STUDIES ON ALKALINE PROTEASES FROM CONIDIOBOLUS

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DECLARATION

Certified that the work incorporated in the thesis "STUDIES ON ALKALINE PROTEASES FROM CONIDIOBOLUS" submitted by Mrs. Sangita U. Phadatare was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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LIST OF ABBREVIATIONS

BAEE	:	N-Benzoyl-L-arginine ethyl ester
BAPNA	:	N-Benzoyl-DL-arginine-p-nitroanilide
Bis	:	N,N'-methylene-bis-acrylamide
BSA	:	Bovine serum albumin
BTEE	:	N-Benzoyl-L-tyrosine ethyl ester
BTPNA	:	N-Benzoyl-L-tyrosine-p-nitroanilide
CM-Cellulose	:	Carboxy methyl cellulose
DABT	:	3-3-diaminobenzidine tetrahydrochloride
DFP	:	Diisopropyl fluorophosphate
DMSO	:	Dimethyl sulfoxide
EDTA	:	Ethylenediamine tetraacetate
Hb	:	Haemoglobin
MAPI	:	Microbial alkaline protease inhibitor
Mr	:	Molecular weight
PAGE	:	Polyacrylamide gel electrophoresis
PCMB	:	p-Chloromercuric benzoate
pI	:	Isoelectric point
PMSF	:	Phenyl methyl sulfonyl fluoride
SDS	:	Sodium dodecyl sulphate
TAME	:	p-Toluenesulfonyl-L-arginine methyl ester
TCA	:	Trichloroacetic acid
TEMED	:	NNN'N'-Tetramethylethylenediamine
Tris	:	Tris (hydroxymethyl) aminomethane

ABSTRACT

Proteases are hydrolytic enzymes capable of degrading proteins into small peptides and amino acids. They constitute a very large and complex group of enzymes and account for nearly 60% of the total industrial enzyme market. They are obtained from plant, animal and microbial sources. Some of the commercially important proteases are: papain and ficin from plants, trypsin from animals and a large number of acidic, neutral and alkaline proteases from diverse microorganisms such as, Bacillus, Aspergillus, Rhizopus, Mucor and Conidiobolus. Novo Industries A/S (Denmark), Gist-Brocades (Netherlands), Miles Laboratories (USA) are some of the leading protease producing firms.

Proteases are extensively exploited commercially, in the processes like, detergent manufacture, baking, brewing, leather processing, meat tenderisation, peptide synthesis, cheese manufacture, soysauce production, preparation of protein hydrolysates, recovery of silver from waste photographic films, etc. They are also used for medical purposes, for example, as digestive aids in gastro-intestinal disorders like dyspepsia, for treatment of thrombosis and for debridement of dermal ulcers and burns.

Study of proteolytic enzymes is also important from a physiological point of view, as they are involved in many cellular processes like, protein turnover, digestion, bacterial endospore formation and germination, enzyme modification and secretion, fungal hyphal tip growth and yeast

budding.

Despite the industrial and physiological importance of proteases, little information is available on purification and characterization of fungal alkaline proteases. Bacterial proteases, especially those from Bacillus sp., on the other hand, are extensively studied.

The present studies deal with alkaline, serine protease from a novel, little investigated fungus, Conidiobolus coronatus (NCL 86.8.20). The main features of the work are:

1(a) Screening for high protease yielding strain of Conidiobolus and standardisation of fermentation conditions for maximum production of alkaline protease in submerged culture.

1(b) Preliminary evaluation of the compatibility of alkaline protease with some commercial detergents.

2. Investigation of possible involvement of alkaline protease in the violent discharge of conidia, by using following approaches:

(a) Isolation of a specific variant with reduced conidial discharge and its comparison with the parent strain with respect to conidial discharge and protease levels.

(b) Study of effect of protease inhibitors and inducers on conidial discharge.

3. Purification of the low and high molecular weight alkaline proteases.

4. Study of biochemical, physicochemical and immunological

relationship between the two purified alkaline proteases and its implication in physiological regulation of conidial discharge.

1(a)Screening for high alkaline protease producing strain and production of the enzyme in submerged culture

A number of Conidiobolus strains isolated from decomposing plant detritus were tested for alkaline protease production. The isolate C. coronatus (NCL 86.8.20) showing maximum protease activity was selected for further studies.

Effect of various medium constituents, viz. inorganic and organic nitrogen sources, sugars, salts, surfactants, inducers and metal ions on alkaline protease production was studied. Fermentation conditions, such as, pH, temperature, time profile and inoculum size were also standardised for maximum protease production. Maximum protease production was observed at 28°C for 48 h with medium containing sucrose, 3%; ammonium nitrate, 0.38%, tryptone, 2%; potassium chloride, 0.2%; potassium dihydrogen phosphate, 0.1%; casein, 2%; trace elements, initial pH 7 - 7.5.

1(b) Evaluation of compatibility of alkaline protease with commercial detergents

One of the major applications of proteases is in detergents, compatibility of Conidiobolus coronatus (NCL 86.8.20) alkaline protease with commercial detergents was evaluated. Broad substrate specificity, stability at alkaline pH, moderately high temperature (40°C, 1 h) and in presence of various detergents such as SNOWWHITE, NIRMA,

REVAL, SURF AND WHEEL (7 mg/ml) indicated the potential of this enzyme for use in detergents.

2. Involvement of alkaline, serine protease in the conidial discharge

The involvement of protease in the conidial discharge of C. coronatus was investigated by isolating a variant strain with reduced conidial discharge. Except for this difference, it exhibited characteristics similar to the parent strain with respect to nutritional requirements, sensitivity to various antibiotics, germination pattern, growth rate and conidial production. Time course profiles of protease levels and conidial discharge showed that the maximum protease levels coincided with maximum conidial discharge in both the parent and variant strain. Both, protease levels and conidial discharge were higher in parent strain as compared to variant strain. In both the strains, conidial discharge started when the protease level reached 1.0 U/mg protein. Inhibition of the serine protease by phenyl methyl sulfonyl fluoride showed that low protease levels resulted in inhibition of the conidial discharge and a minimum activity of 1.0 U/mg protein is essential for triggering the conidial discharge. Using casein to induce protease, it was further observed that early gain in the protease level (1.0 U/mg protein) leads to early onset of conidial discharge. The above evidence suggests the involvement of protease in the conidial discharge of C. coronatus, thus attributing a new physiological role to serine proteases.

3. Purification of alkaline protease(s)

As a step towards the understanding of the mechanism of regulation of conidial discharge, it was necessary to investigate the kinetics of production and purification of the proteases elaborated by Conidiobolus. The culture filtrate of Conidiobolus showed the presence of two proteases designated as protease I and II. Kinetics of intracellular versus extracellular enzyme production revealed that both the proteases occur intracellularly and protease II is produced later, i.e. at the time coincident with the reduced conidial discharge.

The properties of crude intracellular protease were identical to those of extracellular protease indicating that these proteases are produced intracellularly and then secreted into the culture medium.

The extracellular proteases were purified to homogeneity by (i) ammonium sulphate precipitation; (ii) preparative polyacrylamide gel electrophoresis at pH 7.6 and (iii) ion-exchange chromatography on CM-cellulose at pH 7.0.

Final yields of protease I and II were 24 and 10.7% respectively. Their specific activities were 45 and 30 U/mg respectively.

4. Biochemical, physicohemical and immunological characterisation of the two alkaline proteases

In order to trace origin of protease II, the purified enzymes were compared with respect to their biochemical, physicochemical and immunological properties. The two

proteases possessed identical biochemical properties, viz. optimum pH (9.7 - 10), optimum temperature (40°C), stability pH (7 - 7.5) and temperature stability (1 h at 35°C - 40°C). Both possessed esterase activity and were inhibited by 0.1 mM phenyl methyl sulfonyl fluoride. The amino acid analysis and two dimensional peptide mapping of the two proteins revealed that protease II was completely homologous with protease I, though protease I showed some additional portion not contained in protease II. Western-Blot ELISA, double diffusion and immunotitration experiments with anti-protease I antibody also revealed the structural similarity of the two proteases. The antigenic valencies of protease I and II were 16 and 12 respectively. The proteases differed only in their charge, specific activity and molecular mass. Protease I and II had pI of 9.9 and 9.0 and molecular mass of 23,170 and 19,270 daltons respectively. Specific activities of protease I and II were 45 and 30 U/mg protein respectively, as mentioned previously.

The purified protease I showed partial degradation to protease II *in vitro*, the process being sensitive to PMSF, indicating its proteolytic nature. These results suggest that formation of a less active protease by autoproteolysis represents a novel means of physiological regulation of protease activity which in turn regulates conidial discharge in *C. coronatus.*

A part of these studies has been published/communicated as:

1. Evidence for the involvement of serine protease in the conidial discharge of Conidiobolus coronatus
   Sangita Phadatare, M.C. Srinivasan and Mukund Deshpande
   Archives of Microbiology (1989) **153:** 47-49.

2. Evidence for controlled autodegradation of alkaline protease and its implication in physiological regulation of conidial discharge in Conidiobolus coronatus
   S.U. Phadatare, M.C. Srinivasan and V.V. Deshpande
   (Communicated)

C O N T E N T S

CHAPTER-I

GENERAL INTRODUCTION

Proteases are hydrolytic enzymes capable of degrading proteins into small peptides and amino acids. From commercial point of view, they are the most important group of enzymes and account for nearly 60% of the total industrial enzyme market (1). They are obtained from plant, animal and microbial sources. Some of the commercially important proteases are: papain and ficin from plants, trypsin from animals and a large number of acidic, neutral and alkaline proteases from diverge microorganisms such as Bacillus, Aspergillus, Rhizopus, Mucor and Conidiobolus. Proteases constitute a very large and complex group of enzymes which differ in properties such as substrate specificity, active site and catalytic mechanism and pH and temperature activity and stability profiles. Commercial proteases have applications in a range of processes which take advantage of the unique physical and catalytic properties of the individual enzyme types. Proteases also play an important role in the metabolic and regulatory processes of the cell. The investigations on proteases provide considerable knowledge about the structural and enzymatic nature of the proteases and have also assisted substantially in examination of their multitude of roles in nature. In spite of the extensive studies carried out on proteases, there still exists a lacuna in our knowledge of these enzymes and they still remain the most sought after group of enzymes from commercial as well as physiological points of view.

Historical background

Proteases are the most ancient of the enzymes in use. Milk clotting enzymes have been used to transform milk into products such as cheese since about 5000 BC, when it was observed that milk carried in calf stomach tended to curdle. Pancreatic proteases were used for dehairing and bating of hides and as presoak detergents since about 1910. Use of animal and microbial rennets in cheese making and proteases of Aspergillus oryzae in baking are other two prominent applications of proteases. Fungal proteases have been used for centuries in the orient for production of soy sauce, tamari sauce and miso.

The following are some of the important milestones in modern protease technology:

1) In 1905, Röhm in Germany established the usage of proteases for dehairing and bating of hides.

2) In 1913, the first known enzymatic presoak detergent 'Burnus' was marketed by Rohm and Hass Company.

3) In 1930's, Bio-38 containing pancreatic trypsin was introduced by Jaag in Switzerland.

4) In 1959-60 Bacillus alkaline proteases took over pancreatic trypsin and were found to be more advantageous for use in detergents. Protease containing detergent, 'Biotex' increasingly captured the detergent market.

5) In 1969, the detergent industry met with unfavourable publicity when some detergent workers developed allergies

due to the dusting effect of the enzyme. The problem was overcome by the introduction of dust-free preparations for use in detergents. This was accepted by the FDA in 1971 and sales of enzyme washing powder again began to increase. Alkaline detergent proteases now account for 25% of the total industrial enzyme sales.

Since 1971, the search for protease of improved thermal and alkaline stability has continued. Apart from use of a highly alkaline stable enzyme from Bacillus sp. in detergents and leather dehairing applications, no major developments have occurred in this area. In food processing, establishment of microbial cheese making enzymes as rennet substitutes has been the major recent advance in the application of microbial proteases. Upto 10 years ago, proteases were regarded as degradative enzymes which could only catalyse the total hydrolysis of proteins. However, recent specific and sensitive assay techniques with more selective substrates have demonstrated that proteases carry out highly specific and selective modifications of proteins by limited hydrolysis and they can also carry out reverse reactions, i.e. peptide synthesis instead of hydrolysis under controlled conditions. Thus considerable emphasis is placed on research on proteases. The discovery of new, highly specific proteases and improved enzyme technology such as immobilization and novel purification methods make proteases an attractive group of enzymes from a basic as well as technological viewpoint.

Types of proteases

Based on the type of catalytic mechanism (2) proteases are classified into four groups, viz. serine proteases, cysteine (thiol) proteases, aspartic (acid) proteases and metallo-proteases. This classification is according to the reactivity of the protease towards inhibitors which react specifically with particular residues in the active site region.

1. Serine proteases:

The serine proteases have a reactive serine residue in the active site and their activity is inhibited by the reaction of this particular serine residue with diisopropyl fluorophosphate (DFP) and phenyl methyl sulfonyl fluoride (PMSF). They are the most widely distributed group of proteolytic enzymes in microbial as well as animal systems. They are reported from a number of microorganisms such as, Arthrobacter, Bacillus, Staphylococcus, Saccharomyces, Aspergillus, Neurospora etc. Trypsin, chymotrypsin, subtilisin and elastase are some of the important serine proteases.

Serine proteases are generally active over a range of neutral and alkaline pH, with an optimum between pH 7 - 11. They have broad substrate specificities, including considerable esterolytic activity towards many ester substrates and are generally of low molecular weight (18.5 - 35 KDa). However, the largest serine protease reported is the Blakeslea trispora enzyme with a molecular weight of 126 KDa (3).

Most of them have isoelectric points between pH 4.4 and 6.2. Morihara (4) classified serine proteases into four sub-groups, according to their side chain specificity towards oxidised insulin β-chain, as: trypsin-like proteases, alkaline proteases, Myxobacter α-lytic protease and staphylococcal protease.

2. Cysteine proteases:

The cysteine proteases have a reactive cysteine residue in the active site. They are sensitive to sulphydryl reagents such as parachloromercury benzoate, N-para-Toluene sulfonyl-L-Lysine chloromethyl ketone, iodoacetic acid, iodoacetamide, heavy metals and are activated by reducing agents like potassium cyanide or cysteine, dithiothreitol and EDTA. The occurrence of this type of proteases has been reported in a limited number of fungi. Extracellular cysteine proteases have been reported from fungi viz. Aspergillus, Microsporum, Sporotrichum and bacteria such as Bacteriodes, Closteridium and Streptococcus etc.

Cysteine proteases are generally active between pH 5 to 8. They can be divided into two groups, according to their side chain specificities, viz. clostripain and streptococcal proteases.

3. Aspartic proteases:

The aspartic proteases are characterised by maximum activity at low pH values (3 - 4) and by insensitivity towards inhibitors of serine, cysteine and metallo-proteases.

They have a reactive aspartic acid residue. Most aspartic proteases are sensitive to epoxy and diazoketone compounds in the presence of copper ions. They are also inhibited by pepstatin or Streptomyces pepsin inhibitor. They are widely distributed in fungi, but are rarely found in bacteria or protozoa. They are reported from Aspergillus, Penicillium, Rhizopus, Neurospora, Mucor, Entothia etc.

Most aspartic proteases have molecular weights in the range of 30 - 45 KDa and pI in the range of pH 3.4 - 4.6. These enzymes are specific against aromatic amino acid residues on both sides of the cleavage points. Two types of aspartic proteases have been recognised, viz. pepsin-like and rennin-like. Pepsin-like proteases are used in soy-sauce manufacture and rennin-like proteases, which show milk clotting activity, are used in cheese manufacture.

4. Metalloproteases

Metalloproteases require divalent metal ion for their activity. They are sensitive to metal chelating agents, such as, EDTA and can be reactivated by zinc, calcium or cobalt. Some of the important metalloproteases are reported from Bacillus, Pseudomonas, Serratia, Aspergillus and Penicillium. These enzymes can be divided into five groups viz. acid, neutral and alkaline proteases and Myxobacter alkaline protease I and II. Each group shows different side chain specificity. Optimum pH is 5-6, 7 and 7 - 9 for acid, neutral and alkaline proteases, respectively.

Distribution of microbial alkaline proteases

Alkaline proteases are reported from a large number of bacterial and fungal sources. Among the bacterial alkaline proteases, subtilisins are the most extensively studied proteases. Species of Bacillus (5-8) are among the most potent alkaline protease producers and proteases from this group have found widespread industrial applications. Some of the other bacterial protease producers include Arthrobacter (9, 10), Achromobacter (11, 12), Bacteriodes (13, 14), Actinomycetes (15-17), Pseudomonas (18), Serratia (19), Thermus sp. (20, 21) etc.

Alkaline proteases are also produced by several fungi. North has reviewed comparative biochemistry of proteases of eukaryotic microorganisms (22). Alkaline proteases have been isolated and characterised from a variety of Hyphomycetous fungi, Aspergillus oryzae, A. flavus, A. fumigatus, A. sydowi, A. sojae, A. candidus, A. sulphureus, A. ochraceus Alternaria tenuissima, Alternaria alternata (Fr.), Kiessl, Achremonium kiliense, Achremonium chrysogenum and Cephalosporium sp. Detailed information regarding these proteases is documented in chapter V. Alkaline proteases have also been reported from Fusarium semitectum (23), F. vasinfectum (24), Gliocladium roseum (25), Malbranchea pulchella var. Sulfurea (26-29), Penicillium lilacinum (30), P. roqueforti (31), P. charlesei (32), P. cyaneofulvum (33, 34), Phymatotrichum omnivorum (35, 36), Scopulariopsis brevicaulis (37) and Torula thermophilia (38).

Alkaline protease production by fungi belonging to Phycomycetes has been relatively little investigated. There are very few reports on production and characterization of alkaline proteases from this group of fungi. Proteases from Phycomyces blaklesleeanus (39), Blastocladiella emersoni (40) and Blakeslea trispora (3) have been well studied. Jonsson (41) has reported proteolytic activity by Entomophthora sp. in submerged cultures. Alkaline protease production by E. coronata is described by Hurion and his coworkers (42). A patent granted to American Cyamide Company (43) describes alkaline protease production by Entomophthora, Basidiobolus and Conidiobolus sp. Tokuyama and Asano (44, 45) reported production, purification and characterisation of protease from a Conidiobolus sp. An extracellular protease accompanied by chitin binding hemagglutinin of Conidiobolus lamprauges (45) has been partially purified and characterised.

Physiological functions of microbial proteases

Proteases are also important from physiological point of view. A number of functions have been attributed to proteases. Holzer *et al*. (46) and Ward (47) reviewed the physiological functions of proteases, some of which are discussed here in brief.

1. Protein turnover:

Protein turnover is essential for the adaptation of cells to new environmental conditions, particularly in environments lacking in amino acids, as it provides an amino acid pool for the synthesis of newly required enzymes. *E. coli* deprived of nitrogen can still synthesize β-galactosidase in response to an inducer because of increased protein catabolism, but when protein degradation is blocked, enzyme synthesis is prevented. There is evidence that ATP plays some role in intracellular proteolysis.

2. Nutrition:

A major role of proteases in nature is nutritional, i.e. to hydrolyse large polypeptide chains of proteins into smaller peptides and amino acids which are then utilised by the various cells.

3. Spore formation:

Bacterial endospore formation as well as organisation of slime mould fruiting body has been shown to be induced by nutrient depletion. This involves extensive protein turnover where under endotropic conditions new developmental

proteins in the mother cell and the evolving spore are derived intracellularly. In the bacterial endospore, turnover rates as high as 18% per hour have been observed. Extensive protein turnover also occurs during ascospore formation in yeast diploids. The process of aggregation of slime moulds into multicellular units under starvation conditions and differentiation to a stalk and spore containing fruiting body is also accompanied by extensive proteolysis. Extensive work has been carried out on the role of proteases in Bacillus spore formation with the help of specific mutants and inhibitors (48).

4. Spore germination:

Proteolysis during germination of spores provides amino acids which the spore is unable to synthesize at early stage of germination. This has been well studied in case of Bacillus and Closteridium. Proteases are also involved in eucaryotic germination and hyphal fusion. Macroconidial germination of Microsporium gypseum is controlled by its alkaline protease. A reaction occurring after hyphal fusion of incompatible alleles of Podospora anserina is related to high protease activity.

5. Modulation of gene expression:

Roberts et al. (49) demonstrated derepression taking place as a result of repressor degradation by an ATP-requiring protease. In Bacillus thuringiensis there is strong evidence for an involvement of an intracellular

protease in the modification of the B' sub-unit of RNA polymerase. A possible role of proteases in regulation of translation by modification of ribosomal proteins has also been suggested.

6. Enzyme modification:

Proteases are involved in inactivation of vegetative enzymes during or prior to cell sporulation as seen in case of aspartate transcarbamylase and phosphoribosyl pyrophosphate amidotransferase of B. subtilis and tryptophan synthetase from yeast and Neurospora. Holzer et al. (46) has documented many other examples of proteolytic enzyme inactivations. Other enzymes modified, but not inactivated by proteases are fructose-1,6-diphosphate aldolase of B. cereus, RNA polymerase of B. thuringiensis, etc.

Proteases remove the N-formyl methionine or methionine from nascent polypeptide chains and cause cleavage of translational product of monocistronic mRNA coding for several distinct peptide chains.

7. Enzyme secretion:

Extracellular enzymes are generally synthesized at the cell membrane in a precursor form and then released in the final active form by proteolysis. Peptide extension which is removed in this process is of hydrophobic nature, which facilitates the passage of enzyme through the cell membrane.

Extracellular serine protease of B. licheniformis converts membrane bound penicillinase of this organism into

free extracellular form. Neutral protease of Staphylococcus aureus is activated by its extracellular serine protease.

8. Yeast cell budding:

Model of yeast cell budding is given by Cabib and Farkas (50). Serine protease at the site of septum formation specifically activates a chitin synthetase, which in turn, catalyses synthesis of chitin. A chitin septum is thus formed between mother and bud cell and bud is separated.

Chitin synthetase activation by proteases is responsible for cell wall growth of fungi.

Proteases are also involved in other processes, viz. extension of fungal hyphal tip during growth (51), activation of certain viruses for pathogenicity, protein maturation in viral assembly, mammalian fertilization process, blood coagulation, fibrinolysis and control of blood pressure, hemostasis, inflammation, complement action, cell-cell interactions, gene depression and hormone metabolism.

1. Detergents:

Proteases used in detergents must be stable between pH 9 - 10.5, should possess temperature stability, be compatible with oxidising and chelating agents, and be effective for stain removal at low enzyme level (0.4 - 0.8%) in the detergent solution. The Bacillus alkaline proteases have met with these requirements successfully. Two of the serine alkaline proteases from the alkalophilic bacilli, Esparase and Savinase, along with Alcalase are used in the detergents. The proteases from alkalophilic bacilli are especially advantageous for use in heavy duty and liquid detergents.

2. Cheese manufacture:

Proteases of Mucor miehei, M. pusillus var. Lindt and Endothia parasitica are used as calf rennet substitutes for cheese manufacture. The alkaline protease, subtilisin DY has also been used as rennet substitute. It had an advantage that no bitterness developed in the milk (54). During ripening, cheese proteins are cleaved by the proteases at various sites and protein network which forms the structural component of fresh cheese curd, loses part of its original structure and the rheological properties of cheese are altered. The action of proteases during cheese ripening contributes to the great variety of cheeses produced.

3. Preparation of soy products:

Fungal proteases have been used for centuries in the orient for preparation of soy sauce and other soy products.

Soy sauce is prepared by incubating soybeans, wheat and salt with a mixture of mould, yeast and bacteria and allowing the proteins, carbohydrates and other constituents of soybeans and wheat to be hydrolyzed to peptides, amino acids, sugars and other low molecular weight compounds by the enzymes produced by the microorganisms. Neutral and alkaline proteases of the Aspergillus sp. are mainly used for soy sauce preparation. Nakadai and Nasuno (55) have reported soy sauce production using Aspergillus protease. Immobilized subtilisin was used to carry out limited modifications of soya proteins by Herbert and Dunnill (56) to improve its quality as food.

4. Baking:

Proteases are added to modify wheat gluten and milk proteins during preparation of bread. The limited proteolysis of dough improves the elasticity of the gluten which permits easier machining and consequently results in increased loaf volume, greater symmetry and better grain and texture. The addition of proteases also reduces mixing time by about 30% without detrimental effect on handling properties of the dough. A. oryzae and B. amyloliquefaciens proteases are used for baking. Fungal and bacterial proteases are also used in cracker, biscuit and cookie manufacture, improving the extensibility and strength of the dough, allowing it to be rolled very thinly without tearing.

5. Leather tanning:

Unwanted interfibrillar material of hides is removed

in three step process involving soaking, dehairing and bating, in all of which proteases are used. The purpose of soaking is to swell the hide, which was traditionally achieved by addition of alkali. Currently microbial alkaline proteases like Alcalase, Milezyme are used for this purpose. Dehairing is effected with the help of highly alkaline proteases of Bacillus sp. During bating, collagen of the dehaired hides is delimed and deswollen and protein fibres are partially degraded to make them soft and elastic. A. oryzae, B. licheniformis and B. amyloliquifaciens proteases and trypsin are used for this purpose.

6. Protein hydrolysis:

Proteases are used for meat tenderisation, meat solubalisation and solubilisation of fish protein concentrates, which are of high nutritional value, but are insoluble in water, as a result of the solvent extraction methods used. Protein hydrolysates are extensively used as food for children and invalids. The proteases, especially of Aspergillus, B. subtilis proteases along with papain, ficin and bromelain are used for this purpose. Proteases are also used for decolourisation of blood, which is an important but under-utilised, source of food protein.

7. Medical applications:

Medical applications of proteases are reviewed by Christie (57). The enzyme has been used as digestive aids in gastro-intestinal disorders such as dyspepsia. Streptokinase from Streptococcus pyogenes is used for treatment of

thrombosis. In dental care, a mutanase-protease mixture has been used to reduce dental plaques and improve the clinical condition of palatal mucosa or denture stomatitis. Alkaline protease from B. licheniformis (58) has been used in denture cleanser tablet. Proteases from Serratia marcescense are used for debridement of dermal ulcers and burns. Proteases are also used as potential bacteriocidal agents and for removal of protein contaminants from antibiotic preparations.

8. Peptide synthesis:

The ability of proteases to catalyse peptide bond formation was demonstrated 50 years ago, but it is only in the recent years that, enzymatic peptide synthesis has been used as a preparative method for the modification of proteins. Protease catalysed peptide synthesis has several advantages over the chemical methods. The enzymatic reaction can be carried out in aqueous phase without racemisation, does not require protection of side chain functional groups since the reactions are catalysed at the α-amino and α-carboxyl groups of the amino acids and peptide derivatives. Consequently, a reduced number of side reactions occur, which simplifies the purification procedures and allows the reuse of reaction components. Thus partial or total enzymatic peptide synthesis yields a clean product at a lower cost than the chemical synthesis methods.

Protease catalysed peptide synthesis is carried out by any of the following three methods: 1) reversal of peptide

bond hydrolysis, (ii) aminolysis of esters and (iii) aminolysis of amides (transpeptidation). Literature survey shows that this is the area of major interest today, as judged by the large number of reports on protease catalysed peptide synthesis using trypsin, chymotrypsin, thermolysin, carboxypeptidase Y and subtilisin (59 - 67).

9. Other applications of proteases:

Proteases are used in brewing for body, flavour and nutrient development of beer and as aid in filtration and clarification. They play an important part in chill proofing of beers, preventing precipitation of proteins during cooling of beer preparations.

Other applications of proteases include, recovery of silver from spent photographic films (68), desizing of fabrics, optimal resolution of amino acids, for dissociation of cells from monolayer animal cell culture (69) etc. Proteases are also used for research purposes, such as, trypsin for protein fingerprinting and pronase, protease k for deproteinisation of DNA preparation in molecular biology.

Recent advances in protease technology

Immobilisation:

Immobilisation, i.e. conversion of enzymes from a water soluble, mobile state to a water insoluble, immobile state can be carried out by (i) covalent attachment or adsorption of enzymes on solid supports, (ii) entrapment of enzymes in polymeric gels, (iii) cross-linking of enzymes with bifunctional agents or (iv) microencapsulation of enzymes. This stabilises the enzyme against different kinds of inactivation.

Immobilised proteases have been used in removal of proteins from antibiotic preparations (70) and in peptide synthesis (71). In peptide synthesis, a waterwet Amberlite XAD-7 immobilised thermolysin has been employed. Peptide synthesis is also catalysed by immobilised carboxypeptidase Y and trypsin (72) and Achromobacter lyticus protease 1 (73). Protease immobilisation by microencapsulation also enhances cheese ripening (74). For leather tanning also, the use of immobilised dehairing proteases, with the enzyme bound to clay which allows it to be mixed with the other chemicals needed for dehairing has been suggested (75).

Protein engineering:

Despite the success of proteases in industrial processes only a few naturally occuring proteases are used commercially This is because the requirements of many industrial applications are different from the physiological properties of the enzymes. Moreover, the conditions such as pH, temperature, desired substrate etc. which are used in industry are

non-physiological, which leads to instability or sub-optimal function of these enzymes. Improvement of properties of native enzymes by mutation and selection programmes and chemical modification has been partially effective.

Recent advances in recombinant DNA techniques, site-directed mutagenesis and chemical synthesis of DNA fragments have enabled the systematic alteration in protein structure. As a result, improvements in both the functional and structural characteristics of the enzyme are now possible. Protein engineering is dependent on the prior knowledge of the three dimensional structure of the protein. X-ray crystallographic structures of aspartic protease (76), thermitase (77), proteinase K (78), a protease from B. alcalophilus (79) and protease (80) are reported. Multinucleate magnetic resonance studies are carried out on the Ca^{++} binding sites of trypsin, chymotrypsin and subtilisin.

Subtilisin has been chosen as a model system for protein engineering, since the crystal structure of enzyme and enzyme substrate complex are known, the enzyme has been cloned and expressed in large quantities. One of the interesting modification by side directed mutagenesis of subtilisin is the replacement of methionine by phenylalanine at residue 222, adjacent to active site serine 221. The mutant subtilisin, instead of normal preferential hydrolytic action, exhibits a preference for transesterification reactions (81), which is useful for peptide synthesis. Another example is the enhancement of thermostability of subtilisin E by introduction

of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease (82).

Molecular cloning, expression and characterisation of protease gene from a number of organisms is reported. Protrease gene from Lactobacillus lactis subsp. cremoris H_2 is cloned into E. coli and L. lactis subsp. lactis 4125 (83). A metalloprotease gene from Streptomyces cacoi was cloned in S. lividans (84). The cDNA, encoding subtilisin-like serine proteases TW7 and TW3 is cloned from slow growing fungus Tritirachium album and was sequenced and expressed (85), while protease K from this organism was cloned in E. coli (86). An alkaline protease was cloned from B. pumilus to B. subtilis (87). Another protease from Pseudomonas aerusinosa was cloned in E. coli (88, 89) and transformants were selected using anti-alkaline protease antibody. cDNA clone for alkaline protease of Aspergillus oryzae was introduced into Saccharomyces cerevisiae (90). Achromobacter protease I gene cloned and expressed in E. coli was sequenced (91). Gene responsible for extracellular serine protease of Lactobacillus lactis subsp. cremoris SK11 was cloned in E. coli (92). Gene for alkaline serine exoprotease of Vibrio alginolyticus was cloned in E. coli and its nucleotide sequence was determined. This protease is calcium dependent and detergent resistant (93). Structural gene for α- lytic protease was cloned from Lysobacter enzymogenes (94).

Present investigation

Although, fungal alkaline proteases have been extensively studied, there are very few reports on alkaline protease production from the phycomycetous fungi, especially *Conidiobolus* sp. The present study deals with alkaline, serine protease from the novel, little investigated fungus *Conidiobolus coronatus* (NCL 86.8.20). The main features of the work are:

1. Screening for high protease yielding strain of *Conidiobolus* and optimisation of the fermentation conditions for maximum production of alkaline protease in submerged culture.
2. Investigation of possible involvement of alkaline protease in the forcible discharge of conidia, through the following approaches:

(a) Isolation of a specific variant with reduced conidial discharge by UV-irradiation and its comparison with the parent wild strain with respect to conidial discharge and protease levels.

(b) Study of effect of protease inhibitors and inducers on conidial discharge.

3. Purification of the low and high molecular weight alkaline proteases.
4. Study of biochemical, physicochemical and immunological relationship between the two purified alkaline proteases and its implication in physiological regulation of conidial discharge.
5. Preliminary evaluation of compatibility of alkaline

protease with some commercial detergents.

Conidiobolus species are characterised by the unusual phenomenon of forcible discharge of their mature conidia from the unbranched conidiophores. The biochemical basis of this phenomenon was not known. The present studies show the involvement of serine protease in the conidial discharge, thus attributing a new physiological role to serine proteases. The studies also show that formation of a low molecular weight, less active protease from the larger protease by autolysis provides a novel means of physiological regulation of serine protease in Conidiobolus which in turn regulates the conidial discharge. The homology between the two enzymes has been established by biochemical, physicochemical and immunological comparison of the two purified proteases.

The thesis also describes formulation of a medium for maximum production of the alkaline protease which has potential for use in detergents. Preliminary studies to evaluate the compatibility of the enzyme with some of the commercial detergents marketed in India have shown promising results.

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CHAPTER-II

PRODUCTION AND APPLICATION OF ALKALINE PROTEASE OF *CONIDIOBOLUS CORONATUS* (NCL 86.8.20)

SECTION I

PRODUCTION OF ALKALINE PROTEASE

Summary

A number of Conidiobolus strains isolated from decomposing plant detritus were tested for alkaline protease production. The isolate C. coronatus (NCL 86.8.20) showing maximum protease activity was selected for further studies.

Effect of various medium constituents, viz. inorganic and organic nitrogen sources, sugars, sugar alcohols, salts, surfactants, inducers and metal ions on alkaline protease production was studied. Fermentation conditions, such as, pH, temperature, time profile and inoculum size were also standardised for maximum protease production. Maximum protease production was observed at 28°C and 200 rpm, for 48 h with the medium containing sucrose, 3%; ammonium nitrate, 0.38%; tryptone, 2%; potassium chloride, 0.2%; potassium dihydrogen phosphate, 0.1%, casein, 2%; zinc sulphate, 0.001%; calcium chloride, 0.001%, initial pH, 7-7.5, using 12% (v/v) preinduced inoculum grown in the same medium under similar conditions for 24 h.

Introduction

In order to obtain high and commercially viable yields of a microbial enzyme, it is essential that a suitable over-producing strain is identified and isolated and the factors controlling its growth and production are optimised. Micro-organisms are specific in their nutritional requirements. They exhibit diverse pattern of preferences towards different nutrients and are dependent on various substrates for the synthesis of their cellular constituents. Environmental conditions such as pH of the medium, temperature, aeration, nature and concentration of substrates, presence of salts, metal ions etc. greatly influence the growth and production of a particular compound by the organism. Fungi grow in a variety of media and under various conditions. A typical medium contains a carbon source, a nitrogen source, salts, metal ions and, if necessary, growth factors. A particular culture can be "induced" to synthesize a specific product by manipulation of nutritional conditions. However, due to diverse nutritional requirements of fungi, fermentation conditions standardised for one fungus may not be suitable for the other for the production of the same product. Jonsson (1, 2) studied many fungal cultures for protease production and concluded that the amount of protease produced varies greatly with the strains and media used.

In view of the vast industrial potential of alkaline proteases, the bulk of the literature on proteases is in

patents which tends to concentrate on their commercial production. Table II-1 lists some of the commercially used protease producing strains (3).

Screening for protease producers:

With many extracellular products, it has been possible to relate the level of productivity of the strains to the magnitude of radius of the product's zone of diffusion from microbial colonies grown on agar media. This approach has not been always found to be satisfactory in the screening of proteases. Aunstrup (4) showed that correlation between the diameter of protease zone clearing around a colony on agar medium and capability of the organism for protease production in submerged culture is not mandatory, as seen in the case of Bacillus sp. and Aspergillus sojae.

Semisolid/submerged production of proteases:

Proteases from Aspergillus sulphureus (5), A. oryzae (6), Penicillium cyaneofulvum (7), Fusarium semitactum (8) and Mucor pusillus (3) are produced by semisolid, static fermentations, usually on wheat bran moistened to 50%. It is generally accepted that semisolid cultivation results in higher yields of the enzyme than that in submerged culture. In semisolid fermentation, mycelium is exposed to atmospheric air and is allowed to grow undisturbed in its usual form, which permits development of spores and fruiting bodies to a much greater extent than the submerged fermentation. But this method has one disadvantage viz. it is almost impossible

Table II-1 : Commercially important protease producing microorganisms

Source	Protease (trade name)
Aspergillus niger	Proctase, Pamprosin
Aspergillus oryzae	Veron P, Panazyme, Prozyme, Biozyme A, Sanzyme, Sumyzyme AP, Fungal protease
Rhizopus sp.	Newlase
Bacillus amyloliqueifaciens (*Bacillus subtilis*)	Newtrase, Rapidermase Proteinase 18, Protin, Bioptrase, Nagasse, Rhozyme
Bacillus licheniformis	Alcalase, optimase, Maxatase P
Bacillus thermoproteolyticus	Termoase, Thermolysin
Bacillus sp., alkalophilic	Esperase, Savinase, Highly alkaline protease
Endothia parasitica	Surecurd, Suparen
Mucor miehei	Rennilase, Fromase, Marzyme, Morcurd
Mucor pusillus	Emporase, Meito rennet, Noury lab

to feed ingradients to the medium during fermentation, which means that change of pH value and carbohydrate concentration are predetermined.

The proteases of Mucor miehei and Bacillus sp. are produced in submerged culture.

Continuous or semicontinuous method of protease production are not known to be commonly used in commercial practice.

Composition of media used for protease production

Carbon source:

Various compounds have been used as carbon sources for protease production such as, glucose (9-15), sucrose (16-19), starch (20-23), gram flour (17), wheat bran (18, 24, 25), lactose (26), maltose (27, 28), dextran (29), sweet potato meal (30), corn steep liquor (31), fructose (18), sodium pyruvate (32, 33), methyl acetate (34), barley and potato (35), paraffins (36), liver extract (2), alkanes (37, 38), silk worm powder (38), glycerol (39), mannitol (40).

Glucose seems to be the most preferred carbon source for protease production. However, in case of Aeromonas hydrophila (3), Lagenidium giganteum (41) and Aspergillus nidulans (42) repression of protease production by glucose is reported. While Leptomitus lacteus (43) failed to utilise glucose and preferred fatty acids, organic acids and glycerol as main source of energy, organic acids served as the sole carbon source for protease production by various Bacillus sp., Candida lipolytica and Rhodotorula glutinis (44, 45).

Nitrogen source:

Inorganic compounds like ammonium or nitrate salts, organic compounds like proteins and amino acids serve as efficient nitrogen sources for protease production by many fungi.

Tyrosine stimulated protease production in Alternaria alternata (Fr.) Keissl (18), glutamine served as sole C and N source for Pseudomonas fluorescens (32), while all amino acids except methionine served as nitrogen for the latter. Serine, asparagine, glycine, tyrosine, alanine supported protease production in Entomophthora sp. (46). Leucine enhanced protease production in Serratia (47). Aspartate, methionine, arginine, isoleucine, leucine or valine served as sole source of nitrogen for protease production by Aspergillus terricola (48). Though, on the other hand, amino acids can lead to repression of protease production, as seen in case of Conidiobolus adiaeratus (49), wherein threonine inhibits protease production. Isoleucine and proline have repressive effect on protease production in Bacillus sp. (50). End product repression by amino acids has been reported in case of Sarcina sp.(51) and Arthrobacter sp. (52).

Some organisms prefer proteins and peptides to amino acids as nitrogen sources, for both growth and protease production. In case of Erwinia chrysanthemi replacement of peptone with amino acids results in reduction in protease

production, indicating the possible involvement of intact peptide bonds in the induction process (53).

Various organic compounds have been used as nitrogen source for protease production. Soybean meal (9, 20, 54, 55 56), casein (29, 47, 57), peptone (7, 26, 58-60) and yeast extract (41, 61, 62) are the most widely used nitrogen sources, the other sources being, meat extract, tryptose, tryptone (12), urea (63), gelatin (64), liver digest (18, 65, 66), maize steep liquor (67), fish meal (35), bovine serum albumin (10), skim milk (32, 68), whey (69) etc.

Effect of salts and metal ions:

In addition to carbon and nitrogen, microorganisms require other elements like potassium, magnesium, sulphur, phosphorous and trace elements like manganese, copper, zinc, molybdenum, calcium etc. (70) for growth and protease production.

Magnesium sulphate is used in most of the fermentation media designed for protease production (18, 2). Phosphate ions are known to support protease production in many organisms (12, 20, 33, 71). Manganese chloride stimulated protease production in Streptomyces sp. (23) and calcium ions enhanced protease production in Pseudomonas fluorescens (72).

Compounds like polycations (73) and silicon (74) have been used to promote protease production in Bacillus subtilis and Aspergillus soya respectively.

Growth phase and protease production:

Protease production can occur at stationary phase of growth as in case of thermophilic Streptomyces sp. (23), or in late log and stationary phase as seen in case of P. fluorescens (32). One Bacillus sp. produces protease during post exponential phase of growth (75, 76), some Bacillus sp. however, produce protease during exponential phase of growth (77). Peptidase activity in B.. megaterium cell was present at all stages of growth (78). A lag between growth and protease production was observed in case of Streptomyces sp. (23) and Entomophthora sp. (66).

Recovery of protease from fermentation broth:

As most of the commercially used proteases are extracellular, the most widely employed method for their recovery is filtration or centrifugation. Other methods like liquid-liquid extraction using a reversed micellar organic solvent (79) are also used. Separation is aided by pretreating the solution with a coagulating or flocculating agent. It can be precipitated with inorganic salts, such as calcium sulphate or calcium phosphate. An alternative method is flocculation using synthetic polyelectrolytes such as polyamines. Aluminium and polyacrylamide induced flocculation, followed by filtration is also reported for recovering the enzyme from fermentation broth. In some cases, filter aid, such as diatomaceous earth must be added before filtration.

The present investigation deals with the screening for

high protease yielding strain of Conidiobolus and optimisation of fermentation conditions for the maximum production of alkaline protease from the high yielding strain of Conidiobolus coronatus (NCL 86.8.20).

Materials & Methods

Materials:

Malt extract, yeast extract, peptone and Agar were purchased from Difco Laboratories. All the other reagents used were of analytical grade.

All the buffers and reagents were prepared in double distilled water.

Isolation and screening of *Conidiobolus* strains:

Conidiobolus cultures were isolated from decomposing plant detritus from various places, by the method described by Drechsler (80). Fine particles of decomposing plant detritus were superimposed on MGYP agar (malt extract, 0.3%; glucose, 1%; yeast extract, 0.3%; peptone, 0.5%; agar, 2%, pH 6.5 - 7.0) blocks attached to the inner surface of petri-plate lid and the plates were incubated at 28°C. Single colonies developing from forcibly discharged conidia were picked up and transferred to MGYP agar slants, incubated at 28°C for 72 h. The isolates were stored at 15°C.

The isolates were tested for protease production by inoculating 100 ml MGYP broth with 2% (w/v) casein, in 500 ml Erlenmeyer flasks using 72 h grown cultures as inoculum. The flasks were incubated at 28°C and 200 rpm on a rotary shaker for 48 h. After the fermentation, the cells and insoluble debris in the fermented broth were separated by filtration with Whatman No. 1 filter paper and the filtrate was tested for protease activity. The same method was used

for isolation of the enzyme in all the subsequent studies.

Fermentation:

Preparation of inoculum. C. coronatus culture grown for 48 h on MGYP slants was transferred to 100 ml MGYP broth in 500 ml Erlenmeyer flasks. The flasks were incubated at 28°C and 200 rpm for 24 h. The mycelium was separated from broth by centrifugation, washed with sterile distilled water and resuspended in equivalent amount of sterile distilled water. This was used as inoculum in the fermentation studies.

Protease production. Czapek Dox medium (sucrose, 1%; sodium nitrate, 0.3%; dipotassium phosphate, 0.1%; Magnesium sulphate, 0.05%; potassium chloride, 0.1%, ferrous sulphate, 0.001%, pH 7.0) modified at every step was used as fermentation medium. 10% (v/v) inoculum was used to inoculate 100 ml fermentation medium in 500 ml Erlenmeyer flasks. After incubation at 28°C and 200 rpm for 48 h, the flasks were harvested as described earlier and protease activity in each flask was determined.

Enzyme assay:

The protease activity was determined according to Kunitz (81). The reaction mixture contained an aliquot of suitably diluted enzyme solution and Hammersten casein (10 mg) in 0.1 M sodium carbonate buffer, pH 9.7 in a total volume of 2 ml. After incubation at 37°C for 10 min, the reaction was terminated by addition of 3 ml of 5% trichloroacetic acid (acidified with concentrated hydrochloric acid). The

precipitate thus formed was filtered through Whatman No. 1 filter paper after standing for 30 min at room temperature. The absorbance of trichloroacetic acid soluble fraction was measured at 280 nm.

One unit of protease activity was defined as the amount of enzyme resulting in an increase of 1.0 absorbance per ml of reaction mixture per minute at 37°C.

Results and Discussion

Isolation and screening of Conidiobolus strains:

A number of Conidiobolus strains isolated from decomposing plant detritus were tested for alkaline protease production. The isolate C. coronatus (NCL 86.8.20) showing maximum protease activity (Table II-2) was selected for further studies.

Conidiobolus coronatus is predominantly a saprophytic fungus, associated with plants. It grows very rapidly. Conidiophores are micronemous and indistinguishable from mycelium. At the tip of the conidiophore, large globose conidia are formed, which are forcibly discharged. The size of conidia varies between 35 - 45 microns. Conidia either germinate to give rise to mycelium or to microconidia on radial sterigmata or to succession of secondary conidia. The discharged conidia form a visible whitish deposit on the glass above the growing culture (Fig. II-1).

Preservation and maintainance of conidiobolus:

Lyophilisation, the most widely used method of preservation was unsuitable for Conidiobolus. The culture could not survive at temperatures below 10°C. Preservation in sterile water at 5 and 15°C was also unsuitable for Conidiobolus. The isolate C. coronatus was preserved on MGYP agar slants at 15°C and subcultured after every 3½ months. The isolate was maintained on MGYP agar slants at 28°C.

Table II-2 : Screening for high alkaline protease producing *Conidiobolus*

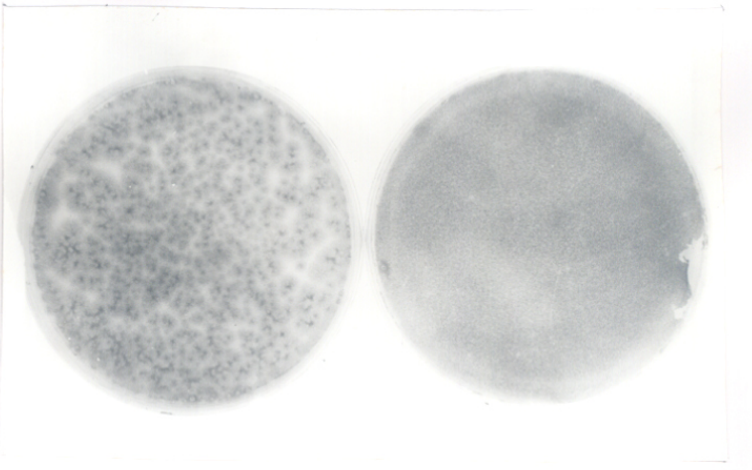
Organism	*Activity (U/ml)
C. mycophilus (NCL 86.6.6) (microconidia forming, zygosporic)	0.160
C. macrosporus (NCL 86.6.13) (microconidia forming, zygosporic)	0.320
C. macrosporus (NCL 86.6.15) (zygosporic)	1.096
C. coronatus (NCL 86.8.20) (Poona strain)	12.0
Conidiobolus sp. (NCL 87.1.13) (Khandala strain)	0.406
Conidiobolus sp. (NCL 87.1.14) (Khandala strain)	0.660
C. coronatus (NCL 87.1.15) (Mahabaleshwar strain)	0.236
C. brefeldianus (NCL 87.5.1) (zygosporic)	0.272
C. brefeldianus (NCL 87.5.2) (zygosporic)	0.224
Conidiobolus sp. (NCL 87.5.16)	0.400
C. nanodes (NCL 88.4.30) (zygosporic)	0

* Protease activity was determined after fermentation in MGYP broth (containing 2% (w/v) casein), at 28°C and 200 rpm for 48 h.

Fig. II-1 C. coronatus (NCL 86.8.20) grown on MGYP medium

A, C. coronatus grown at 28°C for 48 h.

B, Top view of the plate showing discharged conidia



A B

Optimisation of fermentation conditions for maximum protease production:

Alkaline protease production was tested in MGYP broth with 2% casein, under both static and shaking conditions (200 rpm) at 28°C. Protease production under stationary conditions was poorer (5 U/ml) as compared to that under shaking conditions (12 U/ml). Hence, the fermentation conditions for maximum protease production were standardised in submerged culture.

Effect of various medium constituents, viz. inorganic and organic nitrogen sources, sugars and sugar alcohols, surfactants, inducers and metal ions on protease production was studied. Fermentation parameters such as, pH, temperature, time profile and inoculum size were also standardised for maximum protease production. Although, 12 U/ml protease activity was obtained in MGYP broth with 2% casein, for the fermentation studies, a synthetic medium, i.e. Czapek Dox minimal medium was used. Although the activity was poor (0.075 U/ml) in this medium, it was preferred as the basal medium, as it allowed a systematic study of various nutritional and environmental factors on the alkaline protease production. At every step, the medium was modified with respective constituents and the newly modified medium was used as a basal medium for the subsequent studies unless otherwise specified.

Effect of various inorganic nitrogen sources:

Various compounds were tested as sources of inorganic

nitrogen for alkaline protease production by C. coronatus, using Czapek Dox medium (medium A) devoid of sodium nitrate as the basal medium. As seen from Table II-3, maximum protease activity (0.223 U/ml) was obtained with ammonium nitrate. It gave maximum protease activity (0.35 U/ml) at a concentration of 0.38% (w/v), the higher concentrations being inhibitory (Fig. II-2). The medium modified with 0.38% (w/v) ammonium nitrate, in place of sodium nitrate, was used in the subsequent studies (medium B).

Increased production of protease due to incorporation of ammonium salts in the medium was observed in case of Bacteriodes amylophilus (28). It is seen from Table II-3, that protease production was higher in the media containing ammonium salts as compared to those containing respective sodium salts, indicating that in case of C. coronatus, ammonium salts support the protease formation better than the sodium salts. This was also observed in case of protease from Pseudomonas aeruginosa (12). On the other hand, in case of Aspergillus nidulans (42), ammonium ions repressed the protease formation.

Effect of sugars and sugar alcohols:

Medium B (i.e. Czapek Dox medium modified with ammonium nitrate) devoid of sucrose was used as the basal medium. Various sugars and sugar alcohols were added to the basal medium at varying concentrations [1-5%(w/v)]. As seen from Table II-4, except for arabinose, maximum protease activity

Table II-3 : Effect of inorganic nitrogen sources on alkaline protease production

Nitrogen source	Concentration % (w/v)	Activity (U/ml)
Sodium nitrate	0.3	0.074
Ammonium sulphate	0.15	0.140
Potassium nitrate	0.25	0.076
Ammonium nitrate	0.19	0.223
Ammonium chloride	0.13	0.158
Diammonium hydrogen phosphate	0.15	0.150

- Czapek Dox medium devoid of sodium nitrate was used as a basal medium.
- The inorganic nitrogen compounds were added at equivalent nitrogen concentrations.
- The final pH was 7.0, in all cases.

Fig. II-2 **Effect of ammonium nitrate on production of alkaline protease by C. coronatus**

Czapek Dox medium (medium A) devoid of sodium nitrate was used as a basal medium. Flasks were incubated at 28°C and 200 rpm for 48 h for all the experiments concerning optimization of fermentation conditions, unless otherwise stated.

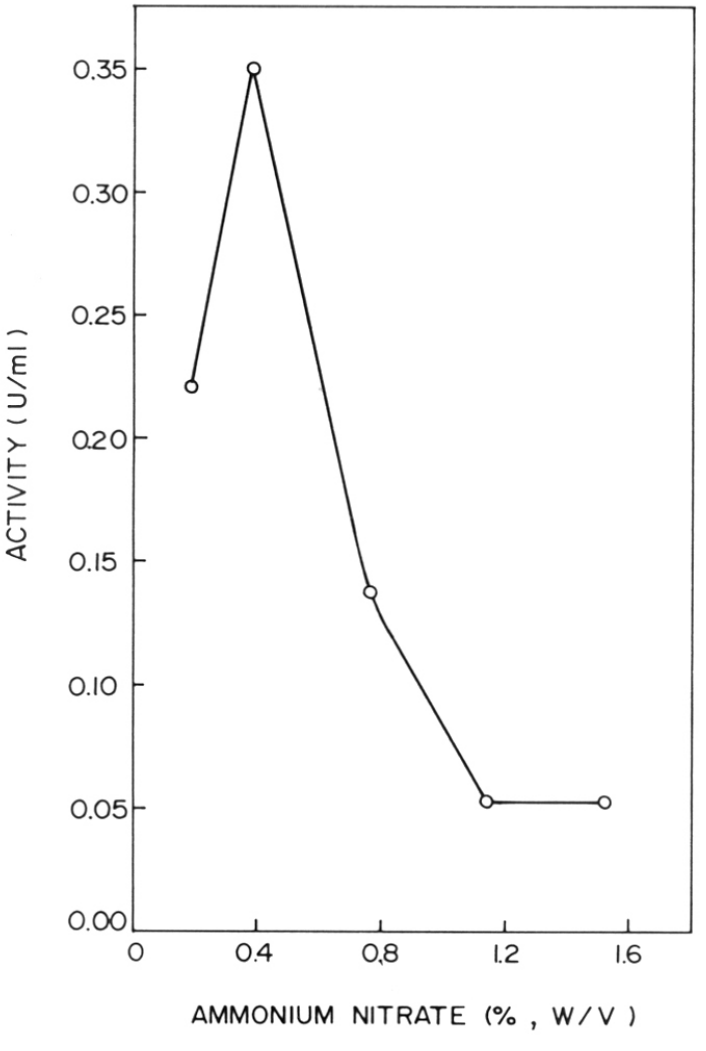

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
ACTIVITY (U/ml)
0
0.4
0.8
1.2
1.6
AMMONIUM NITRATE (% , W/V)

Table II-4 : Effect of various sugars and sugar alcohols on alkaline protease production

Carbon source	Concentration % (w/v)	Activity (U/ml)
Sucrose	1	0.350
	3	0.500
	5	0.500
Glucose	1	0.100
	3	0.120
	5	0.050
Lactose	1	0.150
	3	0.149
	5	0.150
Maltose	1	0.150
	3	0.148
	5	0.145
Fructose	1	0.154
	3	0.138
	5	0.125
Xylose	1	0.180
	3	0.181
	5	0.182

Table II-4 Continued

Carbon source	Concentration % (w/v)	Activity (U/ml)
Arabinose	1	0.400
	3	0.700
	5	0.720
Glycerol	1	0.307
	3	0.480
	5	0.482
Mannitol	1	0.320
	3	0.450
	5	0.460
Sorbitol	1	0.300
	3	0.450
	5	0.460

- Medium B devoid of sucrose was used as basal medium.
- The final pH was 7.0 in all cases.

(0.5 U/ml) and growth were obtained with 3% sucrose and it remained constant with increase in sucrose concentration. Incorporation of arabinose gave 1.5 times higher activity than that of sucrose and seemed to be the most preferred carbon source for alkaline protease production by C. coronatus, but due to its high cost, its use is not commercially feasible.

Sugar alcohols also supported good protease production, but they were less preferred energy sources as compared to sucrose. Hence medium containing 3% (w/v) sucrose as a carbon source was used for further studies (medium C). Glucose seemed to be the least preferred carbon source for alkaline protease production by C. coronatus, while in case of another Conidiobolus sp. (49), it supported the highest production of protease. Influence of carbon nutrition is reported by Latge (82). Eighteen carbon sources including monosaccharides, oligosaccharides, polysaccharides and polyhydric alcohols were tested and glucose, fructose, mannose, trehalose, maltose, glycerol and starch were found to be ideal carbon sources for the growth and production of proteolytic enzymes by Basidiobolus ranarum, Entomophthora obscura, E. thaxteriana, E. coronata, E. destruens and E. virulenta. Mannose was poor growth supporter for C. osmodes Only B. ranarum utilised sucrose efficiently.

Effect of various organic nitrogen sources:

When various compounds (1%, w/v) were used as organic

nitrogen sources for protease production, it was observed that, tryptone, peptone, yeast extract, casamino acids, skim milk, soymeal gave good yields of protease, tryptone being the best out of these compounds. Urea, beef extract, corn steep liquor, on the other hand were comparatively less effective (Table II-5).

Tryptone (1%, w/v) gave 12 times more activity (6.0 U/ml) than control (0.5 U/ml). Increase in tryptone concentration upto 2% (w/v) increased the protease activity to 8 U/ml, however, concentrations higher than 2% did not significantly contribute towards protease production (Fig. II-3). The medium containing 2% (w/v) tryptone (medium D) was used in the subsequent studies unless otherwise specified.

Peptides and proteins were found to be excellent source of nitrogen for both growth and protease production. Murakami (83) suggested that proteins or their large molecular weight fragments play some role in the stimulation of protease formation. In case of Erwinia chrysanthemi, good yield of protease activity was obtained with peptone, but when it was replaced with amino acids, no protease production occurred, indicating the involvement of intact peptide bonds in the induction process (53). On the other hand, low molecular weight ($\lesssim$ 5000 daltons) fractions obtained from skim milk stimulated the enzyme production by Pseudomonas fluorescens (32), whereas large fractions were ineffective, as promoters of enzyme production.

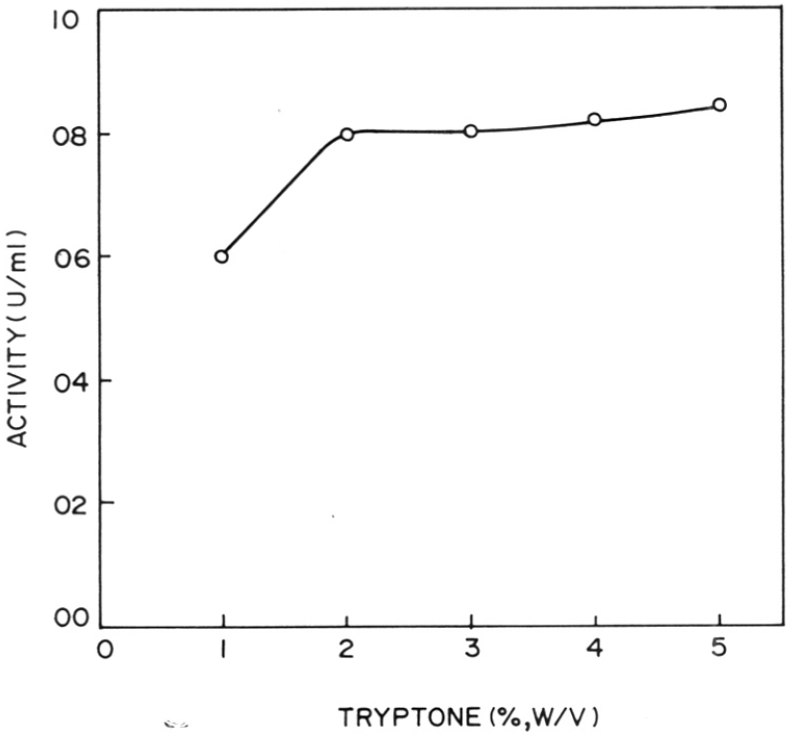
Table II-5 : Effect of organic nitrogen sources on alkaline protease production

Nitrogen source	Concentration % (w/v)	Final pH	Activity (U/ml)
*Basal medium (Control)	-	7.0	0.500
Peptone	1	8.0	5.3
Tryptone	1	8.0	6.0
Yeast extract	1	8.0	5.0
Casamino acids	1	8.0	4.6
Soymeal	1	8.0	4.0
Urea	1	7.5	1.9
Beef extract	1	7.5	1.7
Corn steep liquor	1	7.5	1.0
Skim milk	1	8.0	4.3

* Medium C was used as basal medium

Fig. II-3 Effect of tryptone on the production of enzyme

Medium C was used as a basal medium.


10
08
06
04
02
00
ACTIVITY (U/ml)
0
1
2
3
4
5
TRYPTONE (%, W/V)

Effect of amino acids as nitrogen source:

Medium modified with sucrose (3%, w/v), but devoid of any organic nitrogen source (i.e. medium C) was used as the basal medium. Various amino acids were added at 0.5% (w/v) concentration. As seen from Table II-6, alanine, tyrosine and glycine gave higher protease activity as compared to the control. However, the activity was much lower than that obtained with organic nitrogen sources. Thus it seems that, though, these amino acids can support protease production by C. coronatus to some extent, the organism prefers proteins as sources of organic nitrogen. Hence in the subsequent studies, medium D containing 2% tryptone was used. On the other hand, in case of Entomophthora apiculata and E. coronata (46), amino acids like serine, asparagine, glycine, tyrosine and alanine were the preferred nitrogen sources.

Effect of various salts:

When potassium chloride in the medium D was replaced with sodium chloride (0.1 - 0.3%, w/v), protease production decreased indicating the preference of the organism for potassium ions over sodium ions. Increase upto 0.2% (w/v) concentration of potassium chloride further increased the protease production. However, at higher concentrations it remained constant (Table II-7).

Phosphate ions in the form of potassium dihydrogen phosphate (0.1% w/v), along with 0.2% potassium chloride increased the protease production significantly (11 U/ml).

Table II-6 : Effect of amino acids on alkaline protease production

Amino acid	Final pH	Activity (U/ml)
*Basal medium (Control)	7.0	0.500
L-Arginine	7.0	-
L-Alanine	8.0	1.246
L-Lysine	7.0	-
L-Aspartic acid	7.0	-
L-Glutamine	7.0	-
L-Tyrosine	8.0	2.008
L-Cystine	7.0	-
L-Glycine	8.0	1.426
L-Histidine	7.0	-
L-Threonine	7.0	-
L-Methionine	7.0	-
L-Serine	7.0	-

* Medium C was used as basal medium

• Concentration of amino acids used was 0.5% (w/v)

\- Activities were negligible.

Table II-7 : Effect of various salts on alkaline protease production

Salt	Concentration % (w/v)	Activity (U/ml)
Potassium chloride	0.1	8
	0.2	10
	0.3	9.9
Sodium chloride	0.1	6.8
	0.2	6.95
	0.3	6.5
Potassium dihydrogen phosphate + Potassium chloride	0.1 0.2	11
Potassium dihydrogen phosphate + Potassium chloride	0.2 0.2	11.2
Potassium dihydrogen phosphate + Potassium chloride	0.3 0.2	11.3
Dipotassium hydrogen phosphate + Potassium chloride	0.1 0.2	10
Dipotassium hydrogen phosphate + Potassium chloride	0.2 0.2	10.7
Dipotassium hydrogen phosphate + Potassium chloride	0.3 0.2	11.0

- Medium D devoid of potassium chloride was used as basal medium
- The final pH was 8.0 in all cases

However, higher concentrations of both did not significantly contribute towards the production. Hence the medium was further modified with 0.2% (w/v) potassium chloride and 0.1% (w/v) potassium dihydrogen phosphate for further studies (medium E). Phosphate ions are known to support protease production in Pseudomonas aeruginosa (12), P. fluoroscens (33) and Alternaria tenuissima (2). High phosphate concentrations (0.3 - 0.4% dipotassium hydrogen phosphate) were essential for protease production in a thermophilic Streptomyces (20) and a Bacillus sp. (71).

Potassium chloride supported protease production in Mucor microsporus (27) and Alternaria alternata (Fr.) Keissl (18).

Effect of metal ions:

Various metal ion salts were added to medium E devoid of ferrous sulphate at 0.001% (w/v) concentration and their effect on protease production was compared with control devoid of metal ions. As seen from Table II-8, addition of zinc sulphate and calcium chloride increased the protease production (12 U/ml) and that of magnesium chloride, copper sulphate and cobalt chloride did not have any effect. So the medium was modified with 0.001% (w/v) of each of zinc sulphate and calcium chloride (medium F). Calcium ions increased protease production in Pseudomonas fluorescens (72).

Effect of casein (inducer) on protease production:

Casein was used as an inducer for production of protease

Table II-8 : Effect of metal ions on alkaline protease production

Metal ions	Activity (U/ml)
*Basal medium (Control)	11.0
Ferrous sulphate	10.9
Manganese chloride	10.8
Zinc sulphate	12.0
Copper sulphate	10.8
Calcium chloride	12.0
Cobalt chloride	11.0

* Medium E devoid of ferrous sulphate was used as basal medium and the effect of additives (0.001% w/v) on the production of protease was determined

- The final pH was 8.0 in all cases

by C. coronatus. It was added to medium F at 1-5% (w/v) concentrations. Addition of 2% casein almost doubled the protease production (22 U/ml) as compared to control, devoid of casein (13 U/ml) (Fig. II-4). However, higher concentrations of casein proved to be inhibitory to protease production. Hence the medium was modified with casein (2%) for subsequent studies (medium G).

Both amino acids and proteins have been used as inducers of protease production. Amino acids act as inducers in case of Pseudomonas fluorescens (72). Casein, peptone and soymeal are the most commonly used inducers for protease production.

Effect of surfactants:

Surfactants like oxgall, sodium dodecyl sulphate (SDS) BRIJ, Tween 20 and Tween 80 were added to the medium G at 0.1 - 0.5% (w/v) concentrations. Table II-9 shows effect of these compounds on protease production. Addition of oxgall, BRIJ and Tween 80 had no effect on protease production, while SDS proved to be inhibitory. Tween 20 at 0.1% concentration had no effect, but at 0.3% concentration led to inhibition of protease production.

As none of the surfactants had any stimulatory effect on protease production, medium modified in the previous step i.e. medium G, was used for optimisation of fermentation parameters like initial pH, temperature, inoculum size and time profile of protease production.

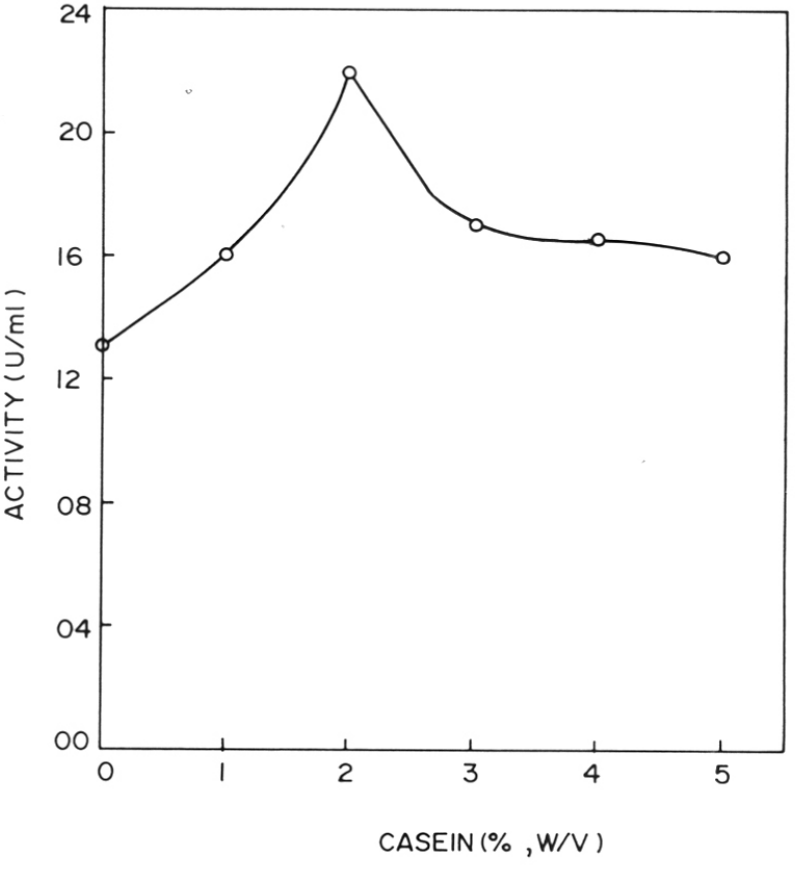

24
20
16
12
08
04
00
ACTIVITY (U/ml)
0
1
2
3
4
5
CASEIN (% , W/V)

Table II-9 : Effect of surfactants on alkaline protease production

Surfactant	Concentration % (w/v)	Final pH	Activity (U/ml)
*Basal medium (Control)	-	8 - 8.5	22
Sodium dodecyl sulphate	0.1	8 - 8.5	18.6
	0.3	7.0	0
BRIJ	0.1	8.0	20.4
	0.3	8.0	20.3
Oxgall	0.1	8 - 8.5	21.3
	0.3	8 - 8.5	22.2
Tween 20	0.1	8.0	21.1
	0.3	8.0	8.2
Tween 80	0.1	8.0	22
	0.3	8.0	22

* Medium G was used as basal medium

Effect of initial pH of the fermentation medium:

The initial pH of the medium G was adjusted in the range of 5.5 - 9.0 with hydrochloric acid and sodium hydroxide. The final pH of the fermentation broth was 8 - 8.5 after 48 h of incubation in submerged cultures. As seen from the Fig. II-5, the initial pH of the medium in the range of 6 - 8.5 did not have much effect on the growth and protease production. Initial pH of 5.5 and 9.0 slightly reduced the protease production. Maximum protease activity was obtained with the initial pH of 7 - 7.5 (22 U/ml).

Tokuyama and Asano (49) observed that protease production by a Conidiobolus sp. was sensitive to initial pH of the fermentation medium, while that by C. coronata (66) was insensitive to initial pH in the range of 4.5 - 9.0.

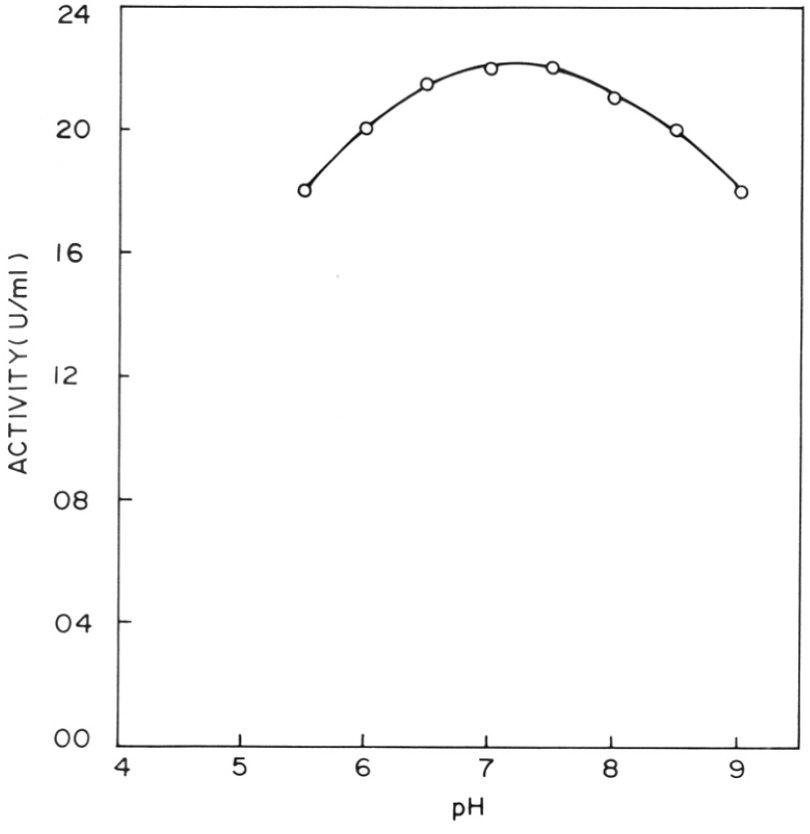
Effect of temperature:

The medium G flasks inoculated with C. coronatus were incubated at 15, 28 and 37°C on a rotary shaker (200 rpm) for 48 h. While, C. coronatus grew slowly at 15°C with poor protease production (6 U/ml), it did not grow at all at 37°C. Optimum growth and protease production (22 U/ml) was observed at 28°C, under shaking conditions. Hence fermentation studies were carried out at 28°C.

Entomophthora virulenta and E. coronata (66) showed optimum growth at 16 - 24°C and optimum protease production at 24 - 32°C, so 24°C temperature was used for their fermentation studies.

Fig. II-5 **Effect of initial pH of the medium on alkaline protease production**

Medium G was used as basal medium


24
20
16
12
08
04
00
ACTIVITY(U/ml)
4
5
6
7
8
9
pH

Effect of preinduced inoculum:

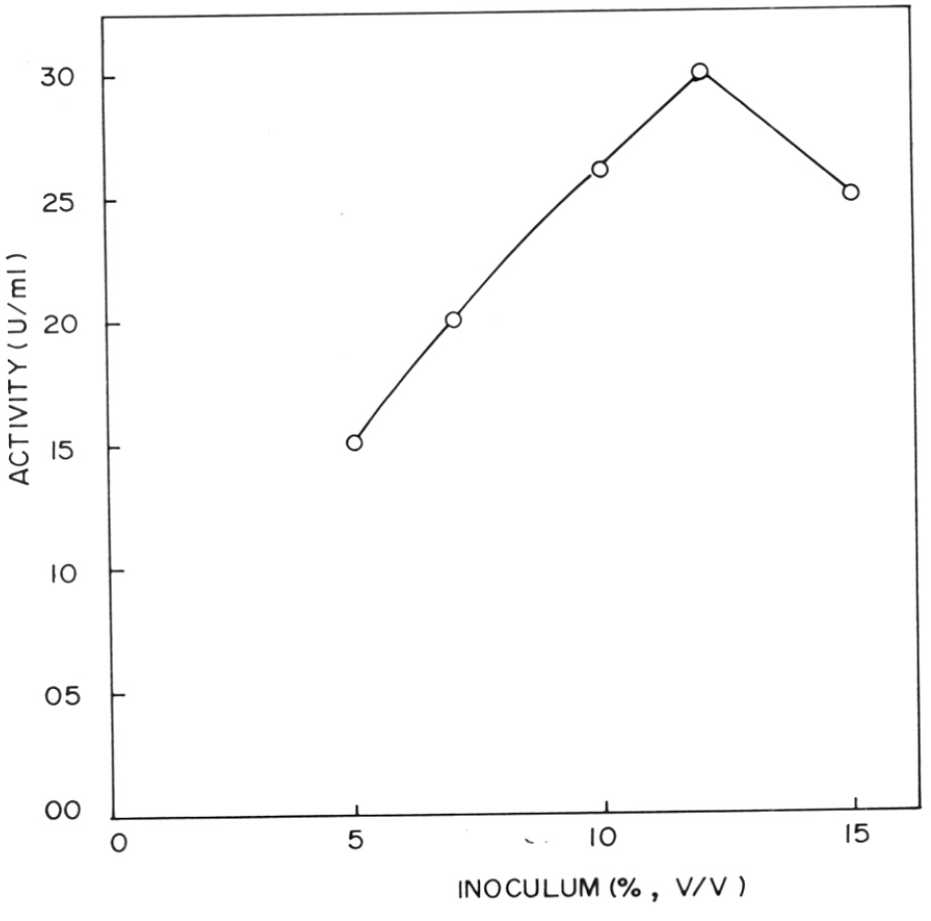
In the previous studies, mycelial inoculum 10% (v/v) grown in MGYP broth at 28°C, for 24 h, washed and resuspended in equivalent amount of sterile distilled water was used.

When the inoculum grown in the same medium, i.e. medium G, at 28°C for 24 h was used for fermentation, it was observed that protease production increased significantly. Fig. II-6 shows the effect of size of preinduced inoculum on alkaline protease production. In these studies, inoculum was used without washing with sterile distilled water. Highest protease activity (30 U/ml) was obtained with 12% (v/v) inoculum. Hence, for the subsequent fermentations, 12% preinduced inoculum was used.

Though C. coronatus has small amount of constitutive protease activity (0.5 U/ml) (Table II-4), protease production is greatly enhanced by the addition of an inducer like casein to the medium. This indicates that protease of C. coronatus is partly constitutive and partly inducible. Constitutive activity, though in small amount, must be responsible for degradation of complex and large substrate like casein, giving rise to smaller peptides, which can enter the cells and in turn effectively induce the protease production. Due to this, when mycelium, preinduced by growing in presence of casein for 24 h is used as inoculum, considerably higher (30 U/ml) protease production is obtained.

Fig. II-6 **Effect of size of preinduced inoculum on production of alkaline protease by C. coronatus**

Inoculum grown in medium G, for 24 h was used for inoculating the fermentation medium flasks of the same composition.


30
25
20
15
10
05
00
ACTIVITY (U/ml)
0
5
10
15
INOCULUM (% , V/V)

Time profile of protease production:

Fermentation medium flasks inoculated with C. coronatus were incubated at 28°C on a rotary shaker, at 200 rpm for different time intervals, ranging from 24 - 96 h. It was observed that at 48 h maximum protease activity (30 U/ml) was obtained, after which it started declining (Fig. II-7).

Optimum fermentation conditions for maximum production of alkaline protease by C. coronatus:

Maximum protease production was observed at 28°C at 200 rpm for 48 h with the medium containing sucrose, 3%; ammonium nitrate, 0.38%; tryptone, 2%; potassium chloride, 0.2%; potassium dihydrogen phosphate, 0.1%; casein, 2%; zinc sulphate, 0.001%; calcium chloride, 0.001%, initial pH 7 - 7.5, using 12% (v/v) preinduced inoculum grown in the same medium under similar conditions for 24 h.

Relationship between growth of C. coronatus and protease production:

The culture was grown in medium at 28°C and 200 rpm, containing a soluble protein source like Edamin S. Mycelial dry weight and protease activity was determined after every 8 h. The protease production was almost parallel to growth during log phase of the organism and reached a maximum at 42 - 48 h, which was the stationary phase of the organism, after which the protease activity started declining slowly (Fig. II-8).

Fig. II-7 **Time profile of alkaline protease production by C. coronatus**

Fermentation medium flasks inoculated with C. coronatus were incubated at 28°C on a rotary shaker, at 200 rpm for different time intervals ranging from 24 - 96 h.

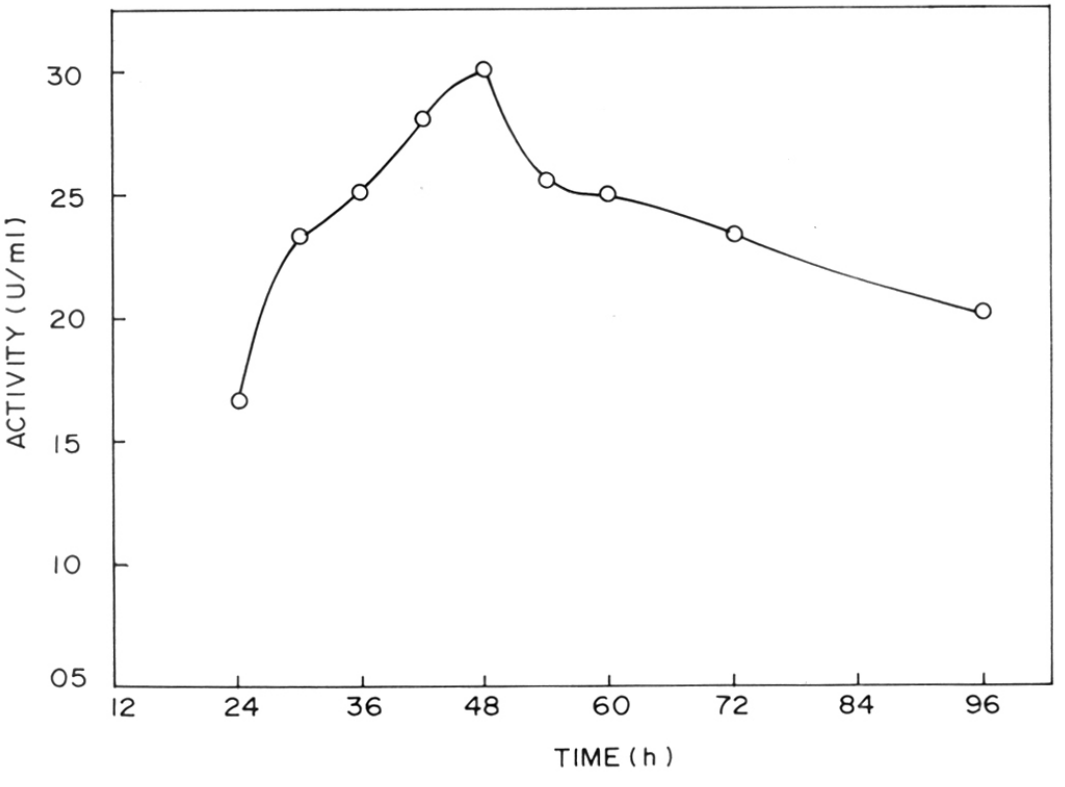

ACTIVITY (U/ml)
30
25
20
15
10
05
12
24
36
48
60
72
84
96
TIME (h)

Fig. II-8 **Relationship between growth and alkaline protease production by C. coronatus**

C. coronatus was grown in medium containing Edamin S. Mycelial dry weight and protease activity was determined after every 8 h.

●, mycelial dry weight (mg/ml)

o, protease activity (U/ml)

SECTION II

APPLICATION OF *CONIDIOBOLUS* ALKALINE PROTEASE IN DETERGENTS

Summary

One of the major applications of proteases is in detergents. Compatibility of *Conidiobolus coronatus* (NCL 86.8.20) alkaline protease with commercial detergents was evaluated. Broad substrate specificity, stability at alkaline pH and moderately high temperature (40°C, 1 h) and stability in presence of various detergents such as SNOWWHITE, NIRMA, REVAL, SURF and WHEEL (7 mg/ml) implied the potential of this enzyme for use in detergents.

Introduction

Proteases were first used in detergents as early as in 1913, when Röhm and Hass from Germany introduced the pancreatic enzymes in a detergent called Burnus, as mentioned in Chapter I. In 1960's, proteases were effectively incorporated into detergents and an alkaline protease from Bacillus licheniformis named subtilisin Carlsberg (Alcalase) developed by Novo Industri A/S, Denmark was marketed in a detergent under the trade name 'Biotex'. In 1969, the detergent industry experienced a set back due to the allergic reactions caused by the enzyme powder. This was soon overcome by introduction of dust-free, encapsulated enzymes. This once again increased the sales of enzyme detergent powder and major application of proteases today is in detergents. The current European market share of protease containing detergents is estimated at approximately 75% (84).

The pH of laundry detergents is generally alkaline and temperature requirements are often high. Detergents also contain oxidising and chelating agents which can be inhibitory to the activity of the enzymes. Some surface active agents, especially cataionic surfactants can also cause denaturation of enzymes. Hence, the ideal enzymes must be stable at alkaline pH, have reasonably good thermal stability, be compatible with common oxidising and chelating agents used in detergents and be effective for stain removal at low enzyme level in detergent solution.

In the present investigation, pH and thermal stability and compatibility of alkaline protease from Conidiobolus coronatus (NCL 86.8.20) with some commercial detergents was tested.

Materials & Methods

Materials:

Popular brands of commercial detergents available in India, examplified by NIRMA, SURF, DET, WHEEL, SNOWWHITE AND REVAL were chosen for the evaluation studies.

pH stability:

Effect of pH on stability of the alkaline protease was studied by incubating the enzyme with buffers covering the pH range of 5.5 to 11.0. Different buffers (0.1 M) used were potassium phosphate (pH 5.5 - 7.5), Tris-HCl (pH 8.0 - 8.5) and sodium carbonate-bicarbonate (pH 9.0 - 11.0).

85 U of enzyme samples were added to different buffers, to make final volume of 0.1 ml. After incubation at 40°C for 1 h, residual activity in each sample was determined by caseinolytic assay and compared with control sample kept at 4°C at pH 7.0.

Temperature stability:

The enzyme samples (85 U) were incubated at various temperatures ranging from 0°C - 55°C, at pH 7.5. After 1 h, residual activities were determined and compared with control kept at 4°C at pH 7.5.

Compatibility with various commercial detergents:

The detergents were diluted in double distilled water to give final concentration of 7 mg/ml to simulate washing conditions. Alkaline protease at a concentration of 0.05 mg/ml was incubated at 40°C in various detergents (7 mg/ml).

After every 10 min interval, 10 µl of the sample were removed The residual activity in each sample was determined at 37°C by caseinolytic assay and compared with control sample incubated at 40°C, without any detergent.

Protease activity on various protein substrates:

The caseinolytic assay was followed for substrates like bovine serum albumin, ovalbumin and Haemoglobin. Units were also calculated as that for caseinolytic assay.

Results & Discussion

When incorporated in detergents, proteases remove not only the obvious stains, such as blood, but also other less obvious material including proteins from body secretions and skin particles and foods such as milk, egg, meat and fish. In absence of proteases, proteinaceous dirt coagulates on the fabric as a result of the washing conditions. The high temperatures and pH and the action of the surfactants and sequestering agents dissolve or disperse most of the dirt components and the bleaching agent decomposes the undissolved dye. The process, however, causes the protein material to precipitate onto the fabric. The coagulated protein also retains other dirt components, such as soil, lipids and carbohydrates and is subsequently difficult to remove. Failure to remove the proteinaceous dirt results in a grey and unclean appearance of the fabric after several washings.

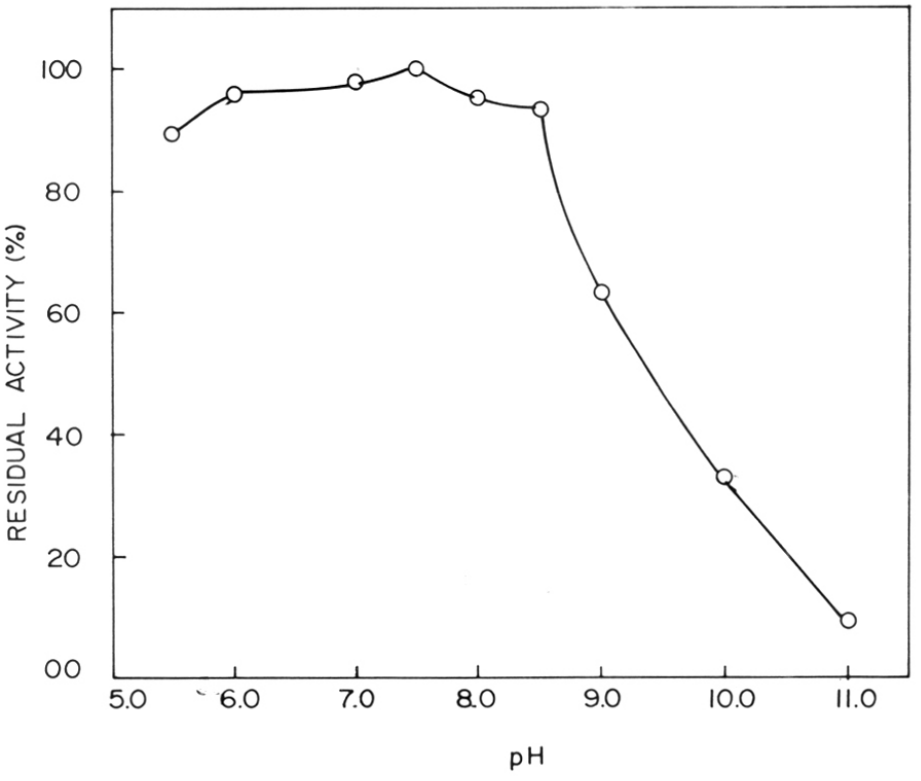
As mentioned previously, an ideal detergent enzyme should be stable at high pH and temperature, be stable with oxidising and chelating agents and be effective at low enzyme level (0.4% - 0.8%) in detergent solution. It should also have a broad substrate specificity.

pH stability:

The pH of the commercial detergent solutions was 8.2 - 8.5. The alkaline protease of C. coronatus was stable at pH 6 - 8.5; pH 7.5 being the maximum stability pH (Fig. II-9).

Fig. II-9 pH stability of alkaline protease

85 U of enzyme samples were incubated in various buffers (pH 5.5 - 11.0) at 40°C for 1 h. After incubation, residual activity in each sample was determined by caseinolytic assay. Activity of control sample incubated at 4°C, at 7.0 pH was taken as 100%.


RESIDUAL ACTIVITY (%)
100
80
60
40
20
00
5.0
6.0
7.0
8.0
9.0
10.0
11.0
pH

Temperature stability:

The thermal stability of Conidiobolus protease is not very high, as it retains 100% activity only upto 40°C for 1 h (Fig. II-10), after which loss in activity is evident. However, as the detergents tested, except for REVAL are used at room temperature, higher thermal stability is not needed in the present case.

Compatibility with detergents:

As seen from Fig. II-11, the protease retained more than 80% of its activity in presence of the detergents, SNOWWHITE, NIRMA, REVAL and more than 56% of its activity in presence of WHEEL and SURF. The maximum stability was seen with SNOWWHITE, as the enzyme retained 90% of its activity after incubation with the detergent at 40°C for 1 h. In presence of DET though, almost 60% of the activity was lost after incubation at 40°C for 1 h.

Proteases should be effective at low enzyme levels. Samal et al. (85) have reported protease from a fungus Tritirachium album Limber which is effective at a concentration of 0.2 mg/ml in the detergent. Conidiobolus protease is effective even at a lower concentration of 0.05 mg/ml in the detergent solutions.

Protease activity on various protein substrates:

Substrate specificity of the alkaline protease was studied using various protein substrates. Though casein was the most preferred substrate, the protease also hydrolysed

Fig. II-10 Thermal stability of alkaline protease

The enzyme samples (85 U) were incubated at various temperatures ranging from O - 55°C, at pH 7.5. After 1 h, residual activity was determined. Activity of control sample kept at 4°C at pH 7.5 was taken as 100%.

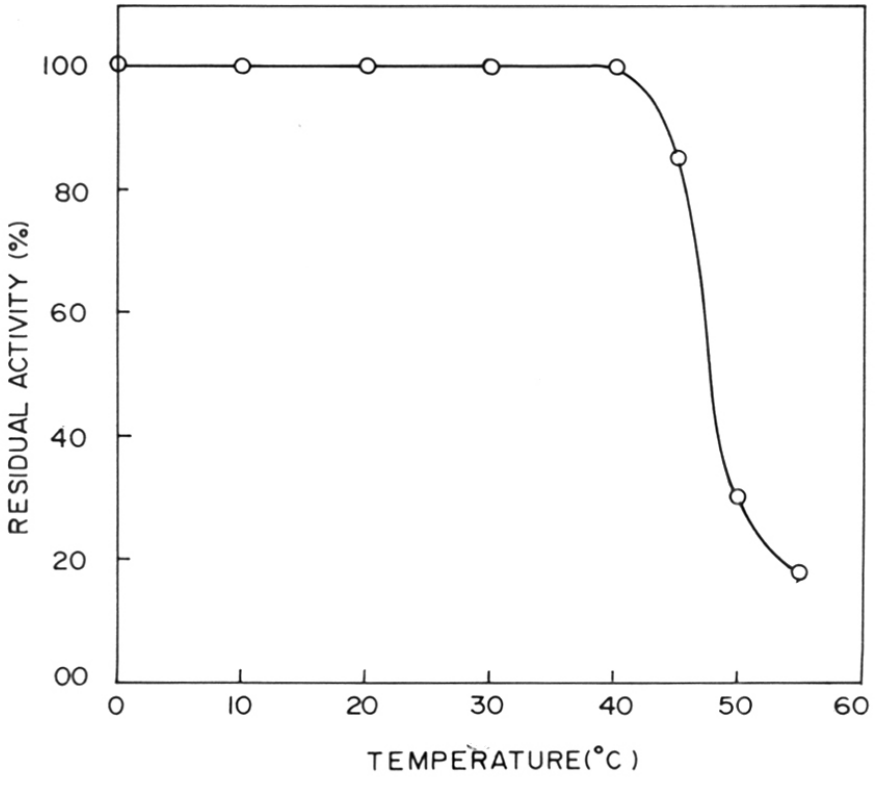
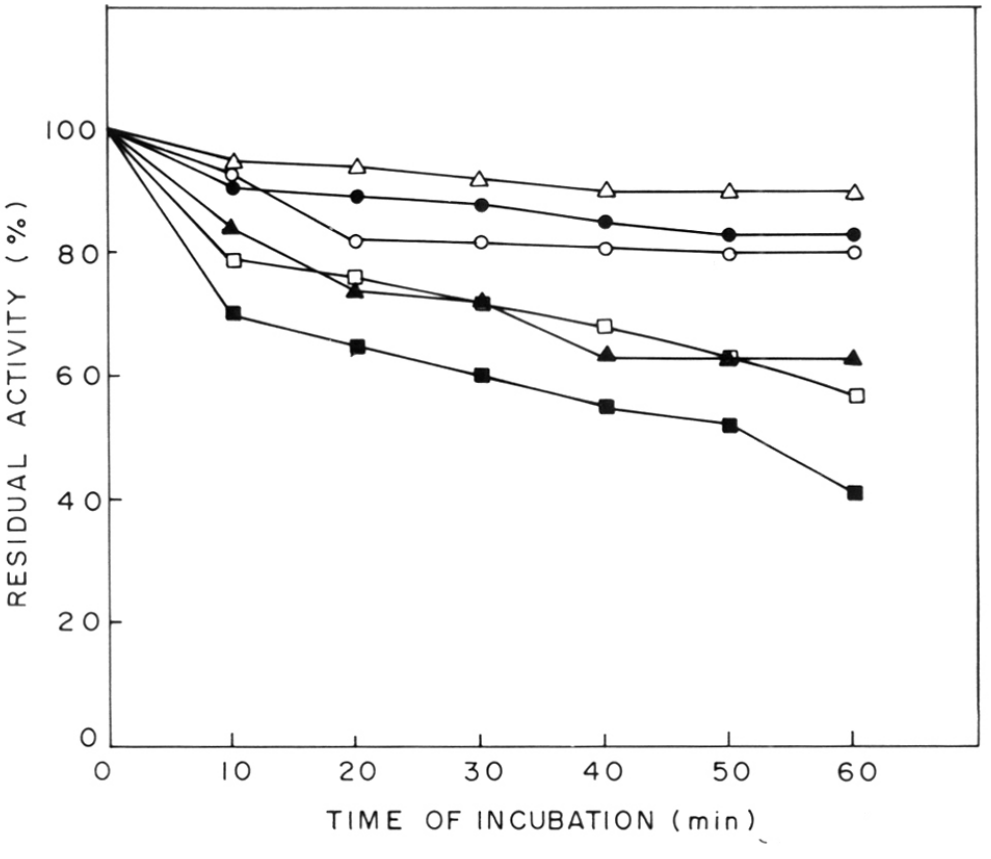

100
80
60
40
20
00
RESIDUAL ACTIVITY (%)
0
10
20
30
40
50
60
TEMPERATURE(°C)

Fig. II-11 Stability of alkaline protease in various commercial detergents

Alkaline protease (0.05 mg/ml) was incubated at 40°C in the presence of various detergents (7 mg/ml). After every 10 minutes, samples were removed and residual protease activity was determined. Activity of control sample devoid of any detergent, incubated under similar conditions was taken as 100%.

△, SNOWWHITE; ●, NIRMA; O, REVAL;

▲, WHEEL; □, SURF; ■, DET.


RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME OF INCUBATION (min)

other proteins such as Haemoglobin, ovalbumin and bovine serum albumin (Table II-10).

The preliminary data on pH and thermal stability, substrate specificity and compatibility of the Conidiobolus protease indicates its potential for application in detergents.

Table II-10 : Alkaline protease activity on various protein substrates

Substrate	Activity (%)*
Casein	100
Ovalbumin	12
Haemoglobin	50
Bovine serum albumin	15

* Activity on casein was taken as 100%

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CHAPTER-III

INVOLVEMENT OF ALKALINE SERINE PROTEASE IN CONIDIAL DISCHARGE OF *C. CORONATUS*

Summary

The involvement of serine protease in the conidial discharge of Conidiobolus coronatus was investigated using the parent wild strain and a variant strain (UV-20) with reduced conidial discharge, selected after UV-irradiation, both the strains were identical in their nutritional requirements, antibiotic sensitivity, colony morphology with respect to conidial germination, rate of growth and extent of conidial formation. Time course profiles of intracellular protease levels and conidial discharge showed that maximum protease levels coincided with maximum conidial discharge in the parent as well as the variant strain. It was also observed that the maximum protease level in the parent strain was approximately 3 times higher (3.4 U/mg protein) than UV-20 strain (1.3 U/mg protein) and the parent strain showed higher level of conidial discharge (> 25 times) than UV-20 strain. In both the strains, conidial discharge started when the protease level was above 1.0 U/mg protein. Inhibition of serine protease by phenyl methyl sulfonyl fluoride (PMSF) showed that low protease levels resulted in inhibition of conidial discharge, and a minimum protease activity of 1.0 U/mg protein appeared to be essential for triggering conidial discharge. Using casein to induce proteases, it was further observed that early gain in the protease levels (1.0 U/mg protein) led to early onset of conidial discharge. The above evidence suggests the involvement of protease in the conidial discharge of

C. coronatus, which has so far not been documented in fungi. The present study thus attributes a new physiological role to serine proteases.

Introduction

In fungi, conidia, the propagules of asexual reproduction are released into the environment for wide dessimination of the fungus. This release can be due to the breakage of supporting cell by lytic enzymes like chitinase or by mechanical means or due to splitting of the double septum at the base of the conidium (1). In the case of Entomophthora and Conidiobolus, members of order Entomophthorales, the splitting of the double septum at the base of the conidium results in its violent discharge from the conidiophore (2). This enables the conidium to transverse greater distance. Forcible discharge of conidia is also observed in case of Basidiobolus (3). Stages involved in the conidial discharge of Conidiobolus have been described by Sawyer (2), but its biochemical basis has not been investigated.

Conidiobolus coronatus (NCL 86.8.20) is characterised by production of a high activity alkaline serine protease and violent discharge of conidia. In a number of cellular differentiation processes viz. macroconidial germination, budding in yeast, hyphal tip elongation and hyphal fusion, proteases, especially serine proteases are known to be involved, where they play either a hydrolytic role or are involved in the activation of cell wall synthesizing enzymes namely chitin synthetases (4-6).

In view of the implications of serine proteases in many cellular differentiation processes, Conidiobolus

coronatus (NCL 86.8.20) isolate was used to explore the possibility of involvement of this enzyme in its conidial discharge, the results of which are discussed in the present chapter.

Materials & Methods

Materials:

Phenyl methyl sulfonyl fluoride (PMSF) and Hammersten casein was purchased from Sigma and MERCK respectively. All the other chemicals and reagents used were also of analytical grade.

All the buffers and reagents were prepared in double distilled water.

Preparation of conidial suspension:

C. coronatus was grown on MGYP agar slants (malt extract, 0.3%; glucose, 1%; yeast extract, 0.3%; peptone, 0.5%; agar, 2%) at 28°C for 48 h. Conidia were harvested by adding sterile distilled water to the slants, followed by gentle scraping with sterile glass spatula. The suspension was then filtered through sterile cotton and the conidial suspension was suitably diluted with sterile distilled water to give required concentration of conidia. Number of conidia was determined using a hemocytometer.

Standardisation of medium for screening of morphological variants of C. coronatus:

MGYP agar plates containing various concentrations (0.05 - 0.5% [w/v]) of either of phenyl ethyl alcohol, sodium dodecyl sulphate, sodium deoxycholate or oxgall were prepared. MGYP agar plates without any of these chemicals served as controls. All the plates were inoculated with conidia (0.1 ml, 10^3 conidia/ml) from an actively sporu-

lating (48 h old) agar slant culture and incubated at 28°C, for 48 h. Growth pattern on all test plates in comparison with control plates was recorded.

Isolation of morphological variants

The conidial suspension (10^6 conidia/ml) was prepared as described previously and was transferred to a sterile petridish and subjected to UV irradiation (Phillips UV germicidal lamp, 15 W) at a distance of 15 cm, for various intervals (1 - 10 min). The suspension was then plated on MGYP agar containing 0.5% (w/v) oxgall and incubated at 28°C for 48 - 72 h. Variants were selected on the basis of their morphological differences from the parent strain, such as, compactness, powdery white surface, reduction in conidial discharge etc. The variant UV-20 characterised by feeble conidial discharge was selected for detailed investigations.

Both the parent and UV-20 strain were maintained on MGYP agar slants at 28°C.

Characterisation of variant UV-20 strain and its comparison with parent wild strain:

1. Nutritional requirements. Czapek Dox minimal medium agar plates and MGYP plates inoculated with 48 h grown parent and UV-20 strain were incubated at 28°C for 48 h.

2. Sensitivity to antibiotics. MGYP agar plates containing various concentrations (50 - 200 µg/ml) of either of griseofulvin, cyclohexamide, aureofungin and kabicidin

were prepared. MGYP agar plates without any antibiotic served as control. The plates were inoculated with parent and UV-20 strain and incubated at 28°C for 48 - 72 h. Two types of inoculum were used. Conidial suspension (0.1 ml, 10^3 conidia/ml) was used as inoculum to study effect of antibiotics on germination of conidia, while mycelial inoculum grown in MGYP broth at 28°C and 200 rpm for 24 h was used to check the effect of antibiotics on overall growth of the organism. As aureofungin and kabicidin were prepared in ethanol and griseofulvin was prepared in dimethyl sulfoxide, effect of equivalent concentrations of these solvents on growth was also determined.

3. Study of growth cycle and measurement of conidial discharge. In case of wild strain, synchronously growing culture was obtained by inverting plate of Conidiobolus culture in stage of active conidial discharge, over a fresh MGYP plate for 1 - 2 minutes. The discharged conidia falling on the agar medium measured similar in size and could be generally assumed to be of the same age and hence predominantly gave synchronous culture. In case of UV-20 strain, which showed considerably reduced conidial discharge, this method could not be applied. Hence washed conidia of young culture with similar dimensions were employed to give an almost synchronous culture.

Conidia (0.1 ml, 10^3 conidia/ml) of synchronously growing culture of parent and UV-20 strain (20 h at 28°C)

were used to inoculate MGYP agar plates, to study asexual growth cycle of C. coronatus. The plates were incubated at 28°C. Growth was followed microscopically. Discharged conidia were collected in distilled water and were counted on a hemocytometer.

The experiment was repeated using MGYP agar plates containing 2% (w/v) casein.

Enzyme assays:

Mycelial growth which could only be seen after 8 h of incubation at 28°C was taken as starting point for the determination of enzyme activities. Samples were removed at different time intervals and the intracellular protease and chitinase activities were estimated. To prepare the crude extracts for enzyme assays the mycelium was scraped with glass spatula and was suspended in distilled water. It was centrifuged at 13,000 g for 5 min. The supernatant was discarded and the process of washing was repeated three times. Intracellular extract was prepared by crushing the mycelium with glass homogenizer. The samples were centrifuged and the supernatant liquid was tested for enzyme activities.

Protease activity was determined as described in previous chapter at pH 9.7 and 37°C using casein as a substrate.

Chitinase activity was measured according to Otakara (7). Suitably diluted enzyme in 0.5 ml of 50 mM sodium

acetate buffer, pH 5.6, was added to 0.5 ml of the substrate, i.e. 1% ethylene glycol chitin. After incubation at 50°C, for 30 min, 1 ml of Nelson Somogyi reagent A was added and the mixture was boiled for 15 min. Then the mixture was cooled and 1 ml of Nelson Somogyi reagent B was added to it. After shaking and filtration through Whatman No. 1 filter paper, the optical density was measured at 520 nm. N-acetyl glucosamine (1 mg/ml) was used as standard. One unit of enzyme is defined as the liberation of 1 µmol of the product per minute.

Protein estimation:

Protein was determined according to the method of Bradford (8) using crystalline bovine serum albumin as standard. The absorbance of the protein- Coomassie Blue G-250 coloured complex was measured at 595 nm.

In all cases, enzyme activities were expressed as specific activity, i.e. units per mg protein.

Effect of PMSF on growth and intracellular protease activity of C. coronatus:

Conidial suspension (0.1 ml, 10^3 conidia/ml) was used to inoculate MGYP agar plates with various concentrations of PMSF (1 - 3 mM). Agar plates without PMSF were used as control. After incubation at 28°C for 48 h, intracellular protease activity was determined as described previously. Growth stages were monitored microscopically.

Results & Discussion

The steps leading to forcible discharge of conidium are described by Sawyer (2) in case of Entomophthora, which is closely related to Conidiobolus. According to this report, a bud, the initial of conidium is formed at the blunt apical portion of the conidiophore. A septum is formed across the base of the spore. This septum consists of two membranes in close apposition, one being the basal wall of conidium, the other being apical wall of conidiophore. As growth continues, the greater hydrostatic pressure within the conidiophore forces the opposed walls to bulge convexly into the conidium. Eventually, the attachment between conidium and conidiophore is ruptured circumferentially and the conidium is violently discharged from the conidiophore. Similar phenomenon was observed in case of C. coronatus (Fig. III-1).

Three approaches can be used to investigate the possibility of involvement of serine protease in conidial discharge of C. coronatus, viz.

(i) isolation of a mutant/variant, specifically affected in conidial discharge and determination of its intracellular protease activity;

(ii) comparison of time profiles of conidial discharge and intracellular protease production; and

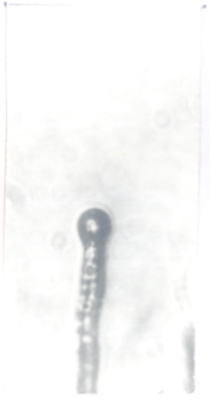
(iii) studying the effect of protease inhibitors and inducers on conidial discharge.

Fig. III-1 Stages in the conidial development and discharge

a - g : sequential stages in conidial development and discharge

Magnification - 1350 X

a



b

c

d

e

f

g

The first type of approach, involving isolation of a specific mutant has been widely used, especially in the study of Bacillus sporulation, wherein the involvement of serine proteases has been established using protease deficient or sporulation negative mutants (9). Involvement of protease in Saccharomyces cerevisiae sporulation was proved with help of low protease mutant showing loss of sporulation (10).

Chen and Miller (11) showed involvement of protease in yeast sporulation by comparing time profiles of protease production and spore formation.

Specific inhibitors of serine protease like PMSF have been used to show its involvement in many cellular processes such as bacterial sporulation (12), macroconidial germination (4), etc. Another inhibitor of serine proteases, microbial alkaline protease inhibitor (MAPI) was also used for this purpose, in study of Bacillus sporulation, by Nishino & Toyokazu (13).

In the present study, all the three approaches were used to study the possible involvement of serine protease in conidial discharge of C. coronatus.

Standardisation of medium for screening of morphological variants of C. coronatus:

In case of mutation of Conidiobolus, the major problem is the rapid development of secondary colonies arising from discharged conidia which fall back onto the plate. Due to

this, identification of mutants becomes very difficult. To overcome this problem, various surface active agents like phenyl ethyl alcohol, SDS, sodium deoxycholate, oxgall were incorporated in the medium. Out of these, 0.5% oxgall was found to be most suitable, since it restricted the growth and reduced the conidial discharge, which in turn facilitated the identification of morphological variants (Fig. III-2).

The other agents, however, inhibited the growth of C. coronatus and hence were considered to be unsuitable.

Isolation of morphological variants:

After UV-irradiation for 2 minutes, 10% of the survivals were obtained as morphological variants. Out of these, variant UV-20 strain, showing reduced conidial discharge, was selected for further studies (Fig. III-3).

Characterisation of variant UV-20 strain and its comparison with parent strain:

To study the possibility of involvement of protease in conidial discharge, a variant which is specifically affected in conidial discharge and which otherwise is very similar to parent strain was desirable. So the variant UV-20 strain was compared with the parent strain with respect to its nutritional requirements, sensitivity to various antibiotics and growth rate.

1. Nutritional requirements. Both the parent and UV-20 strain showed very poor growth on Czapek Dox minimal medium

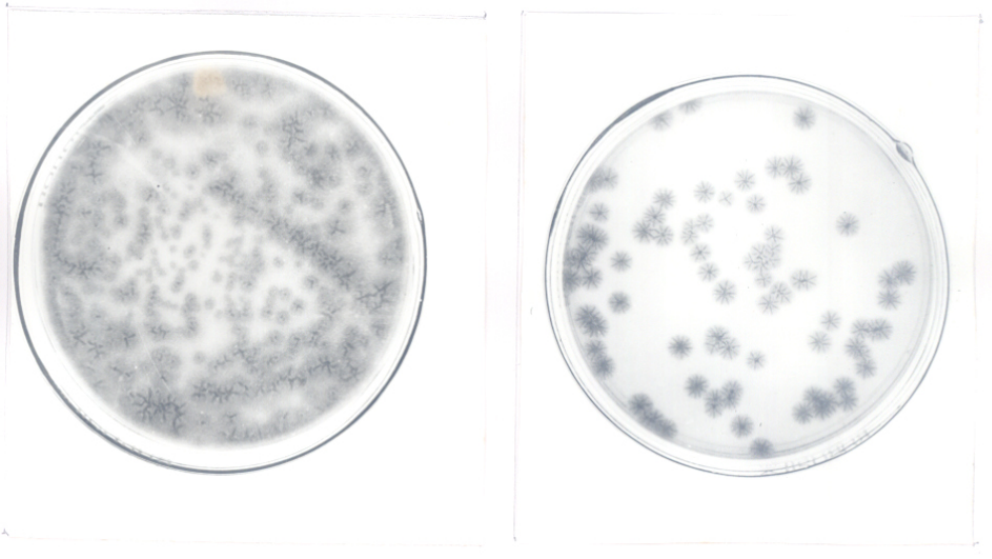
Fig. III-2 **Effect of oxgall on the growth of C. coronatus**

C. coronatus was grown on

(a) MGYP medium

(b) MGYP medium containing 0.5% (w/v) oxgall

The plates were incubated at 28°C for 48 h.

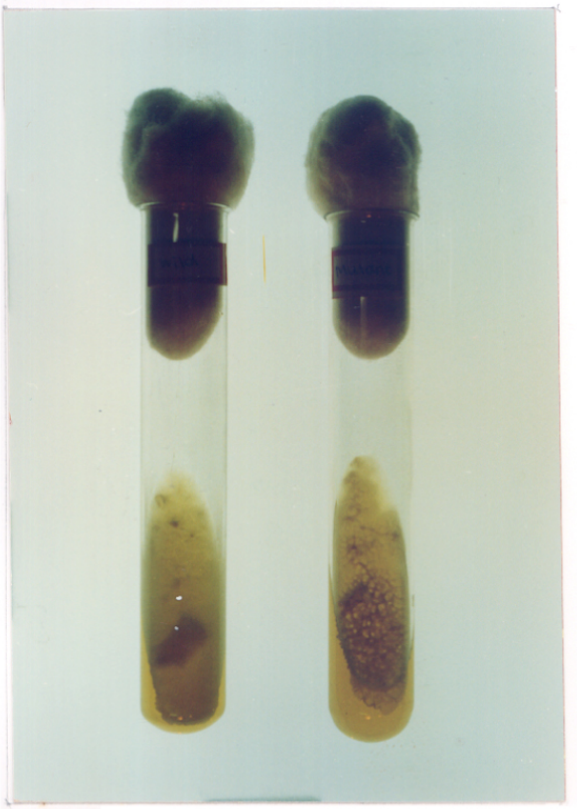


a

b

Fig. II-4 **Effect of casein on production of alkaline protease by C. coronatus**

Medium F was used as a basal medium.



a b

as compared to that on MGYP medium. This is because, Conidiobolus prefers organic nitrogen to amino nitrogen for growth (14).

2. Sensitivity to antibiotics. Change in the sensitivity to various antibiotics means change in the respective target sites in the cell, e.g. a kabicidin resistant mutant has altered cell membrane permeability (15), as cell membrane is the target site of kabicidin. Thus, the comparison of antibiotic sensitivity of parent and the variant strain can give information regarding possible altered structure of the variant.

Table III-1 gives results of antibiotic sensitivity tests of the parent and UV-20 strain. It is clear from the table that the two strains are identical in their responses towards different antibiotics. Both are unaffected by aureofungin and cyclohexamide. Both the conidial germination and mycelial growth is inhibited by kabicidin in the two strains. Griseofulvin also affects the germination and growth in both the strains, but to a relatively lesser degree.

Identical antibiotic sensitivity of the parent and UV-20 strains indicates their identical cellular structure.

3. Study of growth cycle, measurement of conidial discharge and intracellular protease and chitinase levels. The asexual growth cycle of C. coronatus consists of four stages namely, mycelial growth, conidiophore formation, conidia

Table III-1 Effect of antibiotics on conidial germination and mycelial growth of parent and UV-20 strain

Antibiotic	Parent strain		UV-20 strain	
	Conidial germination	Mycelial growth	Conidial germination	Mycelial growth
Control (without antibiotic)	-	-	-	-
Ethanol control (control + ethanol)	-	-	-	-
DMSO control (Control + DMSO)	-	-	-	-
Griseofulvin				
100 µg	-	-	-	-
200 µg	+	+	+	+
Cyclohexamide				
150 µg	-	-	-	-
Aureofungin				
150 µg	-	-	-	-
Kabicidin				
50 µg	+	-	+	-
100 µg	++	++	++	++
150 µg	++	++	++	++

DMSO - Dimethyl sulfoxide

-, no effect; +, restricted; ++, totally inhibited.

development and conidial discharge. Conidia of synchronously growing culture (20 h at 28°C) were used as inoculum to study the asexual growth cycle of C. coronatus. At 28°C, conidia germinated by forming 3-4 germ tubes, within one hour after inoculation. While mycelial growth was visible at 8 h, conidiophore formation and conidia development started after 10 and 12 h, respectively. Discharge of mature conidia occurred after 13 h. When intracellular protease level was determined at various growth stages, it was observed that it remained unchanged (0.9 U/mg protein) during the mycelial growth. It increased to 1.1 U/mg during conidiophore formation and conidia development. The protease level showed a further increase (1.5 U/mg protein) prior to active conidial discharge (Fig. III-4, inset).

In view of the apparent relationship between protease levels and growth cycle stages in the parent strain, an attempt was made to explore this phenomenon by studying the intracellular protease levels in UV-20 strain, showing considerably less conidial discharge compared to the wild strain. Except for this difference, both the strains exhibited similar characteristics with respect to germination pattern, growth rate and conidial formation. Time profile of protease activity and conidial discharge in parent strain showed that protease activity started increasing after 11 h and reached a maximum at 28 h. Though, the conidial discharge started only after 13 h, it also showed maximum at 28 h. Both, the protease level and conidial

Fig. III-4 **Time course observations of intracellular protease activities and conidial discharge of parent and UV-20 strain of C. coronatus**

Conidial suspension (0.1 ml, 10^3 conidia/ml) was used to inoculate MGYP agar medium. Plates were incubated at 28°C.

O, protease activity, parent strain; ●, protease activity, UV-20 strain; △, conidial discharge, parent strain; ▲, conidial discharge, UV-20 strain.

The maximum discharge obtained in case of the parent strain was taken as 100%.

Inset: Time course observations of intracellular protease activities and conidial discharge of C. coronatus parent strain, from 8-15 h.

O, protease activity; △, conidial discharge.

Growth phases: A, mycelial growth; B, conidiophore formation; C, conidia formation; D, conidial discharge.

Data shown is a mean of four sets of experiments.

DISCHARGED CONIDIA, %
PROTEASE ACTIVITY, U / mg PROTEIN
TIME, h
PROTEASE ACTIVITY U/mg PROTEIN
DISCHARGED CONIDIA, %
A
B
C
D
TIME, h

discharge declined after 28 h. A similar pattern was observed in case of UV-20 strain, except that for both protease level and conidial discharge to reach the maximum, it took 30 h (Fig. III-4). Maximum protease level in the parent strain was approximately three times higher (3.4 U/mg protein) as compared to UV-20 strain (1.3 U/mg protein). In addition, the parent strain also showed higher level of conidial discharge (> 25 times) than UV-20 strain. It was also observed that in both strains, the onset of conidial discharge occurred when the protease level was above 1.0. Thus, the increased time required for the onset of conidial discharge in UV-20 strain could be due to the longer time required for the protease level to reach 1.0.

Chitinases have been implicated in the spore release in fungi (16). Hence the chitinase levels in all the growth stages of parent and UV-20 strain were determined. As seen from Fig. III-5, no increase in the chitinase levels is observed at any of the growth stages, in both strains and level of chitinase in UV-20 strain is identical to that in parent strain. Therefore, it can be assumed that chitinase does not have a role in the conidial discharge of C.coronatus.

Effect of PMSF on growth and intracellular protease activity of C. coronatus:

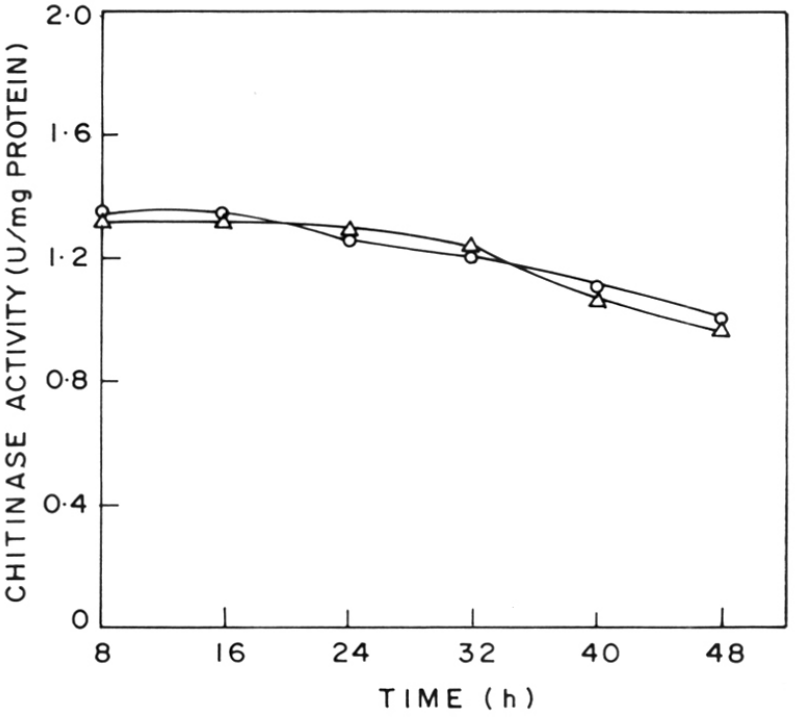
PMSF is a specific and powerful inhibitor of serine proteases (17). In the present studies, PMSF at concentration of 5.0 mM brought about a complete inhibition of

Fig.III-5 **Time course observations of intracellular chitinase activities of parent and UV-20 strain of** *C. coronatus*

Conidial suspension (0.1 ml, 10^3 conidia/ml) was used to inoculate MGYP agar medium.

Plates were incubated at 28°C.

O, chitinase activity, parent strain; △, chitinase activity, UV-20 strain.


CHITINASE ACTIVITY (U/mg PROTEIN)
2·0
1·6
1·2
0·8
0·4
0
8
16
24
32
40
48
TIME (h)

protease activity in the crude extract, indicating the presence of only serine proteases. PMSF has been used to show involvement of serine proteases in many cellular differentiation processes, like, macroconidial germination, hyphal fusion (4) and bacterial sporulation (12). Hence, a similar approach was used to assess the involvement of serine protease in conidial discharge of C. coronatus. Response of the organism with respect to its growth and intracellular protease activity, to different concentrations of PMSF was studied. PMSF at a concentration of 3.0 mM when used in the growth medium, inhibited the mycelial growth in case of the parent strain. While 2.5 mM PMSF led to the inhibition of conidia formation, lower concentration (2.0 mM) did not affect conidia formation, though the conidial discharge was completely inhibited. This was accompanied by decrease in protease activity from 3.75 U/mg protein to 0.6 U/mg protein. Hence the inhibition of conidial discharge can be correlated to decrease in the protease level ($\lesssim$ 1.0 U/mg protein) due to PMSF. Similar results were obtained with UV-20 strain, though corresponding concentrations of PMSF required to bring about the inhibition were lower (Table III-2). From these results, it can be concluded that a minimum protease activity of 1.0 U/mg protein is necessary to trigger conidial discharge in both parent and UV-20 strain.

Further evidence for the involvement of protease in conidial discharge was studied by using a protease inducer

Table III-2 : Effect of PMSF on growth and intracellular protease activity of Conidiobolus coronatus

PMSF (mM)	Protease (U/mg protein)		Effect on growth phases	
	Parent	UV-20	Parent	UV-20
Control (without PMSF)	3.75	1.30	-	-
1.0	2.66	0.90	-	+
1.5	1.30	0.45	-	++
2.0	0.60	0.20	+	++
2.5	0.45	ND	++	+++
3.0	ND	ND	+++	+++

_, no effect; +, conidial discharge completely inhibited; ++, conidia formation restricted; +++, mycelial growth inhibited; ND, not detected.

(casein) in the growth medium. Addition of 2% (w/v) casein, brought about an early increase in the protease levels (1.0 U/mg protein), resulting in an early onset of conidial discharge both in the parent and UV-20 strain, as compared to the controls (without casein). This observation can be correlated to the early gain of protease levels necessary to trigger the conidial discharge.

All the above results indicate that serine protease of C. coronatus is involved in its conidial discharge, thus attributing a totally new physiological role to serine proteases.

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CHAPTER-IV

PURIFICATION OF ALKALINE SERINE PROTEASES OF *C. CORONATUS*

Summary

Serine protease of Conidiobolus coronatus (NCL 86.8.20) was shown to be involved in the onset and regulation of conidial discharge (Chapter III). As a first step towards the understanding of the mechanism of its regulation, it was necessary to investigate the kinetics of production and purification of the protease elaborated by Conidiobolus.

The culture filtrate of Conidiobolus showed the presence of two proteases designated as protease I and II. Kinetics of intracellular versus extracellular enzyme production revealed that both proteases occur intracellularly, though protease II is produced later, i.e. at the time coincident with the reduced conidial discharge.

The properties of crude intracellular protease were identical to those of extracellular protease, indicating that these proteases are produced intracellularly and then secreted into the culture medium.

The extracellular proteases were purified to homogeneity by (i) ammonium sulphate precipitation; (ii) preparative polyacrylamide gel electrophoresis at pH 7.6 and (iii) ion-exchange chromatography on CM-cellulose at pH 7.0.

Final yields of protease I and II were 24 and 10.7% respectively. Their specific activities were 45 and 30 U/mg respectively.

Introduction

The serine protease of Conidiobolus coronatus (NCL 86.8.20) is responsible for triggering the violent discharge of its conidia (Chapter III). This observation has suggested a totally new physiological role to serine proteases. Since the extent of conidial discharge depends on the level of protease, a mechanism to reduce the enzyme activity would eventually regulate the conidial discharge. However, a certain minimum level of protease is indispensible for the survival and the growth of the organism (Table III-2). To deduce the mechanism by which the level of protease is so critically controlled, kinetics of intracellular protease production was studied, which revealed that the organism produces two electrophoretically separable proteases, protease I in the initial period of growth and protease II at the time coincident with the reduced conidial discharge.

The properties of crude intracellular protease were exactly identical to those of extracellular culture filtrate, indicating that Conidiobolus serine protease is produced intracellularly and then secreted into the culture medium. In order to study the structural relatedness of these proteases and to understand their role in conidial discharge, it was essential to purify the two proteases. Taking advantage of the partly inducible nature of the Conidiobolus protease, the enzyme was produced using casein as an inducer and the purification of the corresponding extracellular proteases was undertaken as a pre-requisite to discern the

mechanism of regulation of their activity and conidial discharge in turn.

Majority of purification procedures for proteases reported in literature use affinity chromatographic techniques (1-7). Ion exchange chromatography and gel filtration techniques have also been applied (8-15). Slab polyacrylamide gel electrophoresis is reported for purification of alkaline proteases from Monascus sp. (16).

This chapter deals with the purification of extracellular alkaline, serine proteases of C. coronatus to homogeneity.

Materials & Methods

Materials:

Coomassie Brilliant Blue G-250, CM-cellulose, NNN'N'-tetramethylethylenediamine (TEMED), NN'-methylene bisacrylamide were purchased from Sigma. All the other chemicals used were also of analytical grade.

All the buffers and reagents were prepared in double distilled water.

Enzyme production:

Enzyme was produced in 500 ml Erlenmeyer flasks containing 100 ml standardised protease production medium (Chapter II). Vegetative inoculum was grown in the same medium for 24 h. Inoculum (12% v/v) was used for inoculating the experimental flasks which were incubated at 28°C on a rotary shaker at 200 rpm for 48 h. The cells and the solid residues were removed from culture broth by filtration through Whatman No. 1 filter paper and the clear filtrate was used as the source of enzyme.

Caseinolytic enzyme assay:

The assay was performed as described in chapter II.

Protein estimation:

Protein was determined according to the method of Bradford (17) as described in previous chapter. Since acrylamide impurities in the purified enzyme (after preparative PAGE) do not interfere in this method, it was preferred to the method of Lowry *et al.* (18).

Analytical polyacrylamide gel electrophoresis:

Electrophoresis was carried out by using cationic system C described by Zuidweg et al. (19), with some modifications. The separation gel contained 0.1 M KOH and 0.5 M Boric acid, pH 7.6; 7.5% acrylamide; 0.2% Bis; 0.125% TEMED and 0.07% ammonium persulphate. Electrode buffer contained 0.13 M Tris and 0.2 M Boric acid, pH 7.8. Anode was at the top and cathode at the bottom of the apparatus.

Basic fuchsin was used as a tracking dye. Electrophoresis was run with 4 mA current per gel tube for a period of 150-180 min.

Location of protease activity in the crude enzyme extract after analytical PAGE:

The culture filtrate concentrated by lyophilization was subjected to analytical gel electrophoresis at pH 7.6. After electrophoresis, one gel was stained with Coomassie Brilliant Blue G-250 prepared according to Blacksley and Boezi (20) and the other was cut into 20 pieces of 0.5 cm each. The gel pieces were crushed in distilled water and were directly estimated for protease activity. Graph of gel fractions was plotted against their protease activity.

Time profile of intracellular protease production:

C. coronatus was grown on MGYP slants at 28°C for 12, 24 and 36 h. Mycelium was scraped with glass spatula and was suspended in distilled water. It was centrifuged at 13,000 g for 5 min. The supernatant was discarded and the

process of washing was repeated three times. Intracellular extract was prepared by crushing the mycelium with glass homogeniser. The samples were centrifuged and the supernatant liquid was subjected to analytical electrophoresis. After electrophoresis, bands were visualised by staining with Coomassie Brilliant Blue G-250.

Kinetics of production of intracellular versus extracellular activity:

C. coronatus was grown in MGYP broth with and without casein (2%) at 28°C, at 200 rpm on rotary shaker for 72 h. Samples were removed after every 12 h. Mycelium was separated from fermentation broth by centrifugation at 13,000 g for 5 min. The supernatant was treated as an extracellular enzyme. The mycelium was thoroughly washed and suspended in distilled water as described previously. Intracellular extract was prepared by crushing the mycelium with glass homogeniser. Both intracellular and extracellular extracts were estimated for protease activity.

Comparison of intracellular and extracellular proteases:

(i) Optimum pH. Optimum pH of the enzymes were determined by estimating the enzyme activity at different pH values ranging from pH 6.0 - 11.0. Potassium phosphate (pH 6.0 - 7.5), Tris-HCl (pH 8.0 - 8.5) and sodium carbonate-bicarbonate (pH 9.0 - 11.0) were the buffers used.

(ii) Optimum temperature. Activities of the proteases were determined at different temperatures ranging from 25 - 55°C, at pH 10.0.

(iii) pH stability. To check the effect of pH on stability of the proteases, various buffers were used, viz. sodium citrate-citric acid (pH 4.5), sodium acetate-acetic acid (pH 5.0), potassium phosphate (pH 5.5 - 7.5), Tris-HCl (pH 8.0 - 8.5) and sodium carbonate-bicarbonate (pH 9.0 - 11.0).

70 U of individual enzyme samples were added to different buffers, to make final volume of 1.0 ml. After incubation at 40°C for 1 h, residual activity in each sample was determined by caseinolytic assay and compared with control sample kept at 4°C, at pH 7.0.

(iv) Temperature stability. The enzyme samples (70 U) were incubated at various temperatures ranging from 0°C - 60°C, at pH 7.5. After 1 h, residual activities were determined and compared with control sample kept at 4°C at pH 7.5.

(v) Effect of EDTA. 70 U of enzyme samples were incubated in sodium carbonate-bicarbonate buffer, pH 10.0, with 20 mM EDTA, at 30°C for 20 min. After incubation, residual activity was determined and compared with control sample incubated under similar conditions, but without EDTA.

(vi) Effect of active site specific reagent. 70 U of enzyme samples were incubated with 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), prepared in dimethyl sulfoxide (DMSO), at room temperature for 10 min. Controls with only DMSO and without either DMSO or PMSF were also incubated simultaneously. After incubation, residual activities were determined.

Enzyme purification:

The proteases were purified from crude enzyme preparation by ammonium sulphate precipitation, preparative PAGE and CM-cellulose chromatography. All operations were carried out at 4°C, unless otherwise stated.

(a) Ammonium sulphate precipitation. The culture filtrate (1000 ml) was concentrated by precipitating the enzyme to 0.9 saturation with ammonium sulphate.

Ammonium sulphate was added slowly to the culture filtrate with constant stirring and was allowed to stand overnight. Precipitated enzyme was collected by centrifugation at 20,000 g in Sorvall centrifuge. The precipitate was dissolved in 10 mM potassium phosphate buffer, pH 7.0 and was then dialysed against the same buffer to remove ammonium sulphate. The dialysed enzyme was concentrated by lyophilisation and was subjected to preparative PAGE.

(b) Preparative PAGE & electrophoretic elution of the enzymes. The cationic system C described by Zuidweg et al. (19) was used with previously described mofidications. Electrophoresis was carried out in the apparatus constructed according to Ghadge et al. (21), wherein gel making and subsequent electrophoresis is carried out in the same apparatus. The apparatus was fabricated from perspex sheets (Fig. IV-1).

Two slab gels (18 x 11 x 0.9 cm) were prepared. 50 mg protein, with basic fuchsin as a tracking dye was loaded on

Fig. IV-1(A) Dimensions of the preparative electrophoresis unit

(a) Chamber with slanting wall, with 2 x 8.5 cm base

(b) Second chamber, 3 x 8.5 x 19 cm

(c) Common middle wall, 8.5 x 18.2 cm height

(d) Two support strips each 5.5 x 0.5 x 1 cm

(e) 0.8 cm gap below the common middle wall

(f) Slanting wall of chamber 'a'

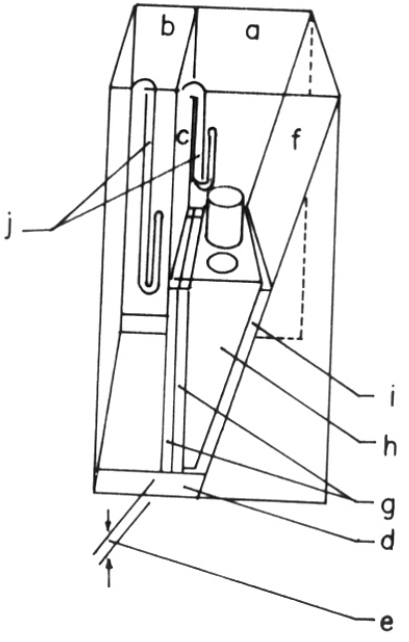
(g) Gel cassettes

(h) Wedge

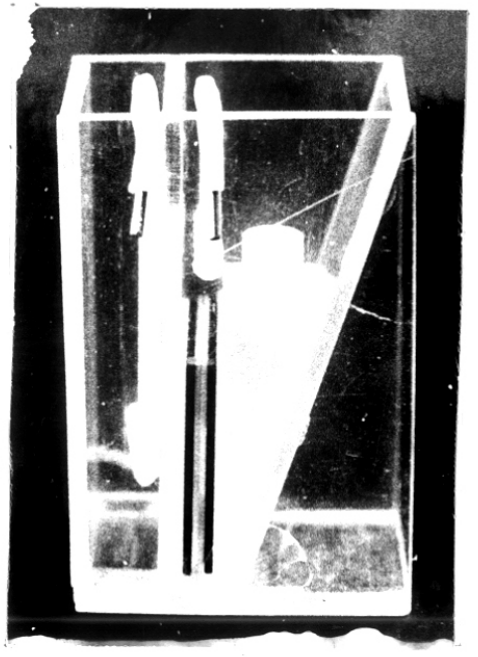
(i) Spacer or spacers, if necessary, each 8.5 x 9.5 cm height, thickness from 0.3 to 0.5 cm

(j) Two 'L' shaped platinum electrodes

Fig. IV-1(B) Preparative electrophoresis unit



A



B

each of the slab gel. Electrophoresis was carried out for 28 h at 100 V and 40 mA at 4°C. An empty cassette, sealed at the bottom and filled with bath buffer, was placed between the two gel cassettes to act as a cooling device during the run.

After the run, the two protease bands were located on the gel, by cutting a vertical side strip of each slab gel and staining it with Coomassie Brilliant Blue G-250. Based on the results of the analytical electrophoresis, the proteases could be easily detected on the strips, as they are the prominent protein moieties of the crude extract. The strips were then aligned to respective slab gels, the corresponding bands were cut and the enzymes were eluted by the method described by Bodhe et al. (22). Gel slices of respective enzymes were placed on the recovery cells containing foam pieces soaked in KOH-Borate buffer, pH 7.6. Vertical downward electrophoretic elution was carried out through the thickness of the gel. Tris-Borate buffer, pH 7.8 was used as bath buffer. The enzyme gets collected in the foam piece, its escape being prevented by semipermeable membrane made of cellulose caseing at the bottom of the recovery cell. Elution was carried out at 60 mA for 6 h. After the run, the enzymes were collected by squeezing the foam. The eluates were dialysed against 10 mM potassium phosphate buffer, pH 7.0 and were concentrated by lyophilisation. Both the eluates, showing single bands after gel electrophoresis were subjected to ion exchange

chromatography on CM-cellulose, to remove ionic acrylamide impurities.

(c) Ion-exchange chromatography on CM-cellulose. CM-cellulose (2.0 g) equilibrated with 0.01 M potassium phosphate buffer, pH 7.0 was packed in a glass column (1.2 x 15 cm). Protease I (20 mg) was adsorbed on CM-cellulose at pH 7.0 and column was washed with same buffer to remove unadsorbed impurities. Adsorbed protein was then eluted with 0.08 M phosphate buffer, pH 7.0. Fractions of 2 ml were collected at a flow rate of 12 ml/h and were assayed for protein and enzyme activity.

Protease II was treated similarly except that the adsorption and elution were carried out in 0.02 M and 0.06 M buffer respectively.

Results & Discussion

The analytical electrophoresis of crude enzyme culture filtrate showed two peaks of protease activity corresponding to two prominent protein bands on the gel, indicating the presence of two electrophoretically separable, extracellular proteases (slow moving, designated as protease I and fast moving, as protease II) (Fig. IV-2). In order to verify whether the two proteases were products of a post-secretional event or they occurred intracellularly, the time profile of their intracellular production was investigated.

Gel electrophoresis of 12 and 24 h samples of the intracellular enzyme showed only a single protease band corresponding to protease I, while 36 h sample showed two bands analogous to those obtained for the culture filtrate (Fig. IV-3). Intracellular occurrence of the two proteases rules out the possibility of their formation as a result of post-secretional event.

Fig. IV-4 shows the kinetics of production of intracellular verus extracellular protease activity. Both intra and extra-cellular proteases were inducible by casein. However, amount of extracellular protease was approximately three times more than the corresponding intracellular activity with casein.

The properties of crude intracellular protease such as optimum pH and temperature, stability at different pH and temperature, sensitivity towards EDTA and PMSF and electrophoretic patterns were exactly identical to those of

Fig.IV-2 **Location of the protease bands after analytical PAGE**

Crude culture filtrate was subjected to analytical PAGE. After electrophoresis, one gel was stained with Coomassie Brilliant Blue G-250 stain and other was cut into 20 pieces and estimated for protease activity. Drawing of the gel at the bottom of the figure is a schematic representation of the corresponding positions of the two proteases on the gel.

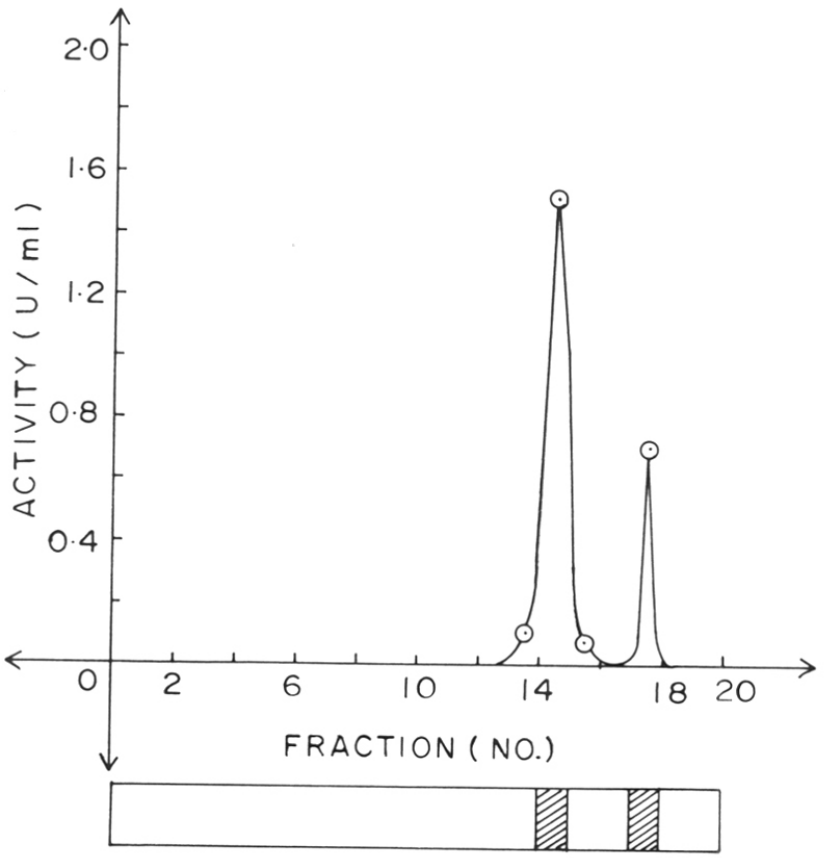

2·0
1·6
1·2
0·8
0·4
ACTIVITY (U / ml)
0
2
6
10
14
18
20
FRACTION (NO.)

Fig. IV-3 **Time profile of intracellular protease production**

C. coronatus was grown on MGYP slants. Crude extracts prepared by crushing the washed mycelia were centrifuged and subjected to electrophoresis.

Lane 1, extracellular enzyme preparation

Lane 2, 12 h sample

Lane 3, 24 h sample

Lane 4, 36 h sample

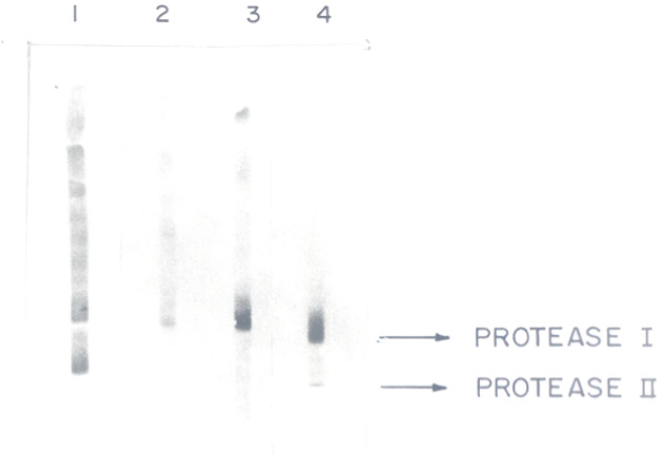
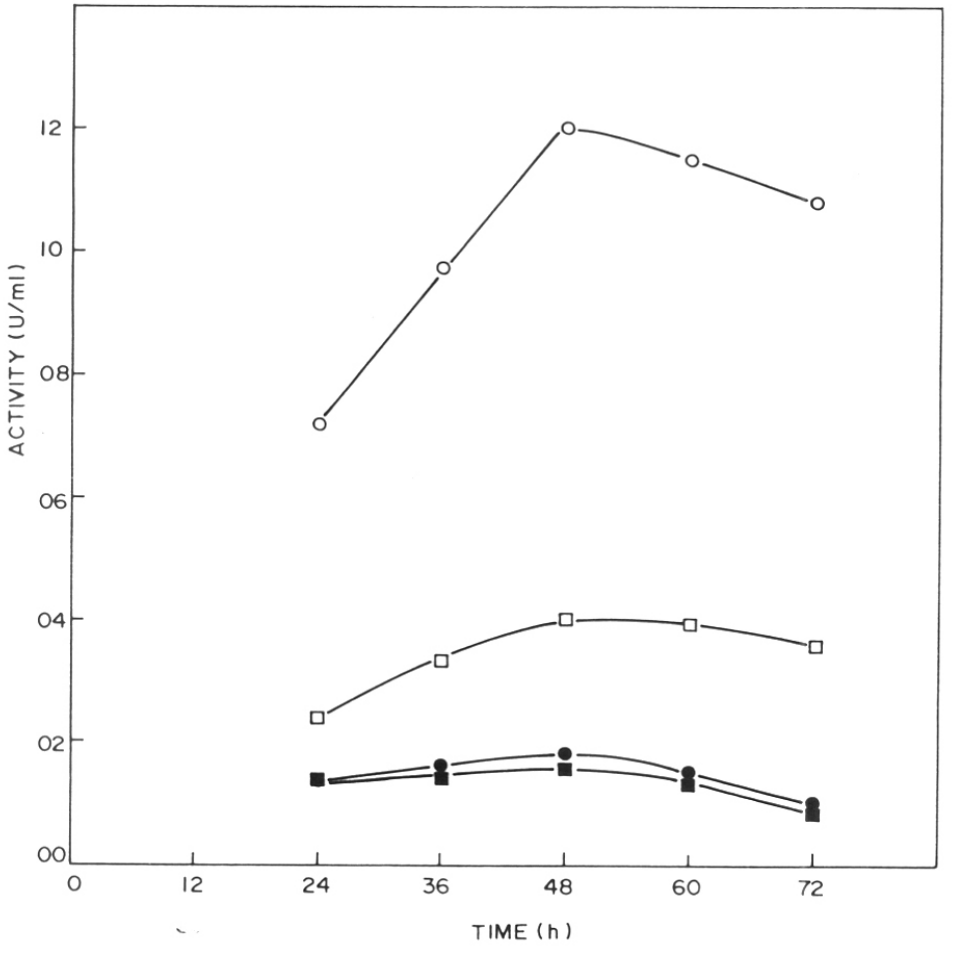

1
2
3
4
PROTEASE I
PROTEASE II

Fig. IV-4 **Kinetics of production of intracellular versus extracellular protease**

The organism was grown in submerged culture in MGYP medium, ●, extracellular; ■, intracellular or MGYP + casein medium, o, extracellular; □, intracellular.


ACTIVITY (U/ml)
12
10
08
06
04
02
00
0
12
24
36
48
60
72
TIME (h)

extracellular culture filtrate, indicating that these proteases were produced intracellularly and then secreted into the culture medium (Table IV-1).

The two extracellular proteases were purified by ammonium sulfate precipitation, preparative PAGE and ion exchange chromatography. Table IV-2 shows summary of the purification of the two proteases. The progress of purification was followed by analytical PAGE. The purified enzymes were electrophoretically homogenous (Fig. IV-5). The final recovery of protease I and II was 24% and 10.7%, and fold purification was 17.3 and 11.5 respectively. Specific activities of protease I and II were 45 and 30 U/mg respectively.

The method of purification by preparative PAGE is simple, fast and almost a single step purification process. The elution of the proteins is also rapid as it is carried out through the thickness of the gel and the distance travelled by proteins is very short. However, in this procedure it is essential to remove the non-protein impurities of acrylamide and acrylate, which was achieved by further purification of enzyme on CM-cellulose column.

Though fold purification of protease I and II is considerably less, the resulting specific activities are high. This shows that ratio of proteases to other proteins in culture broth of C. coronatus is high.

Table IV-1 : Comparison of the properties of crude extracellular and intracellular protease preparations

Parameter	Value	
	Extracellular Protease	Intracellular Protease
Optimum pH	9.7 - 10	9.7 - 10
Optimum temperature (°C)	40	40
Stability pH	7.0 - 7.5	7.0 - 7.5
Temperature stability (°C)	35 - 40, 1 h	35 - 40, 1 h
EDTA (0.2 M) sensitivity	insensitive	insensitive
PMSF (0.1 mM) sensitivity	totally inhibited	totally inhibited

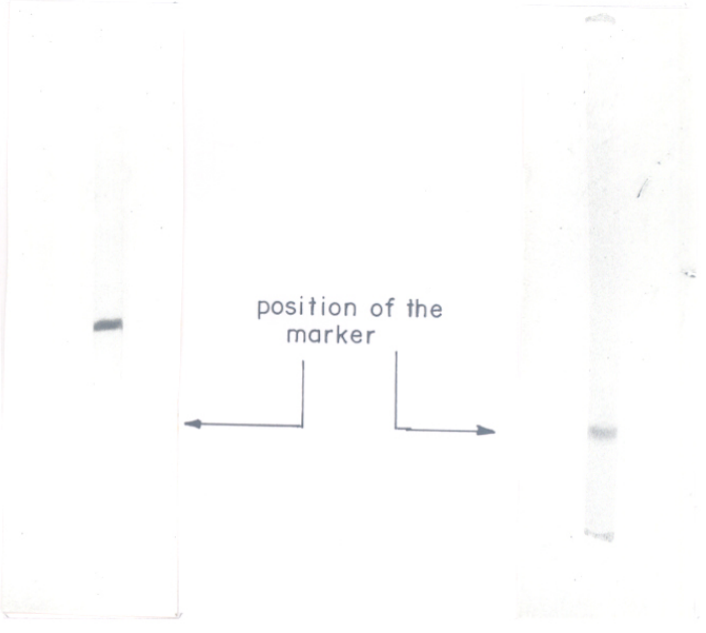
Table IV-2 : Purification of *C. coronatus* proteases

Step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
I. Culture filtrate	1000	11,500	30,000	2.6	1.0	100
II. Ammonium sulphate precipitation	18	750	18,000	24.0	9.2	60
III. Preparative PAGE						
i) protease I	250	202	9,000	44.5	17.1	30.0
ii) protease II	250	135	4,000	29.6	11.4	13.3
IV. CM-cellulose chromatography						
i) protease I	10	160	7,200	45.0	17.3	24.0
ii) protease II	10	107	3,210	30.0	11.5	10.7

Fig. IV-5 Analytical PAGE of protease I and II at pH 7.6

a) Protease I

b) Protease II



a b

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CHAPTER-V

BIOCHEMICAL, PHYSICOCHEMICAL AND IMMUNOLOGICAL COMPARISON OF THE TWO PROTEASES AND ITS IMPLICATION IN PHYSIOLOGICAL REGULATION OF CONIDIAL DISCHARGE

Summary

To understand the regulation of conidial discharge, the mechanism of control of protease activity was investigated. Preliminary studies on proteases revealed the presence of two electrophoretically separable intracellular proteases (protease I and II). Formation of smaller and less active protease II coincided with the decrease in conidial discharge. In order to trace the origin of protease II, the corresponding purified extracellular enzymes were compared with respect to their biochemical, physicochemical and immunological properties. The two proteases possessed identical biochemical properties, viz. optimum pH (9.7 - 10), optimum temperature (40°C), stability pH (7 - 7.5) and temperature stability (1 h at 35 - 40°C). Both possessed esterase activity and were inhibited by 0.1 mM phenyl methyl sulfonyl fluoride (PMSF). The amino acid analysis and the two dimensional peptide maps of the two proteins revealed that protease II was completely homologous with protease I, though the latter showed some additional portion not contained in the former. Western-Blot ELISA, double diffusion and immunotitration experiments with antiprotease I antibody also confirmed the structural similarity of the two proteases. The antigenic valencies of protease I and II were 16 and 12 respectively. The proteases differed only in their charge, specific activity and molecular mass. Protease I and II had pI of 9.9 and 9.0 and molecular mass of 23,170 and 19,270 daltons respectively. Specific activities of protease I and

II were 45 and 30 U/mg respectively.

Purified protease I showed partial degradation to protease II *in vitro*, the process being sensitive to PMSF, indicating its proteolytic nature. These results suggest that formation of a less active protease by autoproteolysis represents a novel means of physiological regulation of protease activity which in turn regulates conidial discharge in *C. coronatus.*

Introduction

As described in previous chapter, Conidiobolus coronatus produces two electrophoretically separable proteases, protease I in the initial period of growth and protease II at the time coincident with the reduced conidial discharge stage. This observation arose several questions viz. (i) whether the two proteases are homologous and if so (ii) whether one is produced from the other by post-translational modification and (iii) what is their significance in controlling the conidial discharge. To gain insight into the mechanism of regulation of protease activity and consequent conidial discharge, the biochemical, physicochemical and immunological comparison of the two proteases was carried out.

As compared to the vast literature available on fungal proteases, there are very few reports on purification and characterization of proteases of fungi belonging to Phycomycetes, especially Conidiobolus (1, 2).

Table V-1 gives the summary of biochemical and physicochemical properties of proteases from some of the fungal, bacterial and actinomycetes species. Amino acid compositions of some of the fungal, alkaline proteases and subtilisins are given in table V-2.

Table V-1 : Summary of properties of fungal and bacterial alkaline proteases

Microorganism	Ref.	Optimum pH	Optimum Temp.(°C)	pH Stability	Heat Stability	Mol. Wt. (KDa)	pI	Inhibitors	Metal ion Inhibition
Aspergillus flavus	3	7.0-9.0	-	-	-	23-27	-	-	-
A. flavus var columnaris	4	8.0-11.0	50-55	-	-	-	-	PMSF	-
A. sojae	5	11.0	-	4.5-10	$\angle$60°C	-	-	DFP	Hg^{2+}, Fe^{2+}, Cu^{2+}
A. oryzae	6								
Pro I		-	-	5.0-8.5	$\angle$60°C	-	-	-	Ni^{2+}, Cu^{2+}, Zn^{2+}
Pro II		-	-	4.5-10.5	$\angle$60°C	-	-	-	Cu^{2+}, Zn^{2+}
Pro III		-	-	3.0-6.0	$\angle$60°C	-	-	-	Ni^{2+}, Cu^{2+}, Zn^{2+}
A. oryzae (aspB)	7	10.3-10.4	-	-	$\angle$55°C	-	-	DFP	-
A. oryzae (aspC)	8	9.0-10.0	-	-	-	-	-	DFP, PMSF	Hg^{2+}, Zn^{2+}, Cu^{2+}
A. oryzae	9	9.3-9.5	40-42	5.0-8.0	45°C	-	-	-	-
A. oryzae 460	10	11(polyLys) 4(PolyGlu)	-	5.0-8.5	50°C	23	-	DFP	-
A. oryzae E1212	11	10.0	-	6.0-8.0	4°C	35	-	DFP	-
A. sulphureus	12	7.0-10.0	50	6.0-11.0	$\angle$37°C	-	-	DFP	Zn^{2+} Cu^{2+} Co^{2+} Ni^{2+}
A. sydowi	13	8	40	6.0-9.0	$\angle$45°C	-	-	DFP	Zn^{2+}, Ni^{2+}, Hg^{2+}

Table V-1 Continued

Microorganism	Ref.	Optimum pH	Optimum Temp. (°C)	pH Stability	Heat Stability	Mol. Wt. (KDa)	pI	Inhibitors	Metal ion inhibition
A. candidus	14	11.0-11.5	47	5.0-9.0	40°C	22-23	4.9	DFP	-
A. fumigatus	15	7.5,10.0 (casein) 4.0,9.0 (Hb)	40	-	∠60°C	-	-	DFP	-
A. tenuissima	16	9.5	-	-	-	23-24	∠10.5	-	-
A. sojae	17	9.0-10.0	-	-	-	25	5.1	-	-
A. niger	18	7.8	45	5.5-7.5	∠40°C	21	4.1	PMSF, EDTA	Cu^{2+} Mn^{2+} Co^{2+} Hg^{2+}
Acremonium kiliense	19-21	10.5	-	3.0-7.0	∠55°C	28.5-31	-	DFP, PMSF	-
Alternaria alternata (Fr.) kiessel	22	8.0-9.5	-	wide range	-	-	-	-	-
Cephalosporium sp	23,24	11.0	50	4.0-11.0	45°C	22.5	10.5	DFP	Fe^{3+}, Hg^{2+}
Penicillium cyaneofulvum	25	9.5-11.0 (casein) 6.5-8.5 (Hb)	-	4.0-11.0	∠60°C	45	-	DFP	Hg^{2+}, Zn^{2+}
P. lilacinum	26	11.0	50	4.0-11.0	∠40°C	24-26	-	PMSF	Hg^{2+}, Ag^{2+}, Cu^{2+}
P. charlesei	28	7.0-9.0	45	6.0-10.0	55°C	44	8.5-9.0	PMSF	-

Table V-1 continued

Microorganism	Ref.	Optimum ph	Optimum Temp(oC)	pH Stability	Heat Stability	Mol. Wt. (KDa)	pI	Inhibitors	Metal ion inhibition
P. cyclopium	29	7.0	-	-	-	32	5.0	PMSF	-
Phymatotrichum omnivorum	27	5.0 (Hb)	-	-	-	33	-	PMSF, EDTA	-
Yeast proteinase C	30	9.0-11.0	-	-	-	61	3.6	-	-
Conidiobolus sp.	1	9.0	40-50	7.0-8.0	40°C	-	-	EDTA	Hg^{2+}, Ba^{2+}, Ag^{2+}, Al^{3+}, Cu^{2+}
Scopulariopsis brevicaulis	31	10.5-11.0 7.0-9.5 (Hb) 6.0-8.0 (gelatin)		-	-	-	-	DFP, PMSF	-
Streptomyces griseus	32								
Pro I		9.5	40	-	-	18.5	9.6	DFP, I_2, N-bromosuccinimide	-
Pro II		9.5	50	-	-	15.5	8.8	"	-
S. rimosus	33	4.0-4.5, 10.5-11.0	50	4.0-9.0	40°C	22	4.9	DFP, PMSF	-
Thermophilic Streptomyces sp.	34	-	-	-	-	21.5	9.5	-	-
Subtilisin Carlsberg	35	10.5	60	-	$\lesssim$ 50°C	27.3	9.4	-	-

Table V-1 Continued

Microorganism	Ref.	Optimum pH	Optimum Temp(°C)	pH Stability	Heat Stability	Mol. Wt. (KDa)	pI	Inhibitors	Metal ion inhibition
Subtilisin BPN'	35	10.5	60	-	$<$ 50°C	27.5	7.8	-	-
Bacillus No. 221	36	11.5-12.0	60-65	4.0-11.0	60°C	30	$>$ 9.4	DFP, Urea	-
B. subtilis IFO 3027	37	11.0	55	5.0-7.0	50°C	540	-	PMSF, MAPI, chymostatin	-
B. pumilus	38	9.0	50	6.0-10.0	-	-	-	DFP,N-bromo-succinimide	Cu^{2+}, Fe^{2+}, Hg^{2+}
Bacillus sp.	39	11.5	60	5.0-9.5	$<$ 50°C	17.5	10.6	DFP	Zn^{2+}, Hg^{2+}
B. thuringiensis var. kurstaki	40	8.5-9.0	70	7.0-11.0	60°C	34	9.0	PMSF, PCMB	
Alkalophilic Bacillus sp NKS21	41	10.2	-	-	40°C	32	2.8	-	-
B. subtilis A-50	42	7.0-10.0	40	6.0-7.0	60°C	30	4.3	PMSF, EDTA EGTA	-
Pseudomonas maltophila	43	10.5	55	5.0-12.0	$<$ 60°C	46	$>$ 10.6	EDTA, PMSF, chymostatin	-
Rhodocyclus gelatinosus	44	7.8	50	7.0-7.2	50°C	80	6.3	DFP, PMSF EDTA, chymo-statin	-

Table V-1 Continued

Microorganism	Ref.	Optimum pH	Optimum Temp.(°C)	pH Stability	Heat Stability	Mol. Wt. (KDa)	pI	Inhibitors	Metal ion Inhibition
Thermus aquaticus	45								
T351		9.6	-	9.0-9.6	85°C	23	8.5	DFP, PMSF	-
RT6		8.8	-	9.0-9.6	∠ 85°C	27	8.5	DFP, PMSF	-
TOK3		9.6	-	9.0-9.6	85°C	25	8.9	DFP, PMSF	-
41A		9.6	-	9.0-9.6	85°C	28	8.9	DFP, PMSF	-
Thermus TOK3	46	9.5	-	-	80°C	25	8.9	DFP, PMSF EDTA, EGTA, O-phenanthro-line	-

_ Not cited in the reference.

Table V-2 : Amino acid compositions of fungal alkaline proteases and subtilisins

Amino acid	Alkaline proteases											Subtilisin	
	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)	(m)
Lysine	11-12	12	17	11	14	12	14	4.7	1	17	12	9	11
Histidine	4	4	5	3-4	5	5	4	4.4	1	7	5	5	6
Arginine	2	3	3	2	3	4	3	10	10	8	9	4	2
Aspartic acid + Asparagine	21	21-22	26	21	31	27	25	28.1	18	54	35	28	28
Threonine	11	13	14	11	18	19	15	23	33	17	20	19	13
Serine	19	23	24	20	28	20	23	36	35	27	31	32	37
Glutamic acid + glutamine	12	13	14	12-13	19	12	15	10	12	36	13	12	15
Proline	4	5-6	7	4-5	6	5	7	13.8	4	23	11	9	14
Glycine	19	21	25	20	27	31	22	39.5	55	32	32	35	33
Alanine	23	23	29	23	32	31	24	33.9	22	20	38	41	37
Cysteine	0	0	ND	0	2	0	0	5.9	6	*7	18	0	0
Valine	15	16	16	15	18	21	16	13.5	19	24	8	31	30

Table V-2 Continued

Amino acid	Alkaline proteases											Subtilisin	
	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(1)	(m)
Methionine	0	1	1	1	2	0	2	2	0	4	12	5	5
Isoleucine	9-10	10-11	12	9-10	14	10	11	16.2	7	19	12	10	13
Leucine	9	10	12	9	14	14	10	17.7	7	30	14	16	15
Tyrosine	5	4-5	7	5	8	7	6	9.5	13	24	8	13	10
Phenylalanine	5	6	6	5	7	5	7	6.7	6	21	7	4	3
Tryptophan	2	2	2	2	2	2	2	2.8	4	11	ND	1	3

a: Aspergillus oryzae (47); b: A. oryzae (8); c: A. oryzae (10); d: A. flavus (3); e: A. sojae (48); f: A. sulfureus (12); g: A. candidus (49); (h) Acremonium kiliense (20); (i) Alternaria tenuissima (50); j: Yeast proteinase c (30); k: Penicillium cyclopium (28); (l) Subtilisin Carlsberg (51); m: Subtilisin BPN' (52).

ND = Not determined *Half cystine detected.

Materials & Methods

Materials:

Bovine serum albumin (BSA), anti-rabbit-IgG-horse radish peroxidase conjugate, PONCEAU S concentrate, Coomassie Brilliant Blue G-250 and R-250 and molecular mass markers were purchased from Sigma. The suppliers of the following chemicals are indicated in parentheses: SDS (Koch Light Laboratories), Sephadex G-50 (Pharmacia), Ampholines (LKB), Freund's adjuvant (Difco), nitrocellulose membrane (Schleicher and Schull, Keene N.H., USA), 3-3-diaminobenzidine tetra-hydrochloride (DABT) (Sisco Research Laboratories, India). All the other chemicals used were also of analytical grade.

All the buffers and reagents were prepared in double distilled water.

Details of enzyme assay (Chapter II), protein estimation (Chapter III) and PAGE (Chapter IV) are described previously.

Enzyme assays:

(a) Protease activity on various protein substrates. The usual caseinolytic assay was followed for substrates like bovine serum albumin, ovalbumin and Haemoglobin.

Activity of enzyme on azocasein and azoalbumin was determined by the method of Ansari and Stevens (53). One ml of the chromogenic substrate (2.5 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.0 was incubated with 0.1 ml of suitably diluted enzyme for 30 minutes at 37°C. The reaction was stopped by addition of 0.04 ml of 50% (w/v) TCA. After centrifugation,

the absorbance was measured at 440 nm. Units were calculated as that for caseinolytic assay.

(b) Esterase activity. The esterase activity of enzyme with BAEE, BTEE, TAME was determined spectrophotometrically at 253 nm (54), 256 nm (55) and 247 nm (56) respectively. Potassium phosphate buffer (0.1 M) pH 7.5, 0.1 M Tris-HCl, pH 7.8 with 0.1 M $CaCl_2$ and 0.05 M Tris-HCl, pH 7.8, with 0.01 M $CaCl_2$ were the buffers used for BAEE, BTEE and TAME respectively. The reaction was initiated by adding 0.1 ml of suitably diluted enzyme to 1.0 ml of various concentrations of substrate solution and the rate of change of absorbancy was measured.

(c) Assay using p-nitroanilide substrates. The method of Ansari and Stevens (53) was followed. 0.1 ml of suitably diluted enzyme was added to 5.0 ml of 0.001 M substrate (BAPNA, BTPNA or L-leucine-p-nitroanilide dissolved in DMSO) in 0.05 M Tris-HCl buffer, pH 8.0. Reaction was carried out at room temperature for 30 min and terminated by adding 1.0 ml of 30% acetic acid and absorbance at 410 nm was determined. One unit of enzyme activity was defined as the amount of enzyme which released 1 µmol of p-nitroanilide per minute at 30°C. Appropriate control blanks containing enzyme or substrates alone were also simultaneously run.

Optimum pH and temperature, stability at different pH and temperature and effects of PMSF and EDTA on the activity of purified enzymes were determined as described earlier (Chapter IV).

Effect of metal ions:

Effect of various metal ions on protease activity was determined by preincubating the enzymes with various metal ions and then determining the residual activity.

90 U of individual protease samples in 1.0 ml of 0.1 M Tris-HCl buffer, pH 8.4, containing 10 mM metal ion were incubated at 30°C for 15 minutes, after which the residual activities were determined by adding 1.0 ml of 1% casein in Tris-HCl buffer, pH 8.4. Reaction was carried out as usual at 37°C for 10 min. Activities were compared with the control incubated under similar conditions, but devoid of any metal ion. As some of the metal salts get precipitated in carbonate-bicarbonate buffer, pH 10.0, Tris-HCl buffer was used for this experiment.

Effect of subtilisin inhibitor:

Protease I and II samples were incubated in potassium phosphate buffer, pH 7.0, containing subtilisin inhibitor from Dolichos biflorus, in various inhibitor to enzyme molecular ratios ranging from 1:1 to 10:1, at room temperature for 20 minutes. After incubation, residual activity was determined and compared with control incubated under similar conditions, without inhibitor.

Determination of molecular weight:

1. Gel filtration method. The molecular weight of the enzymes was determined by gel filtration according to the method described by Andrews (57), using Sephadex G-50 packed

in a column (1.6 x 100 cm) with 0.05 M potassium phosphate buffer, pH 7.0, as the eluting buffer. Caliberation curve was prepared by loading separately, 2 mg each of cytochrome c (12,400), myoglobin (17,000), chymotrypsinogen (25,800) and ovalbumin (43,000) as marker proteins. Fractions of 2 ml were collected at a flow rate of 12 ml/h and were assayed for respective proteins. Void volume was determined with Blue Dextran. Cytochrome c was estimated at 550 nm by reducing it with dithionite at pH 8.0 (58). Myoglobin was estimated at 415 nm in 5 mM 2-mercaptoethanol at pH 8.0 (59). Chymotrypsinogen and ovalbumin were estimated by the method of Warburg and Christian (60). Purified protease (0.3 mg) I and II were loaded on the column separately. Molecular weights of enzymes were calculated by extrapolating their elution volumes on a graph of logarithm of molecular weight against elution volume for standard proteins.

2. SDS-PAGE method. SDS-PAGE was carried out according to Laemmli (61). The principle of this method is that treatment of protein with SDS minimises the native charge differences of the proteins, as a result of which they migrate as anion under the influence of electric field. The rate of migration of protein under these circumstances is proportional to its molecular size. SDS treatment also causes extensive disruption of hydrogen and hydrophobic bonds. Treatment with 2-mercaptoethanol breaks all the disulfide linkages. The SDS treated lysozyme (14,300), β-lactoglobulin (18,400), pepsin (34,700) and egg albumin (45,000) were used as standard

protein markers. 50 µg each of the two proteases was treated with SDS and loaded on gel alongwith standard proteins. After the electrophoresis, protein bands were visualised by staining the gels in 0.2% Coomassie Brilliant Blue R-250 in ethanol:acetic acid:water (4:1:5) overnight and destaining in the same solvent. The rate of flow (R_f) for each protein was calculated as the ratio of the distance travelled by protein to that by the marker dye. R_f values of protease I and II were extrapolated on the graph of R_f versus logarithm of molecular weights of standard proteins and their molecular weights were determined.

Subunit detection

The presence of subunits in the enzyme was tested by its migration in SDS-PAGE (62, 63) with and without treatment of enzyme with 1% SDS in combination with 1% B-mercaptoethanol and 0.5% iodoacetamide at 100°C for 5 minutes.

Determination of isoelectric point (pI):

Isoelectric focusing was carried out by method described by Pawar et al. (64) over the pH range of 6 - 11.

U tube column designed by Pawar et al. (64) was used for this experiment. The density gradient was prepared with glycerol using an automatic gradient mixer. Ampholine carrier ampholytes and enzyme solutions were mixed in light and dense gradient solutions. Electrofocusing was carried out at 4°C at a constant voltage of 400 V. The run was complete in 30 h as was indicated by the drop in amperage

from 5 mA to zero. However, the run was continued upto 40 h to ensure the formation of a stable gradient.

After completion of the run, fractions of 0.15 ml (approximately 3 drops) were collected by adjusting the flow rate suitably with the help of a pinchcock. The pH of each fraction was measured using a surface electrode and the individual fractions were assayed for protease activity.

Determination of glycoprotein nature:

Glycoprotein nature of the enzyme was tested by staining the protein on gel by Schiff's staining method (65). After electrophoresis, the gels were immersed in 12.5% TCA for 30 min followed by incubation in 1% periodic acid (in 3% acetic acid) for 50 min. Excess of iodic ions was removed by stirring the gel tubes in water overnight with three changes. The gel tubes were then immersed in fuchsin sulfite stain in the dark for 50 min and washed with freshly prepared 0.5% potassium metabisulfite for 30 min. The gel tubes were rinsed in water for removing excess stain and stored in 7% acetic acid.

Amino acid analysis:

Samples of proteins (0.5 - 1.0 mg) were hydrolysed in 6 M HCl at 110°C for 24 h and 48 h in evacuated sealed pyrex tubes in a block heater. The hydrolysates were evaporated to dryness in a vacuum desiccator. The residual HCl in the hydrolysates was removed by dissolving the residues in 1.0 ml of deionised water followed by drying in vacuum desiccator.

The residues were then dissolved in citrate buffer, pH 2.2 and aliquots were analysed on Beckman automatic amino acid analyser (Model 120 B) by the method of Spackman et al. (66).

Half cystine content was determined as cysteic acid after oxidation with performic acid (67) followed by hydrolysis and amino acid analysis.

Tryptophan is completely destroyed during the acid hydrolysis and thus could not be detected in amino acid analysis by this method. So it was determined by the method of Goodwin and Morton (68) by determining the absorbance of protein at 294.4, 257 and 280 nm.

Two dimensional peptide mapping:

Enzyme preparations (500 µg) were hydrolysed with trypsin at 37°C for 24 h in 0.05 M potassium phosphate buffer, pH 7.5, in a trypsin to enzyme ratio of 1:50. Finger printing of tryptic digests was carried out according to Ingram (69).

The technique involved high voltage paper electrophoresis in one direction followed by ascending chromatography in a perpendicular direction. Paper electrophoresis was carried out using Whatman No. 3 filter paper, cut into T shape (30 x 28 cm with 13 cm long and 6 cm broad sleeves on either side of the length). Michl's buffer (70) (pyridine: acetic acid:water, in the ratio of 10:0.4:90 by volume, pH 6.4) was used for electrophoresis. The filter paper was

soaked in the buffer, blotted to remove excess buffer and was placed on horizontal glass plate. The concentrated tryptic digest of enzyme was loaded on paper. The paper was covered with another glass plate and sleeves on either side were dipped in the electrophoration chambers. Electrophoresis was carried out at a constant voltage of 1000 volts for 2 hours. After electrophoresis, sleeves were cut off and paper was dried. Then it was subjected to ascending chromatography for 12 h, using pyridine:n-butanol:acetic acid:water (20:30:6:24) solvent system (70). The paper was then removed, dried and peptide maps were visualised by spraying the paper with 0.3% ninhydrin solution in acetone. Colour of spots was intensified by heating the paper at 80°C for 10 min. The colour was fixed by a fixing reagent (1% cupric nitrate and 0.05% nitric acid in acetone).

Preparation of antibodies:

Antibodies against electrophoretically pure protease I were raised in a Newzealand white rabbit, by subcutaneously injecting, 2 mg of protein emulsified with equal volume of complete Freund's adjuvant. Booster injections were given at fortnightly intervals for 10 to 12 weeks. The rabbit was bled when the antibody titre reached to 32, as checked by double diffusion.

The antibodies were precipitated to 0.9 saturation with ammonium sulphate. The precipitate was dissolved and dialysed against phosphate buffer saline, pH 7.2 (0.1 M Na_2HPO_4, 0.4 M NaH_2PO_4, 0.14 M NaCl). For quantitation of antibodies,

absorbance of the suitably diluted samples was measured at 280 and 310 nm. Antibody concentration was calculated using the following formula:

$$\frac{O.D._{280\ nm} - O.D._{310\ nm}}{1.5} \times \text{dilution factor}$$

Oüchterlony double diffusion:

Double diffusion was carried out in 1% agarose gel in phosphate buffer saline (PBS), pH 7.2, at 4°C for 24 to 48 h according to Oüchterlony (71). 50 - 100 µg of respective proteins and 0.5 - 0.7 mg of antibody was used. Precipitin lines formed were visualised after drying the gels and staining with 0.25% Coomassie Brilliant Blue R-250 in ethanol:acetic acid:water (4:1:5).

Western blotting:

Crude enzyme preparation and purified protease I and II samples were subjected to SDS-PAGE, using 10% gels according to Laemmli (61). After the run, proteins from the gel were electrophoretically transferred to nitrocellulose membrane (72) over a period of 2 h at 150 V in a buffer containing 20 mM Tris, 50 mM glycine, 20% methanol, at pH 7.8 at 4°C. Protein bands on nitrocellulose membrane were visualised by staining with 1:10 diluted PONCEAU S concentrate.

Enzyme linked immunosorbent assay (ELISA)

The blot was treated overnight with 10% goat serum in PBS at 4°C and washed thoroughly with PBS containing 0.1%

Tween-20. It was then treated with anti-protease antibodies (30 mg/ml) diluted 250 fold with 1% BSA in PBS, for 2 h at room temperature, followed by incubation with 1000-fold diluted goat anti-rabbit IgG horse radish peroxidase conjugate in 10% goat serum for 2 h. After washing with PBS, bound peroxidase was detected with 0.5% DABT in 0.5 M sodium citrate buffer, pH 5.0, containing 0.03% H_2O_2. Due to light sensitivity of DABT, the reaction was carried out in dark.

Immunotitration:

85 U of each of the two proteases and subtilisin were added separately to various amounts of antiserum (0 - 625 µg) in 0.05 M potassium phosphate buffer, pH 7.0. After incubation at 37°C for 1 h, protease activity in each reaction mixture was determined by caseinolytic assay.

To check the protection conferred by substrate against neutralisation of protease activities by antibody, different concentrations of casein (1 - 5%), in potassium phosphate buffer, pH 7.0 were added to enzyme aliquots just before addition of antibody. Antibody concentration was chosen to give 100% inhibition of both proteases in 15 min. An enzyme-substrate control without antibody and antibody-enzyme control without substrate were also incubated. After incubation, at 37°C for 15 minutes, protease activities in all samples were determined.

Determination of antigenic valency:

Varying amounts of proteases ranging from 0 - 20 µg were added to a series of tubes containing 25 µl of antibody (30 mg/ml) to the final volume of 500 µl in PBS. The tubes were incubated at 37°C for 1 h followed by an overnight incubation at 4°C. The precipitate was separated by centrifugation, washed twice with cold PBS and dissolved in 0.1 M sodium hydroxide. Optical densities of all samples at 280 nm were determined. Extinction coefficient values of both proteases were determined at 280 nm. Antigenic valencies were determined according to Heidelberger and Kendall (73).

Quantitative precipitin test developed by Heidelberger and Kendall is the basis of quantitative studies of antigen-antibody interaction. Increasing amounts of antigen are added to a constant amount of antibody and the weight of precipitate formed in each tube is determined. From this, a precipitin curve is plotted as optical density of precipitate at 280 nm against amount of added antigen. Point of equivalence occurs just before maximum precipitation. At this point, no free antigen or antibody molecules can be detected. The amount of precipitate increases after the equivalence point, because of continued incorporation of antigen into the complex. Eventually, soluble complexes are formed in antigen excess and the amount of precipitate decreases.

From extinction coefficient values of antigen and

antibody, one can calculate amount of antibody present in each precipitate.

When the antigen concentration is low there is a relative antibody excess. At the other extreme, at high antigen concentrations, there is free antigen and so each combining site of the antibody is occupied. At both these points, the complexes formed are relatively small. At equivalence, however, there is much cross-linking between molecules and large complexes are formed.

Every antigenic determinant is likely to be covered by a separate antibody molecule at extreme antibody excess. If the amount of antibody in the precipitate at this point is calculated, then the ratio of antibody to antigen and so the relative numbers of molecules of each in the precipitate can be determined, i.e.

$$\frac{\text{Weight of antigen}}{\text{Molecular weight of antigen}} : \frac{\text{Weight of antibody}}{\text{Molecular weight of antibody}}$$

In hyperimmune serum the major proportion of antibody will be of the IgG class with a molecular weight of 1,50,000 daltons.

To obtain the best estimate of antigenic valency, the ratio of antibody to antigen in the precipitate is plotted against the amount of antigen added. If the graph line is extended to the antibody: antigen axis the intercept will give the ratio at infinite antibody excess.

Results & Discussion

Conidiobolus coronatus produces two electrophoretically separable intracellular proteases. As compared to protease I, protease II is produced late, i.e. at the time coincident with the reduced conidial discharge stage. This suggests two possibilities viz. either (i) the protease II is produced by the organism at a later stage of growth or (ii) it is derived from protease I, as a result of post-translational modification. To explore these possibilities and their implication in regulation of conidial discharge, biochemical, physicochemical and immunological properties of the two corresponding purified extracellular proteases were compared.

Biochemical properties

Optimum pH:

Optimum pH of the enzymes were determined by estimating the enzyme activity at different pH values ranging from pH 6.0 - 11.0. Fig. V-1 shows activity profiles of the two proteases at different pH values. Both proteases showed maximum activity at pH 9.7 - 10.0.

Alkaline proteases, as the name indicates, show maximum activity at higher pH values, in the wide range of 7.0 - 11.0, for casein (Table V-1). An alkaline protease from Streptomyces however, has an optimum pH of 13.0 (74). In some cases, the pH optimum is substrate dependent.

Optimum temperature:

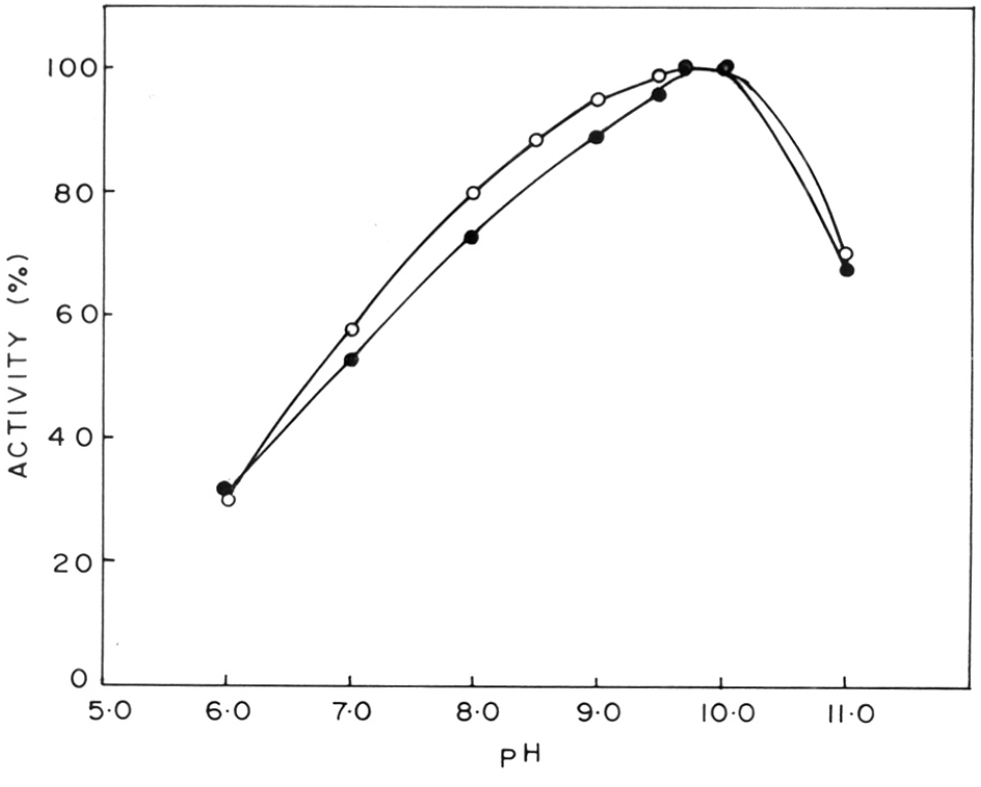
Activities of protease I and II were determined at

Fig. V-1 **Optimum pH of protease I and II**

The protease activity was determined at different pHs (6.0 - 11.0) by the caseinolytic assay. The maximum activity obtained was taken as 100% for each enzyme.

O, protease I

●, protease II


100
80
60
40
20
0
ACTIVITY (%)
5·0
6·0
7·0
8·0
9·0
10·0
11·0
pH

different temperatures ranging from 25 - 55°C, at pH 10.0. Optimum temperature of both proteases was found to be 40°C (Fig. V-2).

Alkaline proteases from various fungi exhibit optimum temperature of 40 - 50°C, while those from bacteria have higher temperature optima (Table V-1). A hyperthermoactive serine protease from archaebacterium Pyrococcus furiosus has optimum temperature of 115°C (75).

Enzyme stability:

Stability studies of proteases are important in the light of their industrial importance. Purified protease I and II were stable, when stored at -20°C, at pH 7.0 - 7.5, at the concentration of at least 1 mg/ml.

(i) pH stability. Effect of pH on stability of the two proteases was studied by incubating the proteins with buffers, covering pH range of 4.5 - 11.0. Protease I and II were stable at pH 6.0 - 8.0; pH 7.5 being the maximum stability pH (Fig. V-3).

(ii) Temperature stability. Fig. V-4 shows thermostability curves of both the proteases. They were stable upto 40°C, after which there was a rapid loss in activity.

Table V-1 shows stability values of many alkaline proteases. Most of the fungal proteases are stable in the pH range of 5.0 - 10.0, especially at neutral pH and at temperatures below 55 - 60°C. Broader range of pH stability, i.e. 4.0 - 11.0 is exhibited by fungi like Penicillium lilacinum

Fig. V-2 **Optimum temperature of protease I and II**

The protease activity was determined at pH 10 with casein as substrate at different temperatures (25 - 55°C). The maximum activity obtained was taken as 100% for each enzyme.

O, protease I

●, protease II

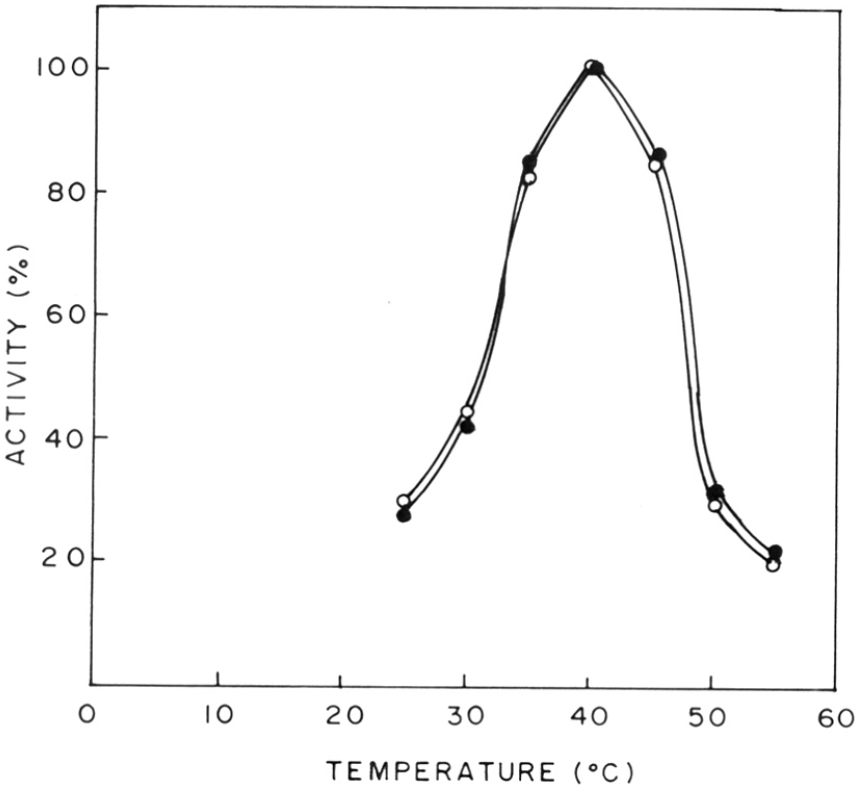

100
80
60
40
20
ACTIVITY (%)
0
10
20
30
40
50
60
TEMPERATURE (°C)

Fig. V-3 **pH stability of protease I and II**

90 U of enzyme samples were incubated in various buffers (pH 4.5 - 11.0) at 40°C for 1 h. After the incubation, residual activity in each sample was determined by caseinolytic assay. Activity of control sample incubated at 4°C, at pH 7.0 was taken as 100%.

O, protease I

●, protease II

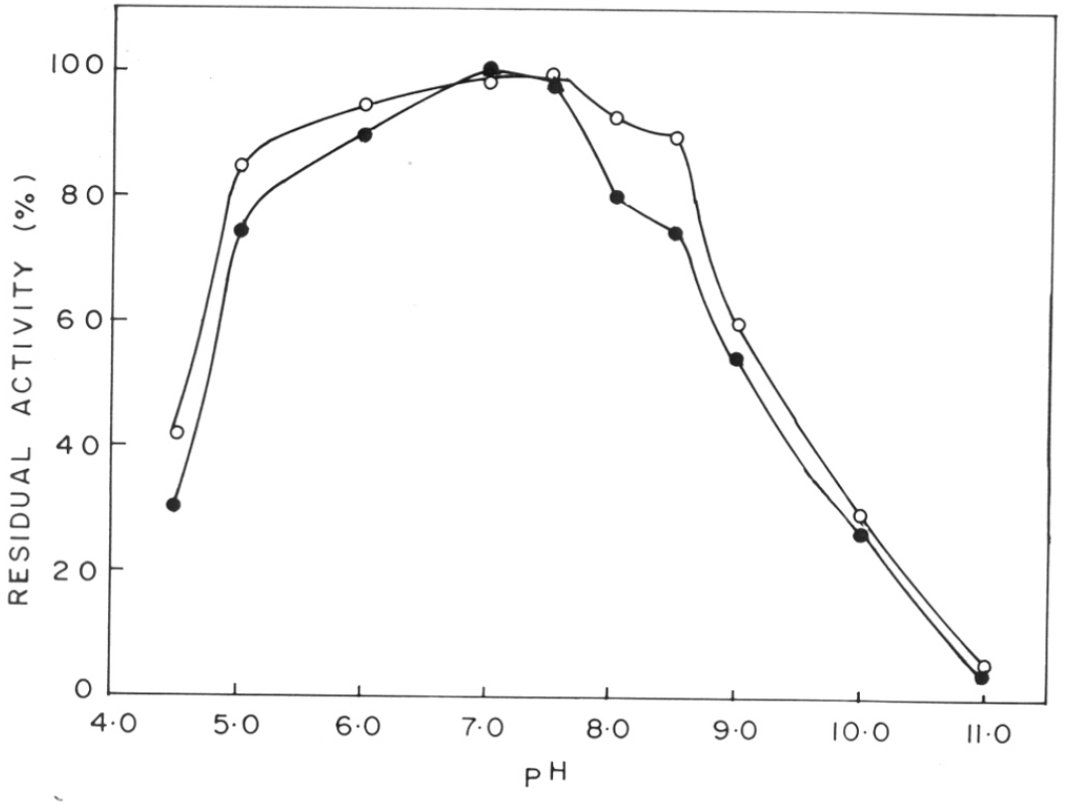
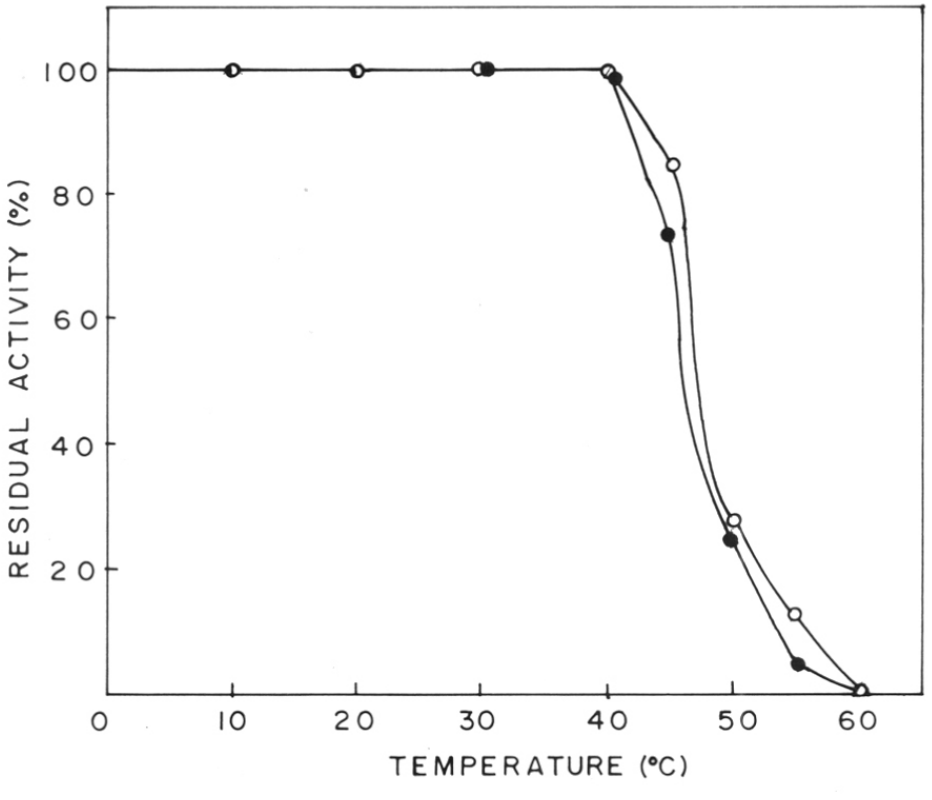

RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
4·0
5·0
6·0
7·0
8·0
9·0
10·0
11·0
pH

Fig. V-4 Thermal stability of protease I and II

The enzyme samples (90 U) were incubated at various temperatures ranging from O - 60°C, at pH 7.5. After 1 h, residual activities were determined. Activity of control sample kept at 4°C at pH 7.5 was taken as 100%.

O, protease I

●, protease II


RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
10
20
30
40
50
60
TEMPERATURE (°C)

(26) and Cephalosporium sp. (23). Alkaline protease of Acremonium kiliense (21) is more stable in acidic pH range, i.e. 3.0 - 7.0.

Bacterial alkaline protease like subtilisin Carlsberg is more stable in acidic range probably due to autolysis at alkaline pH, while that from Pseudomonas maltophila (43) is stable upto pH 12.0. Bacterial alkaline proteases show higher thermal stabilities as compared to fungal proteases, this being one of the main reasons for their great potential as "detergent enzymes".

Some of the proteases like semialkaline protease of Aspergillus melleus (76) are stabilised against thermal inactivation by calcium ions. A serine protease from Thermomonospora fusca Yx is stable upto 80°C, the thermal stability being highly pH dependent (77).

Effect of metal ions:

Effect of various metal ions on protease I and II is summarised in Table V-3. They were unaffected by metal ions like Ca^{++}, Mg^{++}, Cu^{++}, Ba^{++}, Co^{++} and Mo^{++}, while they were inhibited by Zn^{++}, Hg^{++} and Mn^{++} and activated two fold by Fe^{+++}. Table V-1 shows sensitivity of various alkaline proteases to metal ions. Zn^{++}, Hg^{++} and Cu^{++} are common inhibitors of alkaline proteases. Stabilisation or activation of proteases by Ca^{++} ions is reported (13, 35, 78, 79), but activation by Fe^{+++}, as with protease I and II has not been reported hitherto.

Table V-3 : Effect of metal ions on protease activity

Metal ion (10 mM)	Residual activity (%)*	
	Protease I	Protease II
Control (without metal ions)	100	100
Ca^{++}	100	100
Mg^{++}	100	100
Cu^{++}	100	100
Zn^{++}	57.6	42.6
Ba^{++}	100	100
Mn^{++}	54.6	100
Hg^{++}	3.0	0
Fe^{+++}	252	273
Co^{++}	100	95
Mo^{++}	100	100

* Activity of control sample devoid of any metal ions, incubated under similar conditions at 40°C as those of test samples was taken as 100%.

Effect of inhibitors:

(i) Effect of EDTA. There was no effect of EDTA on activity of protease I and II, indicating that they did not require metal ions for their activity. Some of the alkaline proteases sensitive to EDTA are listed in Table V-1.

(ii) Effect of subtilisin inhibitor. Protease I and II showed very weak susceptibility to subtilisin inhibitor. They were inactivated in a 1:8 enzyme to inhibitor molar ratio. Thus although some of their properties were similar to subtilisin, their response towards its specific inhibitor was less.

(iii) Effect of active site serine specific reagent - PMSF. Both proteases were completely inhibited by 0.1 mM PMSF. They were not affected by DMSO. Sulfonylation by PMSF, occurs exclusively at the active site of serine residue and it does not appear to react outside the reactive site under normal conditions of pH and temperature, even when high concentrations of PMSF are used for extended periods (80). Inactivation of protease I and II by PMSF, suggested the involvement of serine residue in the catalytic function of these enzymes, hence they appear to be serine proteases. Inactivation of other alkaline proteases by DFP or PMSF, indicating their serine protease nature is documented in Table V-1.

Substrate specificity:

Substrate specificity of the two proteases was studied

using various protein and synthetic substrates. They hydrolysed a variety of proteins such as casein, Haemoglobin, azo-casein, ovalbumin, azo-albumin and bovine serum albumin. Specific activities of the two enzymes on different protein substrates are shown in Table V-4. Casein was the most preferred substrate.

Their esterase activity was indicated by hydrolysis of synthetic substrates like BAEE, BTEE, TAME, though it was poor as compared to that of trypsin (54). They showed preference for esters containing arginine and tyrosine residues. They lacked amidase activity as suggested by the lack of hydrolysis of BAPNA and Leucine p-nitronilide (Table V-5).

Physicochemical properties

Molecular weight:

(i) Gel filtration. Molecular weights of protease I and II were determined by comparing their elution volumes with those of marker proteins with known molecular weights, from Sephadex G-50 column (1.6 x 100 cm). Molecular weights were calculated from the graph of Ve/Vo against log molecular weight plotted according to Andrews (57) (Fig. V-5). Molecular weights of protease I was 23,170 daltons and that of protease II was 19,270 daltons, i.e. only 4,000 daltons less than that of the former.

(ii) SDS PAGE. Molecular weights of protease I and II were determined from their migration in the SDS-PAGE. A graph of log molecular weights against relative mobilities of marker

Table V-4 : Specificity of protease I and II for various protein substrates

Substrate	Activity (%)*	
	Protease I	Protease II
Casein	100	100
Azocasein	31.78	32.64
Azoalbumin	48.03	43.38
Bovine serum albumin	13.07	11.15
Ovalbumin	10.02	2.88
Haemoglobin	49.60	50.10

*Caseinolytic activity of the proteases was taken as 100%

Table V-5 : Specificity of protease I and II for various synthetic substrates

Synthetic substrate	Activity (U/ml)	
	Protease I	Protease II
BAEE	2.00	0.85
BTEE	4.71	2.00
TAME	1.50	1.00
BAPNA	Nil	Nil
Leucine-p-nitroanilide	Nil	Nil

'ig. V-5 **Molecular weight determination of protease I and II by gel filtration on Sephadex G-50 column**

The column was calibrated with following standard proteins

(1) Ovalbumin (Mr 43,000)

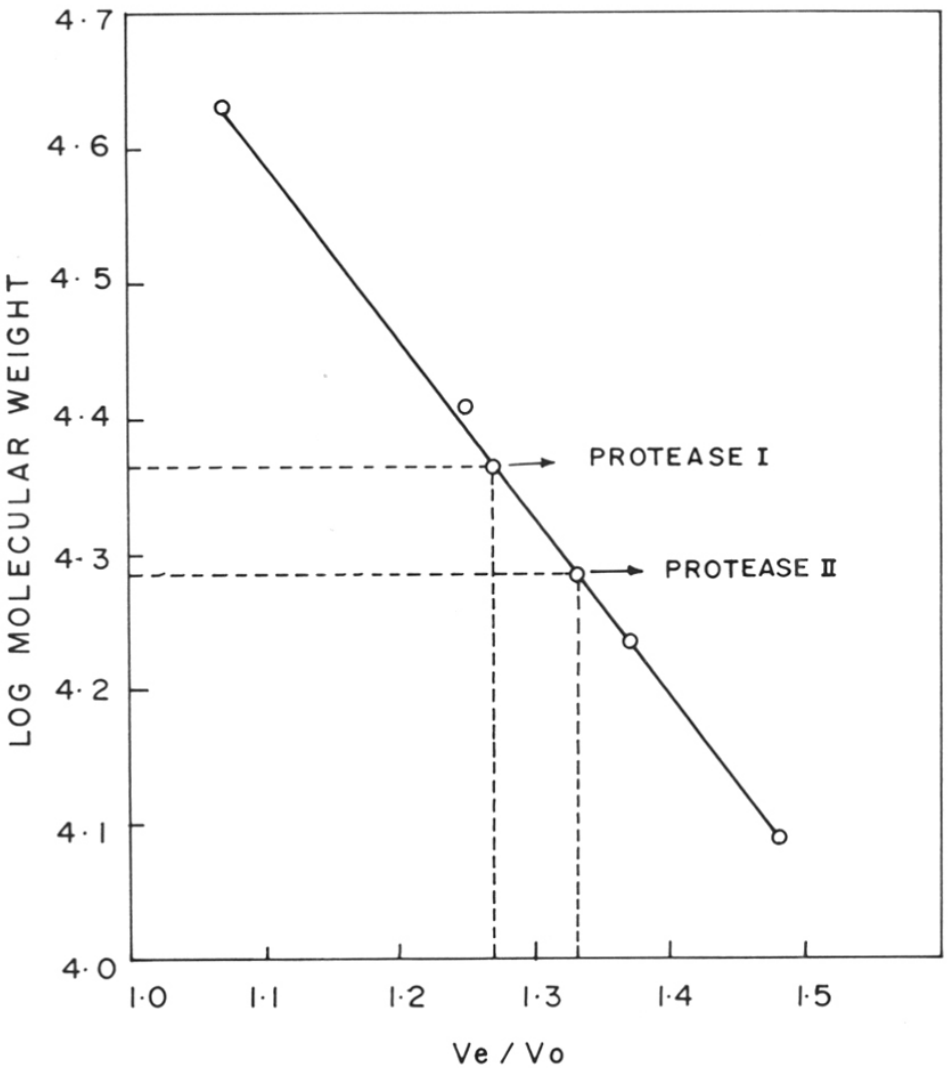
(2) Chymotrypsinogen (Mr 25,800)

(3) Myoglobin (Mr 17,000)

(4) Cytochrome c (Mr 12,400)

The column was equilibrated and eluted with 0.05 M potassium phosphate buffer, pH 7.0

V_o - void volume V_e - elution volume


4·7
4·6
4·5
4·4
4·3
4·2
4·1
4·0
LOG MOLECULAR WEIGHT
PROTEASE I
PROTEASE II
1·0
1·1
1·2
1·3
1·4
1·5
Ve / Vo

proteins (Fig. V-6) indicated that the molecular weights of protease I and II were 24,000 and 19,500 daltons respectively. 1% SDS and 1% BME treated enzyme samples also showed single protein bands in SDS-PAGE corresponding to that of native protein, indicating presence of single polypeptide chain.

Molecular weights of most of the fungal proteases are in the range of 20,000 - 30,000 daltons (Table V-1), though from Penicillium (25, 28, 29), high molecular weight proteases are reported, molecular weights being 45,000, 44,000 and 35,000 daltons respectively. A protease of 61,000 daltons molecular weight is also reported from a yeast (30). Serine protease of Blackslea trispora is reported to have a molecular weight of 1,26,000 daltons (81). A high molecular weight protease, i.e. of 5,40,000 daltons has been reported from B. subtilis (38).

Isoelectric point (pI):

Isoelectric focusing of protease I and II was carried out using ampholine carrier ampholytes of a pH range 6.0 - 11.0. pIs of protease I and II were 9.9 and 9.0 respectively (Fig. V-7). This difference in charge made their electrophoretic separation possible. pIs of various alkaline proteases are reported in Table V-1. pI as low as 2.8 (41) and as high as 10.6 (39) has been reported for alkaline proteases.

Glycoprotein nature:

Glycoprotein nature of the two proteases was examined

Fig.V-6 Molecular weight determination of protease I and II by SDS-PAGE

Relative mobilities (R_f values) were plotted against logarithm of molecular weights. SDS-treated marker proteins used were :

(1) egg albumin (Mr 45,000)

(2) pepsin (Mr 34,700)

(3) β-lactoglobulin (Mr 18,400)

(4) lysozyme (Mr 14,300)

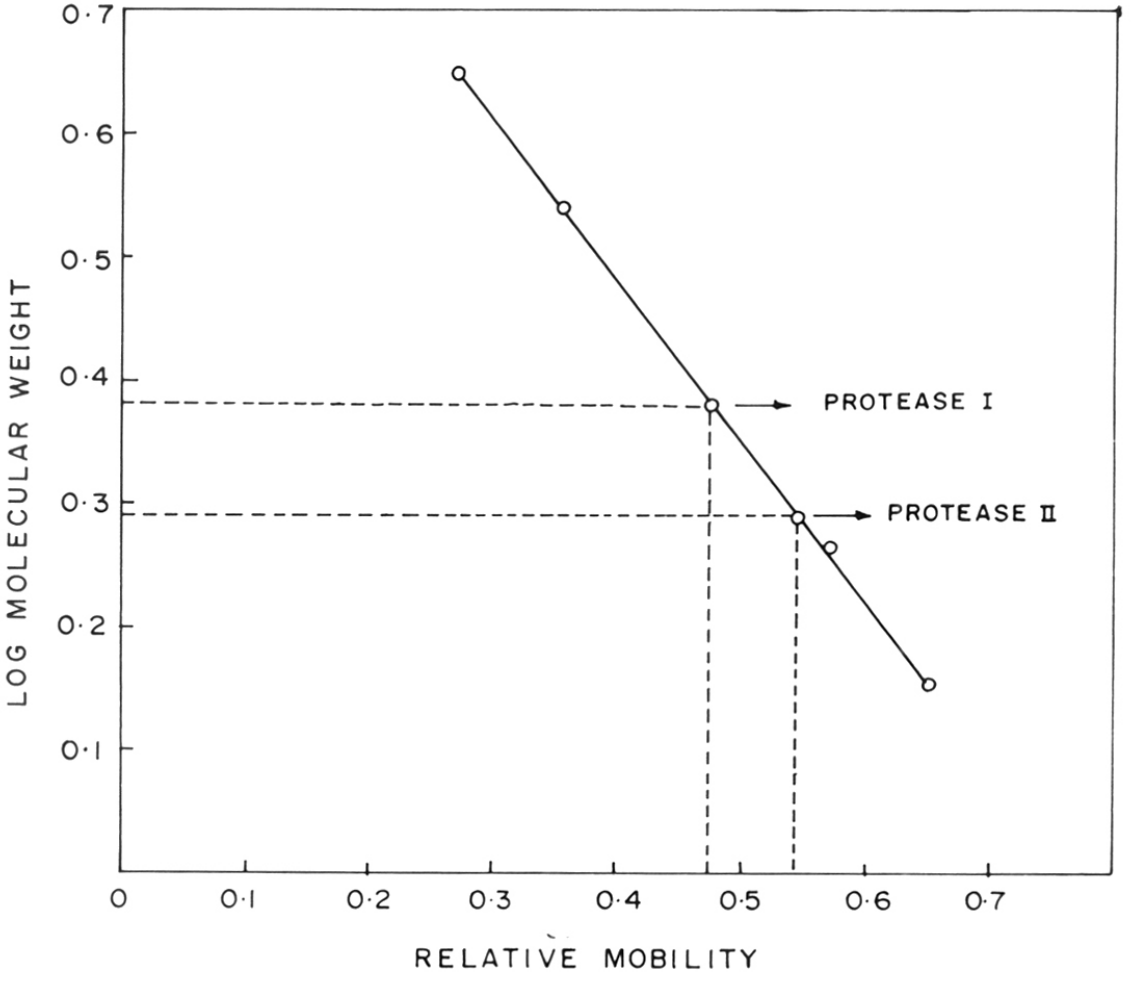
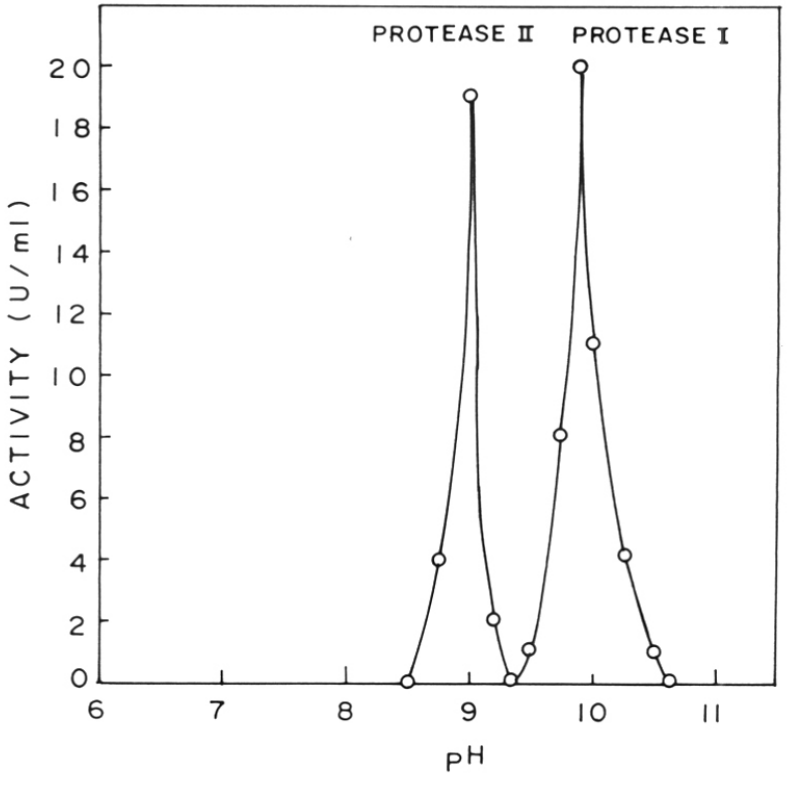

0·7
0·6
0·5
0·4
0·3
0·2
0·1
LOG MOLECULAR WEIGHT
PROTEASE I
PROTEASE II
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
RELATIVE MOBILITY

Fig. V-7 Isoelectric focusing profiles of protease I and II

Isoelectric focusing of proteases was carried out using ampholine carrier ampholytes of a pH range 6.0 - 11.0.


PROTEASE II
PROTEASE I
ACTIVITY (U/ml)
0
2
4
6
8
10
12
14
16
18
20
6
7
8
9
10
11
pH

by Schiff's staining (65). Both the enzymes did not show purple-red bands corresponding to protein band in PAGE, indicating that they did not contain any covalently bound carbohydrate moiety. Alkaline proteases containing small amount of carbohydrate are reported from A. flavus (3), Thermus Tok3 (46) and B. subtilis (82).

Characteristics of protease I and II are summarised in Table V-6. Both the proteases were very similar in several biochemical and physico-chemical properties. They differed in three aspects viz. molecular weight, specific activity and charge, protease I being the larger and the more active enzyme of the two.

In view of the apparent similarity of these enzymes, an attempt was made to study their structural homology by comparing their amino acid compositions, peptide maps of their tryptic digests and their responses towards the specific antibody.

Amino acid composition:

Analysis of amino acid compositions (Table V-7) revealed that amino acid composition of protease II was totally contained in protease I, but protease I had some additional residues. As seen from the table, there is predominance of basic amino acids over acidic residues, especially in case of protease I. This predominance is also revealed by its higher pI value of 9.9. As compared to other alkaline proteases (Table V-2), lysine content of protease I is the highest. Amino acid analysis also showed high content of

Table V-6 : Properties of protease I and II

Parameter	Value	
	Protease I	Protease II
Specific activity (U/mg) (on casein)	45	30
Optimum pH	9.7 - 10	9.7 - 10
Optimum temperature (°C)	40	40
Stability pH	7 - 7.5	7 - 7.5
Temperature stability (°C)	35 - 40, 1 h	35 - 40, 1 h
EDTA (0.2 M) sensitivity	insensitive	insensitive
PMSF (0.1 mM) sensitivity	totally inhibited	totally inhibited
Glycoprotein nature	Nil	Nil
Molecular weight (daltons)		
by Sephadex G-50	23,170	19,270
by SDS PAGE	24,000	19,500
pI	9.9	9.0

Table V-7 : Amino acid compositions of protease I and II

Amino acid	Compositions (mol/mol)	
	Protease I	Protease II
Lysine	23	15
Histidine	9	5
Arginine	4	3
Aspartic acid + Asparagine	23	23
Threonine	19	15
Serine	21	17
Glutamic acid	3	3
Proline	5	4
Glycine	34	28
Alanine	40	32
Cysteine*	1	1
Valine	18	17
Methionine	1	1
Isoleucine	7	5
Leucine	8	7
Tyrosine	3	2
Phenylalanine	4	3
Tryptophan	3	3

*Determined as cysteic acid after performic oxidation.

neutral (glycine and alanine) and hydrophilic amino acids (serine and threonine) in case of both the proteases. This observation is similar to almost all alkaline proteases whose compositions are reported (Table V-2), except that the Conidiobolus proteases appear to be rich in lysine.

Presence of cysteine residue, as in case of protease I and II, seems to be uncommon in case of many alkaline proteases, though protease from Penicillium cyclopium is reported to contain 18 cysteine residues (29).

Two dimensional peptide mapping:

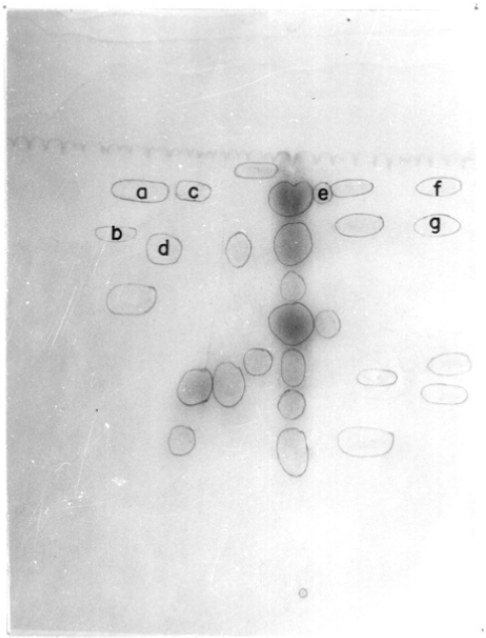
Due to the highly specific nature of trypsin action, peptides generated in tryptic digest of a protein are characteristic of distribution of lysine and arginine residues in that protein. This provides a very efficient means for carrying out structural homology studies between two proteins. By comparing, peptide maps of tryptic digests of two proteins, definite conclusions can be drawn regarding similarities or dissimilarities in their primary structures. Such comparison was made to study homology of subtilisin Novo with subtilisin BPN' (83).

Results of peptide mapping of tryptic digests of protease I and II are shown in Fig. V-8. As protease I contained 23 lysine and 4 arginine residues, 28 peptides were expected, which is the case as shown in Fig. V-8[A]. 19-21 peptides were obtained in case of protease II which contained 15 lysine and 3 arginine residues (Fig. V-8[B]). The digestion patterns of both proteins were essentially identical,

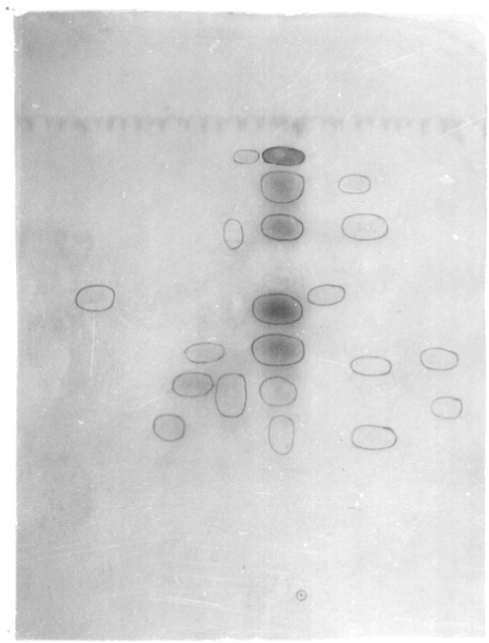
Fig. V-8 Peptide maps of tryptic digests of protease I and II

A, protease I

B, protease II



A



B

except that peptides designated as a, b, c, d, e, f, g in peptide map of protease I were absent from that of protease II. All peptides of protease II, on the other hand were homologous with those of protease I.

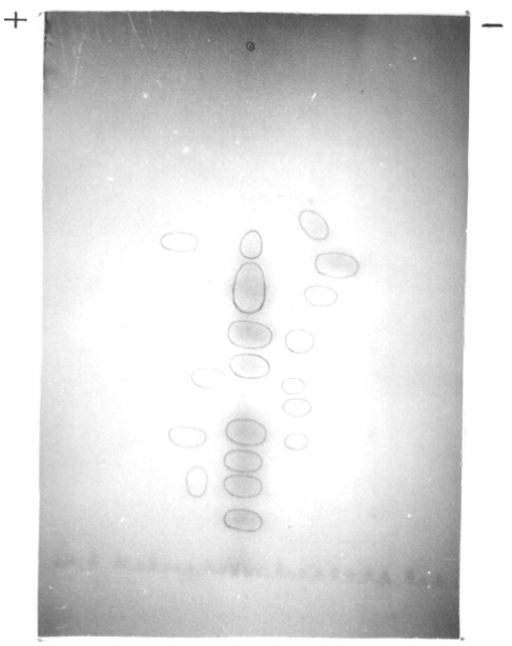
Results of amino acid analysis and two dimensional peptide mapping furthered the speculation that primary structure of both the proteases was similar, but protease I had some additional portion not contained in protease II, which might be contributing towards its being more active.

Tryptic peptide maps of protease I and II were compared with that of subtilisin and were found to be different (Fig. V-9). This indicated their distinctness from subtilisin, in contrast to their apparent subtilisin like biochemical properties. This was consistent with the fact that these two proteases were feebly sensitive to specific subtilisin inhibitor as mentioned earlier.

Immunological properties:

Specificity of antigen-antibody reaction provides a very valuable means of studying homology between two antigens. As antibody reacts with specific antigenic determinants on the surface of an antigen, it can be used to compare topological structure of two antigens. Hence for further study of homology between the two proteases, polyclonal antibodies were raised against purified protease I and its cross reaction with protease II in Western-Blot ELISA, Oüchterlony double diffusion and immunotitration was determined.

Fig. V-9 Peptide map of tryptic digest of subtilisin



Comparisons based on immunological specificity are reported in literature. Antibodies against intracellular serine protease and extracellular subtilisin BPN' of Bacillus amyloliquifaciens (84) were raised in rabbits and it was shown that the proteases of subtilisin family have a pronounced immunological variability. Structural relationship between proteosome complex of Saccharomyces cerevisiae and ATP-dependent protease Ti of E. coli (85) was demonstrated with the help of antibodies. Using antibodies, two proteases (ALPase I and II) of Bacillus sp. NKS-21 (41) were shown to be distinct. Immunological specificity has also been used for comparing Bacillus proteases (35), subtilisins (86) and various proteases of Pseudomonas sp. of dairy origin (87). Specificity of antibody has also been used as a basis for classification of proteases from Streptococcus cremoris (88) and from Bacillus sp. (89). The role of calcium in the thermal stability of semialkaline protease from Aspergillus melleus was investigated with the help of antibodies. Immunological properties of intact protein were compared with those of the protein with one calcium atom bound (76). Immunological characterisation of protease from bacterial strain (90), Mucor pusillus (91), A. fumigatus (92) and B. stearothermophilus (93) is also reported.

Western Blot-ELISA:

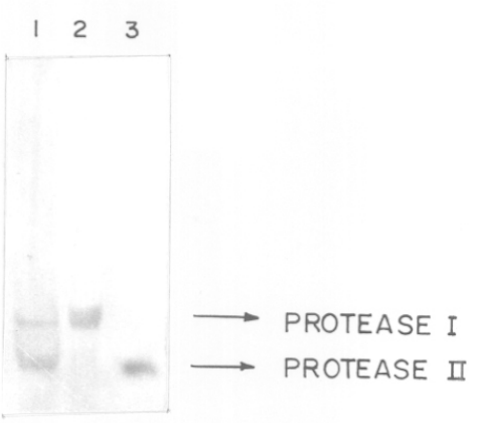
Crude enzyme preparation and purified protease I and II were transferred to nitrocellulose paper after SDS PAGE, and probed with antiprotease I antibody (Fig. V-10). The crude

Fig. V-10 Westernblot of proteases

Lane 1, crude enzyme preparation

Lane 2, purified protease I

Lane 3, purified protease II


1 2 3
PROTEASE I
PROTEASE II

sample showed only two bands of antigen-antibody reaction, corresponding to purified protease I and II, indicating specificity of the antibody towards both the proteases. Also this showed the highly specific nature of the antibody, as it did recognise only other protein in the crude enzyme preparation.

Ouchterlony double diffusion:

In the double diffusion test, the precipitin lines of protease I and II merged with each other, indicating the presence of identical antigenic sites on both enzymes. The crude enzyme preparation on the other hand, showed only single arc, confirming the same (Fig. V-11).

Antiprotease I antibody did not react with subtilisin, trypsin, chymotrypsin, pronase and protease K, indicating that protease I and II are immunologically distinct from these proteases (Fig. V-12).

The precipitin lines of intracellular and extracellular crude protease preparations merged with each other (Fig. V-13) confirming the speculation that these enzymes are formed inside the cell and then secreted out.

Immunotitration:

The study of immunocomplex formation on enzyme activity helps to understand the possible modification of the enzyme as a result of the complex formation with specific antibodies. Neutralisation of both proteases with antibody resulted in 100% loss of activity (Fig. V-14). The loss in activity could be due to either of the two reasons; (i) due to binding

Fig. V-11 Oüchterlony double diffusion of proteases with antiprotease I antibody

1, protease I

2, protease II

3, crude enzyme preparation

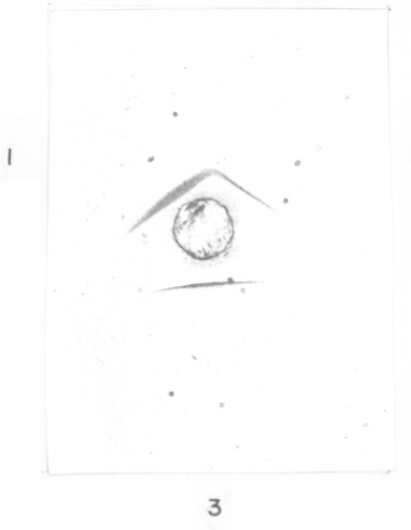

1
2
3

Fig. V-12 Oüchterlony double diffusion of various proteases with antiprotease I antibody

1, protease I

2, pronase

3, protease K

4, subtilisin

5, chymotrypsin

6, trypsin

Central well, antiprotease I antibody.

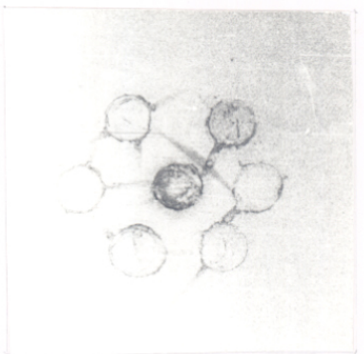

2
1
3
6
4
5

Fig. V-13 Oüchterlony double diffusion of crude intracellular and extracellular protease with anti-protease I antibody

1, intracellular enzyme preparation

2, extracellular enzyme preparation

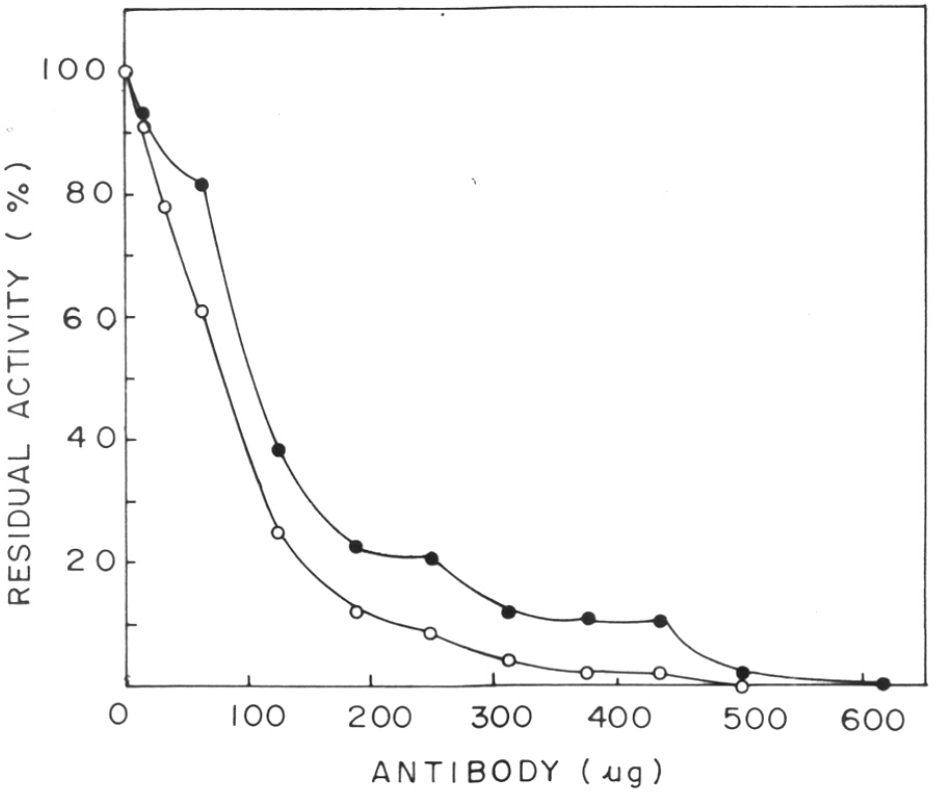

1
2

Fig. V-14 **Neutralisation of protease I and II with anti-protease I antibody**

Equal units (85) of protease I or II were added to different amounts (0 - 625 µg) of antiprotease I antibody. After incubation at 37°C for 1 h, each reaction mixture was assayed for protease activity.

O, protease I

●, protease II


RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
100
200
300
400
500
600
ANTIBODY (ug)

of antibody to enzyme, its conformation changes, so that it can no more bind substrate, though binding sites of antibody and substrate could be physically apart from each other, (ii) antigen binding sites overlap the sites needed for activity of enzyme.

In the present case, preincubation of the enzymes with substrate prior to treatment with antibody, protects the enzyme against neutralisation by antibody. Maximum protection given by substrate i.e. 1% casein in 0.05 M potassium phosphate buffer, pH 7.0, was 56.5% in case of protease I and 55.2% in case of protease II. Increasing substrate concentration did not further contribute towards protection. This shows that, in case of protease I and II, antigen binding sites at least partially overlap substrate binding sites, thus when the later are blocked by substrate, efficient binding to antibody to enzyme can not take place, which in turn results in partial neutralisation of enzyme activity.

Total neutralisation of protease II with antiprotease I antibody indicated that it had similar antigenic determinants as that of protease I. Antiprotease I antibody failed to neutralise subtilisin, even at high concentrations, thus reconfirming its immunologically distinct nature from protease I and II.

Determination of antigenic valency:

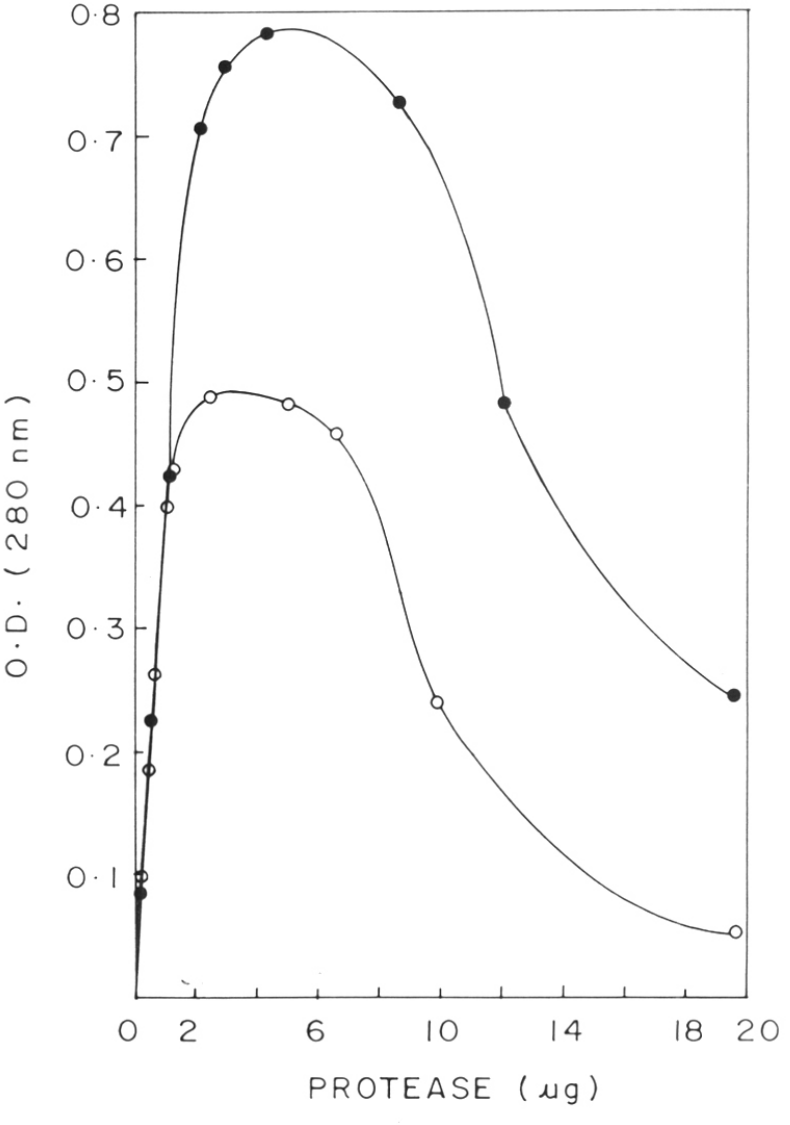
Fig V-15 shows, the graph of antigen-antibody complex (O.D. at 280 nm) against amount of protease I/II. Antigenic determinants were calculated in the antibody excess region

Fig. V-15 **Determination of antigenic valency**

Antibody-antigen complex (O.D. at 280 nm) was plotted against amount of antigen (protease I & II) Varying amounts of protease (I/II) were added to a fixed amount of antiserum and incubated at 37°C for 1 h and at 4°C for 18 h. The precipitate was collected by centrifugation, washed and redissolved in 0.1 M NaOH and O.D. at 280 nm was determined.

O, protease I

●, protease II


0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
O·D· (280 nm)
0
2
6
10
14
18
20
PROTEASE (µg)

and a graph of ratio of antibody to antigen molecules against the amount of antigen used was plotted (Fig. V-16) for both the proteases. The point, at which the straight line intercepts the Y axis gives the number of antigenic determinants on enzyme molecule. It is seen from graph, that, antibody recognises 16 determinants on protease I and 12 on protease II. This again points towards the fact that protease II lacks certain part contained in protease I, which contributes significantly towards the topology of the enzyme, which in turn, may be responsible for the higher specific activity of the protease I.

In literature, many reports are cited where an organism produces more than one protease, but only few of them produce two proteases with great degree of homology with each other. In such cases, question arises regarding the origin of these enzymes. Two possibilities existing in this respect are: (1) production of two proteases by homologous genes or (2) production of one protease from the other by post translational modification.

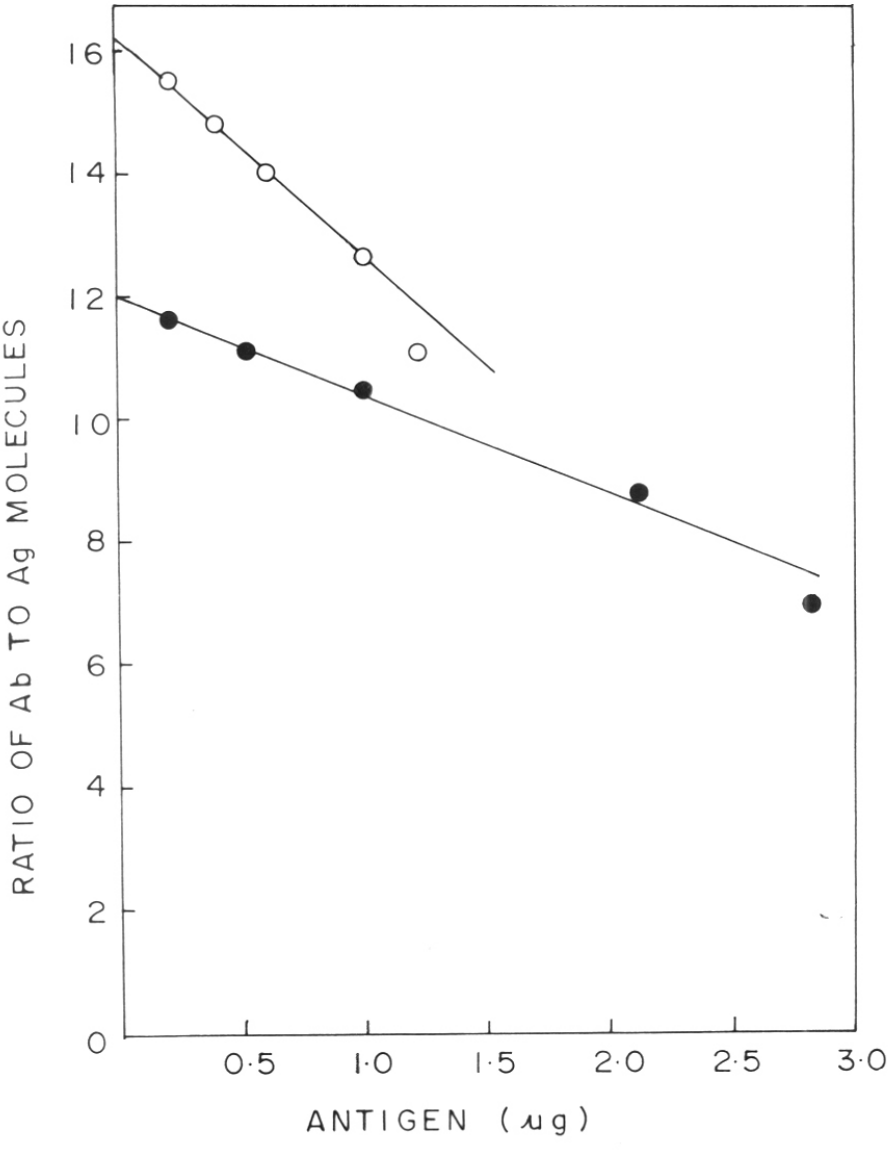
Post translational modification of protease can be non-enzymatic or enzymatic. Non-enzymatic conversions are mainly due to deamination of asparginyl and glutaminyl residues in the protein, which are unstable and if hydrolysed, would leave a new negative charge on protein. Therefore electrophoretically more anodic species are formed (94, 95). Such conversions are reported in case of Streptococcus

Fig. V-16 **Ratio of antibody to antigen molecules at different levels of antibody excess**

The point at which the regression line calculated from the antibody to antigen ratios over a range of antigen concentrations, all in antigen excess intercepts the y axis, gives the number of antigenic determinants on the protein molecules, by antiserum.

O, protease I

●, protease II


RATIO OF Ab TO Ag MOLECULES
16
14
12
10
8
6
4
2
0
0·5
1·0
1·5
2·0
2·5
3·0
ANTIGEN (μg)

cremoris (96) and Streptomyces sp. (34) which produce two homologous proteases identical in all properties except amide content. Therefore, one enzyme could be arising by deamination of asparinginyl or glutaminyl residues of the other. In A. sojae, different forms of serine protease arise by similar type of modification of parent protein (94). In case of C. coronatus, this possibility is ruled out, as the daughter protein i.e. protease II is smaller and more cathodic in nature.

Enzymatic modification could mainly be due to auto-degradation, especially in case of proteases. Such phenomenon is well studied for chymotrypsin (97 - 99). Auto-digestion target sites for neutral protease of B. subtilis are identified (100).

The homology studies of protease I and II suggest the possibility that the two proteases may essentially be the product of the same gene, but after formation, a small fraction of protease I may be getting cleaved to form protease II, which retains part of its activity and shows many properties similar to the intact protein.

This fact was also supported by the observation that after incubation at room temperature (28°C) for 24 - 48 h, the purified protease I showed autodegradation resulting in many electrophoretically separable protein fragments. Out of these, the prominent band corresponded to protease II. PMSF treated protease I did not show such degradation,

indicating that the conversion is proteolytic in nature. Thus protease I must produce protease II by autoproteolysis and their production by two homologous genes does not seem plausible.

Autodegradation is also reported for collagenase of Achromobacter iophagus (101). Collagenase autodegradation gives rise to many electrophoretically separable low molecular weight forms from a parent enzyme showing single band of protein on PAGE. Many of these daughter species are less active or totally inactive. Conversion of enzyme to low molecular weight forms is also reported in case of autolysis of subtilisin (102). In case of B. subtilis (82), many daughter protease species are formed by autodegradation, the process being sensitive to PMSF. In fungi though, such phenomenon is not common as compared to bacteria.

Autodegradation in case of C. coronatus protease is unusual in that the parent protein is converted to a daughter protein of definite mass with lesser activity and there is an equilibrium between parent and daughter protein. Protease I is never totally converted to protease II and ratio of protease I to protease II always remains constant, i.e. 69:31, irrespective of initial concentration of protease I. This indicates that the process is finely controlled.

A new physiological role was attributed to C. coronatus protease; i.e. to trigger conidial discharge (Chapter III). Though serine proteases are known to be involved in many physiological functions, involvement in conidial discharge

was reported for the first time.

The physiological activity of proteases is usually controlled by protease inhibitors (103), as is seen in case of septum formation, during yeast budding. When function of protease, i.e. to activate chitin synthetase is over, protease inhibitors present in the cell inactivate the protease. In case of Conidiobolus sp., presence of such systems is not yet known. In addition, this organism always needs some basal level of protease for its survival and growth, as shown in Table III-2. Hence total inactivation of protease after conidial discharge is not desirable.

It is seen from graph III-4, that upto 30 h, protease activity increases, with concomittant increase in conidial discharge. Protease II appears only after 30 - 36 h, coincident with the decline in conidial discharge. Decline in protease activity occurring after 30 h coincides with appearance of protease II. As this process is finely controlled, autoproteolysis of protease I to form a less active protease may represent a novel means of its regulation in vivo, in case of C. coronatus.

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Evidence for the involvement of serine protease in the conidial discharge of *Conidiobolus coronatus*

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Abstract. The involvement of serine protease(s) in the conidial discharge of *Conidiobolus coronatus* was investigated using the parent strain and a variant strain with reduced conidial discharge. Time course profiles of protease levels and conidial discharge showed that maximum protease levels coincided with maximum conidial discharge in both the parent and variant strains. Inhibition of serine protease(s) by phenyl methyl sulfonyl fluoride showed that low protease levels resulted in inhibition of the conidial discharge and a minimum activity of 1.0 U/mg protein is essential for triggering the conidial discharge. Using casein to induce proteases, it was further observed that early gain in the protease level (1.0 U/mg protein) leads to early onset of conidial discharge. The above evidence suggests the involvement of protease(s) in the conidial discharge of *C. coronatus*.

Key words: *Conidiobolus coronatus* – UV-20 variant strain – Conidial discharge – Serine proteases – Phenyl methyl sulfonyl fluoride inhibition

In fungi, the conidial release can be due to the breakage of supporting cell by lytic enyzmes like chitinases or by mechanical means or due to splitting of the double septum at the base (Smith 1977). In case of *Entomophthora* and *Conidiobolus*, members of order Entomophthorales, the splitting of the double septum at the base of the conidium results in its violent discharge from conidiophore (Sawyer 1931), the biochemical basis of which is not well understood. In a number of cellular differentiation processes viz. macroconidial germination, budding in yeast, hyphal tip elongation and hyphal fusion, proteases, especially serine proteases, are known to be involved, where they play either a hydrolytic role or are involved in the activation of cell wall synthesizing enzymes namely chitin synthetases (Holzer et al. 1975; Cabib and Farkas 1971; Smith and Berry 1974). In our laboratory, we have isolated several *Conidiobolus coronatus* strains which produce high levels of serine proteases (Srinivasan et al. 1983). Since serine proteases have been implicated in many cellular differentiation processes, one of the *Conidiobolus* isolates was used to study the possible involvement of this enzyme in conidial dicharge, the results of which are reported in the present communication.

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Materials and methods

Organism

Conidiobolus coronatus (NCL 86.8.20) isolated from plant detritus was identified by its typical villous conidia, production of microconidia on radial sterigmata and the forcible discharge of conidia. Taxonomically *C. coronatus* is classified in the order Entomophthorales, family Entomophthoraceae.

C. coronatus parent strain and the variant strains isolated after UV mutagenesis of the parent strain conidia, were maintained on MGYP agar slants (malt extract, 0.3%; glucose 1.0%; yeast extract, 0.3%; peptone, 0.5%, agar, 2%) at 28° C.

Isolation of morphological variants

C. coronatus parent strain conidia were harvested by adding sterile distilled water to MGYP agar slant of 48 h grown parent strain, followed by gentle scraping with sterile glass spatula. The suspension was then filtered through sterile cotton and conidial suspension (10^6 conidia ml) was transferred to a sterile Petri dish and subjected to UV irradiation (Phillips UV germicidal lamp, 15 W) at a distance of 15 cm, for different time intervals. The suspension was then plated on MGYP agar plates containing 0.5% (w/v) oxgall (a surfactant) and incubated for 48 h at 28° C. Growth was significantly restricted and conidial discharge was also reduced due to the presence of oxgall, which in turn facilitated the identification of the variants. Variants were selected on the basis of their morphological differences from the parent strain such as compactness, dryness, reduction in conidial discharge etc. After 2 min exposure, 10% of the survivals were obtained as morphological variants.

Measurement of conidial discharge

MGYP agar plates (10 cm diameter) inoculated with conidia (10^3 ml^{-1}) harvested in sterile distilled water, were incubated for 48 h at 28° C. Growth was followed microscopically. Discharged conidia were collected in distilled water and were counted on a hemocytometer.

Enyzme assays

Mycelial growth which could only be seen after 8 h of incubation at 28° C, was taken as starting point for the determi-

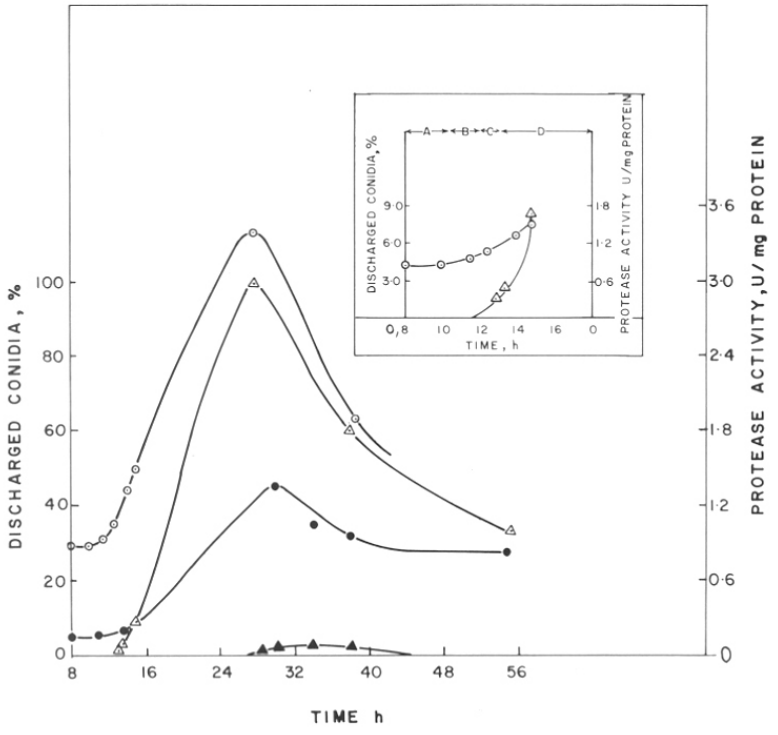

Fig. 1
Time course observations of intracellular protease activities and conidial discharge of parent and UV-20 strain of *Conidiobolus coronatus*. Conidial suspension (0.1 ml, 10^3 conidia/ml) was used to inoculate MGYP agar medium. Plates were incubated at 28° C. Protease activity, parent strain (○); protease activity, UV-20 strain (●); conidial discharge, parent strain (△); conidial discharge, UV-20 strain (▲). The maximum discharge obtained in case of the parent strain was taken as 100%. *Inset:* Time course observations of intracellular protease activities and conidial discharge of *C. coronatus* parent strain, from 8 h to 15 h, protease activity (○); conidial discharge (△). Growth phases: A, mycelial growth; B, conidiophore formation, C, conidia formation; D, conidial discharge. Data shown is mean of four sets of experiments

nation of enzyme activities. Samples were removed at different time intervals and the intracellular protease and chitinase activities were estimated. Crude extracts for enzyme assays were prepared by crushing the mycelium in presence of distilled water in a glass homogenizer.

Protease activity was determined at pH 9.7 and 37° C using casein as substrate (Sutar et al. 1986). One unit of enzyme activity is defined as the liberation of 1 µmol of tyrosine per minute at 37° C.

Chitinase activity was measured according to Otakara (1964). One unit of enzyme is defined as the liberation of 1 µmol of the product per minute using ethylene glycol chitin as substrate.

Protein estimation

Protein was estimated according to the method of Bradford (1976) using crystalline bovine serum albumin as standard.

In all cases enzyme activities were expressed on the basis of units per mg protein.

Table 1. Effect of PMSF on growth and intracellular protease activity on *Conidiobolus coronatus*[a]

PMSF (mM)	Protease (U/mg protein)		Effect on growth phases	
	Parent strain	UV-20 strain	Parent strain	UV-20 strain
Control (without PMSF)	3.75	1.30	−	−
1.0	2.66	0.90	−	+
1.5	1.30	0.45	−	++
2.0	0.60	0.20	+	++
2.5	0.45	ND	++	+++
3.0	ND	ND	+++	+++

−, No effect; +, conidial discharge completely inhibited; ++, conidia formation restricted; +++, mycelial growth inhibited; ND, not detected. *C. coronatus* grown on MGYP medium plates with PMSF at 28° C for 48 h. Intracellular protease activities were estimated as mentioned in Materials and methods. Growth stages were monitored microscopically

[a] Data shown is mean of four sets of experiments

Results and discussion

The asexual growth cycle of *Conidiobolus coronatus* consists of 4 stages, namely, mycelial growth, conidiophore formation, conidia development and conidial discharge. Conidia of synchronously growing culture (20 h at 28° C) were used as inoculum to study the asexual growth cycle of *C. coronatus*. At 28° C, while the mycelical growth was visible at 8 h, conidiophore formation and conidia development started after 10 h and 12 h, respectively. Discharge of mature conidia occurred after 13 h. While the intracellular protease levels remained unchanged (0.9) it increased to 1.1 during conidiophore formation and conidia development. However, protease level showed a further increase (1.5) prior to active conidial discharge (Fig. 1, inset).

In view of the apparent relationship between protease levels and growth cycle stages in the parent strain, attempts were also made to explore this phenomenon by studying the intracellular protease levels in variants showing reduced conidial relase. The variant designated UV-20, obtained by the mutagenesis of parent strain conidia, showed considerably reduced conidial discharge as compared to the parent strain. Except for this difference, both the parent and UV-20 strains exhibited similar characteristics with respect to growth rate and conidial production. When the protease levels were determined at various growth stages of the parent and UV-20 strain, it was observed that the maximum protease level in the parent strain was approximately 3 times higher (3.4) as compared to the UV-20 (1.3). In addition the parent strain also showed higher level of conidial discharge (> 25 times) than UV-20 strain. In case of parent strain, the protease activity started increasing after 11 h and reached a maximum at 28 h. Though the conidial discharge started only after 13 h, it also showed a maximum at 28 h. Both the protease level and conidial discharge declined after 28 h. A similar pattern was observed in case of UV-20, except that for both protease level and conidial discharge to reach the maximum, it took 30 h (Fig. 1). It was also observed that in both strains, the onset of conidial discharge occurred when the protease level was above 1.0. From these observations, it can be concluded that the increased time required for the onset of conidial discharge in UV-20 can be due to the longer time required for the protease level to reach 1.0.

Chitinases have been implicated in the spore release in fungi (Iten and Matile 1970). Since no changes in the chitinase level were observed at any of the growth stages in both the parent and UV-20 strains, it can be assumed that chitinase does not have a role in the conidial discharge in *C. coronatus*.

Phenyl methyl sulfonyl fluoride (PMSF) is a powerful inhibitor of serine proteases (Gold and Fahrney 1964). In the present studies, PMSF at a concentration of 5.0 mM, brought about a complete inhibition of protease activity present in the crude extract, indicating the presence of only serine proteases. Using PMSF, involvement of serine protease(s) has been shown in case of macroconidial germination as well as in hyphal fusion (Holzer et al. 1975). Hence, a similar approach was made to assess the involvement of serine protease(s) in the conidial discharge in *C. coronatus*. PMSF at a concentration of 3.0 mM when used in the growth medium, inhibited the mycelial growth in case of the parent strain. While 2.5 mM PMSF led to the inhibition of conidia formation, lower concentration (2.0 mM) did not affect conidia formation, though the conidial discharge was completely inhibited. Hence the inhibition of conidial discharge can be correlated to decrease in the protease level (< 1.0) due to PMSF. Similar results were obtained with UV-20 strain, though the corresponding concentrations of PMSF required to bring about the inhibition were lower (Table 1). From these results, it can be concluded that a minimum protease activity of 1.0 is necessary to trigger conidial discharge in both parent and UV-20 strains. Further evidence for the involvement of protease in conidial discharge was studied by using a protease inducer (casein) in the growth medium. Addition of 2% (w/v) casein, brought about an early increase in the protease levels (1.0), resulting in an early onset of conidial discharge both in the parent and UV-20 strains as compared to the controls (without casein). This observation can be correlated to the early gain of protease levels necessary to trigger the conidial discharge. All the above results indicate that serine protease(s) is involved in the conidial discharge in *C. coronatus*.

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