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Purification and Some Properties of Acid Protease from *Penicillium Expansum*

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Abstract: An acid protease was isolated from the culture broth of Penicillium expansum grown on rice husk (40 mesh fine powder) mineral medium. The protease enzyme was purified on Sephadex G-100 and DEAE Sephadex A-50 with recovery of 7.52%. Purified enzyme shows a single band on SDS polyacrylamide gel electrophoresis. The optimum activity of protease was found at pH 3.5 and temperature 30°C. The activation energy for the hydrolysis of casein by acid protease was 93 KJ/mol. The enzyme activity was highly increased in the presence of $CoCl_2$ (71.57%), cysteine (54.23%), mercaptoethanol (44.70%), $ZnCl_2$ (34.78%) but slightly activated by $CaCl_2$ (10.24%) and $MnCl_2$ (1.37%). Whereas protease activity was reduced on the addition of EDTA (55.88%), $AgNO_3$ (67.85%) and $HgNO_3)_2$ (64.71%). The thermal stability of protease was increased in the presence of $ZnCl_2$. The half-life of acid protease in the absence and presence of $ZnCl_2$ at 50°C was determined 17 and 28 minutes respectively.

Key Words: Acid protease, Penicillium expansum, Rice husk

Introduction

It is well known that a large number of thermophilic fungi secrete soluble extracellular acid proteases. Acid proteases are now called aspartic proteases. Of the aspartic proteases, pepsin (EC 3.4.23.1) acts on hemoglobin and synthetic substrate acetyl -L-phenylalanyl-diiodotyrosine (Ac-Fy-I2) Majima *et al.*, (1988). Pepsin exhibit a milk clotting action at pH 5.3 while the aspartic proteinase from Aspergillus saitoi (EC 3.4.23.6) does not hydrolyze Ac-Fy-I2 but it is capable of activating trypsinogen and chymo-trypsinogen in the acidic pH range. The proteinase was inactive as to the milk clotting (Ichishima, 1970). Microbial acid proteases isolated from *A. oryzae* Tsujita and Endo, (1977), Scytalidium lignicolum ATCC 24568 Oda *et al.*, (1986) and Xanthomonas sp. bacterium Oda *et al.*, (1987) are capable to hydrolyze milk casein well to produce a large number of amino acids.

Several workers Arvidson *et al.*, (1973) Laughlin and Faubert, (1977), Takeuchi and Ichishima, (1986), Murakami *et al.*, (1991) and Vasantha *et al.*, (1984) have purified more than one or two type of extracellular proteases (acid, neutral and alkaline) from culture broth of fungal and bacterial species. The main object of this study was to utilize rice husk as carbon source instead of pure sugars for the growth and production of industrially important enzymes. Previously, the properties of purified extracellular neutral Dahot, (1995) and alkaline protease Dahot, (1994) from the culture broth of Penicillium expansum have been reported. The present paper deals with some properties of purified acid protease of Penicillium expansum.

Materials and Methods

Strain: Penicillium expansum strain CMI 39761 was used which was obtained 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.* (1966).

Inoculum: A spore suspension of the fungus was prepared as reported earlier, Dahot, (1987).

Cultivation conditions:800ml of basal medium without glucose supplemented with 8.0g of rice husk fine powder (40 mesh) was taken in 2000 ml conical flask plugged with cotton wool and autoclaved at 1.5kg/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 50 x 10^6 spores/ml. The flasks were incubated in a cooled orbital shaking incubator (Gallenkamp) at 220 rev m^{-1} at 35°C. After 48, the cells were removed 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 Dahot, (1987), Dahot, (1993) with slight modification, casein was dissolved in Glycine-HCl buffer pH 3.5.

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

Enzyme purification: 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. Precipitates were collected by centrifugation at 5000 x g for 20 minutes (Kubota refrigerated centrifuge at 4°C) and were dissolved in small volume of 0.1M Tris-HCl buffer pH 7.5. Dialysis was carried out against same buffer at 4°C for over night.

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 same buffer at a flow rate of 28 ml/hour and the fraction volume of 4.0ml/tube was collected. Pooled fractions showing enzyme activity was rechromatogram 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. The enzyme was eluted pH gradient with the same buffer from pH 7.0 to 9.0 containing 0.2 M NaCl. The flow rate was adjusted to 20 ml/hour with a fraction volume of 5ml/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 (1964), 7.5% SDS-polyacrylamide disc gel at pH 8.3 (Tris - Glycine buffer). 50µl of sample was loaded with sample gel buffer and a constant current supply of 4mA 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 destained 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 gel electrophoresis using a series of protein with known molecular weight as standard.

Determination of protein: The absorbance was measured at 280 nm to monitor the protein during chromatography separation. The protein content was measured by the method of Lowry *et al.*, (1951), using bovine serum albumin as a standard.

Results and Discussion

An acid protease was purified from culture broth of Penicillium expansum by acetone precipitation, Sephadex G - 100 and DEAE Sephadex A - 50. Fig. 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 fractions were dialyzed and again adsorbed on DEAE Sephadex A-50 column (Fig.2). The steps of purification procedures and recovery of enzyme at each step are presented in Table 1. The recovery of purified acid protease from Penicillium expansum culture broth was achieved 7.52% and the purified enzyme had specific activity 77.45units/mg protein. The purified acid protease migrated as a higher protein band (Fig. 3) and its apparent relative molecular weight was estimated to be 20500 on SDS-polyacrylamide gel electrophoresis.

The effect of hydrogen ion concentration on the activity of purified protease was determined with casein over the pH range of 2.0 to 7.0 using Universal buffer (0.2 M). The maximum reaction product was occurred at pH 3.5 as shown in Fig. 4. Furthermore, the effect of temperature on protease activity of Penicillium expansum is shown in Fig. 5. The optimal temperature of incubation at pH 3.5 for casein digestion was found to be 30°C. The activation energy of Penicillium expansum acid protease was found to be 93 KJ/mol (Fig. 5b).

The protease enzyme of Penicillium expansum was heated at various temperatures ranging from 30 to 90°C for 10 minutes in the absence and presence of $ZnCl_2$. The remaining activity was assayed at 30°C. The protease activity was found heat stable and retains more than 15 and 35% at 80°C with in 10 minutes in the absence and presence of $ZnCl_2$. The stability of acid protease of Penicillium expansum was increased in the presence of $ZnCl_2$ and at pH 3.5, in the absence and presence of $ZnCl_2$, the Tm of the acid protease increased from 62 to 73°C (Fig. 6). At 50°C, the $t_{½}$ in the absence and presence of $ZnCl_2$ was 17 and 28 minutes respectively (Fig. 7). Stabilization of proteases can be result either prevention of protein unfolding or a decrease in autolytic degradation (or both). It is reported that stabilization of enzymes was increased by the addition of salts, ligands (including substrates), monosaccharides, polyols, organic polymers and by other mechanisms Cowan *et al.*, (1987).

Table 2 shows the data of divalent cations and reducing agent effect on protease activity. Protease activity was strongly activated with Zn2+, Co2+, cysteine and mercapto-ethanol whilst Mn2+ did not show any significant effect. However, enzyme activity was strongly inhibited by EDTA (specific inhibitor of metal protease) to the same level as the Sporotrichum pulverulentum

Table 1: Purification of protease from culture broth of Penicillium expansum

Purification steps	Total protein mg	Total protease activity µg	Specific activity	Purification fold µg/mg	% Yield
Culture broth	2400	7450	3.10	1	100.00
After dialysis	260	5900	22.69	7.32	79.19
Sephadex G-100	79	2120	26.83	8.65	28.46
DEAE Sephadex A-50	7.23	560	77.45	24.98	7.52

Table - 2: Effect of various reagents on acidic protease activity of Penicillium expansum at 30°C

Reagents	activity Units/ml	% Relative activity	%Activation /(Inhibition)
Control	25.50	100.00	-
$CaCl_2$	28.12	110.24	10.24
$MnCl_2$	25.85	101.37	01.37
$ZnCl_2$	34.37	134.78	34.78
$CoCl_2$	43.75	171.57	71.57
EDTA	11.25	44.12	(55.88)
$AgNO_3$	8.20	32.15	(67.85)
$Hg(NO_3)_2$	9.37	35.29	(64.71)
Cysteine	39.33	154.23	54.23
Mercapto-ethanol	36.90	144.70	44.70

Eriksson and Peterson, (1982). However, it is not a member of metallo protease class but inhibition due to the chelation of stabilizing divalent cation (Zn2+ of Co2+) ions rather than the loss of a catalytic active site metal can not be ruled out at this stage Toogood *et al.*, (1995). On the other hand, enzyme activity was strongly stimulation in the presence of reducing agent to the same extent as the Penicillium duponi Hashimoto *et al.*, (1973) and this indicates that acid protease possess - SH group at active site. The inhibition effect of Ag+ and Hg2+ support thus assumption Arvidson *et al.*, (1973) and Laughlin and Faubert, (1977). This result indicates that Penicillium expansum acid protease possess two or more active sites. Further work on substrate specificity,

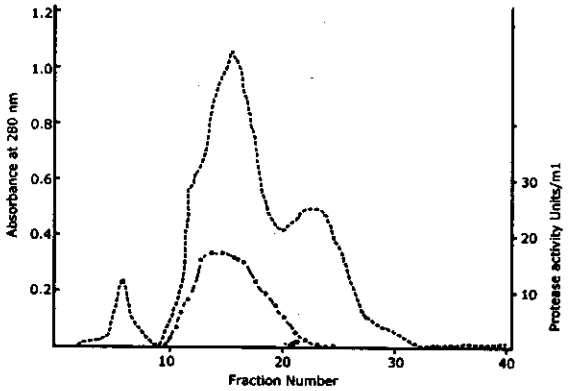

Fig. 1: Elution profile in Sephadex G-100 gel filtration of extracellular protease of Penicilliumexpansum .-.-.-.-. absorbance, o-o-o-o-o Protease activity

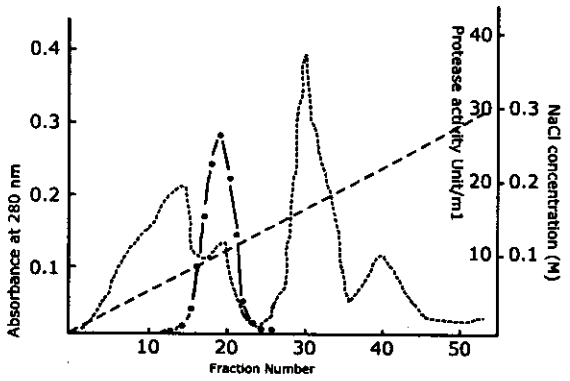

Fig. 2: Ion-exchange chromatography of extracellular acidic protease of Penicillium xpasum on DEAE Sephadex A-50 .-.-.-.-.- Absorbance, v-v-v-v-v Protease activity .....Nacl gradient

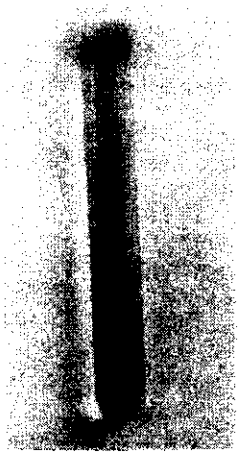

Fig. 3: SDS-polyacrylamide disc gel Electrophoresis of purified acid protase of *Peicillium expansum*

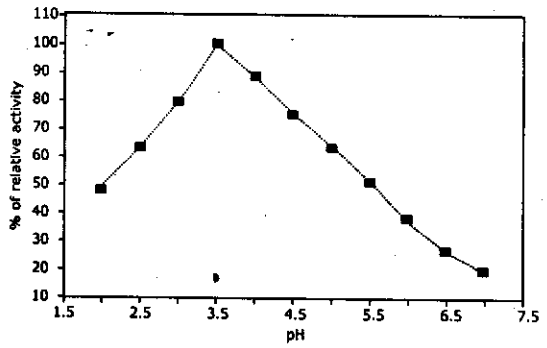

Fig. 4: Effect of pH on protease activity of Penicillium expansum

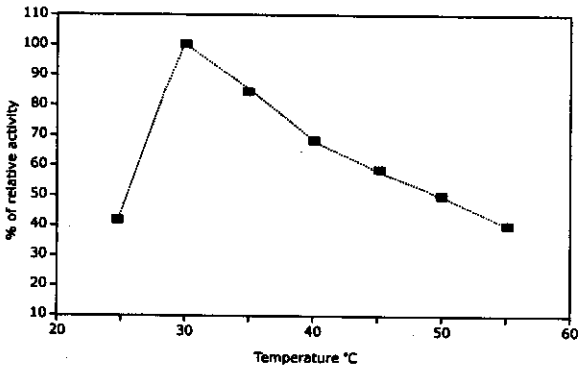

Fig. 5: Effect of temperature on protease activity of *P. expansum*

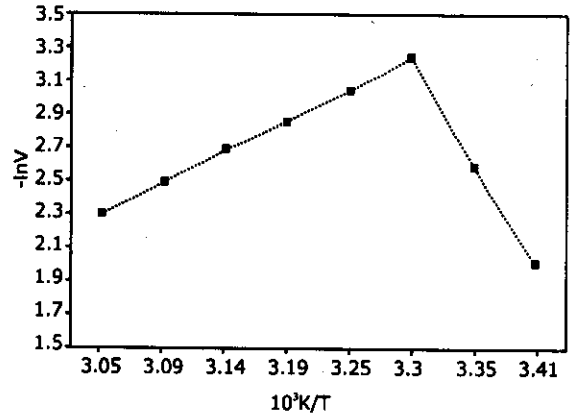

Fig. 5b: Arrhenius plot for the determination of activation energy of hydrolysis of casein by acid protease. Activation energy (Ea) = Slop x R, Where R = 8.314 JK^{-1} mol = 93KJ/Mol^{-1}.

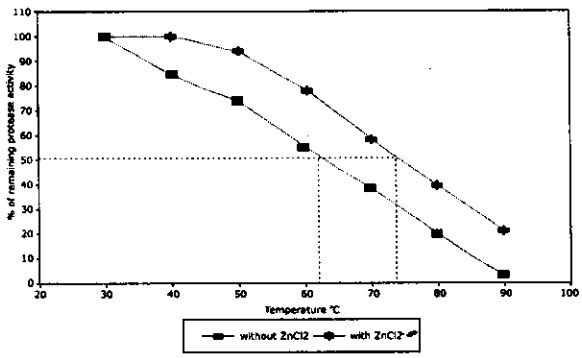

Fig. 6: Thermal stability of acid protease activity of *P.expansum*

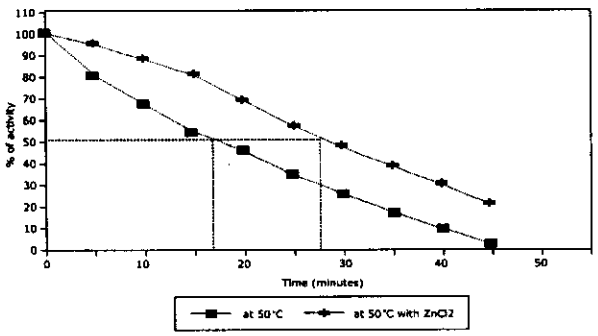

Fig. 7: Stability of acid protease activity of *P.expansum*

N-terminal amino acid is under investigation and will be reported later on.

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