volume of 0.1 M Tris-HCl buffer pH 7.5 and dialyzed against the same buffer at 4°C for overnight.

Sephadex G-100 column chromatography

The dialyzed sample was then applied to the Sephadex G-100 column (30 x 2.5 cm) previously equilibrated with 0.1 M Tris-HCl buffer pH 7.5. The enzyme was eluted with the same buffer at a flow rate of 28 ml/hour and the fraction volume of 4.0 ml/tube was collected. Pooled fractions showing enzyme activity was rechromatographied on DEAE column.

DEAE Sephadex A-50 chromatography

The above pooled and dialyzed sample was applied to DEAE Sephadex A-50 column (14 x 2.5 cm) previously equilibrated with 0.1 M Tris-HCl buffer pH 7.0 to 9.0 containing 0.2 M NaCl. The flow rate was adjusted to 20 ml/hour with fraction volume of 5 ml/tube. Fractions showing protease activity were pooled and checked for homogeneity and to study the enzymatic properties.

Disc gel electrophoresis

The homogeneity of the purified enzyme was confirmed by polyacrylamide disc gel electrophoresis by the method of Davis (7). 7.5% SDS-polyacrylamide gel at pH 8.3 (Tris-Glycine buffer). 50 μg of protein was loaded with sample gel buffer and a constant current supply of 4 mA per gel rod (13 x 0.6 cm) was applied for 4 hours. After electrophoresis run, gels were stained with 1% Coomassie brilliant blue R-250 and were distained with acetic acid: methanol (7.5:5.0 v/v) till the appearance of blue band against a clear background. The Molecular Weight of the purified alkaline protease was measured by SDS-polyacrylamide disc electrophoresis using a series of protein with known molecular weight as standard.

Determination of protein

The absorbance was measured to monitor the protein during chromatographic separation. The protein content was measured by the method of Lowry *et. al.* (14), using bovine serum albumin as a standard.

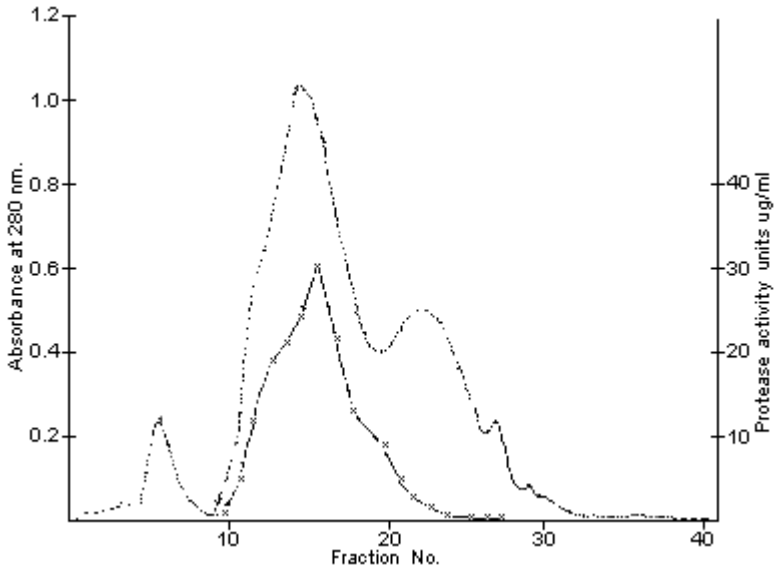
RESULTS

Alkaline protease was purified from culture broth of Penicillium expansum by the procedure described in materials and methods. Figure 1 shows the elution pattern of the enzyme and a broad active peak was obtained when the dialyzed sample was passed through a Sephadex G-100 column (30 x 2.5 cm) with 0.1 M Tris-HCl buffer pH 7.5. The pooled fractioned were dialyzed and again adsorbed on DEAE Sephadex A-50 column (Figure 2). The steps of purification procedures and recovery of enzyme at each step are pre-

Table 1: Purification of alkaline protease of *Penicillium expansum.*

Procedure	Total protein (mg)	Total activity (PU)	Specific activity PU/mg protein	Purific. fold	Recovery %
Culture broth	2400	1550	0.6	1	100
Dialyzed	260	1350	5.19	8.10	87.09
Sephadex G-100 gel filtration	79	808	10.22	15.98	52.12
DEAE Sephadex A-50 chromatography	12.08	744	61.59	96.23	48.00

Figure 1: Elution profile of Sephadex G-100 chromatography of Extra-cellular alkaline protease of *Penicillium expansum.* .....Absorbance at 280 nm; x-x Alkaline protease.



sented in Table 1. The recovery of purified alkaline protease from Penicillium expansum culture broth was achieved 48% and the purified enzyme had specific activity 61.6 units/mg protein. The purified alkaline protease migrated as a higher protein band (Figure 3) and its apparent relative molecular weight was estimated to be 20500 on SDS-polyacrylamide gel electrophoresis.

Table 2: Results showing the effect of various reagents on purified alkaline protease activity of *Penicillium expansum* at 35°C.

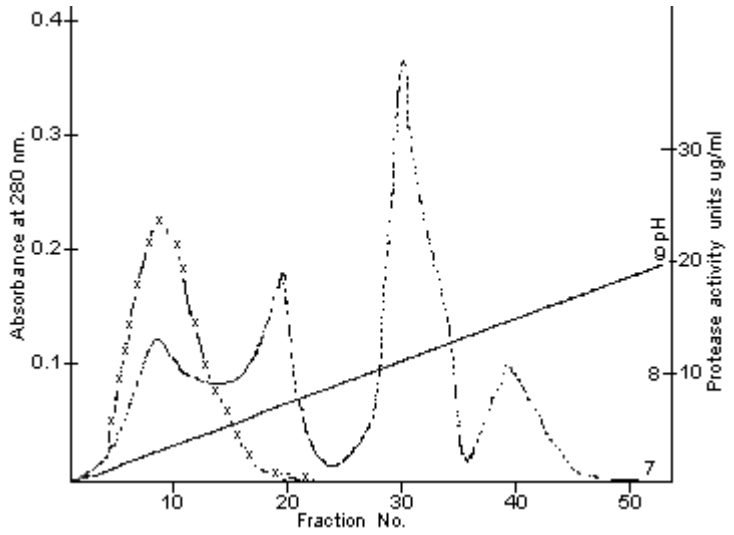
Reagent	Conc.	Activity units/ml	% Relative activity	% Activation (inhibition)
Control	-	20.30	100.00	-
$CaCl_2$	5mM	20.57	101.33	1.33
$MnCl_2$	5mM	19.89	87.98	(2.02)
$ZnCl_2$	5mM	20.97	103.30	3.30
$CoCl_2$	5mM	47.37	234.43	134.43
$AgNO_3$	5mM	7.10	34.95	(65.05)
$Hg(NO_3)_2$	5mM	8.12	40.00	(60.00)
SDS	5mM	17.59	86.65	(13.35)
Cysteine	5mM	88.26	434.77	334.77
2-Mercaptoet.	5mM	95.03	468.12	368.12

SDS: Sodium dodecylsulphate.

Figure 4 illustrates the activity response to pH and maximum activity was found at pH 10.5. While Figure 5 shows the temperature at which maximal enzyme activity obtained was 35°C. An abrupt decrease in rate of reaction was observed when the reaction temperature was further increased. This seems to be due to enzyme denaturation. Purified alkaline protease was heated at various temperature for 10 minutes. The activities remaining after heat treatment were assayed by standard method. Heat stability tests showed that purified enzyme was fairly stable up to 50°C but looses its complete activity at 80°C with in 10 minutes as shown in Figure 6.

The effect of some divalent cations and reducing agents on the enzymatic activity are summarized in Table 2. Alkaline protease activity was slightly stimulated in the presence of Ca^{2+} and Zn^{2+} whilst Mn^{2+} did not show any significant effect on enzyme activity. However, Ag^{+}, Hg^{2+} and SDS reduced the activity of alkaline protease but the activity was significantly increased by free reducing agents such as cysteine and mercaptoethanol.

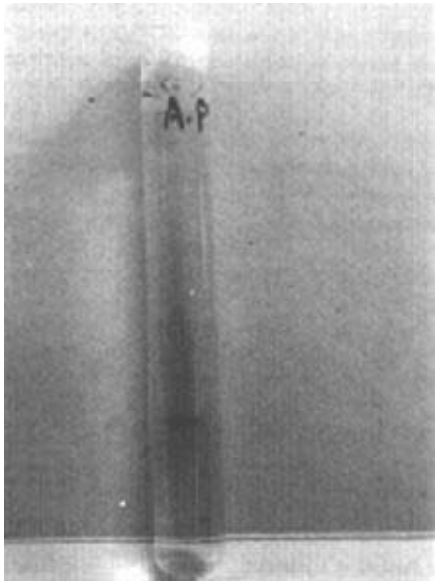
Figure 2: Elution pattern of Ion-exchange chromatography of extra cellular alkaline protease of Penicillium expansum on Sephadex A-50 ..... Absorbance at 280 nm; x-x-x Alkaline protease; ..... pH gradient.



DISCUSSION

A study was designed to investigate the possible use of rice husk as carbon and energy source for the growth of Penicillium expansum and production of protease. In recent years, much interest has been focused to search new and cheapest carbon sorue due to the rice pure sugar and hydrocarbon prices. We have observed that greater amount of proteases are produced by Penicillium expansum grown on rice husk fine powder mineral medium (6). In the present paper, work was undertaken to purify and to study some basic properties of Penicillium expansum extra-cellular protease. In this study an approximately 96 fold purification was achieved with a yield of about 48.0%. The purified enzyme was shown to be homogeneous by polyacrylamide disc gel electrophoresis. The estimated molecular weight of this enzyme (20500) is similar with the values reported for alkaline proteases (21).

Figure 3: SDS-Polyacrylamide disc gel electrophoresis of the alkaline protease of *Penicillium expansum.*



Maximum protease activity was found at pH 10.5. In view of optimum pH, Penicillium expansum enzyme activity can apparently be classified as alkaline protease according to the suggestion of Keay (11) and Fogarty *et. al.* (8). This observation is favorably comparable with the finding of Lambert *et. al.* (12) and Stevens (22) in case of alkaline protease of Verticillium dahliae (pH 9.5) and Aspergillus nedulans (pH 11.0) respectively.

The stimulation of activity in the presence of reduc-

Figure 4: Effect of pH on the activity of Alkaline protease of *Penicillium expansum*.

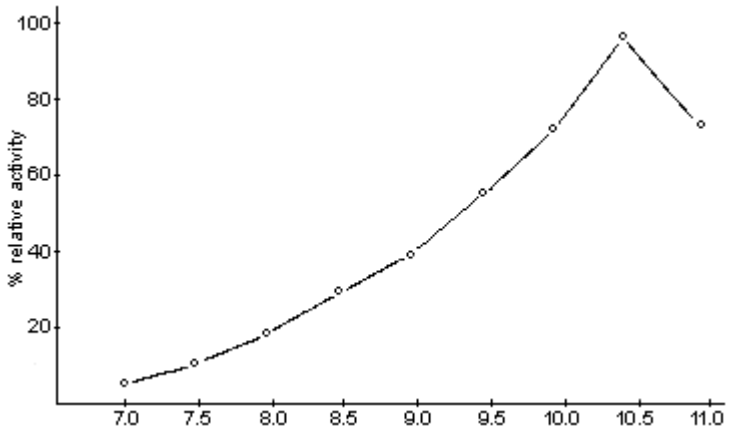
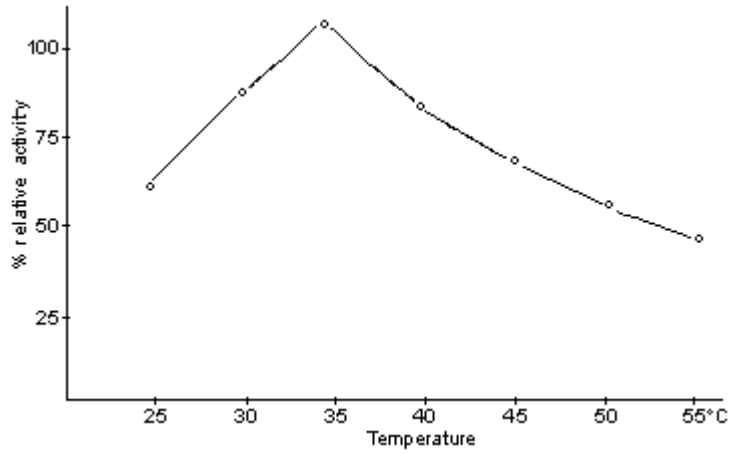


Figure 5: Effect of temperature on alkaline protease activity of *Penicillium expansum.*



ing agents indicates that alkaline protease possess -SH groups at the active site. The inhibitory effect of Hg^{2+} and Ag^{2+} supported this assumption (1,9,13). Penicillium expansum alkaline protease is different in several respects from other fungal alkaline proteases, mainly by its appearance as a cysteine enzyme. Mostly fungal alkaline proteases are generally serine enzyme and are inhibited by PMSF (20).

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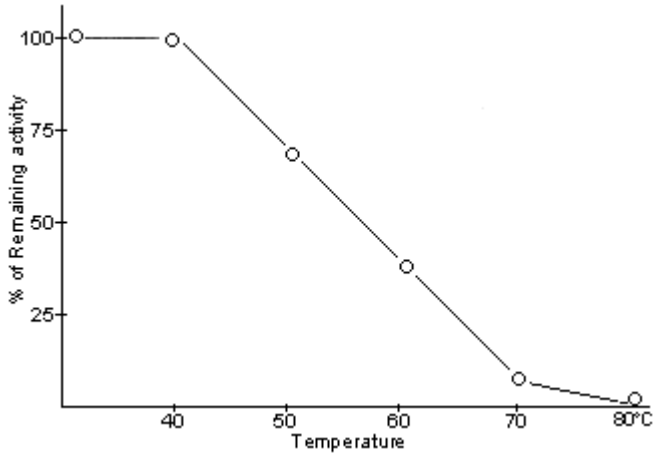
Figure 6: Effect of heat on the stability of alkaline protease from *Penicillium expansum.*



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