Acid protease from *Aspergillus oryzae*: Structure-stability and enhancement of the activity by physical, chemical and molecular biological approaches

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DECLARATION

I hereby declare that the thesis entitled "Acid protease from *Aspergillus oryzae*: Structure -stability and enhancement of the activity by physical, chemical and molecular biological approaches" submitted to the University of Mysore for the award of degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY is the result of the research work carried out by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under the guidance of Dr. Sridevi Annapurna Singh during the period of 2004 – 2009.

I further declare that the research work embodied in this thesis has not been submitted for the award of any other degree.

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SRIDEVI ANNAPURNA SINGH

(Guide)

Acid protease from *Aspergillus oryzae*: Structure-stability and enhancement of the activity by physical, chemical and molecular biological approaches

ABSTRACT

The focus of this investigation has been to screen fungal sources for aspartate proteases, which are used in food industries as food protein modifiers and as replacers of animal rennets in cheese making. The isolation, characterization and stability parameters of two high active aspartic proteases from *Aspergillus oryzae* MTCC 5341 are reported for the first time.

Aspergillus oryzae MTCC 5341 produced maximum aspartic protease production (8.6 $\times 10^5$ U/g bran of acid protease and 40, 000 U/g bran of milk clotting enzyme.) by solid state fermentation. Process parameters for solid state fermentation for production of these enzymes have been optimized and downstream parameters standardized. The acid protease from A. oryzae has a molecular weight of 47 kDa and is rich in β structure (~60%) like other aspartic proteases. Milk clotting enzyme from *A. oryzae* is a 34 kDa aspartic protease with 49°C as its midpoint of thermal denaturation.

Pepstatin inhibited acid protease completely; other class specific protease inhibitors had no effect on proteolytic activity. Pepstatin is a competitive inhibitor with a K_i of 3.7×10^{-7}M. Acid protease activated trypsinogen to active trypsin, Val-$(Asp)^4$-Lys, a hexapeptide towards N-terminal of precursor trypsinogen is cleaved to release trypsin.

Acid protease was stable in a pH range of 2-6 and on either side, enzyme lost activity and structure due to collapse in hydrophobic interactions. Temperature induced aggregation of the molecule due to autolysis. Chaotropic salts induced unfolding of the molecule and thereby resulting in aggregation, molecular chaperons like SCN^{-1}, β and α- casein gave thermal protection- involvement of ionic interactions in maintenance of structure

Chemical denaturants induced unfolding curves were cooperative and biphasic- N→U. GuHCl obeyed "two fold" rule in unfolding of the molecule ([denaturant]1/2 were Urea- 1.74 M and GuHCl- 0.85 M). Loss of proteolytic activity upon unfolding coincides with loss in tertiary and secondary structure reflecting good correlation between activity and structural integrity of the molecule

Rennet hydrolyzed all the three casein fractions (α-, β- and κ- casein) showing little specificity for hydrolysis. Higher rate of hydrolysis towards κ – Casein than α and β caseins. Hydrolysis of α-, β- caseins by the protease action adds bitter peptides essential for flavor developing during ripening. Cheese preparation compared with control (*Mucor* rennet). Clotting time, yield and surface characteristics were on par with control These studies on aspartic proteases from *A. oryzae* may lead to new sources of high activity enzymes for use in food industry. These enzymes with improved stability help in understanding the structure-function relation of aspartic proteases.

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ABBREVIATIONS

ANOVA	Analysis of variance
ANS	8-anilinonaphthalene 1-sulphonic acid
AOAP	*Aspergillus oryzae* acid protease
AP	Acid protease
bp	Base pair
$CaCl_2$	Calcium chloride
CAP	Commercial acid protease
CD	Circular Dichroism
DEAE	Diethylaminoethyl cellulose
DSC	Differential scanning calorimetry
DSF	Defatted soyflour
Ea	Energy of activation
EDTA	Ethylene diamino tetra acetic acid
GuHCl	Guanidine hydrochloride
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
kcal	Kilo calories
kDa	Kilo daltons
K_m	Michelis constant
K_{sv}	Stern-Volmer constant
Mb	Mega base
min	Minutes
MTCC	Microbial type culture collection
NaCl	Sodium chloride
NBS	N-bromosuccinimde

PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric pH
PMSF	Phenyl methyl sulphonyl fluoride
RSM	Response surface methodology
SCN	Sodium thio cyanate
SDS	Sodium dodecyl sulfate
SmF	Submerged fermentation
SMS	Sodium meta bisulphate
SSF	Solid state fermentation
TCA	Trichloro acetic acid
TEMED	N,N,N′,N′- tetramethylethylenediamine
Tm	Transition temperature
UV	Ultraviolet
V_{max}	Maximum velocity

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AIM AND SCOPE OF PRESENT INVESTIGATION

Proteases occupy a central position in commerce for their applications constituting nearly 70% of the global enzyme market. Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. Microorganisms elaborate a large array of proteases, which are intracellular or extracellular. Amongst proteases, aspartate proteases find application in industry for cheese making, as digestive aids, beer clarifiers, as food protein modifiers and debittering protein hydrolysates preparations.

Filamentous fungi are exploited for the production of industrial enzymes due to their ability to grow on solid substrate and produce a wide range of extracellular enzymes. Solid state fermentation is most opted in production of industrial enzymes from fungal source, since the substrate provides nutrition besides being a solid support for mycelia growth.

Each application of protease requires unique properties with respect to specificity, pH dependence, temperature and stability. Screening of various sources with higher protease activities could therefore, facilitate the discovery of novel proteases suitable to new industrial applications.

Aspartate proteases play an important role in the manufacture of cheese in dairy industry. Increased demand for cheese, coupled with ethical and low availability of rennet has led to replacement of calf rennet by microbial milk clotting enzymes. Microbial milk clotting enzymes constitute ~33% of total protease utilization; largely replace animal rennet for milk clotting. However, these microbial milk coagulants are associated with non-specific and heat stable proteases which lead to the development of bitterness in cheese after storage and a poor yield. Search is on for the low cost methods of producing enzymes that are completely inactivated at the normal pasteurization temperatures and contain very low levels of contaminating proteases. Microbial milk coagulants from fungal sources can be conveniently produced by solid state fermentation and are useful in the cheese making industry owing to their narrow pH and temperature specificities. Cheese industry has a great demand for aspartate proteases, since they assist in clotting milk apart from playing a key role in flavor and texture development.

Attempts are being made to stabilize novel enzymes against extreme environmental conditions by genetic and protein-engineering techniques by targeting random sites in the gene or protein molecule. However, to successfully achieve this it is necessary to understand the structure – function and stability relationships of these enzymes under different conditions.

The main objective of the present investigation has been to screen fungal strains for aspartate proteases, which are to be used either in baking industries as

protein modifiers or as substitute to animal rennet in dairy industry and to study the structure-function and structure-stability relationships as well as the unfolding and aggregation behavior of proteases which can lead to the development of more efficient and diverse applications.

With the above objectives the following studies were undertaken.

1. To isolate, purify and characterize microbial aspartate proteases and to evaluate mechanism of action with defined end applications and
2. To understand the structure and stability of aspartate protease from *A. oryzae*.

An attempt has been made to understand the interactions that contribute to the stability of aspartate protease. These studies have been carried out by employing different spectroscopic and activity measurements.

The salient findings of the present investigation are

1. Isolation, purification and characterization of acid protease and milk clotting enzyme from *Aspergillus oryzae* MTCC 5341 and molecular characterization of both the enzymes
2. Conformational stability measurements of acid protease –effect of pH, temperature and denaturants
3. Preparation of bread, crackers and cheese with improved qualities by utilizing these enzymes in the process.

These studies have yielded high activity acid protease and milk clotting enzymes and the conformational stability studies have established role of hydrophobic and ionic interactions in maintaining structure-function of the molecule.

Chapter 1

Introduction

INTRODUCTION

Proteases are a group of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. These enzymes catalyze the cleavage of peptide bonds in proteins. They execute a large variety of complex physiological functions including protein turn over, sporulation and conidial discharge, germination, enzyme modification and in nutrition. Proteases assist the hydrolysis of large polypeptides into smaller peptides and amino acids, thus facilitating their absorption by the cell. Proteases play a major role in nutrition due to their depolymerizing activity. These enzymes are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition. Proteases find huge potentials in a variety of industrial applications and thus rank first in terms of demand. Of the industrial enzymes, 75% are hydrolytic in nature. Proteases represent one of the three largest groups of industrial enzymes and account for about ~70% of the total worldwide sale of enzymes (Figure. 1). The current estimated value of the worldwide sales of industrial enzymes is US $ 2-3 billion (Chandel et. al., 2007).

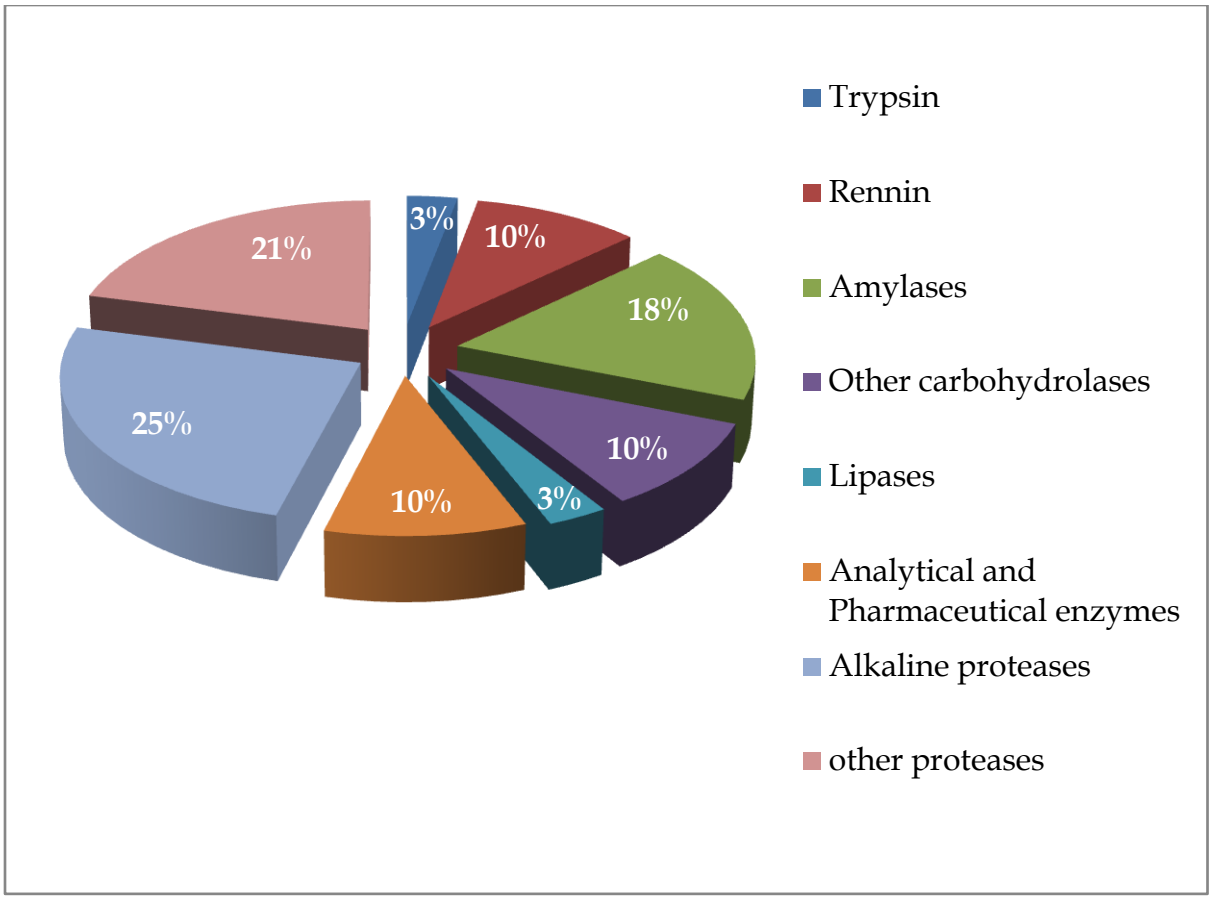


Figure 1. Market share of industrial enzymes. The contribution of different enzymes to the total sale of enzymes is indicated (Chandel et al., 2007).

Sources of proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. Important enzymes from each source are given in Table 1.

Table 1. Sources of some important proteases

Source	Important enzymes	Remarks
Plant	Papain, Bromelain	Limited availability (Rao et al., 1998)
	Keratinase	
	Ficin	
Animal	Trypsin	Limited availability and ethical issues (Boyer, 1971).
	Chymotrypsin	
	Pepsin	
	Rennin	
Microbial	Acid proteases	Account for 40% global enzyme sales (Godfrey and West, 1996). Broad biochemical diversity and amenability to genetic manipulations. Easily produced and in large amounts.
	Rennets	

Microbial enzymes are obtained chiefly from bacteria and fungi. Proteases from these sources are preferred to the enzymes from other sources since they possess almost all the characteristics desired for their biotechnological applications. Filamentous fungi are exploited for the production of industrial enzymes due to their ability to grow on solid substrate and produce a wide range of extracellular enzymes. Among the many advantages offered by the production of enzymes, through fungi, are low material costs coupled with high productivity, faster production and ease with which the enzymes can be modified. Further, the enzymes being normally extra-cellular are easily recoverable from the media. Fungi elaborate a wider variety of enzymes than do bacteria. The fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. In view of the accompanying peptidase activity and their specific function in hydrolyzing hydrophobic amino acid bonds, fungal proteases supplement the action of plant, animal, and bacterial proteases in reducing the bitterness of food protein hydrolysates (Rao et al., 1998)

Production of industrial enzymes from *Aspergillus sp.*

The *Aspergillus sp*. was first recognized as an organism in 1729 by P. A. Micheli. The binomial nomenclature of the fungus is as given below,

Kingdom: Fungi

Division: Ascomycota

Class: Erotiomycetes

Order: Eurotiales

Family: Trichocomaceae

Genus: *Aspergillus*

Species: *oryzae (Aspergillus oryzae)*

The genus *Aspergillus* is found worldwide and consists of more than 180 officially recognized species, and comprises a particularly important group of filamentous ascomycete species. Although it includes the major filamentous fungal pathogen of humans—*Aspergillus fumigatus* (Brookman and Denning, 2000; Latge, 1999) most of the members are useful microorganisms in nature for degradation of plant polysaccharides (de Vries, 2003; de Vries et al., 2000), and they are important industrial microorganisms for the large-scale production of both homologous and heterologous enzymes (Fawole and Odunfa, 2003; Wang et al., 2003). Among them, ***Aspergillus oryzae*** and ***Aspergillus niger*** are on the Generally Recognized as Safe (GRAS) list of the Food and Drug Administration (FDA) in the United States (Tailor and Richardson, 1979). Since *Aspergillus*

species are used in the commercial production of industrially valuable enzymes and other products (Nutan et al., 2002; RaviKumar et al., 2004; van Kuyk et al., 2000), their genes and genomes are being extensively investigated in an effort to understand the associated cellular mechanisms and to expand these applications (Aleksenko et al., 2001; Mabey et al., 2004; van den Hombergh et al., 1997).

Due to their high capacity for producing and secreting extracellular enzymes, *Aspergilli* play an important role in production of industrial enzymes (de Vries et al., 1999; Lockington et al., 2002). *Aspergillus* species are also important microorganisms in the fermented food industry and produce a variety of amylases and proteases (MacKenzie et al., 2000; Petersen et al., 1999). *Aspergillus* species, especially GRAS-designated strains, produce and secrete a variety of industrial enzymes including α-amylases, proteases, glucoamylases, cellulases, pectinases, xylanases and other hemicellulases.

Enzyme production methodologies

Traditionally the industrial enzymes are produced by either solid state fermentation (SSF) or submerged fermentation (SmF).

Solid-state fermentation

Solid state fermentation is defined as a fermentation process in which microorganisms grow on solid material without the presence of free liquid (Cannel and Young, 1980). Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, cheaper costs, less effluent generation and requirement for simple fermentation equipments (Krishna, 2005).

Production of enzymes in solid state fermentation systems

A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. For example, it has been reported that while a strain of *Aspergillus niger* produced 19 types of enzymes, α-amylase was being produced by as many as 28 microbial cultures (Pandey, 1992). Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Generally, hydrolytic enzymes, e.g. cellulases, xylanases, pectinases, etc. are produced by fungal

cultures, since such enzymes are used in nature by fungi for their growth. *Trichoderma* spp. and *Aspergillus* spp. have been widely used for these enzymes. In order to achieve high productivity with less production cost, apparently, genetically modified strains would hold the key to enzyme production. However, widespread opposition exists in the minds of the consumer with regard to any genetic modification. Hence, the search for better and efficient enzyme from nature continues.

Substrates used for the production of enzymes in SSF systems

Agro-industrial residues are generally considered the best substrates for the SSF processes. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used included sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soy hull, sago hemps, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch, etc.(Mitra et. al., 1994; Selvakumar et. al., 1996; Babu and Satyanarayana, 1995; Nigam and Singh, 1994; Tengerdy, 1998). Wheat bran however is the commonly used substrate.

The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related to cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with these. Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size and moisture level/water activity are the most critical. Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor.

Proteases produced by solid state fermentation processes

In recent years, there have been increasing attempts to produce different types of proteases (acid, neutral, alkaline) through SSF route, using agro-industrial residues. Although a number of substrates have been employed for cultivating different microorganisms, wheat bran has been the preferred choice in most of the studies. Malathi and Chakraborty (1991) evaluated a number of carbon sources (brans) for alkaline protease production and reported wheat bran to be the best for cultivation of *A. flavus* IMI 327634. Studies were carried out to compare alkaline protease production in SmF systems and SSF systems. The total protease activity present in one-gram bran (SSF) was equivalent to 100-ml broth

(SmF). A thermostable alkaline protease was reported to be produced by a novel *Pseudomonas* sp. in SSF system (Chakraborty and Srinivasan, 1993).

A new strain of *A. niger,* Tieghem 331221, produced large quantities of an extra-cellular acid protease when grown in SSF system using wheat bran as the sole substrate. Various carbon sources inhibited protease synthesis, indicating the presence of catabolic repression of protease biosynthesis (Chakraborty et. al., 1995). High enzyme activities were obtained in a medium containing high carbon and low nitrogen content. Addition of a suitable phosphate in the medium further improved the enzyme titres. Villegas et. al., (1993) studied the effects of O_2 and CO_2 partial pressure on acid protease production by a strain of *A. niger* ANH-15 in SSF of wheat barn. Results revealed a direct relationship between pressure drop, production of CO_2, and temperature increase. Acid protease production increased when the gas had 4% CO_2 (v/v), and it was directly related with fungus metabolic activity, as represented by the total CO_2 evolved.

Germano et al., (2003) used a strain of *P. citrinum* for serine protease production using agro-industrial residues. The strain also exhibited lipase activity. Datta (1992) used aspen wood for the production of protease from the fungal strain of *P. chrysosporium* BKM-F-1767. Study of this enzyme's characteristics showed that this protease had properties of aspartate-type protease as well as of thiol-type protease.

Optimization of media components: Response surface methodology

In the conventional method, media is optimized by changing one variable at a time while keeping other factors at a constant level (Senthilkumar et al., 2005), which is laborious and often leads to wrong conclusions. Multivariate experiments are designed not only to reduce the number of experiments necessary in the optimization process but also to produce more defined results than those available by univariate strategies (Dutta et al., 2004). Response surface methodology (RSM) is one such multivariate analysis tool comprising mathematical and statistical techniques for generating empirical models. It evaluates the effects of the individual factors and provides optimal levels of variables for desirable responses. RSM is customarily used as a statistical tool in majority of media optimization studies (Dahiya et al., 2005). It is interesting to note that a number of substrates have been tried for cultivating microorganisms; wheat bran has been the preferred choice in many cases. But in many reports wheat bran is used solely for the production of proteases, which resulted in low enzyme activities (Pandey et al., 1999; Germano et al., 2003; Krishnan and Vijayalakshmi 1985; Krishna, 2005). In another report of acid protease production by *Aspergillus niger* Tieghem 331221, addition of protein sources like Casitone, casein, peptone and Trader's protein increased activity levels of the enzyme whereas, various carbon sources inhibited protease biosynthesis thereby indicating the presence of catabolic repression of protease biosynthesis

(Chakraborty et al., 1995). In yet another study of utilizing cheap protein sources for protease production, *Mirabilis jalapa* seed powder was used for increasing the alkaline protease activity yield (*Aspergillus clavatus* ES1) by 14 fold increment (56 U/ml) over un optimized media (Mohammed et al., 2008). Optimization of media parameters by RSM was done by adding glucose (12.5 g L^{-1}) and tryptone (12.5 g L^{-1}) to the solid media which increased alkaline protease production by 60% (112.9 U/ml) using *Shewanella oneidensis* MR-1 (Anbu et al., 2008). RSM has been used as a statistical tool for optimizing media components for protease production by other microorganisms like *Neosartorya fischeri* (Wu and Hang, 2000), *Microbacterium* sp. (Thys et al., 2006) and *Bacillus licheniformis* ATCC 21415 (Mabrouk et al., 1999).

Downstream processing

A wide range of techniques is available for the recovery of the enzymes from the fermented substrate and the choice depends on source, i.e. intracellular or extracellular, scale of operation, enzyme stability and final usage of the product.

Extraction and concentration

The moldy bran is generally dried and stored. Extracellular enzymes are extracted from the bran by the addition of the required solvent with or without agitation. The temperature, time, pH and ratio of bran: solvent need to be optimized. Water, aqueous buffers, diluted solutions of salts (0.9% sodium chloride), 1% glycerol (Lonsane and Krishnaiah, 1992) or diluted (0.1%) solutions

of non-ionic detergents such as Triton X-100, Tween 20, Tween 40, Tween 60, Tween 80 are used to leach the enzymes. These detergents act on both cell wall and cytoplasmic membrane, making the cell permeable to certain protein materials, which are useful for extracting membrane bound enzymes in addition to extracellular enzymes.

Generally about 50-60 minute contact period is allowed. The solvent–to-solid ratio ranges from 1 to 8; higher the value, more diluted the solution will be, though the leached product fraction will increase. Extraction temperature is around 30ºC, but a temperature of 4-10ºC can be maintained to avoid denaturation, proteolysis and microbial growth. The optimum pH for the enzyme may not be the same as that required for optimal leaching. *Rhizopus oligosporus* acid protease is best extracted from fermented rice bran at pH 7, though it is most stable at pH 4 (Ikasari and Mitchell, 1996).

Many novel methods of down stream processing have proven to be effective in the recovery of proteases. Acid proteases have been recovered from solid bran fermented by *Mucor meihei* by semicontinuous multiple contact forced percolation method (Thakur et. al., 1993).

Enzyme preparations are usually supplied in liquid or solid form, irrespective of its source. In liquid preparations, the enzyme is stabilized against chemical and microbial denaturation and degradation by the addition of high concentration of salts like ammonium sulphate and preservatives such as glycerol to increase the

shelf life. The pH of the liquid is also adjusted to optimize stability. Enzymes that show greater stability in solid form are spray-dried or freeze- dried to obtain the powdered form of the enzyme (Ward, 1985).

Purification

Enzyme purification is a complex process and a number of methods are generally applied in sequence for the purification of the enzyme. Purification is aimed at (i) high degree of purity (ii) high overall recovery of activity of the enzyme and (iii) reproducibility. The extraction methods release enzyme into solution in addition to various other cell components such as nucleic acids and polysaccharides. These contaminants make the solution more viscous which could be overcome by differential sedimentation or precipitation. Thus this first step of purification results in more or less clear extract.

Further, the solution is subjected to either salt (ammonium sulphate) or solvent (acetone/ethanol) precipitation to remove unwanted contaminants like organic and inorganic molecules, proteins and water. This precipitation is followed by dialysis against its corresponding buffer for removal of the enzyme bound salts. Ultrafiltration is another technique used to separate proteins by passing water and other molecules through a semi-permeable membrane, thereby concentrating the protein molecules in the solution. This technique is faster and easier to handle than two-step process of precipitation and dialysis. Further the

enzymes are purified to homogeneity by following variety of chromatographic techniques of which few are listed in Table 2.

Immobilization of enzymes: Special reference to proteases

Enzymes accelerate different chemical reactions with high specificity without being permanently modified by their participation in reactions. However, enzymes are costlier than chemical catalysts, in general, and cost effectiveness of enzyme-based processes could be achieved by the repeated use of enzymes. Enzymes remain in solution with products and it is not possible to recover them easily from the reaction mixture. Repeated use of enzymes is possible only when they are made insoluble or stationary in active forms. Immobilization is the process by which an enzyme is made insoluble or stationary with the retention of full or substantial activity. Immobilization is also localization or confinement of enzymes during a process, which permits separation of the enzyme from substrate and product for its repeated use.

Immobilization has got many advantages such as,

- Repeated use of the same enzyme as far as practicable,
- Ability to terminate reaction at any stage by the removal of insoluble enzyme,
- Recovery of enzyme free product and,
- General improvement of enzyme stability

Techniques that are used to immobilize enzyme are broadly grouped in to

- Physical adsorption of enzyme on inert insoluble carrier
- Fixing of enzyme on insoluble support by covalent binding
- Entrapment of enzyme activity in polymerized gel
- Insolubilization of enzyme by cross-linking with bi-functional reagent

Upon immobilization, some changes of physical and chemical properties of immobilized enzymes may take place because of the development of new microenvironment around enzyme by the supporting matrix. The changes are usually expressed to various extents by the altered stability and kinetic parameters of the enzymes. Stability of the enzyme either increases or decreases on immobilization depending on the effect of the microenvironment on stability and denaturation of the enzyme.

There is no best-known method for the immobilization of any specific enzyme. The support, the enzyme, the substrate and technique, are all involved in the development of an effective process. The support material should be non-toxic, low cost, maximum biocatalyst loading capacity and with good flow character and operational durability.

Different methods that are developed to immobilize proteases are given in Table 3, describing about type of matrix, changes in kinetic constants and stability achieved upon immobilization.

Molecular characterization of acid proteases

Acid proteases have common characters with respect to optimum pH, temperature and stability. Fungal acid proteases have an optimal pH between 3-4.5 and are stable between 2.5-6.0 (Chitpinityol et al., 1998). Fungal acid proteases have a molecular weight in the range of 35-50 kDa and prefer bulky and hydrophobic residues for cleavage. Majority of these enzymes show low thermo-stability and lose their activity and structure at moderately high temperature (Rao et al., 1998).All aspartic proteases are inhibited by pepstatin, by binding of the hydroxyl group of the statine to the two catalytic aspartates (Chitpinityol et al., 1998). The *pep A* gene encoding the aspartic protease has been isolated and cloned from *Aspergillus sp.* The nucleotide sequence data reveals ORF's encoding aspartic proteases in these aspergilla are composed of four exons. Prepro peptides of 69, 78, 10 aa were found to precede 395-, 326-, and 323-aa mature proteins of *A. awamori, A. oryzae* and *A. fumigates*, respectively (Rao et al., 1998). Some of the characters of acid proteases from different sources are listed in Table. 4.

Table 2. Enzyme purification methods

Purification method	Enzyme	Remarks
Ion exchange and gel permeation	Acid protease (Tsujitha and Endo, 1978)	Conventional and time consuming method
Affinity chromatography (Immobilization)	α-amylase (Sardar and Gupta, 1998) Glucoamylase (Sharma et. al., 2000) Polygalacturonase (Teotia and Gupta., 2001) β-amylase (Teotia, Khare et. al., 2001) Glucoamylase (Teotia, Lata et. al., 2001)	Immobilized enzymes have several advantages, such as easy recovery, simplification of purification procedure, besides affording scale up (James and Simpson, 1996). Purity is achieved in fewer steps (Sommers et. al., 1989; Sadana and Belaram, 1994; Aksoy et. al., 1998). Immobilization increases temperature stability (Linne 1992).
Adsorption chromatography (Amberlite XAD)	Protease from fermented medium (Sidler, 1986)	Less coverage and more specific application
Dye-affinity membrane chromatography	Neutral protease (Wolman et. al., 2006)	Increases cost of purification, not suitable for industrial scale purification
Charcoal treatment followed by acetone precipitation	Protease (Tunga et. al., 1999)	Partial purification, used in many industrial enzyme purifications where the purity of the enzyme is sacrificed for its activity.
Affinity tagging with polyhistidine tagging	Microbial enzymes (Lilius et. al., 1991)	Mainly used in purifying expressed proteins in microbes.

Table 3. Immobilization of proteases on different matrices

Protease	Matrix	Kinetic constants				Stability	Number of cycles	Reference
		V_{max} free	V_{max} imm.	K_m free	K_m imm.			
Acid protease (*Synergistes sp.*)	Mesoporous activated carbon	3.2 Umg^{-1}	4.0 Umg^{-1}	-	-	Retained 50% activity at	5	Kumar et al., 2009
Pepsin	Polymethyl Methacrylate Acrylaldehyde (PMMA) microspheres	52 Umg^{-1}	36 Umg^{-1}	0.25%	0.34%	Stable up to 50ºC and pH up to 2.2	-	Hu et al., 2006
Esperase	Eudragit S-100,	8.2±1.7Uml^{-1}	4.0±0.7 Uml^{-1}	4.2±1.7 mg/ml	5.2±1.6 mg/ml	12.6 times more stable	5	Silva et al., 2006
Protease (Bacillus licheniformis)	Silica supports	-	-	25.8 µM	20.6 µM	Thermal stability up to 50ºC for 2 h.	-	Ferreira et al., 2003
α-Chymotrypsin Trypsin Papain	Tri(4-formyl phenoxy) cyanurate	-	-	-	-	15-20% increased thermal stability	8	Rao et al., 2006
Papain	Cotton fabric	-	-	-	-	Stable up to pH 7.0	6	Yan Li et al., 2007
Pepsin	Chitosan beads	5220 µM	2780 µM	1.56 mM	2.16 mM	Thermally stable up to 55ºC for 3 h	4	Altun et al., 2007
Cathepsin B	CM-cellulose-Ca-alginate capsules.	-	-	1.02 mM	1.52 mM)	Stable up to pH 8.0 and 70ºC.	3	Sharma et al., 2007
Trypsin	Eudragit S-100			1.0 µM	0.7 µM	Stable up to 45ºC	4	Kumar and Gupta, 1998
Neutrase	Alginate-Glutaraldehyde			6.4±0.2 mg/ml	2.4±0.1 mg/ml	Stable up to 65ºC	-	Orega et al., 2009

Table 4. Molecular characters of acid proteases

Source	Optimum pH	Optimum temperature (°C)	Stability (°C)	Molecular weight (kDa)	Inhibitor	Reference
Aspergillus oryzae	3-4	-	-	46	Pepstatin	Gotou et al., 2009
Aspergillus awamori nakazawa MTCC 6652	5	55	40-70	-	Pepstatin & 1 M urea	Negi and Banerjee, 2009
Synnergistes sp.	5.5-6.5	25-35	-	60	Pepstatin	Kumar et al., 2009
Thermoplasma volcanium	3	55	50-70	-	-	Kocabiyuk and Ozel, 2007
Rhizopus oryzae	5.5	30-45	-	34	Pepstatin	Kumar et al., 2005
Cteropharyngodo idellus (Grass carp)	2.5	37	-	28.5	Pepstatin	Liu et al., 2008
Tilapia nilotica (Bolti fish)	2.5	35	-	31	EDTA	El-Beltagy et al., 2004
Centaurea calcitrapa	3.5-4.5	52	-	-	Pepstatin	Salvador et al.,, 2006
Thermoascus aurantiacus	5.5	60	60	-	-	Merheb et al., 2007
Rhizopus oryzae NBRC 4749	3	50	40-50	37	Pepstatin	Chen et al., 2009
Sardinas sagax caerulea	3	45	50	-	Pepstatin	Castillo-Yanez et al., 2004

Protease Assay

Several assays exist depending upon the type of protease and substrate used. Due to their compact conformation, native proteins are generally not very susceptible to degradation by proteases (Robinson and Jencks, 1965). Protein substrates for proteases are most often hemoglobin (Anson, 1938) or casein and must be completely soluble in buffer. Hemoglobin must be denatured before use, either by treatment with acid (if the assay is at acidic pH) or urea (neutral to alkaline pH). Peptide bonds are more exposed and labile to proteases when proteins are unfolded due to denaturation.

The original casein assay was first described by Kunitz (1947) and later modified by Detmar and Vogels (1971). It involved TCA (trichloroacetic acid) precipitation of the undigested substrate, followed by photometric quantification of the released aromatic amino acids, using L-tyrosine as standard.

Denatured hemoglobin is preferred over casein since the complete amino acid sequence is known. Hemoglobin is also advantageous under acidic assay conditions, i. e. acid proteases. The assay is usually done at pH< 4.5. In this method, at the end of incubation of the enzyme with the substrate, the undigested protein is precipitated with double the volume of 5% TCA and filtered through Whatman #1 filter paper. The absorbance of the filtrate is determined at 280 nm and compared to that of tyrosine standard curve. One unit is defined as the amount of enzyme that produces per minute an absorbance at

280 nm equivalent to 0.1μg/ml of tyrosine (corresponding to an absorbance of 0.001 units at 280 nm).

Certain fluorogenic substrates have been used to study enzyme kinetics. Fluorescent peptide substrates such as of the type A-Phe-Phe-B and bearing an amino terminal fluorescent probe group (dansyl or mansyl) have been used to investigate the rate of formation of A-Phe, i.e. the rate of reduction in the fluorescence of the substrate is measured (Sachdev and Fruton, 1975).

Classification of proteases

Proteases do not comply easily with the general system of enzyme nomenclature due to huge diversity of their action and structure. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Barett, 1994). According to the nomenclature committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (International Union of Biochemistry, 1992).

Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate.

The mode of action of these classes of proteases is listed in Table 5. Based on their amino acid sequences, proteases are classified into different families (Argos, 1987) and further subdivided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Rawlings and Barrett, 1993).

Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively (Barett, 1994).

Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases. To facilitate quick and unambiguous reference to a particular family of peptidases, Rawlings and Barrett (1993) have assigned a code letter denoting the catalytic type, i.e., S, C, A, M, or U followed by an arbitrarily assigned number (Rawlings and Barrett, 1993). The general features of these four classes of endoproteases are listed in Table. 6.

Table 5. Classification of proteases

Protease	Mode of action*	EC No.
Exopeptidases		
Aminopeptidases	●↓○○○○○○•••••••	3.4.11
Dipeptidyl peptidase	●●↓○○○○○○•••••	3.4.14
Tripeptidyl peptidase	● ●●↓○○○○○○•••	3.4.14
Carboxypeptidase	•••••••○○○○○○↓●	3.4.16-3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase	•••••○○○○○○↓●●	3.4.15
Dipeptidases	*●↓●•••	3.4.13
Omega peptidases	••●↓○○○○○○•••••	3.4.19
Endopeptidases	•••○○○↓○○○••••	3.4.21-3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24

(Rao et al., 1998)

* Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and stars signify the blocked termini. Arrows show the sites of action of the enzyme.

Table 6. General features of four types of proteases

Type of protease	**EC No.**	**Molecular weight**	**pH optimum**	**Temperature Optimum (ºC)**	**Active site residues**	**Major inhibitors**	**Major sources**	**Reference**
Aspartic	3.4.23	30-45	3-5	40-55	Aspartic acid	Pepstatin	*Aspergillus, Mucor, Endothia, Rhizopus, Penicillium, Neurospora*, Animal tissue (stomach)	North, 1982; Rao et al., 1998; Kovaleva, et al., 1972
Cysteine or thiol	3.4.22	34-35	2-3	40-45	Aspartate or cysteine	Iodoacetamide, *p*- CMB	*Aspergillus*, stem of pineapple, latex of Figureure tree, papaya, *Streptococcus*, *Clostridium*	Keay and wildi, 1970; Keay et al., 1972; Gripon et al.,1980
Metallo	3.4.24	19-37	5-7	65-85	Phenyl-alanine or leucine	Chelating agents such as EDTA, EGTA	*Bacillus, Aspergillus, Penicillium, Pseudomonas, Streptococcus*	Aunstrup, 1980
Serine	3.4.21	18-35	6-11	50-70	Serine, histidine and aspartate	PMSF, DIFP, EDTA, soybean trypsin inhibitor, phosphate buffers, indole, phenol, triamino acetic acid	*Bacillus, Aspergillus,* animal tissue (gut), *Tritirachium album*	Boyer, 1970; Nakagawa, 1970

Amongst all the classes of proteases, aspartic proteases are well studied and characterized owing to their vast industrial and pharmaceutical applications.

Aspartic proteases

Aspartic acid proteases, commonly known as acid proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (Barett, 1995), and have been placed in clan AA. The members of families A1 and A2 are known to be related to each other, while those of family A3 show some relatedness to A1 and A2. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. The members of the pepsin family have a bilobal structure with the active-site cleft located between the lobes (Sielecki et al., 1991). The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr. The aspartic proteases are inhibited by pepstatin (Fitzgerald et al., 1990). Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Microbial aspartic proteases are broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* spp. These enzymes are described in

detail with respect to importance, mode of action and structural aspects in the following sections.

I. Acid protease (pepsin-like)

Active site

The active site aspartates, Asp 33 and Asp 214 are situated on the corner of the two extended lobes in the N- and C- terminal domains. The side chain of these two aspartates are oriented towards each other around a pseudo-interlobe diad axis in a complicated hydrogen-bonding network known as 'fireman's grip' (Pearl and Blundell, 1984) shown in Figure. This network is formed by the interaction of two loops (residues 32-35 and residues 213-218) and a central water molecule. The carboxyl oxygens of Asp33 and Asp214 are hydrogen bonded with nitrogen atoms of the conserved Gly34 and Gly 217, respectively. In addition, the side chains of Ser35 and Thr218 also form hydrogen-bonds with the outer oxygen atoms of Asp33 and Asp214, respectively (Figure 2).

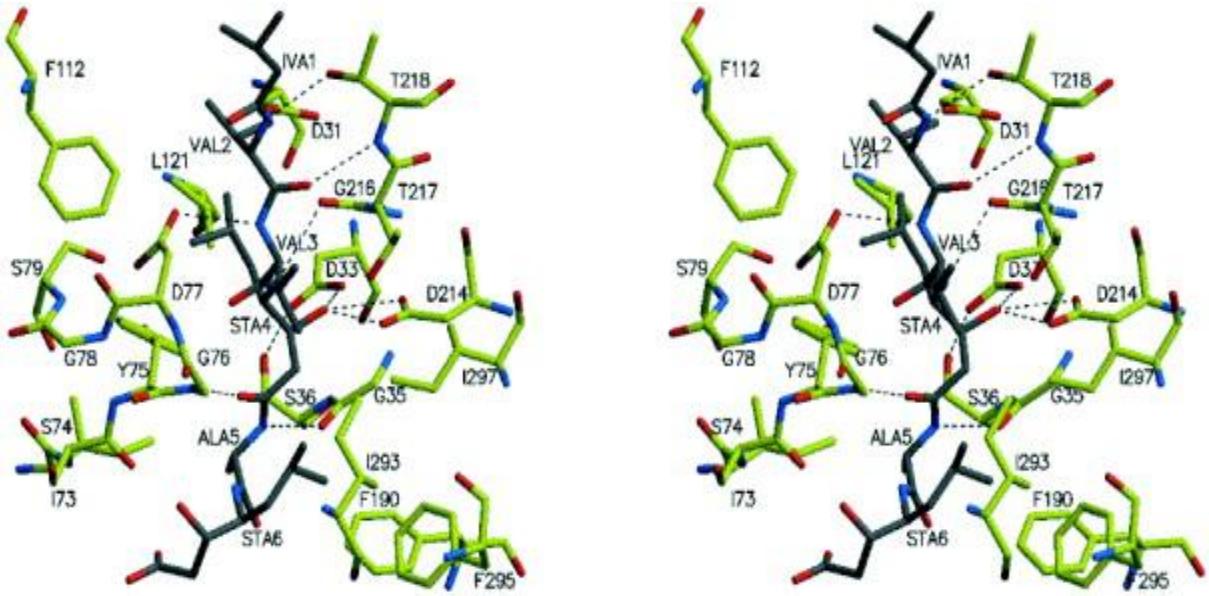


Figure 2. Interaction of active side residues (yellow carbon atoms) of AOAP with pepstatin (grey carbon atoms) illustrated by the programs MOLSCRIPT and Raster3D (Kamitori, et al., 2003). Hydrogen bonds are shown as broken lines.

Mechanism of action of aspartic proteases

Aspartic endopeptidases depend on the aspartic acid residues for their catalytic activity. A general base catalytic mechanism has been proposed for the hydrolysis of proteins by aspartic proteases such as penicillopepsin (James et. al., 1992) and endothiapepsin (Pearl, 1987) (Figure 3). Crystallographic studies have shown that the enzymes of the pepsin family are bilobed molecules with the active-site cleft located between the lobes and each lobe contributing one of the pair of aspartic acid residues that is essential for the catalytic activity (Blundell et. al., 1990). The lobes are homologous to one another, having arisen by gene duplication. The retropepsin molecule has only one lobe, which carries only one aspartic residue, and the activity requires the formation of a noncovalent homodimer (Miller et. al., 1989). In most of the enzymes from the pepsin family, the catalytic Asp residues are contained in an Asp-Thr-Gly-Xaa motif in both the N- and C-terminal lobes of the enzyme, where Xaa is Ser or Thr, whose side chains can hydrogen bond to Asp. However, Xaa is Ala in most of the retropepsins. A marked conservation of cysteine residue is also evident in aspartic proteases. The pepsins and the majority of other members of the family show specificity for the cleavage of bonds in peptides of at least six residues with hydrophobic amino acids in both the Pl and Pl' positions (Keil, 1992).

The specificity of the catalysis has been explained on the basis of available crystal structures (Liu et. al., 1996). The structural and kinetic studies also have

suggested that the mechanism involves general acid-base catalysis with lytic water molecule that directly participates in the reaction. This is supported by the crystal structures of various aspartic protease-inhibitor complexes and by the thiol inhibitors mimicking a tetrahedral intermediate formed after the attack by the lytic water molecule (James et. al., 1992).

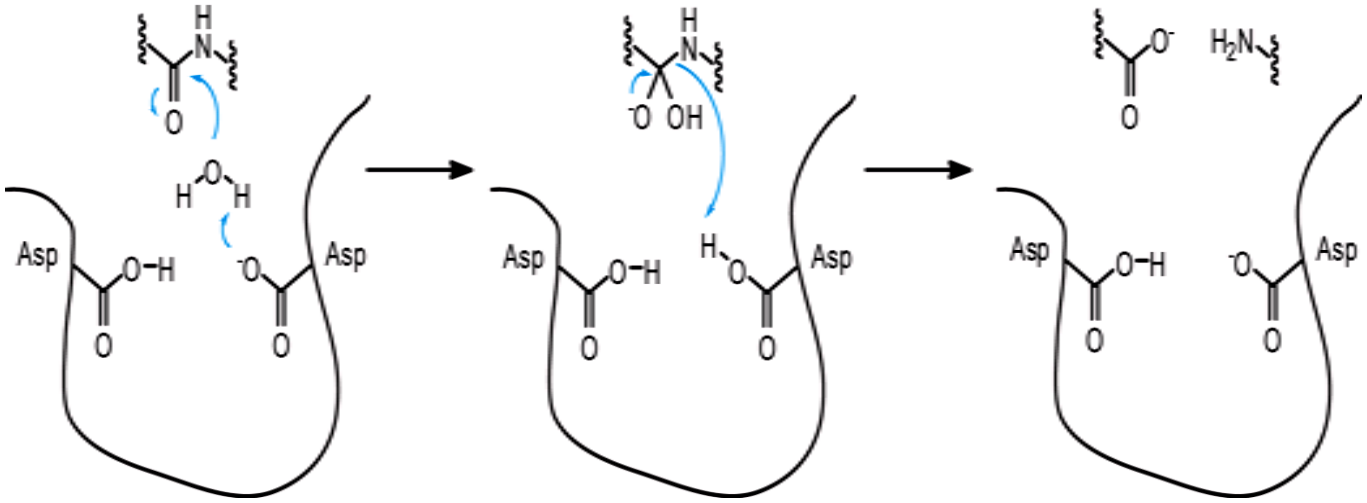


Figure 3. Catalytic mechanism of aspartic proteases

Structure of Pepsin-like aspartic protease

Primary structure

Aspartic proteases consisits of 325-400 amino acid residues with a molecular weight ranging between 35-45 kDa (Shintani and Ichishima, 1994). All mammalian aspartic proteases are synthesized as proenzymes and are subsequently activated.

Secondary structure

High resolution X-ray structures of the enzyme of this class are found in Protein Data Bank (PDB). The enzyme has around 330 amino acids and adopts a crescent shaped structure divided into two lobes, composing the deep active site cleft. Two catalytic Asp residues are located at the centre of the active site cleft, forming the catalytic dyad with hydrogen bonding solvent molecule. The refined model of *Aspergillus oryzae* acid protease (AOAP) has a crescent shaped structure with two lobes (N-lobe and C-lobe) related by a pseudo-2-fold axis, as found in the structures of aspartic protease family. The active site is located on the cleft between two lobes. AOAP has 34 β-strands forming five β-sheet structures with 51% of amino acid residues, and six short α-helices located on the surface of the molecule. β–sheet 1 is extended to N and C-lobes, and a pseudo 2-fold axis in the centre of the sheet. β–sheet 1 with 18 β-strands forms a large twisted β–sheet structure, constructing a hydrophobic core of AOAP. Since each β–strand in β–

sheet 1 rotates anticlockwise relative to the neighboring β–strand, all β–strands in β–sheet 1 are in helical arrangement with an almost complete turn of the left handed helical structure. Although Asp33 is located at the opposite edge of β–sheet 1 to Asp214, both residues are facing each other on the active site cleft, due to helical structure of β–sheet 1. β–sheets 2 and 3 belong to the N-lobe, while β–sheets 4 and 5 belong to the C-lobe. β–sheets 2 and 4 are located on both ends of β–sheet 1, and construct a hydrophobic core of AOAP with β–sheet 1. β–sheet 3 projects across the active site cleft, while β–sheet 5 partially forms the side of the active site cleft (Figure 4).

Tertiary structure

The three dimensional structures of several aspartic acid proteases have been solved by X-ray crystallography. These include porcine pepsin (Andreeva et. al., 1984), pepsinogen (James and Sielecki, 1986), human rennin (Sielecki et. al., 1989), endothiapepsin (Blundell et. al., 1990), penicillopepsin (James and Sielecki 1983), rizopuspepsin and retroviral proteases (Lapatto et. al., 1989; Miller et. al., 1989; Wlodawer et. al., 1989). Structural superimpositions of aspartic proteases reveal that N-terminal domain has greater structural similarity than the C-terminal domain (Gilliland et. al., 1990). The C-terminal domain is more separated from the rest of the molecule than the N-terminal domain and rigid body movement appears in the C-terminal domain (residues 190-302) (Sali et. al., 1992). The greater differences among these proteases are the surface loop regions (Figure 5).

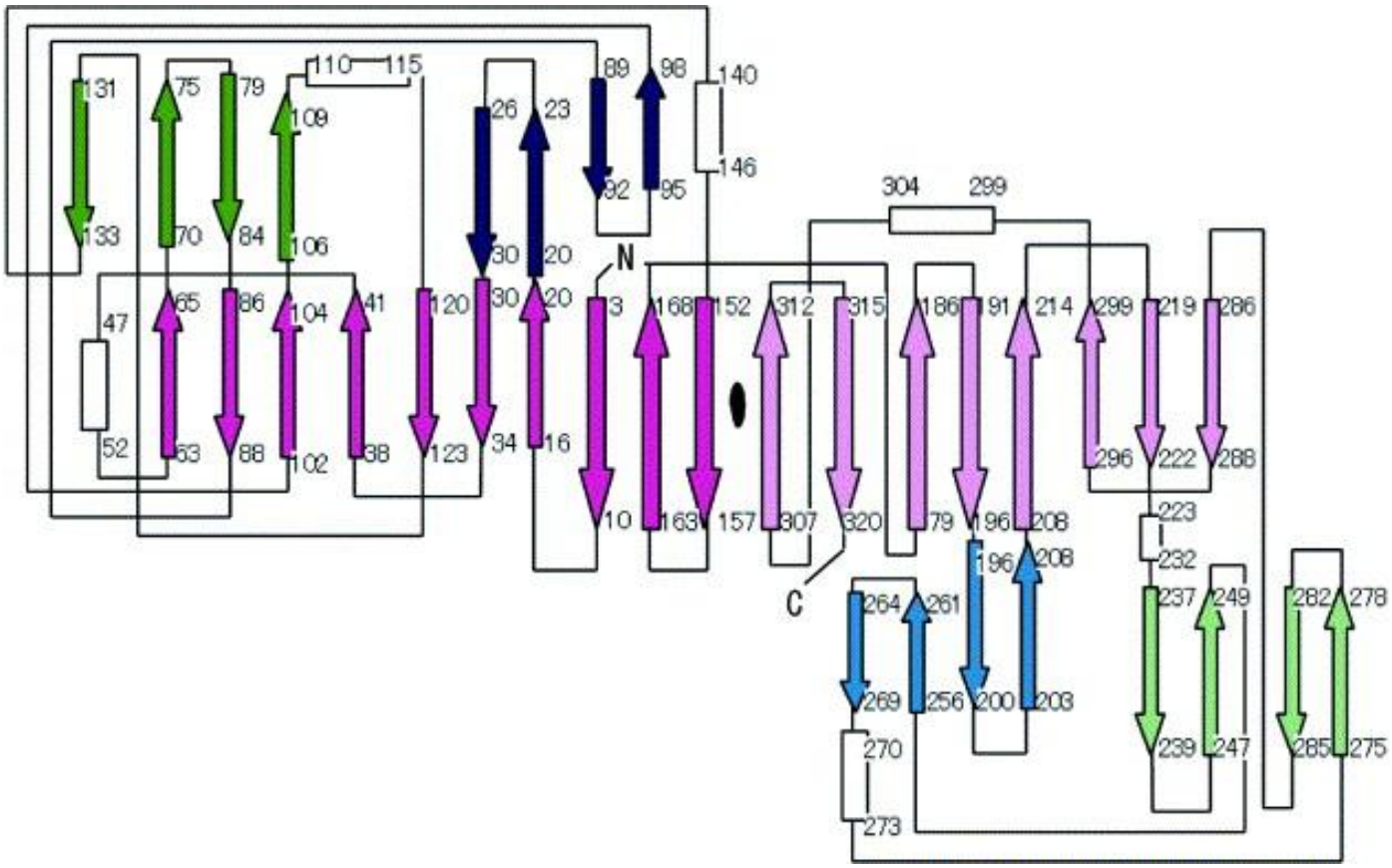


Figure 4. A topographical diagram showing the arrangement of secondary structure elements by acid protease from *A. oryzae*. β-sheet 1 in N-lobe, 1 in C-lobe and sheets 2, 3, 4 and 5 are shown in magenta, light magenta and blue, green, light blue and light green respectively (Kamitori, et. al., 2003).

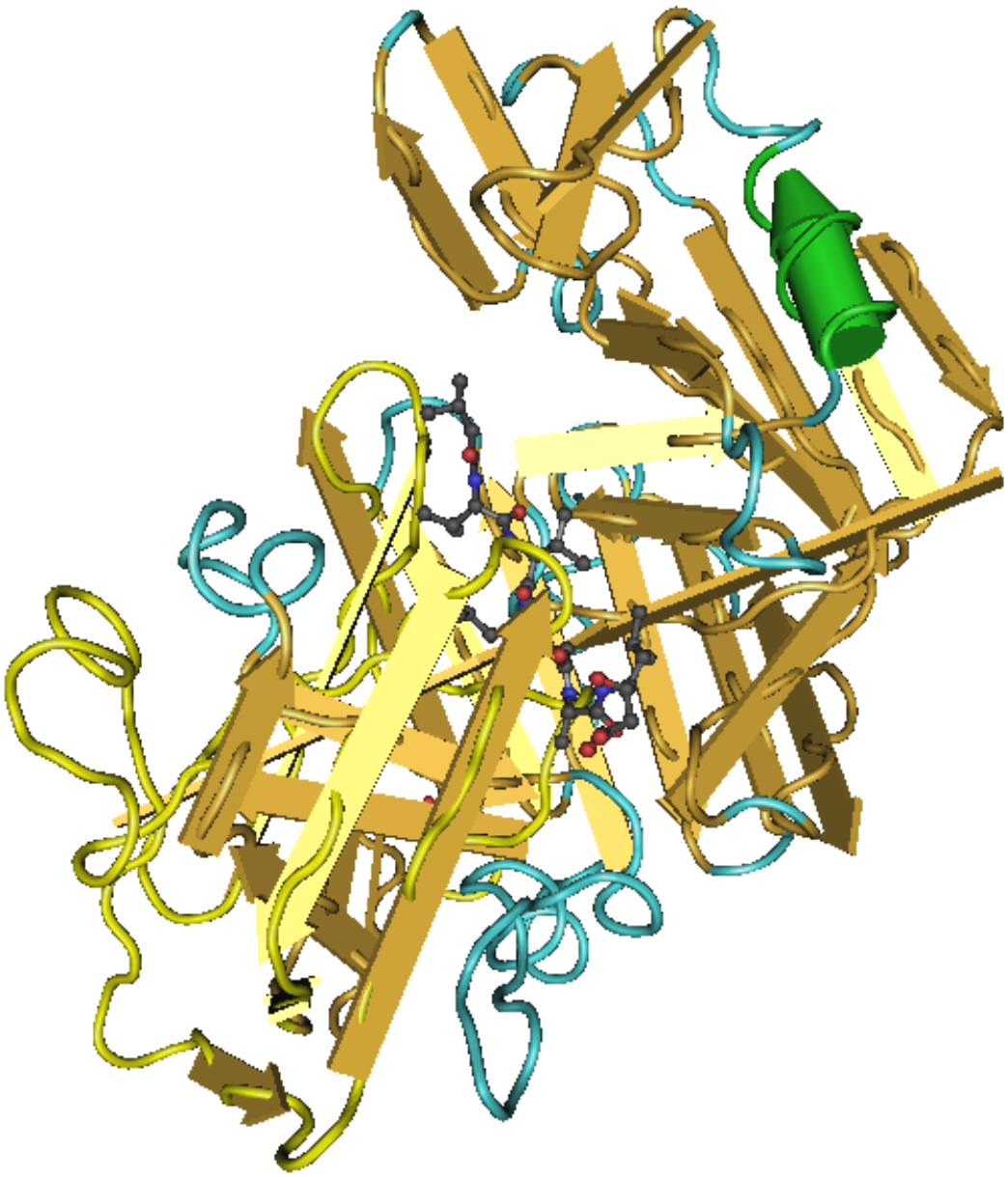


Figure 5. Crystal structure of acid protease from *Aspergillus oryzae* at 1.9A° resolution. Pepstatin molecule at the active site is depicted by ball-stick model (Kamitori, et. al., 2003).

Genomic overview of *Aspergillus oryzae* with reference to aspartic acid proteases

Aspergillus oryzae and *Aspergillus niger* are also natural production hosts, or ''factories,'' in the biotechnology industry for production of fungal and mammalian proteins and metabolites. The genome size of most filamentous fungi is estimated to be 30–40 Mb, encoding 9000—13,000 genes (Machida, 2002). The *A. oryzae* genome consists of eight chromosomes ranging from 2.8–7.0 Mb (Kitamoto et al., 1994). Sequencing of the *A. oryzae* genome was completed by the Japanese National Institute of Technology and Evaluation (Machida, 2002). The total genome size of *A. oryzae* was estimated to be 36.8 Mb, the predicted number of genes is 14,063, and mean gene length is 1178 base pairs (Archer and Dyer, 2004; Machida et al., 2005). Comparison between the genomes of *A. oryzae* and *Saccharomyces cerevisiae* indicated that they share about 4000 common genes, while 9000 and 2400 genes were unique to *A. oryzae* and *S. cerevisiae*, respectively (Machida et al., 2004). Synteny analysis of *A. oryzae, A. nidulans* and *A. fumigatus* indicated that *A. oryzae* has significantly more synteny breaks than exist between *A. nidulans* and *A. fumigatus*. *A. oryzae* had a mosaic structure consisting of loci that were common to the other two species, as well as *A. oryzae*-specific loci. Aspergillus oryzae has the largest expanse of hydrolytic genes among the three aspergilla. The genomes of *A. oryzae*, *A. fumigatus* and *A. nidulans* contain 135, 99 and 90 secreted proteinase genes, respectively, which constitute roughly 1% of

the total genes in each genome. *A. oryzae* possesses more secretory protease genes that function in acidic pH, including aspartic proteases, pepstatin-insensetive protease, serine type carboxypeptidases and aorsin. This increase may reflect A. *oryzae*'s adaptation to acidic pH during the course of its domestication (Machida et al., 2005).

Aspergillopepsins comprise a family of closely related aspartic proteinases produced by fungi of the genus *Aspergillus*. They share significant amino acid sequence homology with penicillopepsins, produced by *Penicillium* species (Stepanov, 1985), and with endothiapepsin from *Endothia parasitica* (Barkholdt, 1987). In addition, aspergillopepsins share limited regions of amino acid sequence identity with aspartic proteinases from other fungi such as *Rhizomucor miehei* and *Rhizopus chinensis* (Graham et al., 1973) and with mammalian gastric proteinases such as pepsin and chymosin (Tang, 1979). Among these aspartic proteinases, the greatest degree of amino acid sequence conservation lies in the regions surrounding the active-site residues.

Acid protease encoding gene (*pep A*) has been cloned and sequenced from *Aspergillus oryzae* (Gomi et al., 1993). The nucleotide sequence of the cloned *pep A* gene has an open reading frame of 1385 bp containing three short putative introns, and encodes 404 amino acid residues. Comparison with other *Aspergillus pep A* gene shows that the inserted positions of the introns are strictly conserved. A full sequence of the *pep A* gene has been given in Figure 6. The deduced amino acid sequence is given in Figure 7.

AAGCTTACTGGATGACAAGGATCTTCGTCGCTCCATGCCTAGACGAGAAACACTGCCTAGTGCTGCTGATGTGGACCGC

TTCTCTCCTGAGCCGGAGTCCGTTGATTATCACCACTACCTTCATGATGACCACCAGAGTAGACAAAGCTATAAAGGAG

CGCCTTCCAGCTGCCCTGAGTAGCTCATCACTCTCCCATCCTCTCCAACAAGAGTCTGAGTTCGTCTAGGCTTGTGCTT

GTCATTCTTTCACATCCAGTCTTTCGTTCCGTCTTCTCCACATTTCGTCTAGAGAGCAATCACTATGGTTATCTTGAGC

AAAGTCGCTGCCGTCGCGGTCGGCCTCTCCACGGTCGCCTCTGCATTGCCCACCGGTCCCTCTCACTCCCCCCATGCTC

GTCGGGATTCACCATCAACCAGATCACCAGGCAGACTGCCCGCGTCGGTCCCAAGACCGCCAGCTTCCCCGCAATCTAC

AGCAGGGCGCTTGCTAAGTATGGCGGTACTGTGCCTGCGCACCTCAAGAGCGCTGTTGCCTCGGGTCACGGTACTGTCG

TGACTTCTCCCGAGCCCAATGACATTGAGTACTTGACTCCTGTCAACATTGGCGGCACGACCCTGAACCTCGACTTCGA

CACTGGCTCGGCCGATCTGTAAGTAATAGACAGTTCTCCACATAAATTCATGACTAATAATAATTCAGCTGGGTCTTCT

CCGAGGAGCTCCCCAAGTCCGAGCAGACCGGCCACGACGTCTACAAGCCTTCTGGAAACGCCTCCAAGATCGCTGGTGC

CAGCTGGGACATCAGCTACGGTGATGGCAGCAGTGCCAGCGGTGACGTTTATCAGGATACTGTCACCGTGGGCGGCGTC

ACTGCCCAGGGCCAGGCCGTCGAGGCCGCTAGCAAGATTAGCGATCAGTTTGTTCAGGACAAGAACAATGACGGTCTGC

TGGGTCTCGCTTTCAGCTCGATCAACACTGGTAAGGCATCCTTCGATTGCACAGTGTTGATGACTGGTGTGTGCTGACA

AAGACTACAGTCAAGCCCAAGCCCCAGACTACCTTCTTCGACACCGTCAAGGACCAGCTGGACGCTCCCCTATTCGCCG

TGACCCTGAAGTACCATGCTCCTGGCTCCTATGACTTCGGCTTCATCGACAAGAGCAAGTTCACTGGTGAACTCGCATA

TGCCGATGTGGACGATTCCCAGGGCTTCTGGCAATTCACTGCTGACGGTTACTCTGTCGGAAAGGGCGACGCCCAGAAG

GCCCCCATCAGTGGTATTGCTGGTGAGTCCCCATCCAATCGCAAATCGACCAGGTCTGGGCATCTACTAACACCTCTCT

CCAGACACCGGTACCACCCTCGTCATGCTCGATGACGAAATCGTCGATGCCTACTACAAGCAGGTCCAGGGCGCCAAGA

ACGACGCATCCGCTGGAGGCTACGTCTTTCCCTGCGAAACCGAGCTCCCCGAATTCACCGTTGTTATCGGCTCCTACAA

CGCCGTCATCCCTGGCAAGCACATCAACTACGCCCCTCTCCAGGAGGGCAGCTCCACTTGCGTTGGCGGTATTCAGAGC

AACTCTGGTCTCGGCCTCTCCATACTGGGTGATGTCTTCCTCAAGAGCCAGTACGTCGTCTTCGACTCCCAGGGTCCCA

GACTCGGCTTCGCCGCCCAGGCTTAAATGCCTGACTAATGCGGGCCCCGTGCTCTGATGCACGGCCTAAGTCTAATGAA

CCGACCCCCTAGCGGGTGATCCGGCTCGATGTTGGAGATGAGTAATCTGATCTACCGATGTATCTATTTCTTCTTGTAT

ATGGTGACTTTGATTTATGAGAGATGGTTTGGTATGCGGGACATGTTGATGGATGATTGCGGCTGTCTTCTTGGACCTC

TGTATATATGACCACGTCGATCGTTACACGAGAGGGCAGCTTTCAATTACATAACACAATTCATCATATTATACACACT

ACCTCATCCATGGACACGAATTTACTA

Figure 6. The nucleotide sequence of *pep A* gene encoding aspartic protease from *Aspergillus oryzae*. The arrows indicate start and stop amino acids for the protein and the nucleotide sequence coding for the mature protein is given in red.

[1]MVILSKVAAVAVGLSTVASALPTGPSHSPHARRGFTINQITRQTARVGPKTASFPAIYSRALAK
YGGTVPAHLKSAVASGHGTVVTSPEPNDIEYLTPVNIGGTTLNLDFDTGSADLWVFSEELPKSE
QTGHDVYKPSGNASKIAGASWDISYGDGSSASGDVYQDTVTVGGVTAQGQAVEAASKISDQF
VQDKNNDGLLGLAFSSINTVKPKPQTTFFDTVKDQLDAPLFAVTLKYHAPGSYDFGFIDKSKFT
GELAYADVDDSQGFWQFTADGYSVGKGDAQKAPISGIADTGTTLVMLDDEIVDAYYKQVQG
AKNDASAGGYVFPCETELPEFTVVIGSYNAVIPGKHINYAPLQEGSSTCVGGIQSNSGLGLSILG
DVFLKSQYVVFDSQGPRLGFAAQA[404]

Figure 7. Amino acid sequence of aspartic protease (44 kDa) deduced from the nucleotide sequence of *pep A* gene from *Aspergillus oryzae* (Gomi et al., 1993).

Aspergillus oryzae produces several aspartic proteases. Most of reports include characterization with reference to molecular weight, optimum pH, optimum temperature and further characterization with respect to cleavage pattern, thermal stability, and structure-stability studies are lacking. Acid protease is secreted as a zymogen like any other proteases and the pro-peptide is cleaved extracellularly making the enzyme active. Comparison of three different studies on amino acid composition of acid protease from *Aspergillus oryzae* is given in Table 7.

Table 7. Comparison of amino acid composition of *A. oryzae* acid protease

Amino acid	Gomi et al., 1993	Tsujita and Endo, 1976	Davidson et al., 1975
Lysine	17	15	22
Histidine	4	4	5-6
Arginine	1	1	1-2
Aspartic acid	29 } 40	36	45
Aspargine	11		
Threonine	24	23	24-25
Serine	29	27	31
Glutamic acid	12 } 30	27	34-35
Glutamine	18		
Proline	15	17	13
Glycine	38	35	43
Alanine	28	26	32
Cysteine	2	3	2
Valine	27	24	29
Methionine	1	1	1
Isoleucine	15	14	16
Leucine	21	19	24
Tyrosine	14	12	16
Phenylalanine	17	15	20
Tryptophan	3	5	6
Total	**326**	**304**	**347-351**

Structure-stability relationship

Stability is a prerequisite for enzymes to be functional at extreme conditions. Studies on protein stability explore the sequence-structure-stability relationship. Sequence defines structure, whose interactions with each of the domains/subunits stabilize the protein (Razvi and Scholtz, 2006). The stability of a protein can be determined by studying the effect of temperature and denaturants on its structure. A precise understanding of thermodynamic and conformational stability contributes to the prediction of enzyme stability.

Conformational stability measurements assume that the molecule may belong only to two thermodynamic states, the folded state (typically denoted N or F) and the unfolded state (typically denoted U). This "all-or-none" model of protein folding, first proposed by Anson (1945), is believed to hold only for small, single structural domains of proteins (50-200 amino acid residues). As the length of the polypeptide chain increases, it becomes energetically unfavorable to form one large domain. Thus, globular proteins with molecular weights over 30 kDa tend to form multi-domain structures with different degrees of inter-domain interactions (Griko et al., 2001).

Some proteins regain their native and functional structure upon removal of the denaturant. This kind of unfolding, also called thermodynamically reversible unfolding comes handy in the determination of the thermodynamic parameters. It is generally accepted that the driving force for the refolding of a protein resides

in its amino acid sequence (Strucksberg et al., 2007). Refolding of small globular proteins, with amino acid residues 100-200, is feasible. One of the major problems in the refolding of multi-domain proteins is related to aggregation of non-native states, which often inherently reduce the efficiency of the refolding process. The proper refolding of these proteins, *in vivo*, takes place under the influence of molecular chaperons, which serve to prevent aggregation of proteins. In other cases, the refolding of a protein cannot be achieved due to covalent modifications during unfolding, such as thiol modification, deamidation, cleavage of labile peptide bonds, removal of prosthetic group, etc. (Sudharshan and Rao, 1999).

Structural basis for thermal stability

Thermal inactivation of enzymes occurs in two steps.

$$N \leftrightarrow U \rightarrow I,$$

Where, N is the native enzyme, U is the reversibly unfolded enzyme and I is the irreversibly inactivated enzyme. The first reversible step is partial unfolding of protein molecules. The second step is irreversible due to conformational and covalent processes. The major conformational processes include aggregation due to enhanced hydrophobic interactions and formation of incorrect structures.

Protein aggregation, the self-association of non-native polypeptide chains to form amorphous or ordered multimeric structures occurs in a wide variety of

conditions. It may appear in the form of inclusion bodies during protein expression in host cells or as a kinetically competing reaction during the recovery of active proteins, thereby diminishing the yield of productive refolding in biotechnological processes (Singh and Panda 2005; Carrio and Villaverde, 2002). Furthermore, protein products which are now increasingly introduced to the pharmaceutical market may undergo non-native aggregation during purification, sterilization, shipping and storage processes (Manning et al., 1989). Although there are some reports suggesting that protein aggregation may proceed through a transiently expanded conformational species within the native state ensemble (Kendrick et al., 1998; Krishnan et al., 2002; Marcon et al., 2006), the first event in the aggregation process is often the unfolding of the native state conformation (Plakoutsi et al., 2004). It is well established that the protein native structures are marginally stable, with free energies about 5–20 kcal mol^{-1} lower than the unfolded "state", so that a relatively small perturbation in the environmental conditions may result in destabilization of the native state (Dill, 1990).

Knowledge on protein folding/unfolding and of denaturation is of great significance for the biotechnology industry as it has a direct effect on the overall stability of an enzyme and therefore on the commercial viability of any process. In what concerns aspartic proteinases, which are of physiological, commercial and pathological relevance, very few studies have been performed on the stability of this family. Studies on pepsin (Campos and Sancho, 2003; Dee et al.,

2006) have demonstrated the formation of inactive intermediaries during pH-induced denaturation that exhibit native-like characteristics.

In the case of pepsin, a typical eukaryote aspartic protease, it has been believed to unfold in two-distinct stages. Calorimetric study revealed that the N-terminal domain was melted first and that the C-terminal domain followed (Privalov et al., 1981). This is in contrast with the results for HIV-1 protease, although the two domains in pepsin correspond to the constitutive subunits of HIV-1 protease in terms of molecular architecture (Lin et al., 1994). An unfolding study in pepsin (Tanaka and Yada, 2001) indicated that the prevention of the dissociation of the N-terminal domain was related to the prevention of unfolding. In HIV-1 protease, the unlinked subunits are rapidly dissociated from each other by diffusion, which may cause its unfolding process to be more cooperative.

DSC study of thermal denaturation of acid protease from *Aspergillus saitoi* indicated a complex unfolding pattern of the enzyme with one or more reversible unfolding transitions followed by an irreversible step (Tello-Sollis and Hernandez-Arana, 1995). Accumulation of partly folded states of ervatamin A (Nallamsetty et al., 2007) were observed induced by all three denaturing agents (pH, temperature and chemical denaturants).

II Milk clotting enzymes (rennin-like)

Cheese is a by-product of dairy industry which is produced by clotting the milk by proteases. Cheese, produced via milk clotting by enzymes from the stomach of unweaned calves, was inadvertently discovered in ancient times, and was employed as a simple and efficient form of preservation of milk nutrients.

All enzymes, employed commercially in milk coagulation are aspartic proteinases. The Rennet or rennin, obtained from the fourth stomach of unweaned calves not only clots milk, but also plays an important role during cheese maturation, a complex process for the balanced development of flavour and texture (Vioque et al., 2000).

Cheese manufacture

The exact process in the making of cheese varies between different varieties. However, the unit operations are similar. The processes involved cheese making (cheddar cheese) are given in the Figure 8.

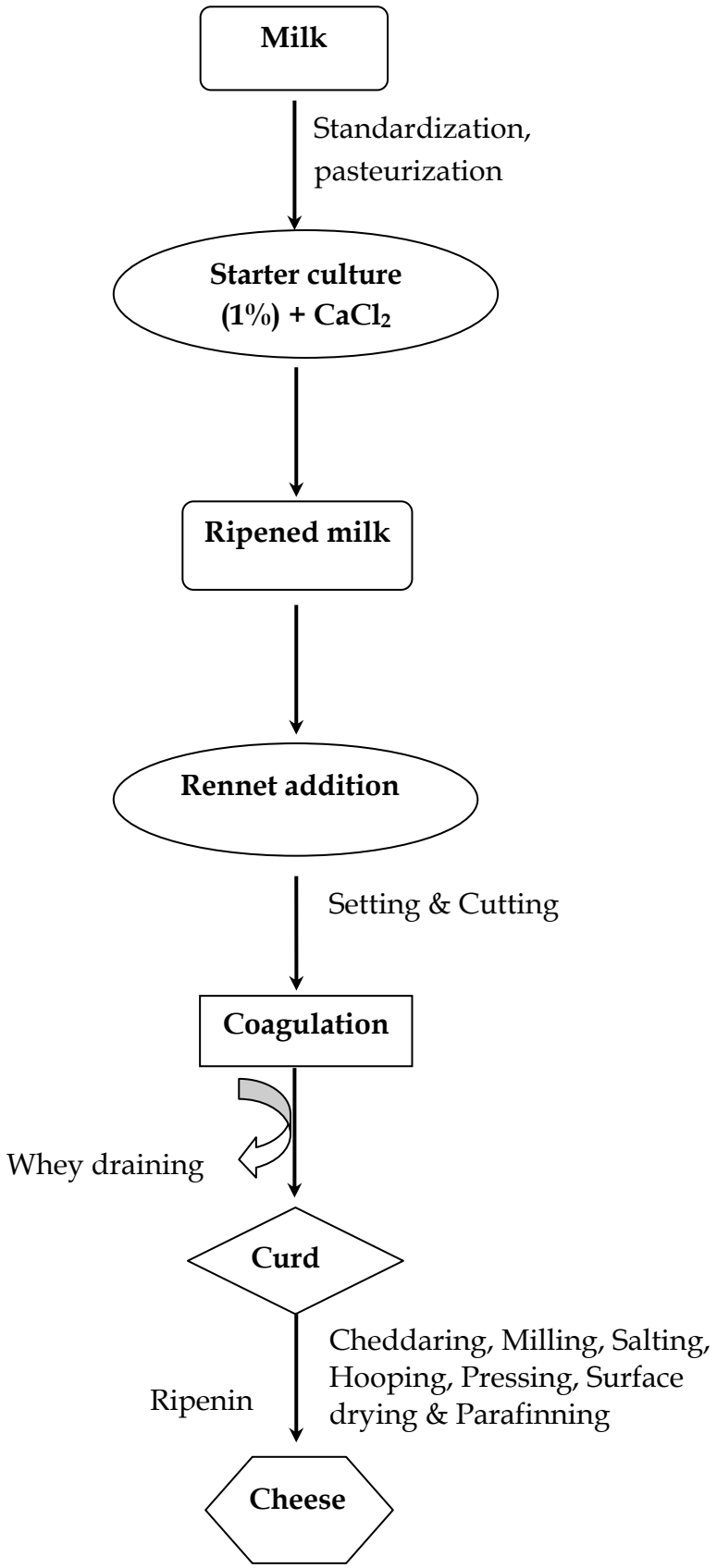


Figure 8. Flow chart for cheese preparation

History of milk clotting enzyme

Since ancient days, extracts of calf stomach have been used for clotting milk in cheese making. The first attempts at isolation of the enzyme were made by Deschamps in 1840, who suggested the name ***chymosine*** (Gr. *chyme*, 'gastric juice'). Lea & Dickinson in 1890 suggested the name ***rennin*** (derived from rennet). This name was popular for many years but was almost indistinguishable from renin from the kidneys, so Foltmann in 1970 suggested a return to the original designation, ***chymosin***, as subsequently adopted by the IUBMB. Hammarstein in 1872 discovered that the enzyme was secreted as an inactive component, which was converted into active enzyme through treatment with acids. He was the first to discover a pro-enzyme. For many years it was generally assumed that chymosin was restricted to young ruminants, but Foltmann & Axelsen, in 1980, showed that chymosin-like enzymes are widely distributed among young mammals. Thus, the chymosin may be characterized as mammalian fetal or neonatal gastric proteinases.

Desirability of rennet

The major application of proteases in the dairy industry is in the manufacture of cheese. The milk clotting enzymes fall into three main categories (i) animal rennets (chymosin) (ii) microbial milk coagulants and (iii) genetically engineered chymosin. Chymosin is extracted from the fourth stomach of unweaned calves. Both chymosin (EC 3.4.23.4) and microbial milk clotting

enzymes belong to aspartic acid protease class and have a molecular weight around 30-40 kDa. Chymosin has high milk clotting to proteolytic ratio, thus making it an ideal enzyme in dairy industry for cheese making.

Milk clotting mechanism

Milk clotting by calf rennet occurs essentially by cleaving the Phe_{105}-Met_{106} bond of *k*-casein, resulting in release of a short hydrophilic glycopeptide (106-169 residues), which passes into the whey. Para-*k*-casein becomes positively charged at neutral pH and causes decrease of the repulsive forces between casein micelles thereby causing aggregation (Green 1973). The general scheme of milk clotting is depicted in Figure 9.

The proteolytic activity of microbial proteases on *κ*- casein has been reported (Yu et al., 1968). The hydrolysis of other proteins in milk including α_S1-casein, α_S2-casein, *β*-casein and α-lactalbumin monomer by chymosin have been reported with much slower rate of proteolysis (Miranda et al., 1989).

Worldwide cheese is a major agricultural product. According to FAO, over 18 million metric tons of cheese were produced worldwide in 2004. The largest producer of cheese is the United States, accounting for 30% of total production followed by Germany and France. A latest report (2008) of cheese production globally is given in Table 8.

Table 8. Cheese production by top producers in 2008 **(http://www.fao.org)**

Country	Production (1,000 metric tons)
United States	4,275
Germany	1,927
France	1,884
Italy	1,149
Netherlands	732
Poland	594
Brazil	495
Egypt	462
Argentina	425
Australia	395

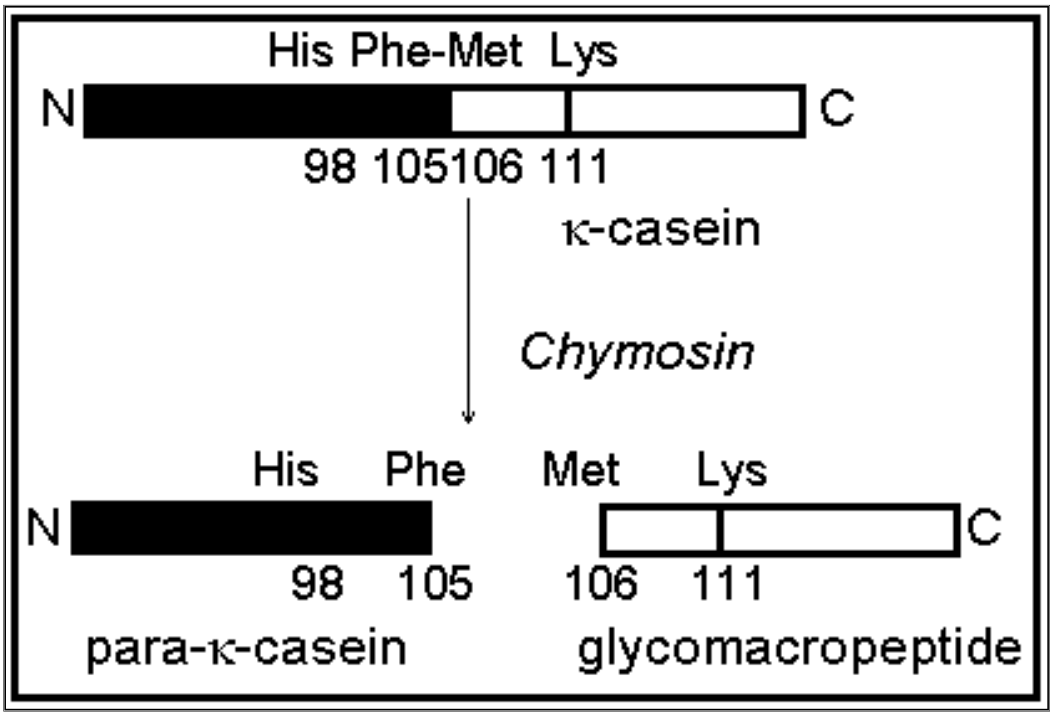


Figure 9. Scheme for the clotting of milk during cheese making

Chymosin Structure

The primary structure of calf prochymosin has been determined both at the amino acid level (Foltmann et al., 1979) and at the nucleotide level (Harris et al., 1982; Moir et al., 1985; Hidaka et al., 1986). All prochymosins consist of about 365 amino acid residues and have M_r values near 40 000. An N-terminal propart is removed during the conversion into mature enzymes that have molecular weight of about 35 kDa. Calf prochymosin has a pI about 5.0 and calf chymosin has a pI about 4.6 (Foltmann, 1966).

The tertiary structure of calf chymosin has been determined by Gilliland *et al.* in 1990 and Newman *et al.* in 1991. As expected, the folding of the peptide chain follows the same pattern as found in all other aspartic proteinases. X-ray analyses of chymosin with amino acid substitutions and loop replacements have been reviewed (Albert et al., 1998). The protein has a bi-lobal folding pattern formed by N-terminal and C-terminal domains divided by a deep active site cleft. A 2.5 Å extended cleft contains the catalytic aspartates and the substrate binding pockets. These two lobes are related by an approximately 2-fold axis which passes between the two catalytic aspartate residues 32 and 215 and forms the approximate intra-molecular symmetry. There are disulphide bridges at position 45-50, 206-210 and 249-282. In addition several ion-pairs are found between Arg59-Asp57, arg157-Glu308, Arg157-Ile326, Arg307-Asp11 and Arg-315-Asp138.

The active site of aspartic proteases is highly conserved and consists of residues Asp-Thr-Gly from each domain. Between N and C domains of chymosin, there is only 9% sequence homology (Newman et al., 1991). A comparison of chymosin structure with other aspartic proteases reveals high degree of structural homology (Gilliand et al., 1990). Chymosin has the closest structure to porcine pepsin of the fungal protease structures, compared to chymosin; the rhizopuspepsin molecule has higher structural homology than penicillopepsin and endothiapepsin. A ribbon structure of bovine chymosin is given in Figure 10.

Of late due to shortage of calf rennet coupled with increased demand from cheese industry and ethical issues associated with chymosin has intensified the search for an alternative to calf rennet (Tubesha and Al-Delaimy, 2003). The replacers generally are associated with (i) poor yield, (ii) non specific proteolytic activity resulting in bitterness after storage and (iii) high thermal stability leading to non specific protein hydrolysis. Research is focused in these areas of finding a new fungal strain with desirable characteristics of low thermal stability and high milk clotting to protease ratio. Microbial substitutes for milk clotting enzymes are gaining popularity as they are easy to obtain in large quantities and are cost effective.

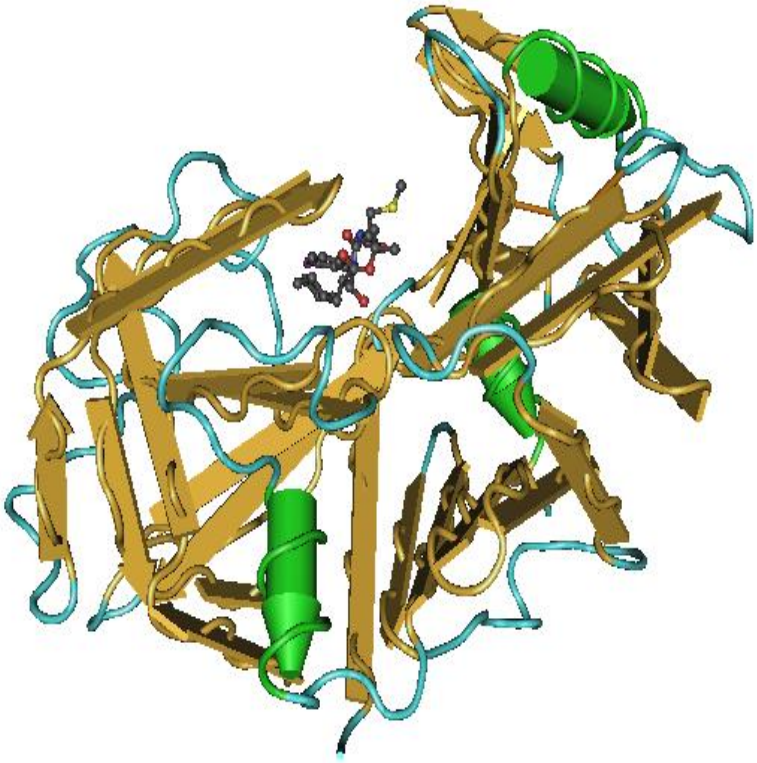


Figure 10. Tertiary structure of bovine chymosin (MMDB ID 73519)

(Gilliand et al., 1990)

Microbial rennets

Cheese industry has a great demand for acid proteases (aspartic proteases), since they assist in clotting milk apart from playing a key role in flavor and texture development (Vioque et. al., 2000). Microbial milk clotting enzymes, which constitute ~33% of total protease utilization, largely replace animal rennet for milk clotting. Fungal substitutes for animal proteases proposed include those from *Mucor pusillus* Lindt (Arima *et. al.,* 1970) *Mucor miehei* (Brown *et. al.,* 1990), *Mucor baciliformis* (Areces *et. al.,* 1992) *Penicillium oxalicum* (Hashem 2000) and *Endothia parasitica* (Sardinas 1968). Microbial rennet preparations are generally associated with proteolytic enzymes, which in turn peptonize milk curd, leading to its dissolution. Removal of non-specific proteolytic activity increases milk clotting/proteolytic activity ratio, bringing to the fore the rennetic properties of the enzyme preparation (Yada and Nakai 1986). Another striking feature that hinders the use of microbial rennets is their high thermal stability. Proteolytic activity, retained at high temperatures (higher than normal pasteurization temperature) causes undesired proteolysis, resulting in flavor loss and bitter taste (Garnot, 1985). Thus, rennets with lower thermal stability are preferred. Extensive cleavage of casein, owing to lesser specificity of microbial enzymes, results in loss of protein and yield, off-flavors and bitter peptides in cheese.

The search for an ideal rennet substitute continues with a number of other strains of fungi being screened for production of milk clotting enzymes. Microbial rennets from different sources differ in their molecular weights, isoelectric

points, specific activity, amino acid composition and glycoprotein nature (Smith and Yada 1991).

Biotechnological applications of acid proteases

The most significant property of acidic proteases is the ability to coagulate proteins, as is evidenced by their widespread application in the dairy industry for their ability to coagulate milk protein (casein) to form curds from which cheese is prepared after the removal of whey (Neelakantan et al., 1999). Acid proteases are also been reported to hydrolyze the peptides found in cheese, which are responsible for the bitter taste, and thus finds application as a debittering agent (Koka and Weimer, 2000).

In baking, these enzymes are used to degrade proteins in flour for biscuits, crackers and cookies. In brewing, they are used for extracting sufficient proteins from malt and barley and for obtaining the desired level of nitrogen nutrients. Acid proteases are also involved in lactose reduction and flavor modification in dairy applications (Sumantha et al., 2006).

Acid protease from *Aspergillus saitoi*, aspergillopepsin I is commercially marketed as Molsin F by Kikkoman Corp., Japan. The enzyme is useful for the production of seasoning materials from the foods containing various proteins, the degradation of the turbidity complex resulting from protein in fruit juices and

alcoholic liquors, and the improvement of quality of protein-rich foods (Sumantha et al., 2006).

Flavourzyme™ is a fungal complex of exopeptidases and endoproteases derived from *A. oryzae* used for extensive hydrolysis of proteins. Kojizyme™ is a similar complex, which finds application in the fermentation of soy sauce. These enzymes are also products of Novozymes, Denmark. Proteases of *Aspergillus* find application as digestive aids in gastro-intestinal disorders such as dyspepsia. Brinase, a plasmin-like acid protease, hydrolyses fibrin and fibrinogen. This is applied on patients on chronic haemodialysis with clotted arteriovenous cannulae (Biesebeke et al., 2002).

Two aspartic proteases from *Aspergillus oryzae* have been isolated and purified. The molecular characteristics of these enzymes are determined. The conformational stability measurements of acid protease have been followed by unfolding, induced by pH, denaturants and temperature. These studies have provided clues to the conformational stability of the molecule and the role of hydrophobic and electrostatic interactions in structure and activity. We have further looked at the biotechnological applications of these enzymes in food industry.

Chapter 2

Materials and Methods

MATERIALS AND METHODS

Fungal strains used in the study were procured from NCIM, NCL, Pune, Microbial Type Culture Collection, IMTECH, Chandigarh and CFTRI culture bank. These cultures were periodically sub-cultured and maintained on potato dextrose agar slants. Inoculum for fermentation was prepared by dispensing fungal spores into sterile saline solution. This spore suspension was serially diluted to get a spore density of 10^5 spores /ml. One mL of this suspension was used to inoculate 100 g dry substrate.

Hemoglobin (acid denatured) was procured from MP Biomedical Inc. (Solon, Ohio); Trichloroacetic acid was from Himedia Laboratories Pvt. Ltd (Mumbai, India). Acrylamide, bis- acrylamide, sodium dodecyl sulfate, TEMED, β- mercaptoethanol, insulin chains (A and B), Mucor rennet, calf rennet, trypsinogen and trypsin were procured from Sigma Chemical Co., USA. Molecular weight marker kit S7 (14.2 – 97.4 kDa) was procured from Bangalore Genie, India. *Aspergillus oryzae*, isolated from the *koji* of soy sauce, is deposited at the Microbial Type Culture Collection, Chandigarh, India under the accession number MTCC 5341. All other chemicals used were of analytical grade.

I **Screening for acid protease activity**

A total of twenty four fungal cultures were screened for acid protease production. Sterile bran was inoculated with the spore suspension containing 10^5 spores/ml. Flasks were incubated at 28 – 30°C and 90 – 95% RH. Extracellular protease activity was checked after extracting moldy bran with water having 0.1 M NaCl at intervals of 24 h. At the end of fermentation, the moldy bran was dried to a moisture content of less than 6% and stored at 4°C.

Substrate preparation and fermentation

Cereal brans, wheat, rice and ragi (*Eleucine coracana*), were purchased from local market of Mysore, India and were of commercial grade. Initial moisture content of the bran was estimated by an infrared moisture meter wherein the bran is dried to a constant weight at 105°C (Sartorius AG, Flawil, Switzerland). Solid state fermentation (SSF) was carried out in 500 mL Erlenmeyer flasks. A mineral solution containing 70 mg each of $CuSO_4$. $5H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ in 100 mL of 0.2 N HCl was added to the bran to obtain a pH of 5.0 (4 mL/ 30 g bran). Erlenmeyer flasks containing 30 g substrate was autoclaved at 121°C for 40 min and inoculated with the spore suspension containing 10^5 spores/ ml. The final moisture content of the bran was adjusted to 60% with mineral solution before autoclaving. The flasks were incubated at 28 – 30°C and 90 – 95% RH. The activity of the acid protease was checked at intervals of 24 h during 192 h of fermentation. The moldy bran, (for storage) with a moisture content of less than 6%,

was stored at 4°C. The bran was extracted with 0.1 M NaCl in water (ratio of bran: solvent, 1:10 (w/v), when required.

Screening of the factors affecting protease production

Effect of carbon sources

Bran from cereals such as wheat, rice and ragi (Finger millet) bran, were used as complex sources of carbon and nitrogen for the fungus to produce acid protease. The brans were autoclaved at 121°C for 40 min before adding fungal spore suspension solution. The pH of bran was 5.0 and was maintained at 30°C. The extent of enzyme production was determined at the end of fermentation.

Effect of nitrogen sources

To further increase the protein content in the solid substrate (bran), complex sources of nitrogen like defatted soy flour, defatted sesame flour, casein and peptone were added to the bran. The effect of individual source on protease production was tested at 5 different levels; 2, 4, 6, 8 and 10% (w/w).

Effect of chemical additives on acid protease production

In order to study the effects of different additives on protease production, the reference media was enriched with additions of phytic acid (0.4%), Al_2Cl_3 (0.1mM), and biotin (0.1%), before subjecting to fermentation for 120 h. At the end of the period, the extent of enzyme production was determined.

Effect of cultivation time, incubation pH and temperature on acid protease production

Wheat bran was moistened to 60%, autoclaved and inoculated with the fungal spores. The extent of enzyme production was determined at every 24 h for a total period of 192 h.

The effect of pH on acid protease production by SSF was carried out in the pH range 3, 4 and 5. Media pH was adjusted using dilute HCl. The activity of the enzyme was monitored every 48 h.

Temperature for growth and production of acid protease was optimized by incubating the flasks containing the inoculated bran at 25, 30, 35 and 40°C. The acid protease activity was monitored every 48 h.

Optimization by response surface methodology

Experimental design

Fermentation parameters were chosen for efficient acid protease production by studying one variable at a time. Accordingly, the parameters that would affect the production of acid protease were: pH, incubation temperature, protein addition and harvesting time. Response surface methodology (RSM) was used for studying effects of interaction among the selected variables. Box- Behnken design (Ferreira et al., 2007) was adopted to optimize the levels of four factors, with three centre points yielding 27 set of experiments. The factors at 3 coded levels: (-1, 0, +1) with minimum and maximum

range of values are as provided in Table 1. The average maximum acid protease activity was taken as the dependent variable (response), with duplicates.

Statistical analysis

The statistical software package Design- Expert® 7.0 (Stat Ease, Inc., Minneapolis, USA) was used for regression analysis to plot table and surface plot. ANOVA was used to estimate the statistical parameters

Production of acid protease by solid-state fermentation

Acid protease was produced extra-cellularly when *Aspergillus oryzae* was grown on wheat bran as media with 60% added moisture. Acidic pH condition was maintained by the addition of a mineral solution containing trace salts $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ (70 mg each in 100 mL of 0.2 N HCl). Defatted soy flour (4%) was added to the bran as a source of additional protein. Spore suspension (10^5 spores/ mL, 100 µL/ 100 g bran) was added as inoculum and the bran incubated at 27°C and 80% R.H. The moldy bran was harvested at the end of 120 h fermentation, dried and stored at 4°C. Extracellular proteases were extracted from the moldy bran by water containing 0.1 M sodium chloride (1: 10, bran: solvent) for 1 h without stirring at 4°C.

Scale-up studies

Tray fermentation was also carried out with 5 kg wheat bran as the substrate. Substrate was prepared as mentioned earlier and inoculated with 2.5% (w/w) moldy bran from

120 h old flask cultures. Bran was equally distributed onto aluminium trays (16″ x 33″ x 12″) and a depth of 1.5″ and incubated in koji room having an RH of 92% and at 30°C for 28 h in a *koji* room. Humidity was maintained using a Humidifier (Idealin Fogging systems, Mumbai). At the end of culture time, bran was harvested and dried at 50°C until the moisture of the moldy bran dropped to 6-8%.

Downstream processing for recovery of protease from moldy bran

Extraction efficiency with different solvents

Extracellular protease was extracted, without stirring, using distilled water, water or 0.1 M acetate buffer (pH 5.0) containing either 0.1 M sodium chloride, 0.1 M sodium sulphate, 0.1 M sodium metabisulphite (SMS) or 0.1% Triton X-100. The extracts were filtered through Whatman no.1 filter paper and the clear extracts were subjected for enzyme recovery, color and activity. The bran to solvent ratio was maintained at 1: 10.

Extraction under static and agitated conditions

The extent of enzyme recovery was studied at moldy bran to solvent ratio of 1:10 (w/w) in Erlenmeyer flasks with or without stirring at 4ºC. The bran was stirred at 200 rpm on an orbital shaker. The extract was filtered through Whatman no.1 filter paper to obtain clear extract. The enzyme activity in aliquots of the extract was determined and expressed as units/g moldy bran.

Extraction time

The optimum time of extraction from the moldy bran using water having 0.1 M NaCl was determined in the range 10-120 min. The bran to solvent ratio was 1:10 and extraction was carried out without stirring.

Repeated extractions

The enzyme was extracted from the moldy bran under optimized conditions using water having 0.1 M NaCl as solvent. The extract was removed after a time interval of 60 min and fresh solvent was added again to the same moldy bran and the efficiency of extraction as a function of the number of cycles for maximum activity recovery from the bran was studied.

Enzyme concentration

The extracted protease was concentrated by lyophilisation, ammonium sulfate precipitation, acetone precipitation and ultrafilteration. Crude extract obtained after separating it from moldy bran was subjected for freeze-drying. Fractional precipitation between 0-40%, 40-60%, 60-80% and 80 - 90% ammonium sulfate was tried. Chilled (-10°C) acetone (2 volumes) was slowly added to precipitate acid protease from the crude extract from moldy bran. After leaving overnight at 4°C, the precipitate was recovered by centrifugation, redissolved in a small volume of 50 mM phosphate buffer, pH 6.0 and dialyzed against the same buffer before assaying activity. Ultrafilteration of the crude extract was done using Amicon UF stirred cell system, using a 30,000 NMWL

PLGC membrane from Millipore, USA. Activity in concentrated enzyme solution was assayed as given above.

Assay methods

Acid protease activity was determined by following the procedure described by Tello-Solis (Tello-Solis et al., 1994). Proteolytic activity was determined using 2% hemoglobin (acid denatured) in Glycine-HCl (pH 3.2, 0.1 M) as substrate. 1 ml of substrate was incubated with 400 μL of appropriately diluted enzyme solution for 10 min at 55°C. At the end of 10 min, 2ml of 5% TCA was added and the tubes are incubated for 20 min to allow unhydrolysed protein to settle down. Unhydrolysed protein was removed by filtration (Whatman No.1). Absorbance of the supernatant was measured at 280 nm. 1 unit is defined as the amount of enzyme that produces an increase in absorbance of 0.001 per min under the above conditions. Protein concentration was determined by the Lowry's method (Lowry et al., 1951) using bovine serum albumin as standard.

Purification of acid protease

The crude enzyme extract, obtained after extracting moldy bran with water containing 0.1 M NaCl, was precipitated with chilled acetone (1: 2, extract: acetone). Two volumes of chilled acetone were slowly added to extract and the precipitate was allowed to settle for 4 h. The precipitated protein was separated by centrifuging at 6000 rpm for 20 min. The pellet was dissolved in minimal volumes of 50 mM phosphate buffer, pH 6.0 and dialyzed against same buffer to remove trace amounts of acetone. The dialysate was applied onto a DEAE-Cellulose ion exchange column (2.5 x 50 cm, 50 mL). Bound

protein was eluted with a salt gradient of 0 – 0.5 M NaCl. The active fractions were concentrated by ultrafilteration (nominal weight cut-off membrane 10, 000 Da, PLGC low protein binding membrane, Millipore Inc., USA) followed by molecular sieve chromatography using G-75 superfine (1 x 100 cm, 100 mL) using 50 mM phosphate buffer (pH 6.0). The active fractions were pooled and the homogeneity was confirmed by SDS-PAGE and HPLC.

Homogeneity of acid protease

Gel electrophoresis

Native PAGE was performed using 12.5% (w/v) acrylamide in gels in absence of sodium dodecyl sulphate and β-mercaptoethanol.

For activity staining, electrophoresis was performed on 12.5% gels in native condition (without SDS and β-mercaptoethanol) containing 1% copolymarised gelatin in separating gel as described previously (Heussen and Dowdle, 1980). After the run, the gel was incubated in glycine HCl buffer (3.2 pH) at 50°C for 30 min. Proteolytic activity *in situ* were visualized as transparent bands against a blue background when stained with 0.1% amido black.

SDS-PAGE was performed using 12.5% (w/v) acrylamide in gels as described previously (Laemmli, 1970). Protein bands were visualized by silver staining. The molecular weight marker standards used were, Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), Ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa).

Size exclusion chromatography on HPLC

HPLC was carried out using TSK G 2000 SW gel column (7.8 mm x 300 mm, 5 μm) at a flow rate of 0.2 ml/min. The column was pre equilibrated with 50 mM phosphate buffer, pH 6, with 0.3 M NaCl. Elution was carried out with same buffer. Purified enzyme was injected and detection was monitored at 280 nm. Molecular weight markers used are alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa)

Characterization of protease from *A. oryzae*

pH optimum and stability and temperature optimum for activity

The pH optimum for activity of protease was determined by incubation in buffer in the pH range 2 – 10. Buffers used were 0.1 M Glycine-HCl (2 – 5 pH), 0.1 M acetate (5 – 7 pH) and 0.1 M Glycine- NaOH (7 – 10 pH). The activity was assayed at 55°C. Maximum activity obtained within the range was expressed as 100% and residual activities calculated.

pH stability of protease was carried out by incubating the enzyme for 24 h in buffer in the pH range 2 – 10 (same buffers as above) at 27°C, followed by activity assay at 55°C. Maximum activity obtained within the range was expressed as 100% and residual activities calculated.

The optimum temperature for activity was determined by assaying protease activity in the range 35 – 70°C. 2% heat denatured hemoglobin in 0.1 M glycine – HCl pH 3.2 was

used as substrate. The maximum activity obtained within the range was taken at 100% and the residual activities calculated.

Determination of K_m and V_{max}

The K_m and V_{max} values for the purified acid protease were determined with acid denatured hemoglobin as the substrate in Glycine-HCl buffer, 0.1 M, pH 3.2. The enzyme was incubated with a range of 0-2 % concentration of the substrate for a period of 10 min at 55°C. A double reciprocal plot was used for estimating K_m and V_{max}.

Spectral characterization of acid protease

UV absorption

The absorption spectrum of acid protease was measured using a Shimadzu UV1601 spectrophotometer. The tryptophan residues in the protein were estimated by earlier reported NBS method (Spande and Witkop, 1967).

Fluorescence measurements

Fluorescence measurements were made using a Shimadzu RF-5000 automatic recording spectrofluorimeter in conjunction with a constant temperature circulating water bath. The temperature of the cell was maintained at 27°C. Protein solution having absorption of 0.075 at 280 nm in 50 mM phosphate buffer, pH 6.0, was used. Excitation spectrum was recorded in the range 200 – 300 nm fixing the emission wavelength at 335 nm. The excitation wavelength was fixed at 278 nm (excitation maximum for the protein) and

emission spectrum recorded in the region 300-400 nm. The spectra were recorded with 5 nm bandwidth for both excitation and emission monochromators.

Circular dichroism (CD) studies

CD measurements were made using Jasco J-810 automatic spectropolarimeter fitted with a xenon lamp. The instrument was calibrated with d (+)-10-camphor-sulphonic acid ammonium salt. The lamp was purged continuously with nitrogen before and during the experiments. The scans were recorded thrice. The far UV CD spectrum was recorded between 190 – 260 nm using a 1 mm path length cell and a protein concentration of 0.22 mg/ ml in 50 mM phosphate buffer, pH 4.5. The near UV CD spectrum was recorded in the range 260 – 320 nm using a 1 cm path length cell and a protein concentration of 1 mg/ ml in 50 mM phosphate buffer, pH 4.5. Secondary structure analysis was carried out using the CDSSTR method (Sreerama, and Woody, 2000; Compton, and Johnson, 1986) with reference database SP175 (Lees et al., 2006) available in DICHROWEB (Lobley et al., 2002)

Thermal stability

The acid protease was incubated in 0.1 M acetate buffer, pH 5, in the temperature range 30°C -65°C for 15 min. The samples were immediately cooled in ice and residual activity was assayed. The un-incubated enzyme activity was taken as 100%.

Inhibition studies

The ability of class specific inhibitors to inhibit protease activity was studied in the range 0–100 μM. The inhibitors used include EDTA (metalloproteases), Iodoacetamide (cystine proteases), PMSF and Antipain hydrochloride (serine proteases) and pepstatin (aspartate protease). After incubating enzyme with the inhibitor for 30 min at 27°C, residual activity was assayed under optimal conditions for activity. The control enzyme activity, without the inhibitor was taken as 100%.

Trypsinogen activation

Trypsinogen activation was studied as reported earlier (Sodek and Hoffmann, 1982). Commercial trypsinogen was prepared at 1 mg/ml concentration in 0.1 M Glycine –HCl buffer, pH 3.2. To this, different aliquots of acid protease were added and incubated at 55ºC for different time intervals. After end of each incubation time, activation is examined by trypsin activity on casein. 20 μl of this was also analyzed for trypsin formation through size exclusion chromatography on HPLC.

Cleavage specificity

Cleavage specificity of acid protease is determined by analysis of peptides in the hydrolyzed insulin chains (A and B). The peptide chains of oxidized insulin (1mg/mL) in 0.1 M glycine-HCl 3.2 pH were subjected to hydrolysis by acid protease at 55ºC for 5 min. At the end of the incubation, the reaction was stopped by 50 μL of 0.01% trifluoroacetic acid (TFA). Peptide fractions were separated by Waters®HPLC system

fitted with a C_{18} column (250 x 4.6 mm, 5μM). A nine step gradient system as described by Stentz (Stentz et al., 1989) having 0.1% TFA/ H_2O, and acetonitrile (ACN) was employed. The gradient system used was: step 1: 10% ACN, 90% 0.1% TFA/H_2O isocratic for 10 min. step 2: 10-min gradient to 20% ACN, 80% 0.1%trifluoroacetic acid/H_2O; step 3: 10-min isocratic at 20% ACN, 80% 0.1% TFA/H_2O; step 4: 20 min gradient to 25% ACN, 75% 0.1% TFA/H_2O; step 5: 10 min isocratic at 25% ACN, 75% 0.1% TFA/H_2O; step 6: 10 min gradient at 28% ACN, 72% 0.1% TFA/H_2O; step 7: 20 min isocratic at 28% ACN, 72% 0.1% TFA/H_2O; step 8: 30 min gradient at 50% ACN, 50% 0.1% TFA/H_2O; step 9: 5 min gradient to initial conditions of 10% ACN, 90% 0.1% TFA/H_2O. Individual peptide peaks were collected for analysis of their molecular weight using mass spectrometry.

Mass spectrometry

The peptides collected after HPLC, are passed into the electro spray ionization source in the negative ion mode of Waters® HPLC mass spectrometer (Model Q-Tof ultima) for their molecular mass analysis. The cleavage points were deduced by comparing the mass of each peptide to their sequence.

Conformational stability studies of acid protease from *A. oryzae.*

Effect of pH and temperature on structure-stability of acid protease

Effect of pH on activity-stability of acid protease from *A. oryzae*

Effect of pH on activity and structure was studied by using buffers in the range of 2-10. Buffers used were Glycine- HCl (pH 2 – 5), acetate (pH 5 – 7) and Glycine- NaOH (pH 7 – 10). Concentrations of all the buffers were 20 mM. A stock solution of the protein was added to the appropriate buffer and the mixture was incubated for 24 h at 25 °C. The final pH and the concentration of the protein in each sample were determined again.

Absorbance spectroscopy

Absorbance measurements were carried out on a Shimadzu 1600 double beam spectrophotometer that was equipped with a constant temperature cell holder. Protein concentrations were between 0.5-1mg/ml for all the experiments. Absorbance spectra were recorded between 200-400 nm.

Fluorescence spectroscopy

Fluorescence measurements were carried out using a Cary Eclipse Varian spectrofluorometer attached with a constant temperature circulating water bath (Huber, Germany). Excitation and emission slit widths were set at 5 nm and 5 nm, respectively, using a 10 mm path length cuvette. Protein concentration used was less than 0.1 O.D at 280 nm to minimize inner filter effects. For a combined tryptophan and tyrosine fluorescence, excitation wavelength of 280 nm was used. The emission was recorded

between 300 to 400 nm. For ANS fluorescence, the excitation was at 380 nm. The emission spectra were collected between 400 and 600 nm with the same slit widths for excitation and emission as above. For measuring temperature dependant changes in the protein, fluorescence emission intensities were measured between 20 and 70 °C with a scan rate of 1°C min^{-1}.

ANS binding assay

Exposure of hydrophobic surfaces in the enzyme was measured by its ability to bind the fluorescent dye ANS. A stock solution of ANS was prepared in methanol and the dye concentration was determined using an excitation coefficient of ε = 5000 M^{-1} cm^{-1} at 350 nm. The protein was incubated with a 100- fold molar excess of ANS for more than 1 h at room temperature in dark and its fluorescence was measured.

Quenching studies by acrylamide

Fluorescence quenching was analyzed according to Stern-Volmer relationship ($F_0/F = 1 + K_{sv}$ [Q]), Where F0 is the fluorescence in the absence of the quencher, F is the fluorescence at molar quencher (acrylamide) concentration [Q], and K_{sv} is the Stern-Volmer constant obtained from the slope of a plot of F0/F versus [Q]. The excitation wavelength was 280 nm and the acrylamide concentration ranged from 0 to 0.22 M.

Circular dichroism measurements (CD)

CD spectra were recorded on Jasco J-810 spectropolarimeter equipped with a constant temperature cell holder. The instrument was calibrated with (+)d-10- camphor sulfonic acid, ammonium salt. All the enzyme solutions were prepared in phosphate buffer (0.02 M, pH 6.0). Conformational changes in the secondary structure of the protein were monitored in the region between 200-260 nm with a protein concentration of using protein concentrations of 0.25 mg/ml in a cuvette of 1 mm path length. Molar ellipticity values were expressed as mean residue ellipticity using a residue weight of 110 g mol^{-1}. The scan speed was 10 nm/min and an average of 3 runs was recorded. The secondary structure of acid protease was analyzed using the computer program of Yang et al. (1986).

Thermal stability

The enzyme was incubated at different temperatures in the range 30-85°C for 15 minutes. Enzyme aliquots were immediately cooled in an ice bath and assayed for activity as described under enzyme assay. T_m, the midpoint of thermal inactivation, was calculated as the temperature at which 50% loss of activity occurred under the test conditions. Inactivation kinetics was carried out by incubating the enzyme at the test temperature. Aliquots of the enzyme were drawn at different time intervals, cooled to 4°C and residual activity assayed. Activation energy (E_a), enthalpy (ΔH^*), entropy (ΔS^*) and free energy change (ΔG^*) were obtained from the Arrhenius plot.

Temperature-scanning spectroscopy

Absorbance measurements were carried out using spectrophotometer from Amersham Biosciences model-ultrospec 4300 Pro, fitted with a temperature programmer to control the speed of temperature change in thermal melting experiments. The concentration of the enzyme was ~3 μM. Thermal unfolding profiles at 280 nm were recorded in the temperature range 25 - 85°C.

Differential Scanning Calorimetry

DSC scans for acid protease (1 mg/ml) (20 mM phosphate buffer, pH-6.0) were performed using the VP DSC ultrasensitive microcalorimeter (Microcal Inc., Northampton, MA) with cell volumes 0.5 ml. The protein concentrations were monitored spectrophotometrically at 275 nm. Heat capacity vs. temperature profiles were obtained for scanning rates 1°C /min in the temperature range 20–90°C at constant pressure of about 1.8 atm over the liquids in the cells. The calorimetric data were corrected for the instrumental baseline water–water. A linear baseline was used to obtain the excess apparent molar heat capacity (Cp^{ex}) related to the denaturation process. DSC curves were analyzed with MicroCal Origin Software.

Turbidity measurements

Turbidity measurements at the specified wavelengths were made using Shimadzu 1601 double beam spectrophotometer. Temperatures were controlled by peltier attachment to the system which maintained at ±0.1°C. Path length of the sample cell used was 10

nm. To determine effect of anti aggregation agents, enzyme was incubated with appropriate concentration of the agent and preheated to 60 ^{o}C. The absorbance values were monitored as a function of time up to 20 min.

Thermal unfolding

Temperature-induced denaturation of the enzyme, under given conditions were performed as a function of increasing temperature. Protein samples were incubated at the desired temperature for 15 min before taking the measurements. The actual temperature of the sample in the cuvette was obtained with a thermocouple using a digital thermometer. Occasionally, samples were also checked for aggregations due to heat by light scattering measurements.

Effect of GuHCl and Urea on structure-stability of acid protease

Phosphate buffer was prepared in double distilled water (0.05 M, pH 6.0). The other buffers used for acid protease stability studies were: glycine – HCl for pH 3.0 and 4.0; sodium acetate for pH 4.8; phosphate buffer pH 6.0; Tris-HCl for pH 7.0 and 8.0; borate buffer for pH 9.0; glycine – NaOH for pH 10.0, 11.0 and 12.0. Molarities of the buffers prepared were 0.05 M.

GuHCl and urea stock solutions were prepared fresh (in buffer). pH of the stock solutions at high denaturant concentrations was corrected by the addition of HCl or NaOH. The denaturant concentrations of stock solutions were determined at 27°C by

refractive index measurements using Eq. 1 and 2 for GuHCl and urea respectively (Pace and Scholtz, 1987).

$$\text{GuHCl molarity} = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3 \quad (1)$$

$$\text{Urea molarity} = 117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3 \quad (2),$$

where ΔN is the difference in the refractive index between the denaturant solution and water (or buffer) at the sodium D line.

Acid protease (10 times the requisite final protein concentration), was prepared in buffer. No change in pH was observed on dilution. Buffer, denaturant stock and 10-20 μl of protein stock solution were added to yield the desired final protein concentrations with the GuHCl and urea concentrations in the range 0.1-6 M and 1-9 M, respectively. Samples containing GuHCl and urea were vortexed and incubated at 27°C for 4 h and 12 h, respectively, to equilibrate. Longer incubation times produced identical signals. Corresponding blanks for all samples were treated in the same manner as described above, except that buffer replaced the enzyme.

Fluorescence spectra

Fluorescence measurements were carried out using a Shimadzu RF-5000 Spectrofluoremeter attached with a constant temperature circulating water bath (Huber, Germany). Excitation and emission slit widths were set at 5 nm and 5 nm, respectively,

using a 10 mm path length cuvette containing buffer. Protein concentration used was less than 0.1 O.D at 280 nm to minimize inner filter effects.

Circular dichroism measurements (CD)

CD spectra were recorded on Jasco J-810 spectropolarimeter, calibrated with d-(10) camphor sulfonic acid, ammonium salt using protein concentrations of 0.094 mg/ml and 0.83 mg/ml, for far-UV and near-UV regions, respectively. All the enzyme solutions were prepared in phosphate buffer (0.05M, pH 4.8). Molar ellipticity values were expressed as mean residue ellipticity using a residue weight of 110 g mol^{-1}. Far – UV CD was recorded in the range 200-260 nm using a 1 mm path length cell while near-UV CD (230 – 320 nm) was recorded using a 10 mm cell. The scan speed was 10 nm/min using a bandwidth of 1 nm. An average of 3 runs was recorded. The secondary structure of acid protease was analyzed using the computer program of Yang et al. (Yang et al., 1986).

Molecular biology experiments

All the techniques of chromosomal DNA isolation, PCR standardization and gel electrophoresis and sequencing are done according to manufacture's instruction.

Agarose gels

Gels for detection of DNA were run at 1.5% and detected under UV light after staining with ethidium bromide. Gels were run in Tris-acetic acid –EDTA Buffer (TAE buffer, 10

mM). 1 kb ladder (λ phage) (Genei, bangalore) was used as the standard at 1 µg/ml concentration.

Preparation of genomic DNA

Genomic DNA was isolated from the fungal mycelia grown on potato dextrose broth for two days. The mycelia were ground in liquid nitrogen and the supernatant was dissolved in TES buffer (100mM Tris, pH 8.0, 10mM EDTA, 2% SDS). After incubating the mixture at 65 ◦C for 10 min, the DNA was extracted with phenol, precipitated with isopropanol and washed with 70% cold ethanol. The DNA was then dissolved in TE buffer. The final yield of DNA obtained was approximately 0.5 µg/µl.

Primer designing

Oligonucleotide primers were designed for conserved regions of *pep A* gene available in NCBI website using PRIMER 3 software. The sequence of the primers is,

Forward- TCCTGGCCCCTGACCCATCG

Reverse- GCCGCGGTCCTGCTTGACTC

Nested- AGCACTGCTGTCCTGGGGGA

The primers were obtained from Sigma-Aldrich, Bangalore.

PCR conditions

The amplifications were performed using a MyCycler, Thermocycler (BIORAD, USA) in a total volume of 50 µl of reaction mixtures containing 0.2 mM of each deoxynucleotide triphosphate, 25 pmol of each primer, 5µl of 10× buffer with $MgSO_4$ (final concentration of $MgSO_4$ was 2.5 mM), 1.25 U of DNA polymerase, and 1µl of diluted genomic DNA (about 50 ng). The initial denaturation step at 95°C for 2 min was followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 40 s, and extension at 72°C for 4 min. The final cycle was followed by additional 10 min elongation at 72°C. The amplified products were visualized by gel electrophoresis at $5Vcm^{-1}$ in a 1.0% agarose gel (containing 1µg/ml ethidium bromide) in 1× TBE (Tris–Borate–EDTA) electrophoresis buffer (10.8 g/l Tris base, 5.5 g/l boric acid, 0.9 g/l sodium EDTA; pH 8) and photographed under UV light.

Nucleotide sequencing and sequence analysis

The nucleotide sequence of the amplified PCR product was determined by VIMTA labs (Hyderabad, India). The basis local alignment tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for database searches. The comparison of acid proteases from *Aspergillus oryzae* with homologous proteins was carried out using the programs Clustal W (version 1.81).

Milk clotting enzyme from *Aspergillus oryzae* MTCC 5341

Substrate preparation for milk clotting enzyme production by SSF

Wheat bran (13% protein content, 11% moisture) was purchased from local market (Mysore, India.). Solid state fermentation (SSF) was carried out in 500 mL Erlenmeyer flasks. Defatted soy flour (4%) and skim milk powder (2%) were mixed with bran. A mineral solution containing 70 mg each of copper sulfate, zinc sulfate and ferrous sulfate in 100 mL of double distilled water was added to the bran and pH of the bran was adjusted with dilute NaOH to obtain a value of 6.3, the optimum for production of the enzyme. Erlenmeyer flasks containing 30 g substrate was autoclaved at 121°C for 40 min and inoculated with the viable spore suspension containing 10^5 spores/ mL. The final moisture content of the bran was adjusted to 60% with mineral solution before autoclaving. The flasks were incubated at 28 – 30°C and 90 – 95% RH in a BOD incubator. The milk clotting activity was checked at intervals of 24 h during 192 h of fermentation. The moldy bran, with a moisture content of less than 6%, was stored at 4°C. The bran was extracted with 0.1 M NaCl in water (ratio of bran: solvent, 1:10 (w/v)), when required.

Scale-up studies

Tray fermentation was also carried out with 5 kg wheat bran as the substrate. Substrate was prepared as mentioned earlier and inoculated with 2.5% (w/w) moldy bran from 120 h old flask cultures. Bran was equally distributed onto aluminium trays (16″ x 33″ x 12″) and a depth of 1.5″ and incubated in koji room having an RH of 92% and at 30°C for

28 h in a *koji* room. Humidity was maintained using a Humidifier (Idealin Fogging systems, Mumbai). At the end of culture time, bran was harvested and dried at 50°C until the moisture of the moldy bran dropped to 6-8%.

Screening for Milk clotting activity

Twelve fungal strains from IMTECH, Chandigarh and four from CFTRI culture bank, were screened for production of milk clotting protease. The strains included in the study were, *Aspergillus awamori* (MTCC 886), *Chaetomium virescens* (MTCC 980), *Dictyostelium discoideum* (MTCC 1579), *Lagenidium giganteum* (MTCC 719), *Metarhizium anisopliae* (MTCC 892), *Penicillium roquefortii* (MTCC 933), *Rhizopus microsporus* var *oligosporus* (MTCC 556), *Trichoderma koningii* (MTCC 796), *Aspergillus niger* (MTCC 2479), *Aspergillus ochraceus* (MTCC 1810), *Mucor fuscus* (MTCC 1462), *Mucor rouxii* (MTCC 386), *Aspergillus oryzae* (CFR 232 or MTCC 5341), *Aspergillus carbonarius (CFR 1047), Aspergillus oryzae (flavus), and Trichoderma spp*. All the strains were grown on wheat bran with 60% added moisture by inoculating a spore suspension having 10^5 spores/ mL (1mL/ 100 g bran). Production of the enzyme was monitored by checking the activity every 24 h during the growth.

Optimization of growth conditions and media composition for production of milk clotting activity from *A. oryzae* MTCC 5341

The effect of incubation pH on milk clotting enzyme production by SSF was studied in the pH range 5.0 to 6.8. Media pH was adjusted using either dilute HCl or dilute NaOH before autoclaving the bran. The activity of the enzyme was monitored at 24 h intervals up to 168 h.

Temperature was optimized, for growth and production of milk clotting enzyme, by incubating the flasks containing the inoculated bran at 25, 27, 30, 33 and 35°C. The milk clotting enzyme activity was monitored every 24 h up to 168 h

Enzyme production at optimum pH and temperature was followed as a function of time from 24 h to 168 h. At the end of each time, extent of enzyme production is determined as described in the following section.

To optimize media conditions for production of milk clotting enzymes from *Aspergillus oryzae* (MTCC 5341), the strain was grown in different culture conditions - wheat bran (WB) alone, WB+ 4% defatted soy flour (DSF), WB+ 4% DSF+ 2% skim milk powder (SMP), WB+ 4% DSF+ 2% skim milk powder (SMP)+0.1% biotin, WB+ 4% DSF+ 2% skim milk powder (SMP)+0.4% phytic acid, WB+ 4% DSF+ 2% skim milk powder (SMP)+ 0.1mM Al_2Cl_3.

Extraction

Moldy bran (6% moisture) was dispersed in water containing 0.1 M NaCl (1:10 ratio of bran: solvent) for 60 min at 4ºC to minimize autolysis. Extract was filtered using Whatman No.1 filter paper and enzyme activity in filtered aliquots was determined and expressed as units/ g moldy bran.

Decolorization

Enzyme extract was mixed with 2.5% (w/v) activated charcoal (in cold) and stirred at 250 rpm for 60 min. Charcoal was removed by centrifuging at 10,000 rpm for 30 min. Activity of the decolorized enzyme and protein concentration of the extract was determined as described in the following section.

Assay methods

Milk clotting activity is determined by following the procedure described by Arima et. al., (1967) 10 % skimmed milk solution is prepared by adding 10 g skim milk powder to 100 mL distilled water containing 0.15% calcium chloride (pH 6.3). 5 ml of this solution is preheated at 55°C for 5 min before an aliquot of appropriately diluted enzyme is added. The time taken for the first appearance of clotting is noted. 400 milk-clotting units is the amount of enzyme that clots the above milk solution in 1 minute.

Proteolytic activity is measured by a modification of Anson (1938) method. To 2.5 mL of 1% casein solution prepared in 20 mM potassium phosphate buffer suitably diluted enzyme solution was added and mixture incubated at 55ºC for 10 min. After incubation,

2.5 mL of 0.44 M trichloroacetic acid solution was added. The mixture was filtered using Whatman no.1 filter paper. To one mL of this filtrate, 2.5 mL of 0.55 M sodium carbonate and 1 mL of three times diluted Folin reagent is added. This solution is kept at 30°C for 30 min and absorbance values are recorded at 660 nm. Proteolytic activity is defined as unit increase in OD at 660 nm. Protein concentration was determined by the Lowry's method (Lowry et al., 1951) using bovine serum albumin as standard.

Lipase activity is determined based on titrimetric method using triacetin as substrate. In brief, a 5% (w/v) solution of triacetin containing 0.1% triton X-100 was prepared in 50 mM sodium phosphate buffer at pH 7.4. The reaction mixture containing 4 mL substrate, 2 mL diluted enzyme (1-2 mg/mL) and 10 µL of 0.1M $CaCl_2$ was incubated at 37°C for 60min. enzyme activity was stopped by addition of 4 mL distilled alcohol. Liberated acid was titrated against 0.05 N NaOH to an endpoint of pH 9.5. One unit of enzyme activity was expressed as micro equivalents of alkali consumed per milligram of protein per hour of incubation.

In order to compare the efficiency of the milk clotting enzyme with standard rennets, milk clotting activity assay is done as described (Harboe et al., 2006). In short, 0.05% $CaCl_2$ is added to reconstituted skimmed milk solution (11%) which is maintained at 32°C in a thermostatic water bath. To 9.7 ml of this milk solution is added 200µl of suitably diluted enzyme solution to give a clotting time in the range 380 to 400 seconds. The clotting times are compared with the standard checks (calf rennet and mucor

rennet) and the milk clotting activities are expressed as % values in comparison to checks.

Purification and molecular characterization of rennet from *A. oryzae*

Entrapment of rennet: Purification

For entrapment procedures, moldy bran was extracted with solvent. Entrapment of rennet was carried out as described previously by Gupta et al. (Gupta et al., 1993). In short, alginate (2.5%, w/v) was dissolved in water. One ml of this alginate solution was mixed with 0.5 ml of protease solution. The above solution was incubated at 25 °C for 30 min. The solution, dropped through a pipette into 0.02 M $CaCl_2$ solution (in water) with gentle stirring, was incubated for 2 h at 25 °C to generate entrapped enzyme in calcium alginate beads. For all the subsequent experiments these beads were considered to be entrapped enzyme. . Pure milk - clotting enzyme was easily recoverable by the addition of 0.5 M NaCl containing 0.2 M $CaCl_2$.

Reusability of the entrapped milk clotting enzyme

To test the reusability, rennet entrapped alginate beads were used to assay enzyme activity. After incubation, the beads were removed from the reaction mixture and reused after washing thrice with double distilled water. Activity was determined in the same manner as described for enzyme assay. The decrease in activity for each cycle was determined assuming activity of beads in the first cycle to be 100%.

Molecular characterization of milk clotting enzyme from *A. oryzae*

Enzyme characteristics

pH optimum and pH stability

The pH optima, of both entrapped and free enzymes were tested by assaying activity in the range 5.5-8.5, by adjusting substrate pH either with 0.1 N HCl or 0.1 N NaOH.

pH stability of milk clotting enzyme was studied by incubating the enzyme for 24 h in buffer in the pH range 3 – 11. Buffers used were 0.1 M glycine-HCl (3 – 4), 0.1 M acetate (4.5 – 5.5) phosphate (6-8) and 0.1 M glycine- NaOH (8 – 11) at 27°C, followed by activity assay as described above. Maximum activity obtained was taken to be 100% and residual activities calculated.

Optimum temperature and thermal stability

Temperature optima, of free and entrapped enzymes, were determined by assaying activity in the temperature range of 35 – 60°C. Skim milk powder (10 %) having 0.15% $CaCl_2$, pH 6.3, was used as substrate. The temperature at which maximum activity was observed was taken to be 100% and relative activities (at other test temperatures) calculated.

The milk clotting enzyme was incubated in 20 mM phosphate buffer, pH 6.0, in the temperature range 30 -65°C, for 15 min. The samples were immediately cooled in ice before assaying residual activity. The un-incubated enzyme activity was taken to be 100%.

Effect of calcium on milk clotting activity

To study the effect of calcium on clotting efficiency, assay was carried out with different concentrations of calcium (0 – 0.25%) in the reaction mixture. Time taken for appearance of first clot was noted down and compared with control.

Inhibition studies

The ability of class specific inhibitors to inhibit protease activity was studied in the range 0–4 mM. The inhibitors used include EDTA (metalloproteases), Iodoacetamide (cystine proteases), PMSF and benzamidine (serine proteases) and pepstatin (aspartate protease). After incubating enzyme with the inhibitor for 30 min at 27°C, residual activity was assayed under optimal conditions for activity. The control enzyme activity, without the inhibitor was taken as 100%.

Analysis of Casein Hydrolysates (CN)

Solutions (0.5% wt/vol) of CN components were prepared by diluting the corresponding substrates (1% wt/vol) with the Na phosphate buffer (100 mmol/L, pH 6.5). The purified enzyme (0.1 mL) was then added to 5 mL of CN, and the mixtures were held at 37°C. Samples (0.5 mL) were taken at 5, 15, 30, 60, 120, 180, and 240 min and 12 h, respectively, and the reaction was also stopped by being mixed with the sample buffer described in Analysis of Substrate Specificity. The degradation of purified enzyme to different CN components was analyzed by using the modified Laemmli

polypeptide fractioning gel system (Ausubel et al., 1995), which was performed under reducing conditions with 20% acrylamide gels (Sigma), and the gels were stained with Coomassie Brilliant Blue G-250, followed by destaining with 20% methanol

Biotechnological application of acid protease: Bakery products

Effect of fungal protease on the quality characteristics of bread and crackers
Ingredients

Wheat flour, compressed yeast, salt, sugar, hydrogenated fat were procured from the local market. The characteristics of wheat flour such as moisture, ash, dry gluten, Zeleny's sedimentation value, falling number, damaged starch, farinograph, extensograph and amylograph characteristics were determined using AACC methods, 2000.

Enzymes

Acid protease from *Aspergillus oryzae* MTCC 5341 and commercial protease *Aspergillus oryzae* (Biocon, Bangalore) was used for the studies.

Rheological Characteristics

Effect of 0.01, 0.02 and 0.03% AP and 0.02% CAP on the farinograph, extensograph and amylograph characteristics of wheat flour was studied according to standard AACC methods (2000). The levels of enzymes used for the experiments were based on preliminary laboratory trials.

Bread making characteristics

Breads were prepared using the following formulation: flour 100 g; AP: 0.01, 0.02, 0.03 g; CAP: 0.02 g; compressed yeast: 2.0 g; salt: 1.0 g; sugar: 2.5 g; hydrogenated fat: 1.0 g and water: farinograph water absorption. Breads were prepared by mixing the ingredients in a Hobart mixer (Model N-50) with a flat blade for 3 min at 61 rpm. The dough was fermented in a chamber maintained at 30° C and 75 % RH for 90 min, remixed, rounded, and again fermented for 25 min, moulded, proofed for 55 min at 30° C, 85% RH and baked for 25 min at 220° C, cooled and packed.

Bread weight was recorded and bread volume was determined using rapeseed displacement method. Breads were evaluated for crust color, shape, crumb color, grain, mouthfeel and taste. Crumb firmness was measured using texture analyzer (Model Tahdi, Stable Microsystems, UK) under the following conditions: sample thickness - 25 mm, load cell – 10 Kg, plunger diameter – 36 mm and plunger speed – 100 mm per minute. Crumb firmness which is a force at 25% compression was measured.

Effect of fungal protease on the quality characteristics of crackers

The effect of 1, 2 & 3% fungal protease on the dough rheological properties and quality of crackers were studied.

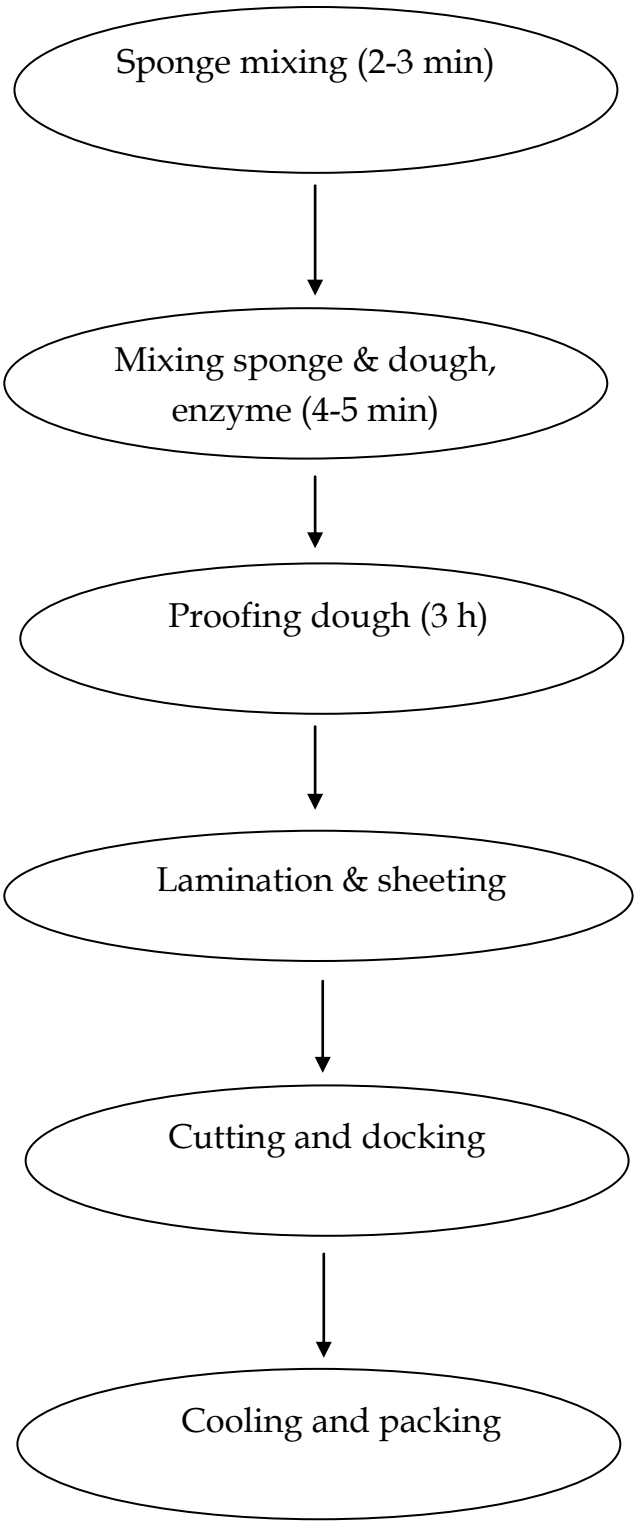
Chemical characteristics of wheat flour such as total ash, dry gluten, SDS sedimentation value, total protein and damaged starch were determined in the wheat flour according to the standard methods (AACC, 2000).

Rheological characteristics of wheat flour dough – Brabender Farinograph (Model No. 810108004, Duisburg, Germany) and Brabender Extensograph (Model No. 996035, Duisburg, Germany) were used for studying the rheological characteristics of wheat flour dough. The tests were carried out as per AACC (2000) methods.

Formulation for preparation of crackers – the following formulation was used

Ingredients	**Sponge (%)**	**Dough (%)**
Flour	30.0	70.0
Yeast	0.5	---
Water	30.0	Variable
Salt	---	1.0
Fat	---	20.0
Sodium bicarbonate	---	0.2
Enzyme protease	---	1, 2 & 3%
Cracker dust formulation:		
Ingredients		**%**
Flour		100
Fat		33.0
Salt		1.0

Flow diagram for preparation of crackers



Measurement of texture

Breaking strength of the crackers was measured by following Triple Beam Snap Technique of Gaines (1991) using TA-HDI texture analyzer (Stable Micro Systems, Surrey, UK). The samples were rested on two supporting beams spaced at a distance of 2.5 cm. Another beam connected to texture analyzer was brought down to break crackers at a cross head speed of 10 mm / min and load cell of 10 kg. Care was taken to see that point of contact was equidistant from both supporting beams. The peak force (in g) at break representing breaking strength was recorded and average value was calculated.

Sensory characteristics of crackers

The crackers were evaluated for sensory characteristics by an eight member trained panel for a total of 100 marks. Different parameters were evaluated with lower scores assigned for crust color (5 marks) and higher scores for crumb layers and texture (20 marks each). Surface character was assigned 10 marks. Flavor, taste, mouth feel and crumb color were assigned 10 marks each.

Statistical Analysis

Statistical analysis of the data was done by using Duncan's New Multiple Range Test as described by Steel and Torrie (1960).

Cheese making trials with milk clotting enzyme from *A. oryzae* MTCC 5341

Milk clotting enzyme from *Aspergillus oryzae* was used to coagulate the milk and to make cheese from bovine milk. Standard procedure was followed to manufacture Cheddar cheese using *Aspergillus* rennet and the results are compared with standard rennet from *Mucor* (Meso™).

Chapter 3

Results and Discussion

Section 1: Isolation, purification and molecular characterization of aspartic proteases from *Aspergillus oryzae* MTCC 5341

Aspergillus oryzae produces a highly active acid protease and milk clotting enzyme under different conditions. This chapter deals/ reports the screening, optimization of the conditions for growth and production of the enzymes, purification and characterization of these enzymes.

1A Screening, Isolation, purification and molecular characterization of acid proteases from *Aspergillus oryzae*

Screening of fungal strains for acid protease activity

Twenty four fungal strains were screened for production of acid protease activity. All the fungal strains were grown on wheat bran by solid state fermentation. The results of the screening experiment are given in Table 9. *Aspergillus oryzae* MTCC 5341 produced the highest extracellular acid protease activity (4.13×10^5 U/g bran) by solid state fermentation under the selected conditions; this strain was selected for further studies.

Optimization of conditions for acid protease production were carried out for the critical parameters including selection of cereal bran, time of fermentation, incubation temperature, initial pH of the media and supplementation of the protein sources in the media.

Table 9. Screening for acid protease activity produced by fungal strains

Strain	Culture no.	Activity units ($\times 10^5$ U/g bran)
Aspergillus oryzae	**MTCC 5341**	**4.13**
Aspergillus carbonarius	CFR 1047	0.86
Aspergillus flaviceps	NCIM 1209	1.38
Aspergillus awamori	MTCC 886	3.31
Aspergillus niger	MTCC 2479	3.43
Aspergillus ochraceus	MTCC 1810	3.45
Aspergillus oryzae	NCIM 1032	0.42
Aspergillus oryzae	NCIM 643	0.32
Aspergillus niger	NCIM 778	0.30
Aspergillus foetidus	NCIM 505	0
Aspergillus giganteus	NCIM 568	0
Aspergillus oryzae	NCIM 637	0.28
Aspergillus oryzae flavus	-	2.95
Aspergillus awamori	NCIM 886	0.39
Penecilium roquefortii	NCIM 712	1.49
Rhizopus microspores var. oligosporus	MTCC 556	3.48
Chaetomium virescence	MTCC 980	2.95
Trichoderma koningii	MTCC 796	3.43
Dictyostelium discodim	MTCC 1579	2.18
Lagenidium giganteum	MTCC 719	2.95
Metarhizium anisopliae	MTCC 892	3.26
Penicilium roquefortii	MTCC 933	1.34
Mucor rouxi	MTCC 386	0.13
Mucor fuscus	MTCC 1462	0.18

Selection of cereal bran for SSF

Wheat bran, rice bran and ragi (*Eleusine coracana*) bran were used as media for solid-state fermentation of *Aspergillus oryzae* MTCC 5341. Wheat bran was the best medium for production of acid protease activity at pH 5 and 30°C (4.8×10^5 units/ g dry bran (100%)). When grown on rice bran or ragi bran, the fungus produced 3.57×10^5 units/ g (74.4%) and 0.5×10^5 units/g bran (11.6%), respectively (Figure 11). The protein content of wheat bran (14-16%) is higher compared to rice bran (7-8%) or ragi bran (5-6%). This could be a factor for lower production of acid protease in rice bran or ragi bran.

Effect of source and amount of additional protein on protease production

Acid protease production was enhanced in presence of protein in the media. Protein sources like defatted soy flour (45-50% protein); defatted sesame flour (50-55% protein), casein (70-73% protein), and peptone (80-85%protein) was added to the bran at 5 different levels: 2, 4, 6, 8, and 10 % (w/w). Higher enzyme titres were obtained in all levels compared to control (only wheat bran). Soy flour at 4% resulted in an increase of ~2 times the activity level from 4.8×10^5 U/ g bran in control to 8.26×10^5 U/ g bran. Sesame flour (7.65×10^5 U/ g bran), casein (6.0×10^5 U/ g bran) and peptone (5.70×10^5 U/ g bran) also resulted in increase protease production, albeit to a lesser extent (Figure 12). Higher levels of defatted soyflour (> 4%) added to wheat bran repressed the activity.

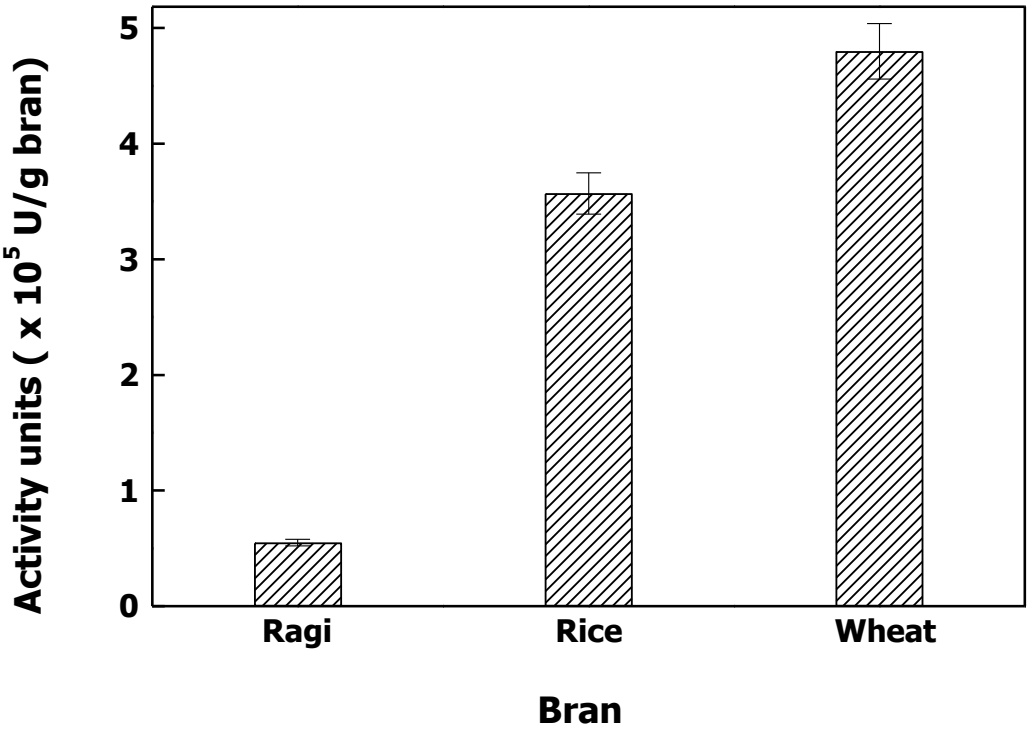


Figure 11. Selection of cereal bran as solid substrate for acid protease production by SSF

Effect of chemical additives on protease production

SSF with supplements like biotin (0.1%), phytic acid (0.4%), and aluminium chloride (0.1mM) reportedly enhance protease production (Tunga et al., 2004). However, in the present study, biotin, phytic acid and aluminium chloride did not significantly influence acid protease production (Figure 12).

Effect of cultivation time, incubation pH and temperature on acid protease production by *A. oryzae*

Acid protease production as a function of the time of cultivation on wheat bran (pH 5) with addition of 4% defatted soy flour (2% additional protein). The temperature of incubation was 30°C. The enzyme production reached maximum at the end of 120 h, followed by a decrease in activity (Figure 13A). Acid protease is an extracellular enzyme and could be degraded.

The optimum pH for extracellular protease activity was 5 (Figure 13B). Temperature of 25°C was suboptimal for growth, whereas growth seized above 35°C. The optimum temperature range for growth and protease production was determined to be 30°C (Figure 13C). It was also observed that when bran was incubated at 25°C, enzyme production gradually increased till the end of 160 h. Fermentation beyond 160 h was not followed in this case since the amount of enzyme produced was less in comparison to fermentation done at 30°C. However when bran was incubated at 30°C, maximum yield was obtained at the end of 120 h, after which, there was a decline.

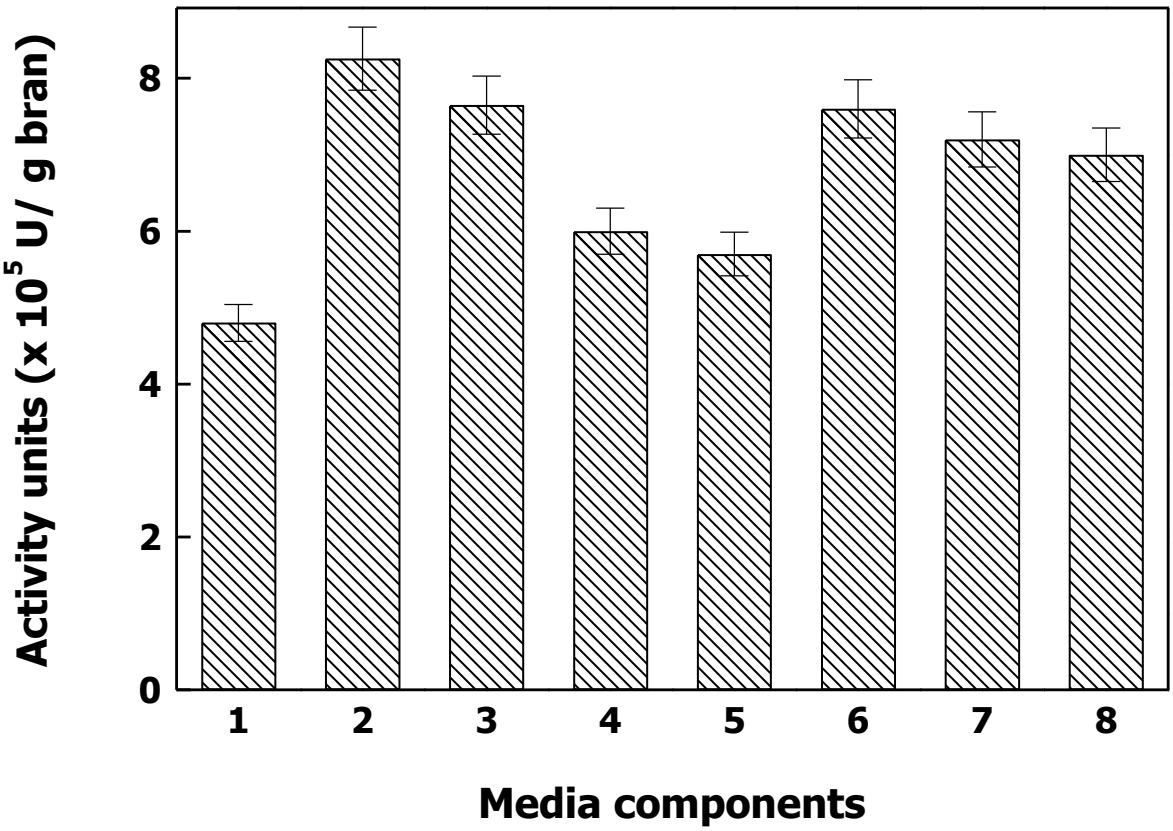


Figure 12. Optimization of media components for growth and production of extracellular acid proteases from *A. oryzae*. Treatments include, wheat bran (WB) (1), wheat bran + 4% defatted soy flour (DSF) (2), wheat bran + 4% defatted sesame flour (3) wheat bran + 4% casein (4), wheat bran + 4% peptone (5) WB + 4% DSF + 0.1% biotin (6), WB + 4% DSF + 0.4% phytic acid (7) and WB + 4% DSF + 0.1mM Al_2Cl_3 (8). Solid state fermentation was carried out at 30ºC and at pH 5.0 for a period of 120 h. Each value is the accumulation of three experiments

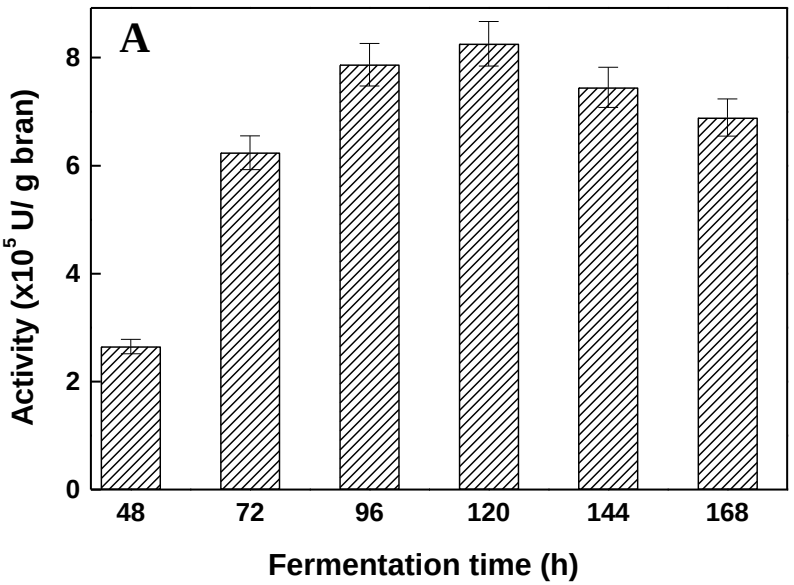


Figure 13A. Production of acid protease by *Aspergillus oryzae* as a function of time. At the end of each fermentation time, moldy bran was extracted with water having 0.1 M NaCl for 60 min. Activity is determined as given under assay method

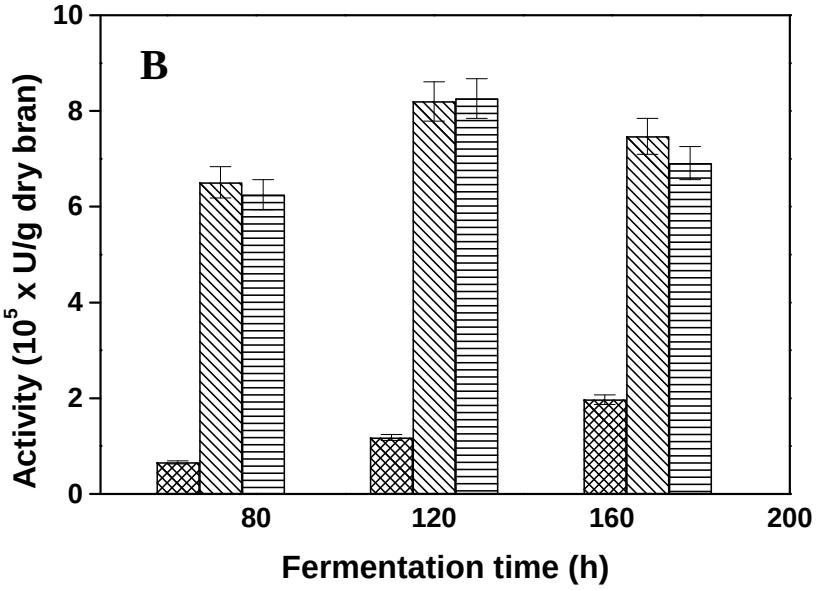


Figure 13B. Effect of pH on growth and production of acid protease from *Aspergillus oryzae* MTCC 5341. pH of solid media during growth was 3 ( ) , 4( ), and 5 ( )

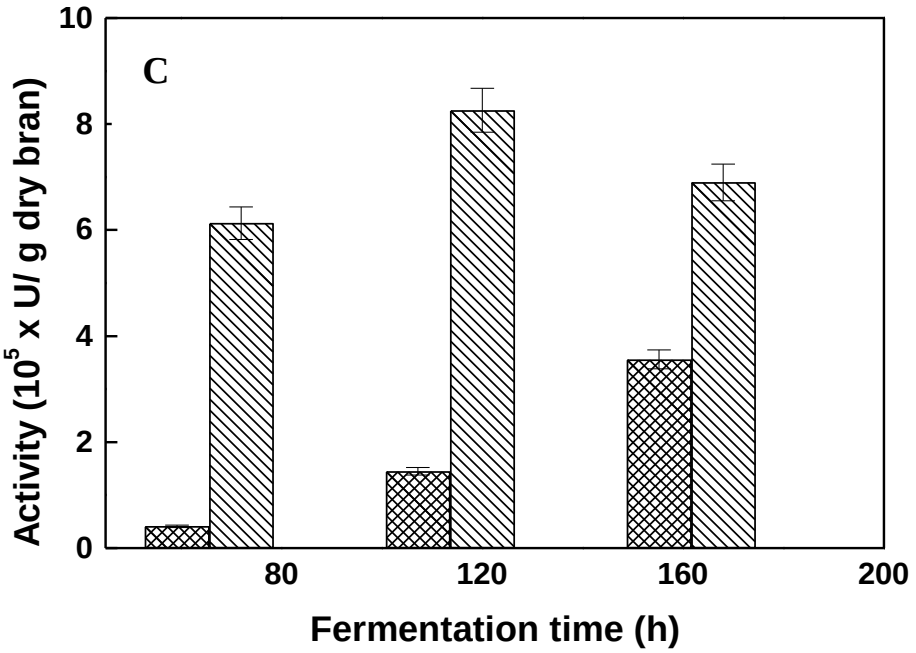


Figure 13C. Effect of temperature on growth and production of acid protease from *Aspergillus oryzae* MTCC 5341. Temperature of incubation during growth was 25ºC ( ) and 30ºC ( )

Based on the above results, it was concluded that pH of the substrate, fermentation time, temperature of incubation and amount of defatted soy flour supplemented in the substrate were the critical factors for acid protease activity.

The influence of the four independent variables on acid protease production was optimized by response surface methodology (RSM). The range and levels of the four independent variables used in RSM in terms of actual and coded factors is given in Table 10.

Table 10. Experimental range and levels of the four independent variables used in RSM in terms of actual and coded factors.

Variables	**Levels**		
	-1	**0**	**+1**
pH	3	5	7
Temperature (°C)	25	30	35
Defatted soyflour addition (%)	0	4	8
Fermentation time (h)	72	120	168

Optimization of factors influencing protease production by RSM using Box-Behnken design

Interactive effects of the factors, viz., pH, incubation temperature, defatted soyflour addition and fermentation time, were examined through RSM using Box - Behnken design. The actual yield of acid protease (response) obtained is given in Table 11. The ANOVA analysis, has yielded the following regression equation in terms of the levels of acid protease produced (Y) as a function of pH (A), temperature of incubation (B), defatted soyflour addition (C) and fermentation time (D).

Acid protease activity (x 10^5 U/ g bran) = 8.65 +1.17 * A+1.21 * B+0.47 * C+0.31 *D+0.13 * A * B+0.49 * A * C-0.13 * A * D-0.27 * B * C+0.31 * B * D+0.02 * C * D-3.31 * A^2 -2.74 * B^2-2.66 * C^2-2.82 * D^2

The subsequent analysis of variance showed aptness of the model for acid protease production. The computed model F-value of 29.911 implies the significance of the model and there is only 0.01% chance that a "model F-value" this large could occur due to noise.

Table 11. Experimental design used in the RSM studies by using four independent variables with three centre points for acid protease production by *Aspergillus oryzae* MTCC 5341 by SSF.

Run order	A pH	B Temperature (°C)	C Defatted soyflour concentration (%)	D Fermentation time (h)	Mean observed response (x 10^5 U/ g bran)
1	+1	0	0	+1	3.6
2	0	0	-1	+1	3.2
3	-1	0	-1	0	1.42
4	+1	0	-1	0	2.35
5	0	+1	0	-1	4.21
6	0	0	+1	+1	4.23
7	+1	-1	0	0	2.82
8	0	0	-1	-1	2.01
9	0	-1	0	+1	1.64
10	+1	0	+1	0	5.21
11	0	+1	0	+1	5.28
12	0	-1	+1	0	2.21
13*	0	0	0	0	8.64
14*	0	0	0	0	8.64
15*	0	0	0	0	8.66
16	-1	-1	0	0	0.43
17	+1	0	0	-1	3.71
18	0	+1	-1	0	4.67
19	0	0	+1	-1	2.96
20	-1	+1	0	0	2.0
21	0	-1	0	-1	1.79
22	-1	0	0	-1	1.01
23	-1	0	0	+1	1.41
24	0	+1	+1	0	4.09
25	+1	+1	0	0	4.9
26	-1	0	+1	0	2.31
27	0	-1	-1	0	1.72

* Central value

The "lack of fit F-value" is not significant and there is only 3.72% chance that a "lack of fit F-value" this large could occur due to noise. Non significant lack of fit shows the model is significant. Pure error is very low, indicating good reproducibility of the data obtained. 'Model P-value' was very small (< 0.0001) and large "lack of fit P-value" (0.3729) from ANOVA (Table 12) and a suitable coefficient of determination (R^2 = 0.9721) and adjusted coefficient of determination ($R^2_{adjusted}$ = 0.9396), the model was highly significant and sufficient to represent the actual relationship between response and the significant variables. The predicted sum of squares (PRESS), a measure of how a particular model fits each point in a design, is 21.48. Seven model terms (A, B, C, A^2, B^2, C^2 and D^2) are most significant. An adequate precision of 19.996 indicates adequacy of signal - to - noise ratio. A ratio greater than 4 is desirable (Table 13). Thus, this model can be used to navigate the design space.

Table 12. Analysis of variance for the fitted second order polynomial model and lack of fit for acid protease production as per Box-Behnken design.

Source	Sum of squares	Df	Mean sum of sqaures	F-value	Prob>F	Significance
Model	130.1695	14	9.297	29.911	<0.0001	Significant
pH (A)	16.356	1	16.356	52.62	<0.0001	
Temperature (B)	17.617	1	17.617	56.677	<0.0001	
Defatted soyflour (C)	2.6508	1	2.6508	8.527	0.0128	
Fermentation time (D)	1.122	1	1.122	3.610	0.0817	
AB	0.065	1	0.065	0.290	0.655	
AC	0.9702	1	0.9702	3.121	0.102	
AD	0.065	1	0.065	0.2091	0.655	
BC	0.2862	1	0.2862	0.9208	0.356	
BD	0.3721	1	0.3721	1.197	0.295	
CD	0.0016	1	0.0016	0.00514	0.944	
A^2	58.417	1	58.417	187.935	<0.0001	
B^2	39.918	1	39.918	128.422	<0.0001	
C^2	37.689	1	37.689	121.249	<0.0001	
D^2	42.550	1	42.550	136.889	<0.0001	
Residual	3.730	12	0.3108			
Lack of fit	3.729	10	0.3729	2797.356	<0.0001	Not significant
Pure error	0.00026	2	0.00013			
Corr. total	133.899	26				

Table 13. Analysis of variance (ANOVA) table for response surface quadratic model.

Parameter	Value
Standard deviation	0.56
Mean	3.52
R^2	0.9721
Adjusted R^2	0.9396
Predicted R^2	0.8395
F-value	29.91
PRESS	21.48
Adequate precision	19.966

The response surface curves for the yields of acid protease as a function of two variables; with the other two kept at their central values, were constructed. The three dimensional response surfaces obtained were convex in nature suggesting well-defined optimum operating conditions. The sharp convexity implied that the responses vary significantly from the single variable optimized conditions. From the response for the interaction of pH with temperature of incubation (Figure 14a), protease yields increased upon increasing pH and temperature up to a value of 5.0 and 30°C, respectively. Decreased enzyme activity was observed at pH above 5.0 and temperatures above 30°C. At optimum levels of these variables (pH of 5.0 and 30°C), a maximum enzyme yield of 8.64 x 10^5 U/ g bran was obtained. Defatted soy flour (50% protein) addition had favorable impact on enzyme yield up to 4% (Figure 14B). Accordingly, 4% protein and pH 5 were optimal for maximum protease production. Any further increase in its concentration repressed the enzyme yield. Similarly, the yields of acid protease were determined to be maximum at fermentation time of 120 h beyond which, activity declined (Figure 14C). Convex shaped response surface curve was obtained when defatted soyflour addition was combined with temperature, indicating a positive influence of these variables for acid protease production (Figure 14D).

Analysis of response surface curves and contour plots indicated optimum levels of the variables necessary to achieve better results. The results predicted by Box –

Behnken design showed that a combination of pH at 5, temperature of 30ºC with 4 % defatted soy flour would favor maximum protease production, yielding 8.64 x 10^5 U/ g bran after 120 h of fermentation. Thus, by optimizing the fermentation parameters using RSM, the yield of acid protease increased from 4.8 x 10^5 U/ g bran to 8.64 x 10^5 U/ g bran.

In a separate set of evaluation experiments carried out to verify the predictions, *Aspergillus oryzae* produces 8.47 x 10^5 U/ g bran which is actually 98 % of the predicted value. Table 14 shows predicted and observed responses to random levels of variables, wherein both the responses are nearly similar in most of the cases.

In an attempt to test the desirability of the model, maximum values of the factors were determined as, pH of 5.4, incubation temperature of 31ºC, defatted soyflour addition of 4.4% and fermentation time corresponding to 123 h yielding a maximum activity of 8.93 x 10^5 U/g bran (Figure 15). Thus the central values framed were close to the values predicted by the statistical model.

Scale up studies and recovery of acid protease

The results were also evaluated through a scale-up study by solid state fermentation, with 5 kg bran as the starting material; the fungi produced 8.02 x 10^5 units of acid protease activity per gram of dry bran. An overall flow diagram for production of acid protease from *Aspergillus oryzae* MTCC 5341 is given in Figure 16.

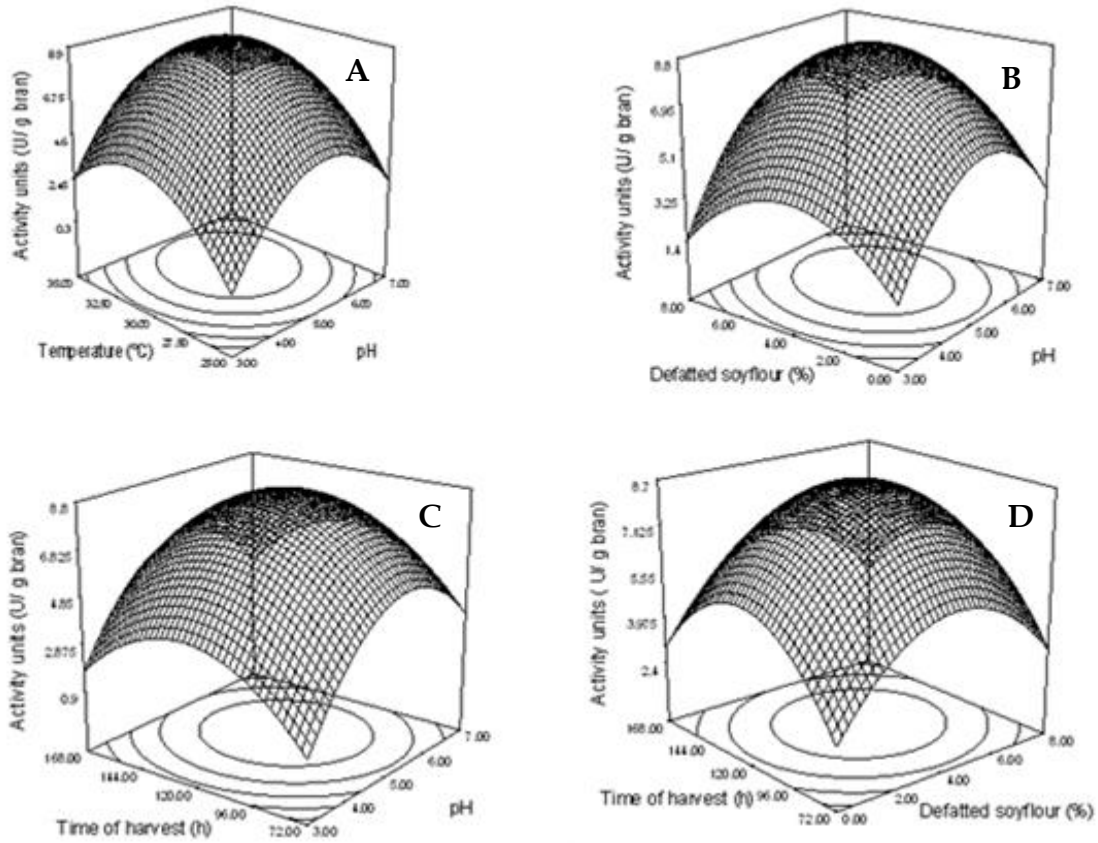


Figure 14A Response surface curve of acid protease production from *Aspergillus* oryzae MTCC 5341 showing interaction of temperature and pH after 120 h of incubation. Defatted soyflour (4%) was added

Figure 14B Acid protease production (× 10^5 U/g bran) observed as a response to the interaction of defatted soyflour addition (%) and pH as variables. Temperature and fermentation time are kept at their central points (30°C, 120 h).

Figure 14C Response surface plot of acid protease production (× 10^5 U/g bran) by *Aspergillus oryzae* MTCC 5341 showing interaction of fermentation time (h) and pH. Temperature of incubation was 30°C and media contained 4% DSF

Figure 14D Acid protease production (× 10^5 U/g bran) observed as a response to the interaction of defatted soyflour addition (%) and temperature as variables. Fermentation time and pH are kept at their central points (120 h, pH 5).

Table14. Design for validation of predictive model using random levels of experimental factors.

Run order	pH	Temperature (°C)	Defatted soy flour addition (%)	Fermentation time (h)	Observed response (x 10^5 U/ g bran)	Predicted response (x 10^5 U/ g bran)
1	4	25	1	100	0.95	0.977
2	6.5	30	6	80	5.17	5.27
3	5	30	4	120	8.47	8.647
4	3	35	4	96	1.13	1.16
5	5	28	4	80	5.51	5.609
6	5.5	32	6	120	8.32	8.36
7	4	25	0	120	0.23	0.20
8	6	30	5	140	8.02	8.02
9	7	28	5	72	2.74	2.72
10	5	30	4	120	8.45	8.64

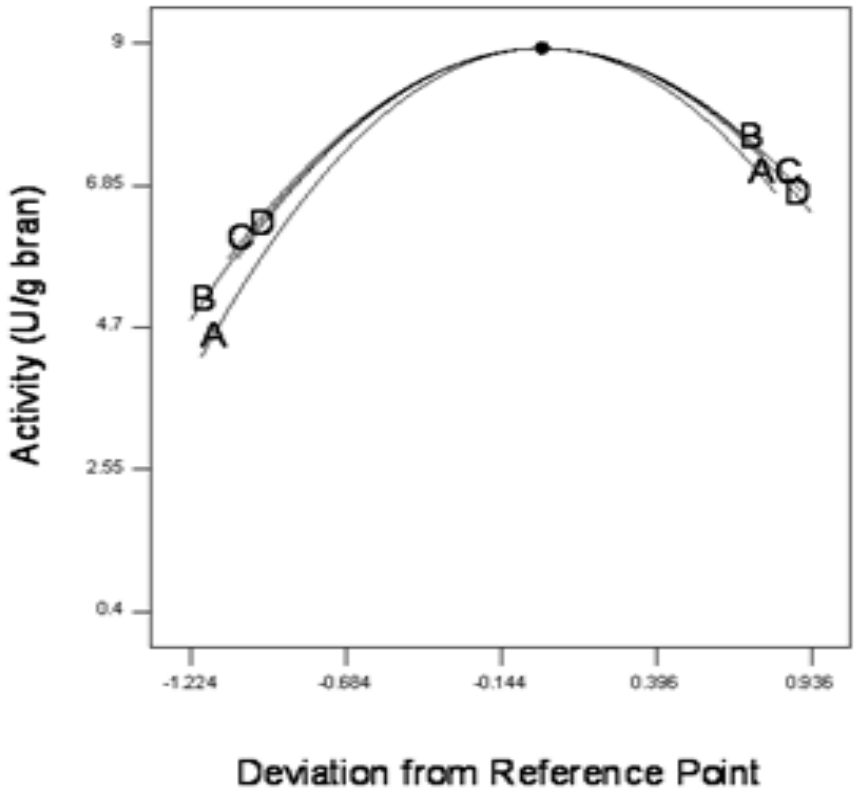


Figure 15. Desirability model graph for production of acid protease from *A. oryzae.* A-incubation pH, B-incubation temperature, C-defatted soyflour addition and D-fermentation time

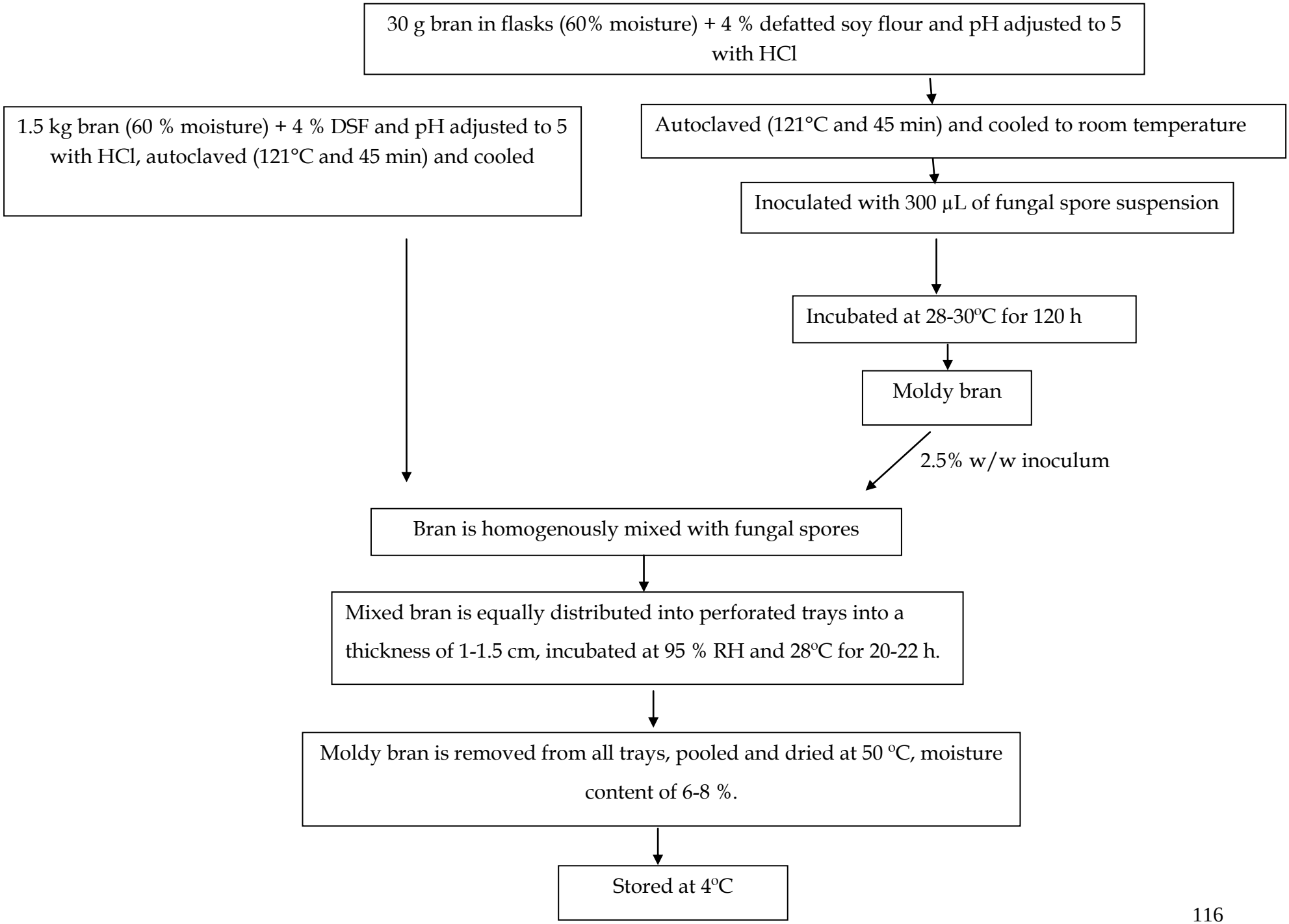


Figure 16. Flow diagram for large scale production of acid protease from *A. oryzae* MTCC 5341 by solid state fermentation

Downstream processing

Downstream processing for recovery of protease from moldy bran

Effect of solvents and time of extraction

The moldy bran (6-8% moisture) was used to recover acid protease. Several solvents were tried separately for maximum recovery of the extracellular acid protease from the moldy bran. Effect of different solvents on enzyme recovery is listed in Table 15. Of all the solvents tried, water or acetate buffer having 0.1 M NaCl were optimum as it leached maximum amount of enzyme (8.3×10^5 U/g bran) into solution. Buffer (acetate) with sodium chloride and Triton X-100 in water also leached out (8.2×10^5 U/g bran and 7.9×10^5 U/g bran respectively) considerable amounts of acid protease into solution. Water containing 0.1 M NaCl was the chosen solvent for the recovery of acid protease from the moldy bran.

To optimize the time of extraction for acid protease activity, moldy bran was extracted with solvent for 10-120 min (Fig 17). Bran to solvent ratio was maintained at 1:10 and extraction was carried out at 4°C without stirring. Extracellular protease activity increased with time of extraction till 60-70 min. Thereafter, the activity remained constant but an increase in gumminess and color was observed. Since wheat bran contains carbohydrates and coloring materials and color and increased viscosity can hinder purification. Longer

extraction times resulted in the leaching into solution. Hence, the time of extraction was optimized as 60 min.

Table 15. Solvents for extraction of acid protease from *Aspergillus oryzae* MTCC 5341

Solvent	**pH**	**Acid protease activity ×10^5 (U/g bran)**
Water	5.3	6.4
Water with 0.1M NaCl	5.3	8.3
Water with 0.1M Sodium sulphate	5.4	5.2
Water with 0.1% Triton X-100	5.3	7.9
0.1 M acetate buffer pH 5.0	4.9	7.6
0.1 M acetate buffer with 0.1M NaCl	4.9	8.2
0.1 M acetate buffer with 0.1 M Na_2SO_4	4.9	5.6
0.1 M acetate buffer with 0.1 M SMS	4.9	5.6
0.1 M acetate buffer with 0.1% Triton X-100	4.9	5.5

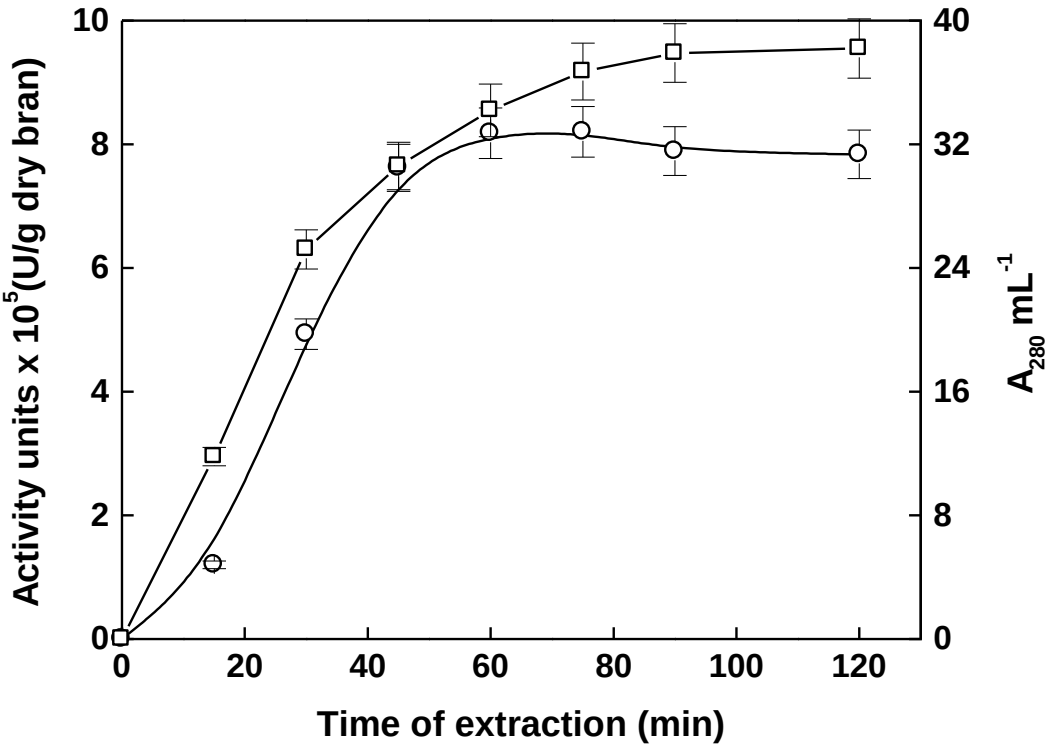


Figure 17. Optimization of extraction time for removing acid protease from *Aspergillus oryzae* MTCC 5341. Enzyme activity (○) and absorbance (□) of the solution after each test time was determined.

Effect of agitation during enzyme extraction

Extraction of enzyme was carried out both under still and agitated condition with the solvent at 4°C. Stirring the moldy bran during extraction did not increase protease activity in the extract. Mere soaking of moldy bran with the solvent was sufficient for maximum enzyme extraction from the bran. Further agitation resulted in increased color and viscosity in the enzyme extract that could interfere with the further purification. Thus, extraction was optimized by soaking the moldy bran with solvent (1:10 ratio), at 4° C for 60 min with intermittent shaking of the flask by hand.

Repeated extraction

The extraction efficiency of acid protease from moldy bran with solvent was studied by carrying out three cycles of extraction for 60 min each. Fresh solvent was added to the same bran after filtering the extract. The efficiency of the first extraction was 78-79% (8.26×10^5 U/ g bran) of the total activity present in the bran. Second extraction showed 15% (1.57×10^5 U/ g bran) activity and third (0.63×10^5 U/ g bran) and subsequent extractions were insignificant in terms of enzyme activity, although color was extracted. Extraction was restricted to only one cycle.

Concentration of the enzyme extract

Ammonium sulphate precipitation and lyophilisation

Concentration of the acid protease in the crude extract was carried out by (a) ammonium sulphate precipitation (b) ultrafiltration (c) lyophilisation and (d) acetone precipitation. When ammonium sulphate precipitation was tried, acid protease could be precipitated at 60-80% concentration but there was loss of enzyme activity up to 40-45% due to denaturation of the enzyme. Thus it was not possible to concentrate acid protease through ammonium sulphate precipitation.

Lyophilization

The crude extract was lyophilized without dialysis or any additives. The lyophilized product was sticky probably due to the polysaccharides in the crude extract. Yield was very low with 23% recovery (Table 16).

Ultrafiltration

Ultrafiltration was carried out using a 10K membrane (PLGC) in a stirred cell. An eight-fold reduction in volume of the crude extract with ~22 – 30% loss in activity was achieved. Thereafter, the viscosity of the solution prevented further concentration due to reduced flow rate and membrane fouling. Yield of the final product was 72% (Table 16).

Acetone precipitation

Acid protease was precipitated using 1: 2 (v/v: enzyme: solvent) of chilled acetone over a period of 4 h. Acid protease could be precipitated out with two volumes of chilled acetone. The recovery was 90-92% (Table 16) and the precipitate could be redissolved completely in buffer. The results of the different methods are summarized in Table 16. Hence, acetone precipitation was the method of choice. Two volumes of chilled acetone were used to achieve a purification fold of 5 (Table 17).

Table 16. Comparison of concentration methods for acid protease from moldy bran from *Aspergillus oryzae* (MTCC 5341)

Concentration method	Yield (%)	Remarks
Lyophilisation	23	Material was gummy and low recovery
Ammonium sulphate precipitation (60-80%)	45	Low recovery due to denaturation
Acetone precipitation	92	High recovery and complete dissolution of precipitate
Ultrafiltration	70-72	Membrane fouling and reduced flow rates

Purification of acid protease

The acetone precipitate was dissolved in 20 mM phosphate buffer, pH 6.0, dialyzed against same buffer for 8 h with 3 changes. This was loaded on a DEAE cellulose column (30 × 2.5 cm) using a linear gradient of 0 - 0.5 M NaCl. Acid protease activity eluted out at 0.3 – 0.35 M sodium chloride. The enzyme was purified 12 times with a yield of 66%. The active pool was concentrated by ultrafiltration and was fractionated on G-75 superfine column (Figure 18A) to a yield of 29%. The acid protease was purified 17 fold with a specific activity of 43, 658 U/ mg (Table 1). Native PAGE ascertained homogeneity of the preparation and *in situ* activity of the acid protease in the gel is shown in Figure 18B.

Homogeneity of acid protease

Gel electrophoresis

Purified acid protease moved as single band on SDS-PAGE (Fig 18C). The protein was homogenous and the molecular weight of acid protease was deduced to be 47,000 Da on SDS-PAGE.

Size exclusion chromatography

Acid protease eluted as a single peak after molecular sieve chromatography on a TSK $G_{2000\ SW}$ column by HPLC (Figure 19A). The molecular weight of the acid protease was determined to be 47, 578 kDa using standard proteins in the range 12.4 – 150 kDa (Figure 19B).

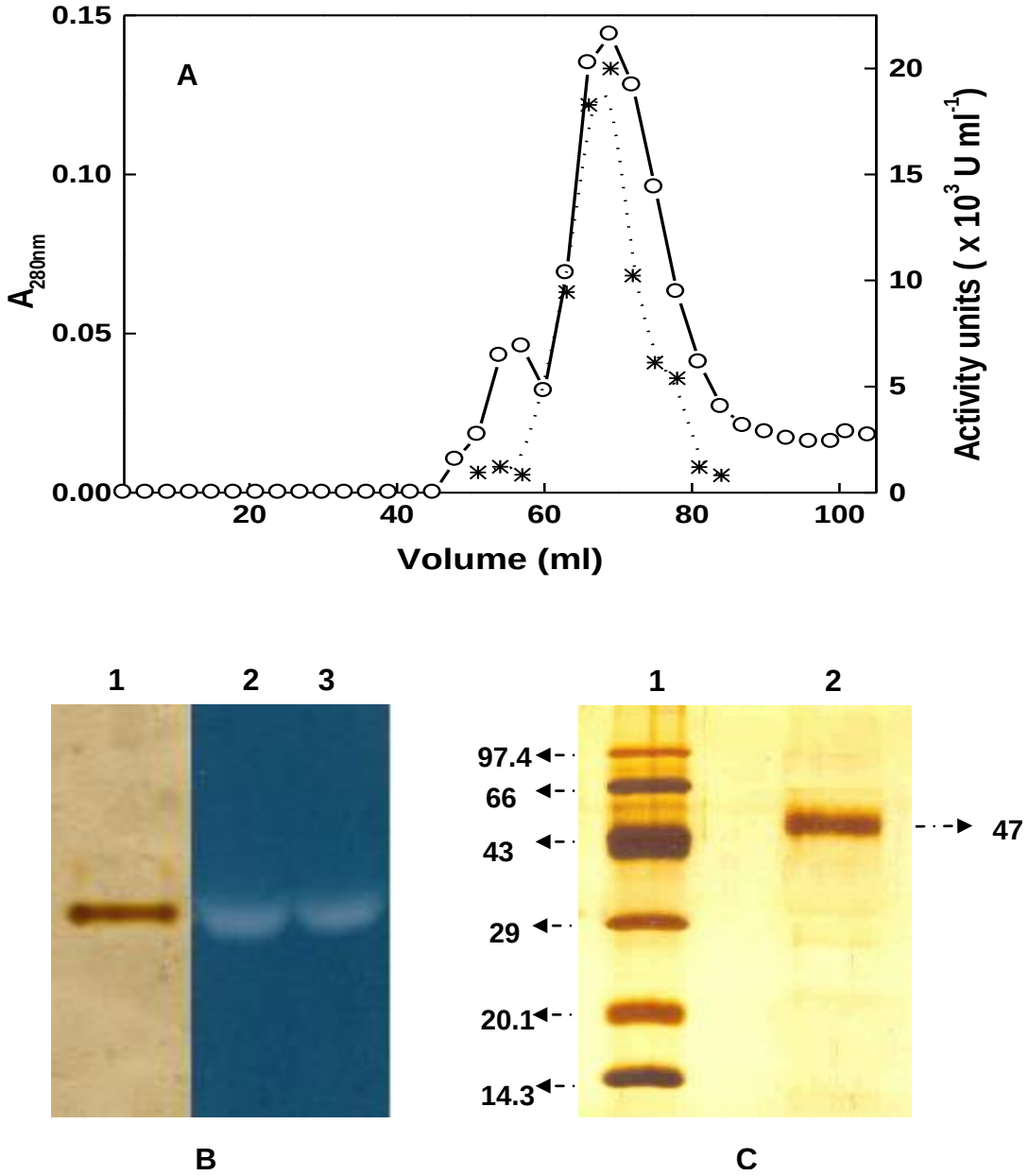


Figure 18. Purification of acid protease from *Aspergillus oryzae* MTCC 5341

A. Purification of acid protease from *Aspergillus oryzae* by molecular sieve chromatography. O—O, protein content; ×---×, protease activity. Experimental details are given under materials and methods.

B. Native PAGE pattern and activity staining in gel electrophoresis for acid protease. Lane 1 – Native gel pattern, Lanes 2 and 3 – Zymogram of acid protease

C. SDS-PAGE pattern and molecular weight determination of acid protease

Lane 1 – molecular weight markers and lane 2 – purified acid protease

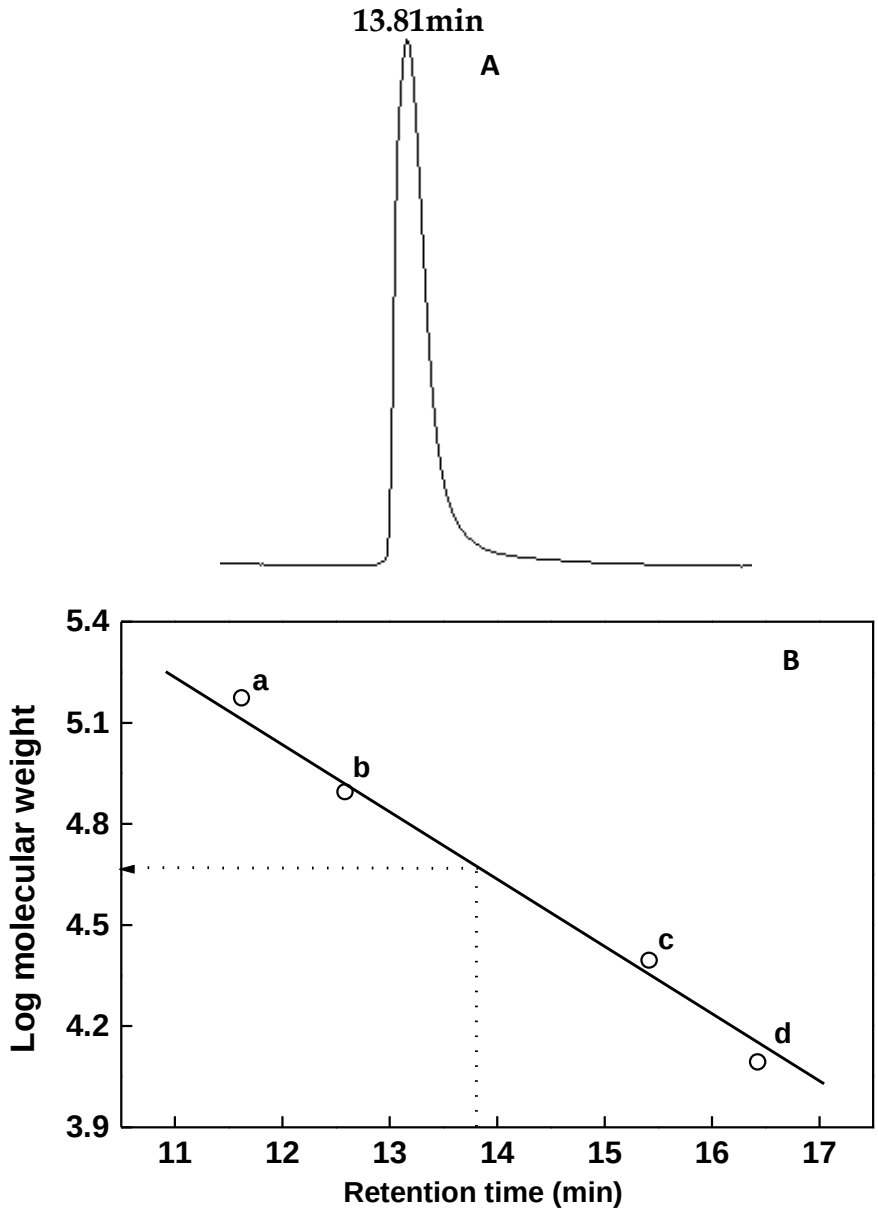


Figure 19. Determination of homogeneity and molecular weight of acid protease through gel filtration by HPLC

A. Chromatogram of acid protease on TSK $G_{2000\,SW}$ gel filtration column
B. Molecular weight determination using standard molecular markers on TSK $G_{2000\,SW}$ column. Standard markers used are, (a) LOX1: 94 kDa (b) bovine serum albumin: 68 kDa (c) carbonic anhydrase: 29 kDa and (d) cytochrome C: 12.4 kDa.

Table 17. Purification of acid protease from *Aspergillus oryzae* MTCC 5341

Purification Step	**Total volume (mL)**	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U/mg)**	**Fold purity**	**Yield (%)**
Crude	30	453	11,91,747	2630	1	100
Acetone precipitation	10	96	11,00,762	11,466	4.35	92
Ion exchange	105	24	7,90,160	32,923	12.5	66
Gel filtration	20	8	3,49,264	43,658	16.6	29

Molecular characterization of acid protease from *A. oryzae*

Effect of pH and temperature on the activity and stability of acid protease

Acid protease from *Aspergillus oryzae* MTCC 5341 was active in the pH range 3 - 4 with an optimum pH of 3.2 (Figure 20A). The enzyme was stable in the pH range 2.5 - 6.0 at 30°C for 24 h. The enzyme was stable in the temperature range 40 - 57°C with optimum temperature for activity of 55°C (Figure 20B).

Determination of K_m and V_{max} for acid protease

K_m and V_{max} were determined to be 0.8% and 89.44 μmol/min/mg protein, respectively, using 0 - 2% acid denatured hemoglobin as substrate.

Spectral characterization

The UV absorption spectrum of acid protease is shown in Figure 21A. The enzyme has an absorption maximum at 278 nm. The number of tryptophan residues was determined to be six by NBS method. The fluorescence spectrum of the enzyme revealed an excitation maximum at 278 nm. The emission maximum was determined to be 335 nm (when excited at 278 nm) (Figure 21B).

The conformation of acid protease was followed by CD measurements at pH 4.5, where the enzyme is in its native form. The near UV CD spectrum is shown as Figure 21C. The enzyme exhibited peaks at 260, 266, 275, 282, 290 and 300 nm.

Minima were observed at 263, 270, 279, 286 and 295 nm corresponding to phenylalanine, tyrosine and tryptophan residues in the enzyme.

The far UV CD spectrum is shown in Fig 21D. The spectrum shows a broad minimum in the region 213 – 216 nm indicative of predominant β structure. Two positive bands are seen at 197 and 230 nm when the spectrum was recorded at pH 4.5. The presence of these bands strongly suggested that acid protease from *Aspergillus oryzae* belongs to family of aspartic proteases. CDSSTR method using SP175 as reference database gave the best fit and was used to estimate secondary structure content (Lees et al., 2006). This method revealed 57% β structure, 4% α helix and 38% aperiodic structure in the protein.

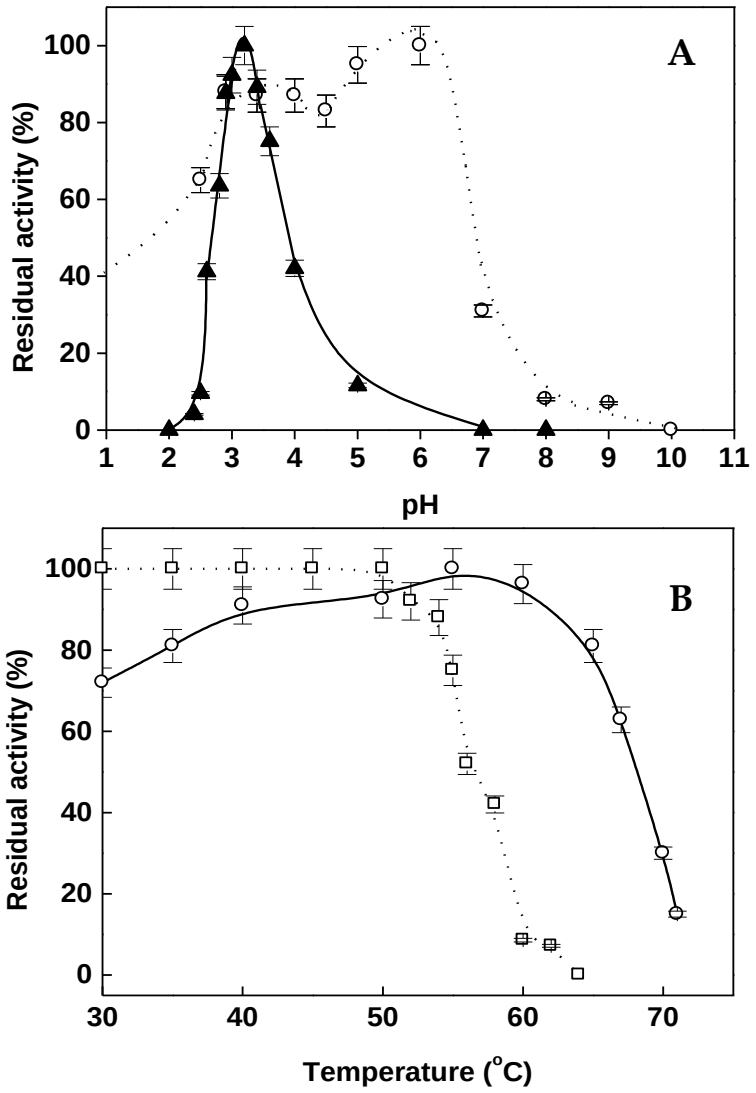


Figure 20. Optimum pH and temperature for acid protease activity

A. Optimum pH (▲—▲) and pH stability (○····○) of acid protease from *Aspergillus oryzae.* Activity measurements were carried out at 55°C. Following buffers (100mM) were used: glycine-HCl (pH 2 – 5), acetate (pH 5 – 7) and glycine- NaOH (pH 7 – 10).

B. Optimum temperature (○—○) for activity and T_m (□····□) for acid protease Activity measurements were carried out at pH 3.2. Temperature was maintained within ± 1°C using a thermostatted water bath.

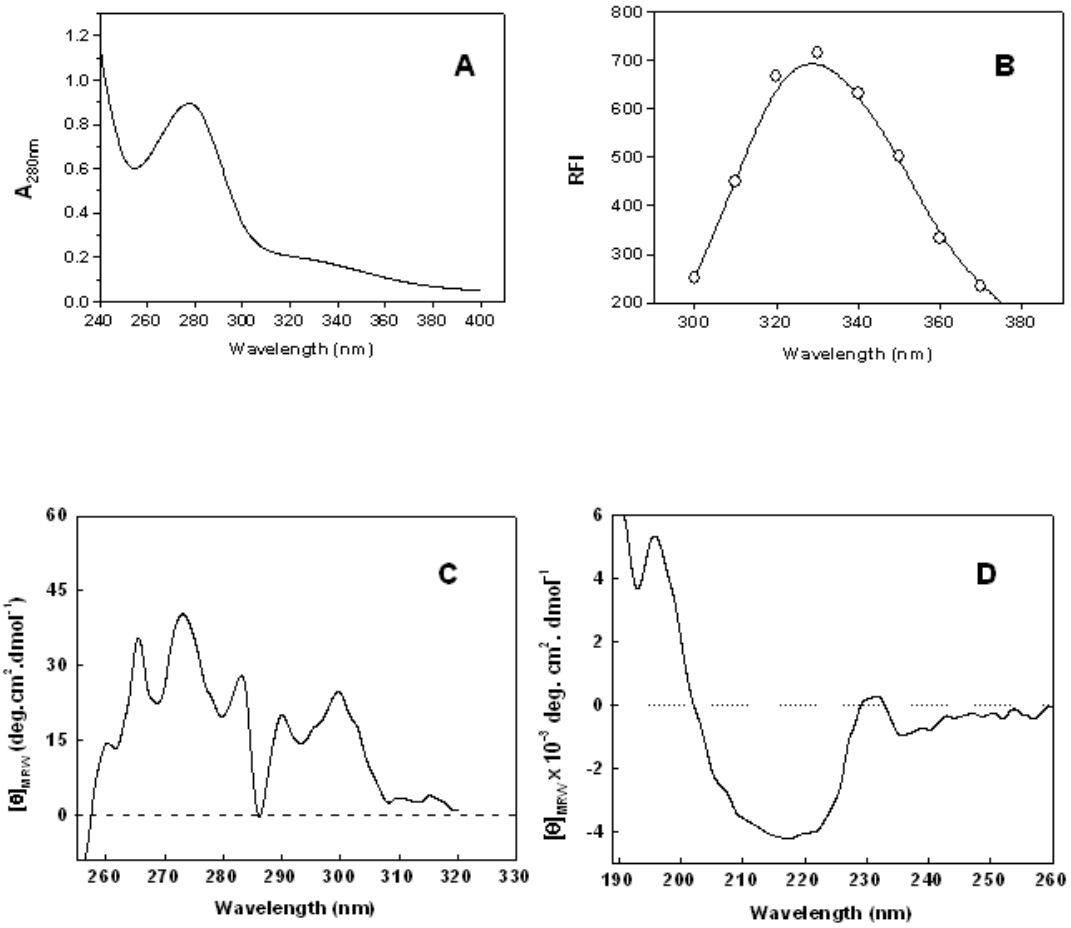


Figure 21. Spectral characterization of acid protease from *A. oryzae*

A. Absorption spectra of acid protease from *Aspergillus oryzae.* The protein was prepared in 50 mM phosphate buffer, pH 6.0.

B. Emission spectrum of acid protease when excited at 278 nm. Emission was monitored from 300-400 nm at 27°C. Protein was prepared in phosphate buffer (pH 6.0, 50 mM)

C. Near UV CD spectra of acid protease from *Aspergillus oryzae*, 1.0 mg/mL protein in 50 mM phosphate buffer pH 4.5. Accumulations of three runs at 10 nm/min are shown. A one cm path length cell was used

D. Far UV CD spectra of acid protease from *Aspergillus oryzae*, 0.22 mg/mL protein in 50 mM phosphate buffer pH 4.5. Accumulations of three runs are shown at speed of 10 nm/min. A one mm path length cell was used.

Inhibition studies

Pepstatin, a hexapeptide inhibitor, specifically inhibits acid proteases by irreversibly binding to the active site. Pepstatin (18.6 μM) inhibits 100 % activity of acid protease while other class specific inhibitors like, EDTA (10 mM), Iodoacetamide (1 mM), PMSF (0.1 mM) and Antipain hydrochloride (20 μM) did not show any enzyme inhibition. Pepstatin inhibited the enzyme competitively with an IC_{50} value of 9.3 μM as shown in Fig 22A. The inhibitor was competitive in nature as demonstrated by Lineweaver-Burk plot (Figure 22B). The inhibition constant, K_i, was determined by Dixon plot to be 0.37 μM (Figure 22 C)

Activation of trypsinogen

Acid proteases possess the characteristic feature of activating native trypsinogen to trypsin at low pH by cleaving between I-K residues in trypsinogen. Acid protease from *Aspergillus oryzae* MTCC 5341 also activates trypsinogen to form trypsin, as confirmed by activity on casein. Acid protease is found to cleave I-K residues to release active trypsin from trypsinogen. Formation of trypsin was also confirmed by HPLC (Figure 23).

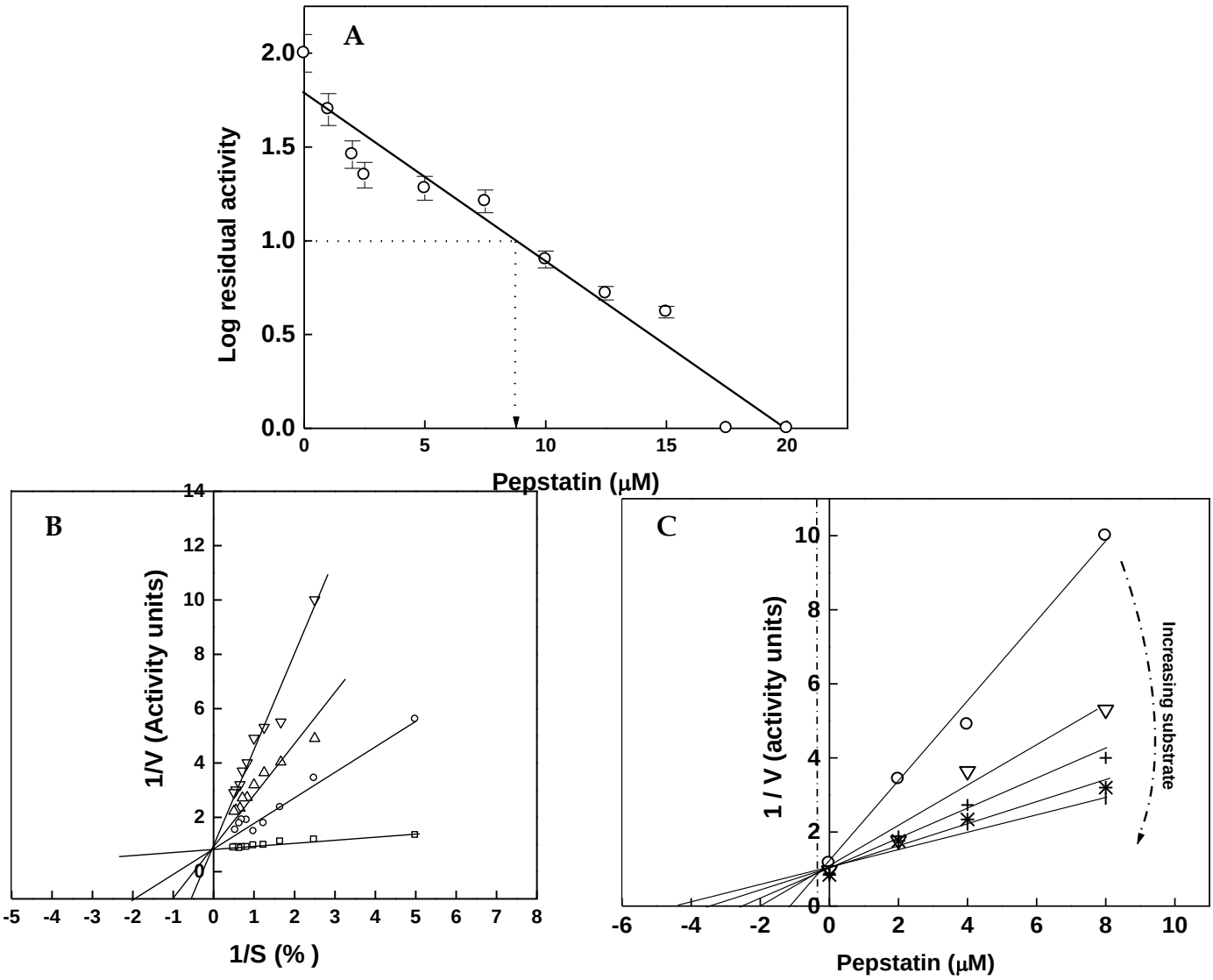


Figure 22. Inhibition of acid protease activity by pepstatin.

- **A.** Concentration of pepstatin used was in the range 0 – 20 μM. Pepstatin was dissolved in alcohol (stock solution). The stock was diluted with buffer (0.05 M phosphate buffer, pH 6.0).
- **B.** Lineweaver- Burk plot analysis for inhibition of acid protease by pepstatin. Enzyme was incubated with 0μM (□), 2 μM (○), 4 μM (△) and 8 μM (▽) of the inhibitor and acid protease activity was determined.
- **C.** Dixon plot: K_i was determined by taking hemoglobin concentrations at 0.2% (○), 0.4% (▽), 0.6% (+), 0.8% (∗) and 1.0% (†).

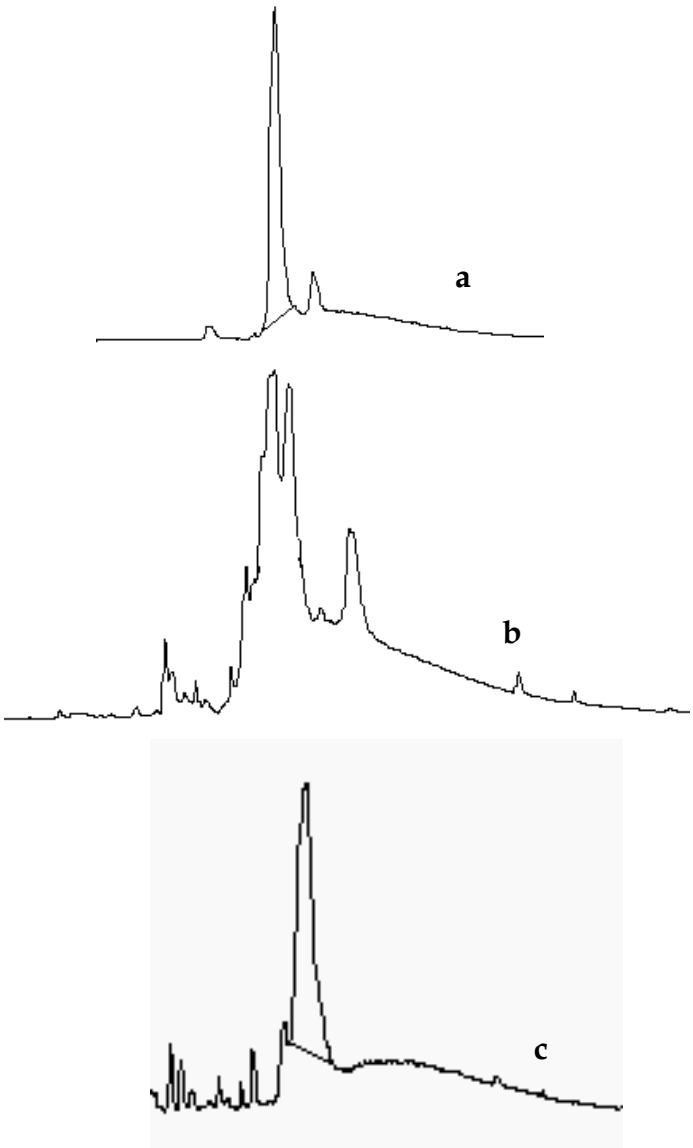


Figure 23. Activation of trypsinogen to trypsin by acid protease by HPLC.

(a) trypsinogen standard (b) trypsinogen + trypsin when incubated with acid protease and (c) trypsin standard. Samples were injected on to a gel filtration column (TSK G_{2000SW}). Standard trypsinogen and trypsin were dissolved (1mg/ml) in Glycine-HCl buffer (0.1M, 3.2 pH) and 20 µl injected. For determining activation of trypsinogen, trypsinogen was incubated in Glycine-HCl buffer pH 3.2 at 55°C with acid protease. After 1min incubation, 20 µl mixture was injected. Flow rate was 1 ml/min with detection at 280 nm.

Cleavage specificity

Acid proteases are endoproteolytic in nature, and show wide range of residue specificity. Acid protease from *Aspergillus oryzae* MTCC 5341 shows endoproteolytic activity and prefers L-V and Y-T residues at S_1 and S_1' positions (Figure 24). This was deduced by allowing acid protease to cleave oxidized insulin B chain for 1 min. It was interesting to note that, the same enzyme did not show any cleavage on oxidized insulin chain A, probably due to lack of above said residue pairs in the sequence. Thus, this enzyme has cleavage specificity between L-V, Y- T and I-K residues (from trypsinogen activation).

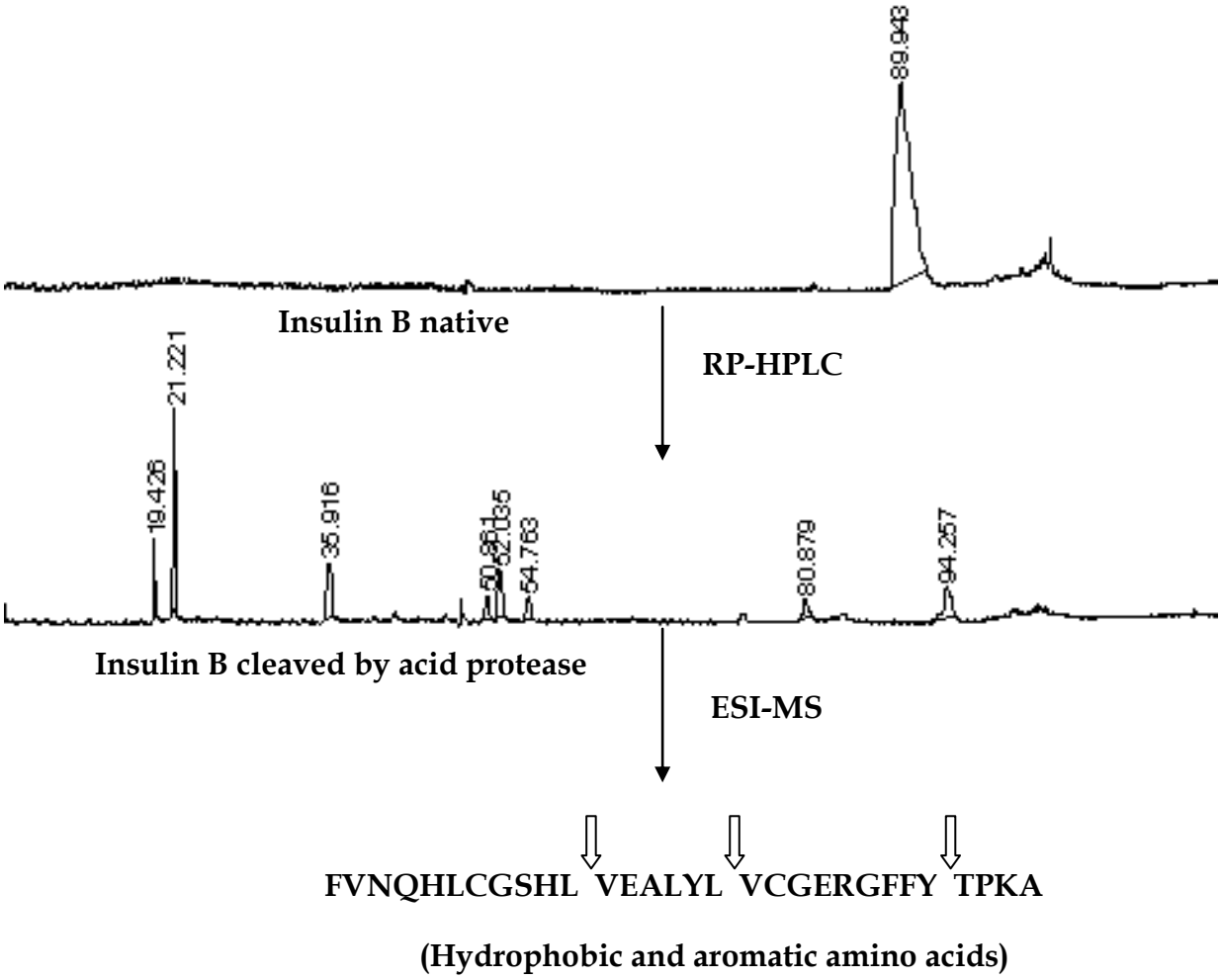


Figure 24. Determination of cleavage specificity for acid protease from *Aspergillus oryzae* MTCC 5341

Molecular biology experiments

Aspergillus oryzae MTCC 5341 was grown on potato dextrose broth for 48 h and chromosomal DNA was isolated using phenol-chloroform method according to manufacture's instructions. The purity of the DNA isolated was checked through agarose gel electrophoresis. Genomic DNA was visible in the gel (Figure 25) as a single band and this preparation was used for further gene isolation experiments.

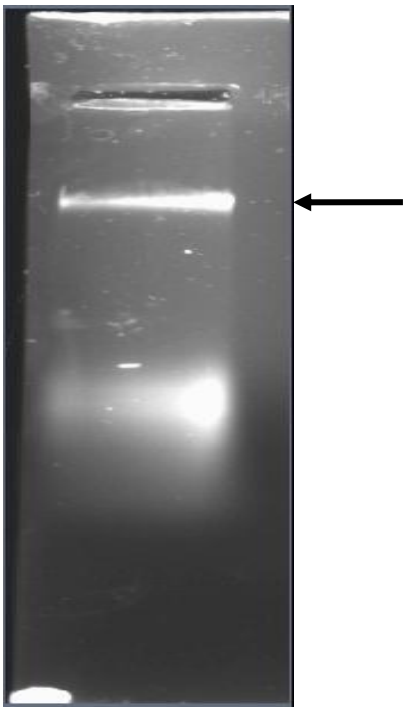


Figure 25. Agarose gel pattern of genomic DNA of *Aspergillus oryzae* MTCC 5341. DNA is indicated by an arrow.

The genomic DNA was used as a template for PCR and the primers were designed for amplifying a part of acid protease gene. PCR conditions were standardized based on Tm values of the primers designed. A fragment of corresponding to 400-450 bp amplicon was obtained. This was subjected to sequencing and the sequence of the amplicon was obtained. To check the accuracy of the amplification, first amplicon was used as a template for second round of PCR amplification. A nested primer which would specifically amplify regions within the first gene product was used in the second round of PCR amplification. An amplicon of 200-220 bp was obtained by this procedure thus confirming the amplification of part of aspartic protease gene (Figure 26).

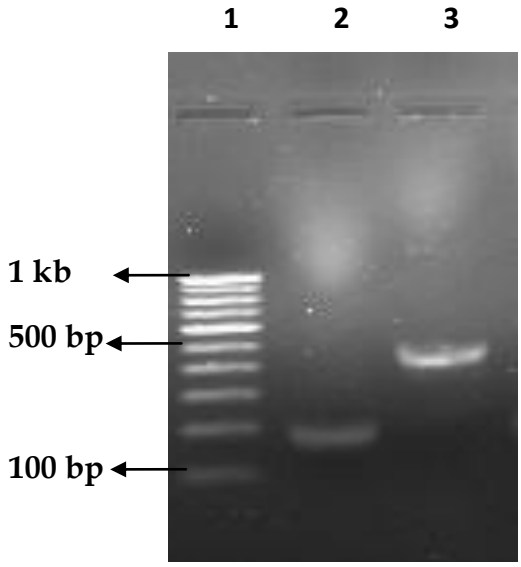


Figure 26. Agarose gel patterns of PCR amplicons obtained from *pep A* gene **Lane 1-** 1 kb DNA ladder, **Lane 2-** amplicon obtained as a result of nested primer amplification and **Lane 3-** amplicon obtained after first cycle of PCR amplification

Based on BLAST analysis of the obtained sequence, the amplified sequence from *Aspergillus oryzae* MTCC 5341 was matching closely with the aspartyl endopeptidase (OpsB) of *Aspergillus oryzae*, and *Aspergillus flavus* NRRL 3357. The amplified sequence of the acid protease and distance tree are represented in Figure 27.

The sequence of the amplified fragment of aspartic protease from *Aspergillus oryzae* MTCC 5341 had 92% homology with reported aspartic proteases of *Aspergillus sp.* It has been reported that *Aspergillus oryzae* produces multiple aspartic proteases which share significant amount (75 – 80%) of structural homology (Ward et al., 2006). The fragment amplified corresponds to a part of the gene coding for an aspartic protease. Further work needs to be carried out to obtain an active aspartic protease in a suitable host organism.

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CATTGACGGGATTTGACTTGGGTGGCTCCAGGGACGGAATCATCGCCAGTTCCAATTTC
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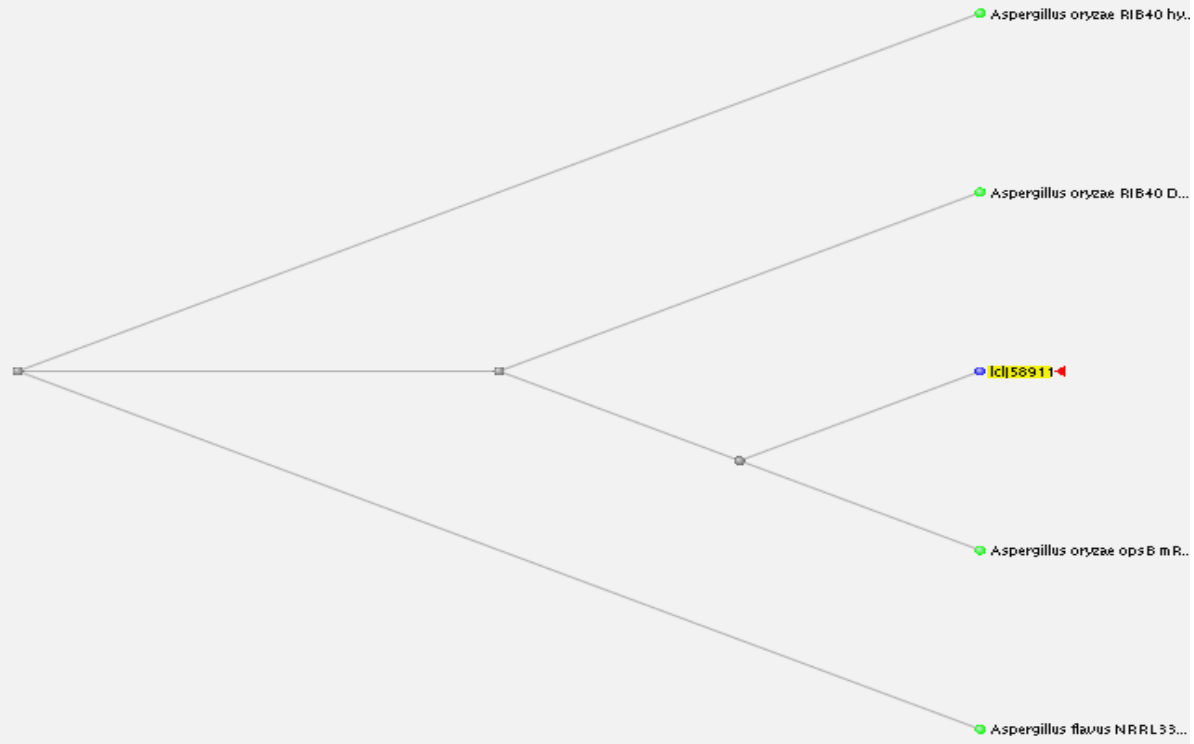


Figure 27. Nucleotide sequence and distance tree of amplified sequence from *Aspergillus oryzae* MTCC 5341 for acid protease

1B: Screening, purification and molecular characterization of milk clotting enzyme from *Aspergillus oryzae* MTCC 5341

Screening for milk clotting activity from fungal strains

Sixteen fungal strains included in the study were screened for production of milk clotting activity by solid state fermentation. Wheat bran was used as the substrate in the fermentation. Of the sixteen fungal strains screened for milk clotting enzyme production, *Aspergillus* oryzae MTCC 5341 produced highest milk clotting activity of 10,000 U/g dry bran though solid-state fermentation (Table 18). Many other fungal strains included in the study also proved to be potential producers of milk clotting enzymes. The milk clotting to proteolytic activity ratio was 500:1. Hence, *Aspergillus oryzae* MTCC 5341 was selected for further experiments

Optimization of process parameters for milk clotting enzyme production by *A. oryzae* MTCC 5341

The substrate pH during growth had a significant effect on the production of milk clotting enzyme. The production of the enzyme was maximum in the pH range 6.3- 6.5 (Fig 28A) and hence, in the subsequent SSF experiments, incubation pH was maintained at 6.3.

Temperature of incubation during growth had a significant influence on the enzyme production. Temperature of 25°C was suboptimal for growth, whereas

growth ceased above 35ºC. The optimum temperature range for growth and milk clotting enzyme production was determined to be 28 ºC -30ºC (Fig 28B).

Maximum production of the enzyme was at the end of 120 h. The enzyme production decreased beyond this period (Fig 29A). Since the enzyme was extracellular in nature, it leached easily into solvent (water having 0.1 M NaCl) during soaking for 60 min at 4ºC. Enzyme was separated from the slurry by filtration using Whatman No.1 filter paper.

In order to optimize fermentation parameters for production of milk clotting enzyme from *A. oryzae*, fermentation was carried out in the presence of different additives to solid substrate (wheat bran). Of the treatments tried, adding defatted soy flour (4%, 50% protein) and skim milk powder (2%, 75-80% protein) to bran as protein supplements increased enzyme production three folds (Fig 29B).

Decolorization using activated charcoal

Pigments from the bran and fungal spores co-leached with the enzyme making the solution brown colored and viscous. The enzyme extract was decolorized using activated charcoal. Charcoal effectively decolorized the material up to 3% as was evident from absorbance values (A_{340}/A_{280} value). Higher charcoal contents resulted in activity loss. Decolorization also reduced contaminating proteins by 30% thereby increasing the specific activity of milk clotting enzyme

to 1474 U/mg. The milk clotting to proteolytic activity increased to 884: 1 (from 500: 1) after decolorization.

Table 18. Screening of fungi for milk clotting activity.

Strain	Culture No.	Milk clotting activity (U/g bran)
Aspergillus awamori	MTCC 886	4285
Chaetomium virescens	MTCC 980	2730
Dictyostelium discoideum	MTCC 1579	3000
Lagenidium giganteum	MTCC 719	4293
Metarhizium anisopliae	MTCC 892	2855
Penicillium roquefortii	MTCC 933	2875
Rhizopus microsporus var. oligosporus	MTCC 556	7750
Trichoderma koningii	MTCC 796	4000
Aspergillus niger	MTCC 2479	525
Aspergillus ochraceus	MTCC 1810	250
Mucor fuscus	MTCC 1462	1938
Mucor rouxii	MTCC 386	5713
Aspergillus oryzae (flavus)	-	1921
Aspergillus carbonarius	CFR 1047	2375
Trichoderma spp.	-	3125
Aspergillus oryzae	**MTCC 5341**	**10,000**

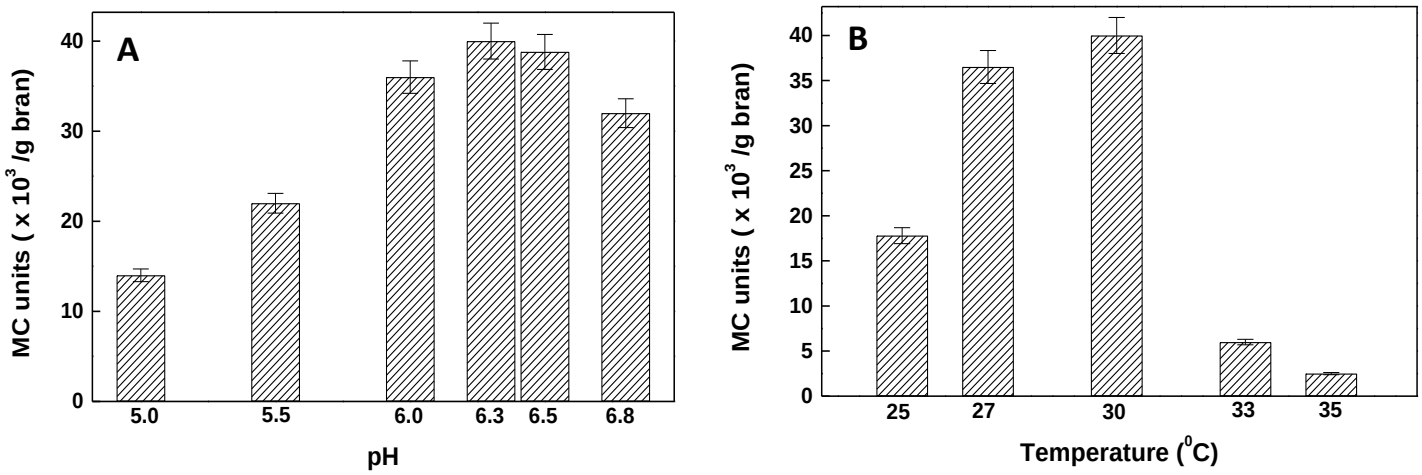


Figure 28. Effect of cultivation pH and temperature on milk clotting enzyme production by *A. oryzae* MTCC 5341

A. Production of milk clotting enzyme by *Aspergillus oryzae* as a function of incubation pH. Activity values are compared at 120 h of fermentation time, where milk clotting activity was highest.

B. Production of milk clotting enzyme by *Aspergillus oryzae* as a function of incubation temperature. Extent of enzyme production is given at 120 h of fermentation time, where *A. oryzae* showed highest milk clotting activity.

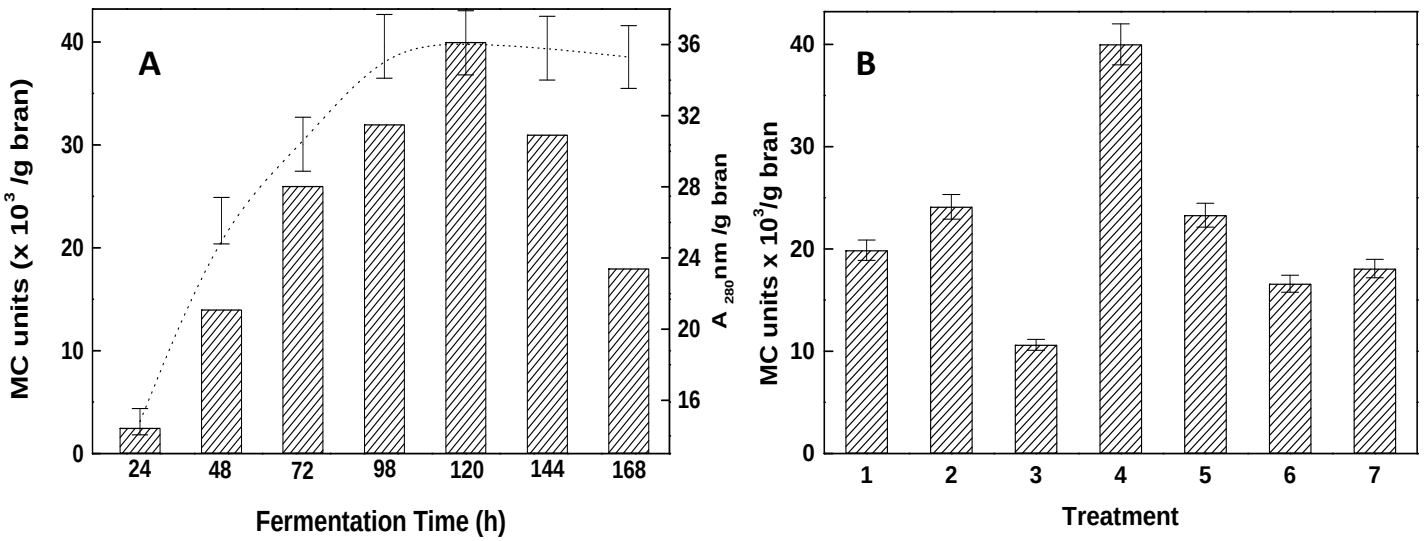


Figure 29. Optimization of fermentation time and substrate conditions for milk clotting enzyme production by *A. oryzae* MTCC 5341

A. Harvesting time for milk clotting enzyme. At the end of each incubation period, enzyme activity was checked, activity units (▨) and absorbance values at 280 nm (·····) were recorded

B. Effect of protein rich supplements on the production of milk clotting enzyme. Wheat bran, 60% moisture content was incubated at 30°C and for 120 h. **1**. Wheat bran (WB); **2**. WB+ 4% defatted soy flour (DSF); **3**. WB + 4% DSF + 2% skim milk powder (SMP) + 0.1% biotin; **4**. WB + 4% DSF + 2% skim milk powder (SMP) cultured at pH 6.3; **5**. WB + 4% DSF + 2% skim milk powder (SMP) + 0.4% phytic acid; **6**. WB + 4% DSF + 2% skim milk powder (SMP) + 0.1mM Al_2Cl_3, and **7**. WB + 4% DSF + 2% skim milk powder (SMP) cultured at 4.8 pH.

Purification and molecular characterization of milk clotting enzyme from *A. oryzae* MTCC 5341

Affinity precipitation with sodium alginate entrapped the decolorized enzyme effectively. The entrapped enzyme could be eluted out with water containing 0.5 M sodium chloride and 0.2 M $CaCl_2$. The enzyme yield was 75 % with a specific activity of 3500 U/mg (Table 19). Immobilization and subsequent purification of milk clotting enzyme also resulted in removal of non specific proteases in the sample, thereby increasing the MC/P ratio from 884 to 1416: 1. Homogeneity of the sample was ascertained by SDS- PAGE and native PAGE, revealed a single band corresponding to 34 kDa (Figure 30A and 30B). The activity of the enzyme was also shown in gels containing co-polymerized gelatin (Figure 30C).

Table 19. Purification of milk clotting enzyme from *A. oryzae* by affinity precipitation

Sample	Total volume (mL)	Total Protein (mg)	Total Milk clotting activity (× 10^3U)	Specific activity (MC) (U/mg)	Proteolytic activity (U/mg)	Yield (%)
Crude	30	120	120	1000	8	100
Charcoal treated	29	78.3	115.4	1474	4.5	96.1
Enzyme recovered after elution with 0.5 M sodium chloride having 0.2 M $CaCl_2$	29	24.6	86.1	3494	2.1	24.7

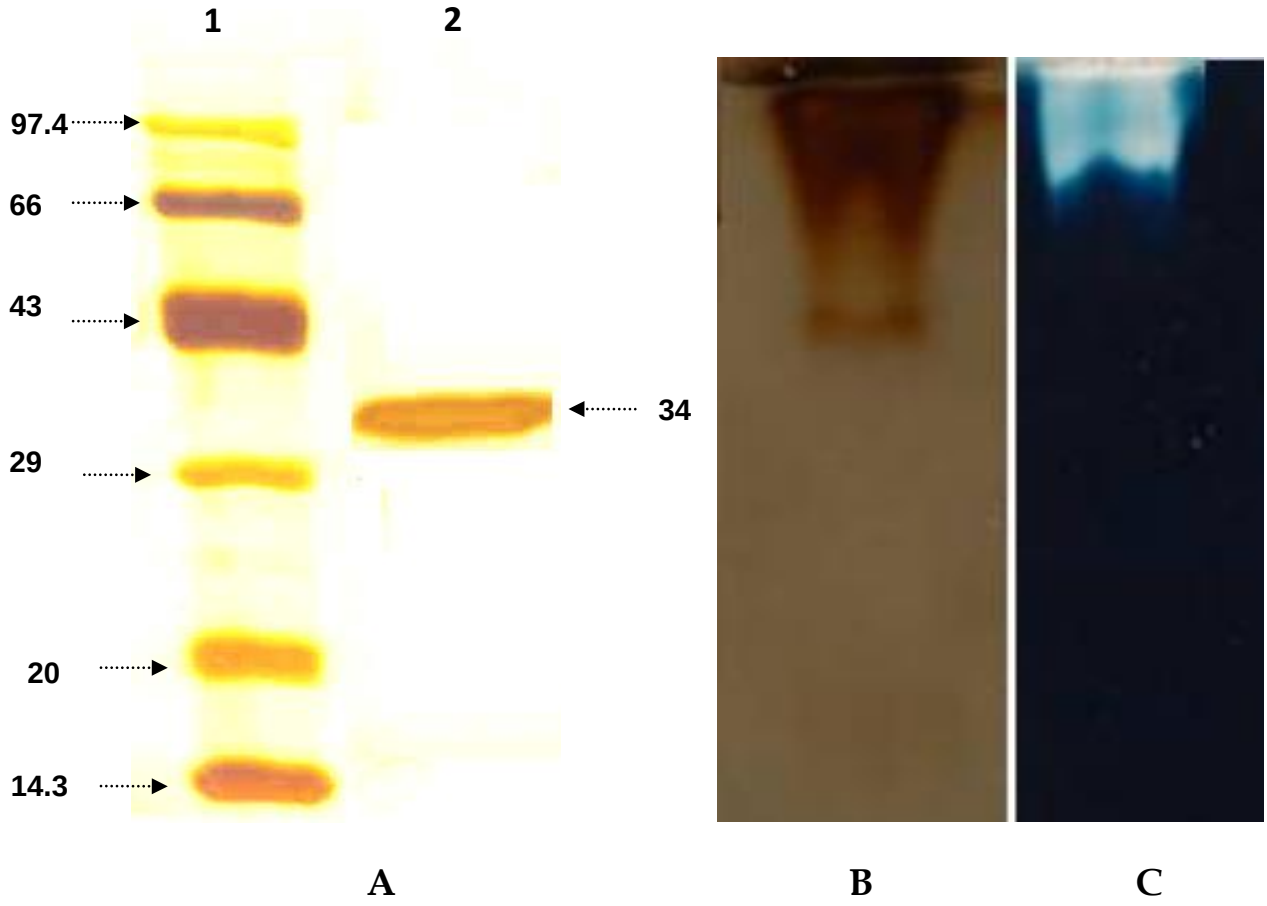


Figure 30. Purification of milk clotting enzyme from *Aspergillus oryzae* MTCC 5341

A. SDS-PAGE pattern and molecular weight determination of acid protease

Lane 1- molecular weight markers and lane 2 – purified milk clotting enzyme

B. Native PAGE pattern of the purified milk clotting enzyme

C. Activity staining in gel electrophoresis for acid protease

Effect of pH on activity and stability of milk clotting enzyme

Optimum pH for activity of the purified milk clotting enzyme from *A. oryzae* was determined to be 6.3. The enzyme was more stable in the pH range 5-8. Beyond this range, on either side, activity of the enzyme reduced drastically. Optimum pH for activity of the entrapped enzyme shifted towards neutral by 0.6 units to 6.9 (Figure 31A).

Effect of temperature on activity and stability of milk clotting enzyme

Milk clotting enzyme purified through affinity precipitation from *Aspergillus* oryzae MTCC 5341 had an optimum temperature of 55°C for activity. The entrapped enzyme was active at a higher temperature range compared to free enzyme. The optimum temperature for activity was shifted by 5 ± 1°C on immobilization to 60°C. The midpoint for thermal inactivation of free enzyme was determined to be 49°C (Figure 31B). Activity was completely lost at 62°C.

Effect of calcium on activity of milk clotting enzyme

Milk clotting enzyme from *A. oryzae* was also responsive to addition of calcium. Coagulation rate was increased with addition of calcium up to 0.2 % (Figure 31C).

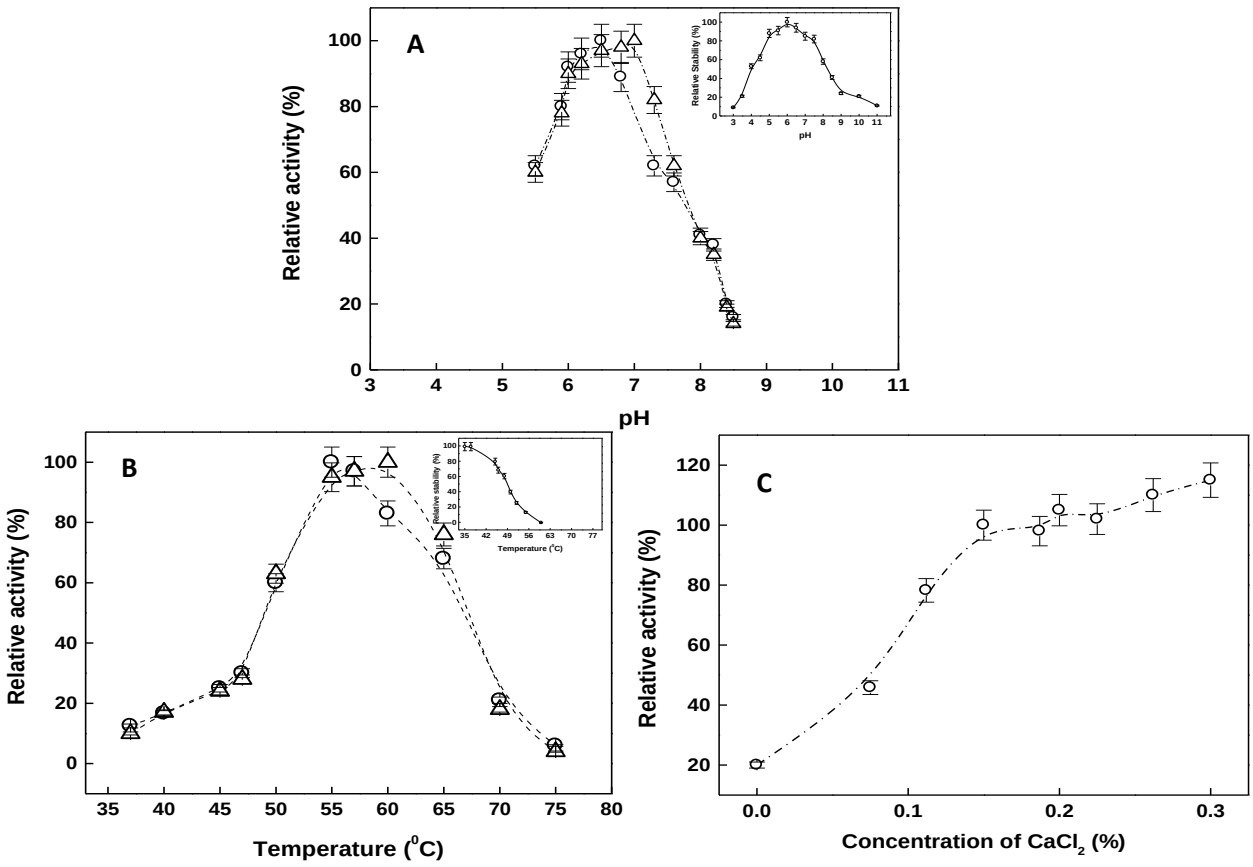


Figure 31. Effect of assay pH, temperature and calcium ions on milk clotting activity of the purified enzyme from *A. oryzae*

A. Effect of pH on activity of purified (O) and entrapped milk clotting enzyme (△). The assay was carried out in buffers in the pH range 5.5-8.5. ***Inset*** Stability of milk clotting enzyme after purification. Enzyme was incubated in test buffers for 24 h at 27°C and residual activity is compared with the control (unincubated enzyme).

B. Effect of temperature on activity of purified (○) and entrapped milk clotting enzyme (△). The assay was carried out at pH 6.3 at the test temperature in the range 35-75°C. ***Inset*** Stability of purified milk clotting enzyme. The enzyme was incubated at the test temperature for 15 min and residual activity is compared with unincubated enzyme.

C. Effect of calcium chloride on the activity of milk clotting enzyme from *A. oryzae* MTCC 5341. The purified milk clotting enzyme was used in the experiment.

Effect of inhibitors on milk clotting enzyme from *A. oryzae*

Protease inhibitors were employed to identify group at the active site of this enzyme (Table 20). The enzyme retained 97% of relative activity in presence of iodoacetamide precluding the role of –SH group in enzyme activity. Similarly, phenylmethane sulphonyl fluoride and benzimidine did not inhibit the enzyme activity; hence involvement of serine residue in the active site has been ruled out. EDTA did not alter the activity of the enzyme showing that the enzyme did not belong to metalloproteases class. The strong inhibition by 78 and 97% at 10 and 20 μm of pepstatin respectively, point towards the presence of aspartate at active site.

Analysis of casein hydrolysis

The casein components were hydrolyzed by milk clotting enzyme from *A. oryzae* MTCC 5341. The enzyme protease showed different degree of hydrolysis towards different components of casein. Analysis of the time course hydrolysis of casein components, by peptide PAGE electrophoresis, revealed that α-casein is hydrolyzed extensively compared to β- and κ- casein. The degree of hydrolysis varied with time, α- and β- casein were degraded increasingly from 5 min to 12 h. The main peptide band, obtained from each of the casein component as a result of hydrolysis, is indicated by an arrow in Figure 32. Alpha casein was completely hydrolyzed compared to β- casein, (partially hydrolyzed) at the end

of 12 h (Fig 32). Most of the κ- casein was hydrolyzed during first 30 min, the degradation was minimal thereafter.

A higher degree of hydrolysis on κ- casein was seen in the primary phase of milk clotting. Alpha and beta caseins showed a higher degree of hydrolysis than κ-casein, α-casein was almost completely hydrolyzed at the end of 12 h reaction, showing the protease has little specificity among casein components.

Table 20 Relative inhibition of milk clotting enzyme from *A. oryzae* by class-specific protease inhibitors

Inhibitor	Concentration (mM)	Relative activity (%)
Iodoacetamide	2	97
	4	94
Ethylenediaminetetraacetic acid (EDTA)	5	100
	10	100
Benzamidine	2	96
Phenylmethane sulphonyl fluoride	1	98
(PMSF)	2	96
Pepstatin	0.01	22
	0.02	3

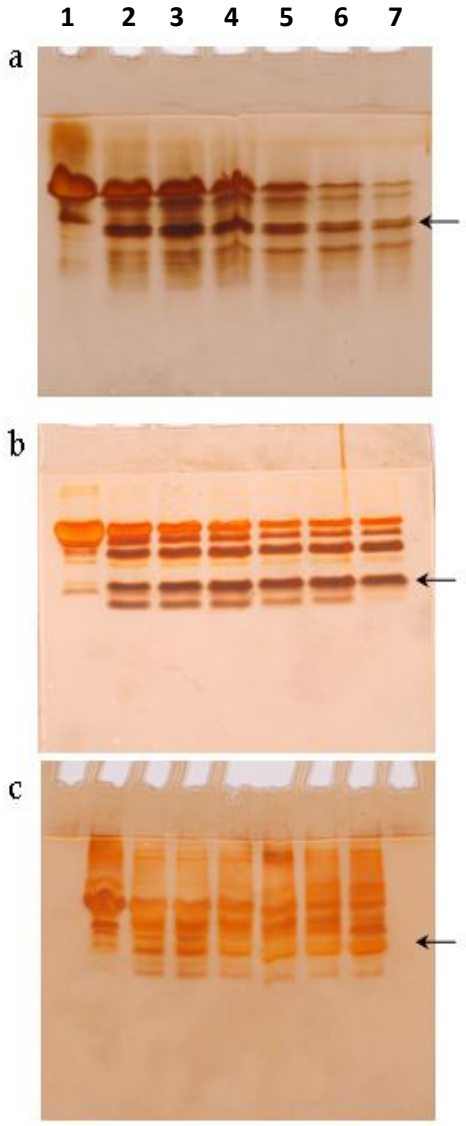


Figure 32. Peptide PAGE electrophoregrams of casein compositions by milk clotting enzyme from *Aspergillus oryzae* MTCC 5341 at times 0, 5, 15, 30, 60, 120 min and 12 h (corresponding lanes labeled from 1 to 7). The main peptide is indicated by an arrow; **a**= *α*-casein; **b**= *β*- casein; **c**= *κ*- casein.

Discussion

Most biotechnological products are proteins and need to be prepared in large volumes with a high degree of purity. Commercial production of proteins is possible by advances in genetic engineering. However, separation and purification techniques for these products have been slow in development. Though the industrial application of these enzymes demands a partially purified enzyme, many biotechnological applications require enzymes in pure form. Thus enzyme purification becomes an integral form of utilization of an enzyme in its fullest extent. Any attempt done in purifying the enzyme should be cost-effective and simple making it economically viable. Though conventional purification methods are tedious, time consuming and expensive, are used in majority of studies of academic interest. Affinity precipitation is an alternative to conventional protein purification techniques and also has several advantages over the other bio-separation strategies for enzymes, being easily scalable and economically viable. The recovery of active protein is high and often the method results in a purification factor of 5–10 (sometimes greater). Further, the process uses inexpensive and easily obtainable materials thus making it suitable for scale-up or commercialization.

In the current study, two aspartate proteases by isolated and purified to homogeneity by conventional and affinity chromatographic methods. The acid was protease purified by conventional chromatographic methods involving ion-

exchange and gel permeation techniques. The acid protease was purified 17 fold with a specific activity of 43, 658 U/ mg. The purified enzyme had the highest reported acid protease activity so far from any fungus (Table 21).

The purified acid protease had an optimum pH and temperature of 3.2 and 55°C for its activity respectively. Acid proteases from *A. oryzae* have been reported to have pH optima in the range 2.5 - 4.0 (Rao et al., 1998). Acid protease was characterized with respect to activity and spectroscopic measurements. Circular dichroism studies of this enzyme are well in accordance with other studies pertaining to acid proteases of different fungal origin (Tello-Solis et al., 1994; Allen et al., 1990; Tello-Solis and Arana, 1995). Pepstatin, a hexapeptide inhibitor, specifically inhibits acid proteases by irreversibly binding to the active site aspartates (Umezawa et al., 1970). Pepstatin inhibited the enzyme competitively with an IC_{50} value of 9.3 μM, which is similar to reported values for acid proteases (Sharma et al., 2005; Kay and Dunn, 1992; Dash et al., 2001; Fusek et al., 1990).

Acid proteases possess the characteristic feature of activating native trypsinogen to active trypsin at low pH (Davidson et al., 1975; Abita et al., 1969; Sanger and Tuppy, 1951). Acid protease from *Aspergillus oryzae* MTCC 5341 activated trypsinogen to form trypsin. Acid protease is found to cleave I-K residues to release active trypsin from trypsinogen.

Acid proteases are endoproteolytic in nature, and show wide range of residue specificity. Most of the acid proteases prefer bulky aromatic and hydrophobic residues at S_1 position (Shintani, and Ichishima, 1994; James et al., 1985; Lowther et al., 1995; Shintani et al., 1996). Acid protease from *Aspergillus oryzae* MTCC 5341 shows endoproteolytic activity and prefers L-V and Y-T residues at S_1 and S_1' positions. Thus the residue specificity for the acid protease was identified as I-K, L-V and Y-T.

Table 21. Comparison of specific activities of purified acid proteases from *Aspergillus sp.*

Sl. no.	Species	Specific activity (U/mg)	Reference
1	A. oryzae	2.54	Davidson et al., 1975
2	*A. oryzae*	**M1**- 16.4	Tsujita et al., 1977
		M2-234	
3	*A. oryzae*	182.5	Kundu et al., 1975
4	*A. niger*	1641	Krishnan et al., 1983
5	*A. kawachi*	144	Yagi et al., 1986
6	*A. oryzae*	**E1**-620	Tsujita and Endo 1977
		E1a-657	
		E1b-644	
		E2-740	
7	*A. oryzae*	660	Tsujita and Endo 1978
8	*A. oryzae*	**F1**-62.4	Tsujita and Endo, 1977
		F2-80.3	
9	***A. oryzae* (MTCC 5341)**	**43,658**	**Current study**

Aspartic proteases also play an important role in the manufacture of cheese in dairy industry. Increased demand for cheese, coupled with ethical and low availability of rennet has led to replacement of calf rennet by microbial milk clotting enzymes. However, these microbial milk coagulants are associated with non-specific and heat stable proteases which lead to the development of bitterness in cheese after storage and a poor yield. Search is on for the low cost methods of producing enzymes that are completely inactivated at the normal pasteurization temperatures and contain very low levels of contaminating proteases.

Microbial milk coagulants from fungal sources can be conveniently produced by solid state fermentation and are useful in the cheese making industry owing to their narrow pH and temperature specificities (Rao et al., 1998). Solid state fermentation offers many advantages over the submerged fermentation method; one of which is the production of milk clotting enzymes with high milk clotting: protease ratio (Pozsar-Hajnal et al., 1974; Ismail et al., 1984). In the present study, *Aspergillus oryzae* produced highest reported milk clotting activity in comparison to available reports (Table 22) and was patented (Vishwanatha et al., 2009).

Table 22. Comparison of milk clotting activity of *Aspergillus oryzae* MTCC 5341 with available reports

Sl.no.	Source	Activity units	Reference
1	*Aspergillus oryzae*	2666.7 U/ g	Shata, 2005
2.	*Mucor bacilliformis*	7500 U/ g	Fernandez et al. , 1999
3.	*Fusarium subglutinans*	78.43 U / mg	Ghareib et al., 2001
4.	*Mucor varianspike*	4000 U/ g	Bernardirelli et al., 1983
5.	*Myxococcus xanthus*	1U/ mL	Poza et al., 2003
6.	*Nocardiopsis sp.*	20U/ mL	Cavalcanti et al., 2004
7.	*Mucor sp.*	700U/mL	Venera et al., 1997
8.	Lettuce	0.19U/mL	Lo Piero, 2002
9.	***Aspergillus oryzae* MTCC 5341**	**40,000 U/g bran**	**Current study**

Cereal bran acts as a nutrient media as well as an anchorage for fungal growth and development. The solid media is supplemented with external nutrient sources for improvement of enzyme yields. In the present study, *A. oryzae* also showed a positive response to the addition of nitrogen sources to the bran. Similar results were also obtained earlier, where addition of organic nitrogen sources like defatted soy flour and skim milk to wheat bran for culturing *M. meihei*, produced a milk clotting activity of 34,000 U/g in comparison to 10,000U/g bran in control (Thakur et al., 1993; Preetha and Boopathy 1997). Optimization of media components such as, incubation pH, temperature and nutrient additions resulted in production of milk clotting enzyme from *A. oryzae* with an activity of 40, 000 U/g bran.

The presence of non-specific proteases poses problem in the effective utilization of microbial milk clotting proteases in cheese-making. Hence, purification of the enzyme is a preferred for better utilization of the enzyme in dairy industry, besides for the functional and structural characterization of the enzyme. There are instances wherein charcoal has been used as the first step of protein purification (Aikat et al., 2001). Decolorization increased specific activity of the milk clotting protease thereby increasing the milk clotting to protease ratio.

The milk clotting enzyme was purified with increased specific activity and milk clotting to protease ratio in a single step purification using sodium alginate as a carrier. Sodium alginate has been used as an affinity matrix of many of the

industrial enzymes (Sardar and Gupta 1998; Teotia and Gupta 2001; Teotia et al., 2001). Alginate being a natural copolymer of mannuronic acid and guluronic acid is a preferred matrix for entrapment of enzymes. It is cost effective and the entrapped enzyme is easily precipitated by addition of a di/trivalent cations. Alginates also offer an inert aqueous environment within the matrix and high gel porosity allows high diffusion rates of macromolecules (Kumar et al., 2006). Immobilization also offered other advantages such as stability against high pH and temperature besides allowing us to reuse the enzyme for many cycles of milk clotting. A comparison of activities of some of the rennets is listed in Table 4. Purified milk clotting enzyme from *A. oryzae* has an MC/P ratio of 4.39 in comparison to 7 in case of recombinant camel chymosin (Kappeler et al., 2006). Other proteases had an MC/P ratio in the range of 0.089 to 2.1. Thus, milk clotting enzyme form *Aspergillus oryzae* showed high activity and high MC/P ratio, when compared to other commercial rennets and other proteases.

pH has a profound impact on milk clotting activity by rennet and pH value less than 5 cause curdling of the milk. Microbial rennets are reported to have an optimal pH between 5.5 -7. In the present study, the milk clotting enzyme has an optimum pH of 6-6.3. This is similar to the pH optimum of chymosin or calf rennet (Okigbo et al., 1985).

Milk clotting enzyme from *A. oryzae* has a midpoint for thermal inactivation of 49°C. This coincides with the midpoint of thermal inactivation for chymosin

(Martin et al., 1980). Thermolability is another preferred quality for microbial milk clotting enzymes as undesired proteolysis of proteins, beyond normal pasteurization temperature, can be avoided.

Calcium ions (up to 0.2%) had a positive effect on increasing the activity of milk clotting enzyme. As reported earlier, calcium ions in the reaction mixture decrease the rennet clotting time (Balcones et al. 1996; Montilla et al., 1995; Storry et al., 1983; Tervala et al., 1985; Kumar et al., 2005). Addition of calcium also reduces pH of milk solution thereby hastening protein aggregation (Gastaldi et al., 1994; Mehaia and Cheryan 1983). Curd firmness, gel strength, aggregation rate and adhesiveness are improved with the addition of calcium (Patel and Reuter 1986; Solorza and Bell 1998; Cavalcanti et al., 2004). Strong inhibition by pepstatin, an aspartate specific protease shows that the milk clotting enzyme from *A. oryzae* has aspartate residues in its active site. Inefficiency of other class specific inhibitors supported the conclusion that the enzyme belong to aspartate protease group.

Casein hydrolysis has been studied with rennet-like enzyme from glutinous rice wine, where the enzyme was more active on α-casein than the other two fractions (Jiang et al., 2007). Caseinolytic specificity among the casein components which readily hydrolyze peptide bonds in α- and β- casein by microbial rennets have been reported (Garg and Johri 1994; Krause et al., 1998). Although the change in cheese properties during ripening and storage is related

to the combined effects of hydrolysis of α-and β- casein, many bitter peptides that are essential to the flavor were found originating from them (Singh et al., 2005).

Our intension was to isolate high activity aspartate proteases from fungal source for the possible application in food industries. We have demonstrated the isolation and purification of two aspartate proteases from A. oryzae MTCC. The current study reports production of milk clotting enzyme from *Aspergillus oryzae* MTCC 5341 with (i) high specific activity (ii) lower thermostability (iii) high MC/P ratio in comparison to existing microbial rennets, making it an ideal enzyme for cheese making industries.

Section 2: Conformational stability of acid protease from *A. oryzae:* Effect of pH, temperature and chemical denaturants on structure-function of acid protease

The stability of acid protease were studied as a function of pH, temperature and chemical denaturants by activity measurements, intrinsic tryptophan fluorescence, circular dichroism and ANS binding studies. The results revealed that hydrophobic and electrostatic interactions of acid protease play an important role in maintaining proper structure for activity and stability.

Effect of pH on conformation of acid protease from *A. oryzae* MTCC 5341

Effect of pH on enzyme activity

Acid protease was incubated at different pH values ranging from 2-10. The effect of pH on enzyme activity was assessed by measuring the residual activity at pH 3.2, under standard assay conditions. The enzyme retained activity in the pH range of 2.5-6.5. Below and above this range, the enzyme was irreversibly inactivated.

Intrinsic Fluorescence properties

Fluorescence emission spectra of acid protease when excited at 280 nm, at various pH values are shown in Figure 33. At the optimum pH of enzyme activity, the emission λ_{max} was 330 nm which was not significantly affected up to a pH of 6.0. At pH values > 6.0, increased fluorescence accompanied by a red shift of +25 nm was observed. At

extremes of pH (2 and 10), the fluorescence emission intensity decreased significantly coupled with a red shift. The acidic and alkaline transitions occurred below 2.0 and above 7.5, respectively. The midpoints of acidic and alkaline transitions were 1.7 and 7.8, respectively.

ANS binding studies

ANS has been shown to bind to hydrophobic regions that become exposed during unfolding of the protein. Figure 34 shows changes in fluorescence emission intensity at 496 nm as a function of pH with corresponding changes in emission $\lambda_{max.}$ At the pH range of 3.5-6.0 there was no change in ANS fluorescence. However increased ANS binding was observed at pH 2.0 with a concomitant blue shift in the emission maximum of 16 nm. Similar results were also noticed in the alkaline region where the amount of ANS molecules bound to the protein significantly increased, indicating the exposure of hydrophobic residues.

Circular dichroism measurements

The far UV CD spectra of acid protease did not change significantly in the pH range of 3-6. At pH 4.5, where acid protease is in its native state, the far UV CD spectrum has a shape typical of a β-sheet-rich protein (Figure 35). In the pH range 8.0–10.0, where acid protease is in alkaline denatured state, the spectrum indicates α-helical character. Analysis of the far UV CD spectra of acid protease in alkaline denatured state indicates that it has higher α-helical and smaller β-sheet content than that observed in the native

state of acid protease (Table 23). The minima shift to 222 nm that is typical of α-helix. The analysis of CD spectra suggested that at high alkaline pH, the loss of activity was associated with the complete loss of tertiary structure and decreased the ordered structure. The acid inactivation was due to disruption of tertiary structure with a significant increase in β-structure.

Table 23. Secondary structure analysis of acid protease incubated at different pH values

pH	α-helix (%)	β-sheet (%)	Random (%)	Total (%)
3.0	9	62	29	100
3.2	8	58	33	99
4	11	58	30	99
5	11	60	29	100
6	11	52	36	99
8	15	56	27	98

Analysis was done using the programme CDSSTR (Dichroweb) as given under materials and methods

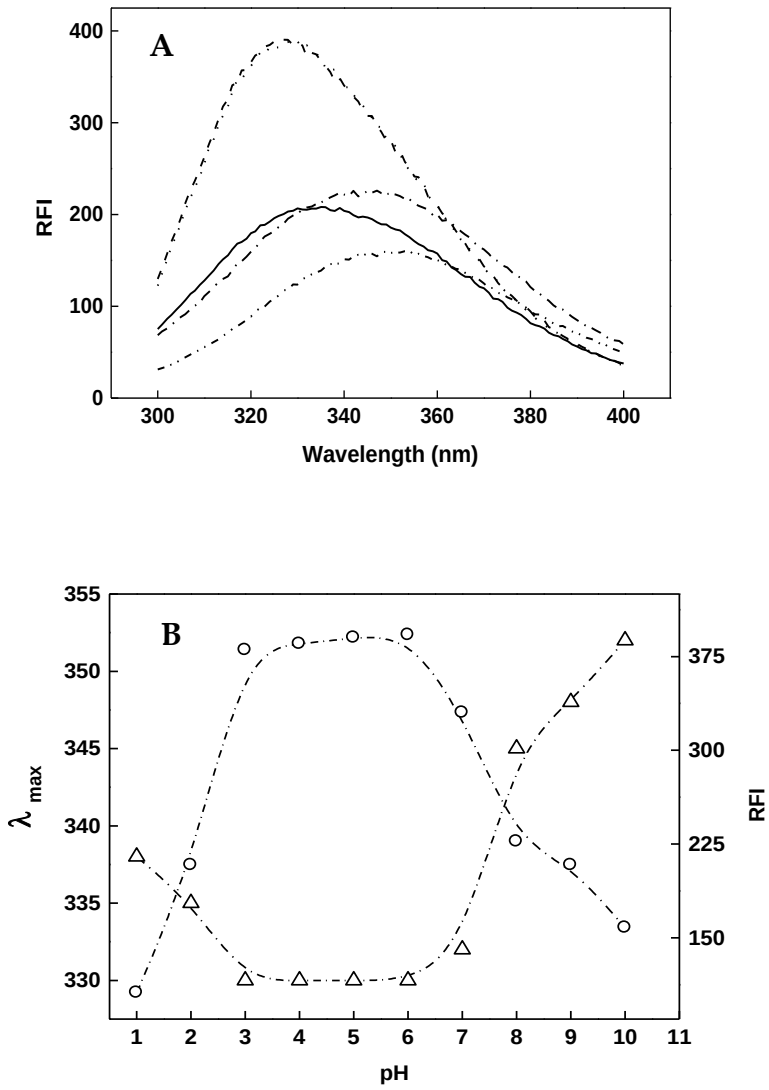


Figure 33. pH dependence of the fluorescence emission spectra of acid protease

A. Acid protease (45μg/ml) was incubated with the same ionic strength buffer solutions of pH: 2 (solid), 4 (dash), 6 (dotted), 8 (dash dot), and 10 (dash dot dot). The enzyme samples were incubated for 2 h at 27 ºC. Measurements were made with excitation wavelength of 280 nm.

B. pH dependence of the fluorescence emission intensity at 330 nm (△) and the respective changes in emission λ_{max} (○).

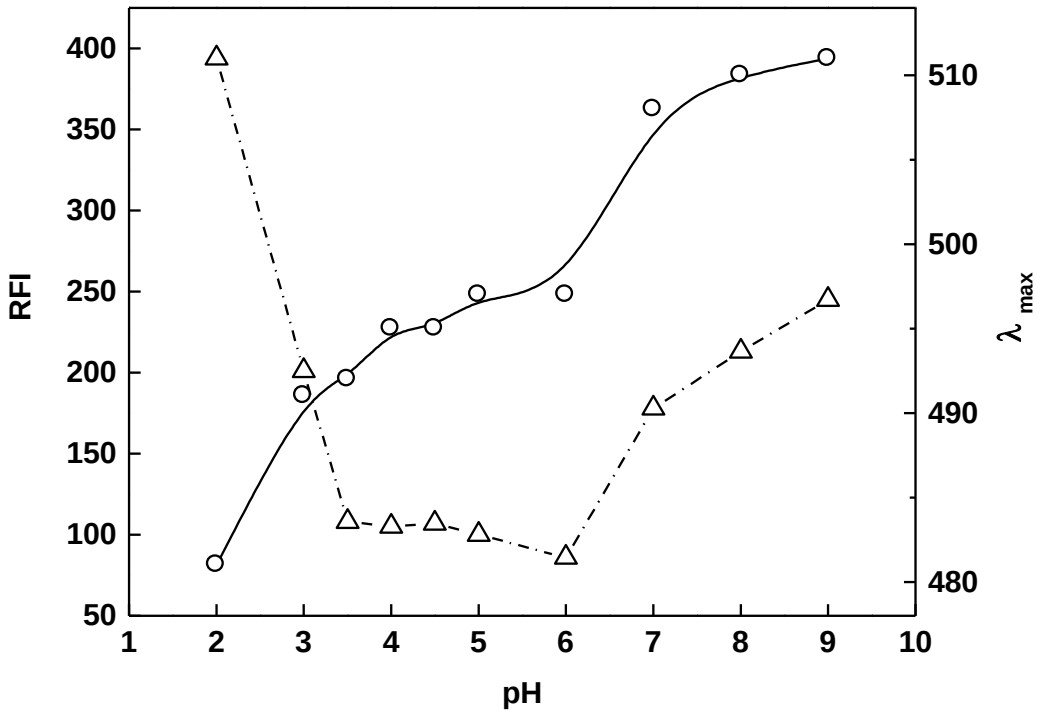


Figure 34. ANS binding with acid protease as a function of pH

Measurements were made from pH 2 to 10 with a protein concentration of 45 μg/ml and ANS concentration of 100 μM. Fluorescence emission intensity (○) of acid protease with corresponding change in λ_{max} (△) is plotted. Acid protease was incubated with ANS in various buffered solutions at 27 °C for more than 1 h in dark and its fluorescence spectra were recorded at an excitation wavelength of 375 nm

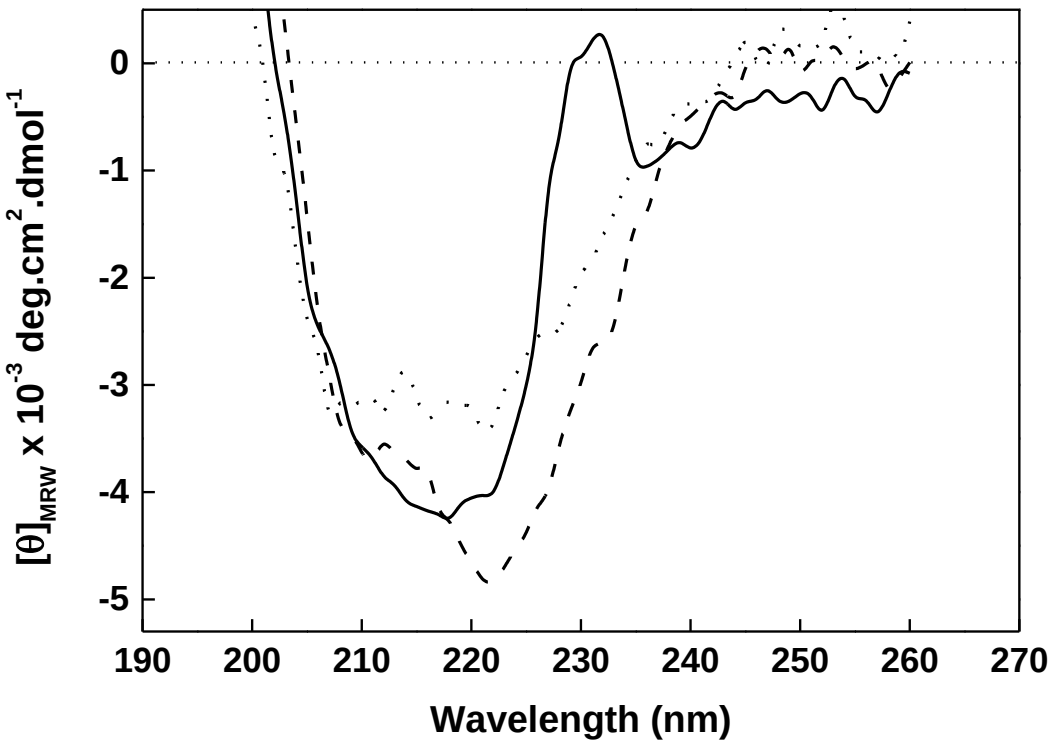


Figure 35. Far UV CD spectra of acid protease at pH 2.5 (dotted line), pH 4.5 (solid line) and pH 8 (dashed line). The spectra were recorded after 24 h incubation at 25 °C. A protein concentration of 12 μM was used. Average of three independent experiments is plotted.

Molecular sieve chromatography by HPLC: Effect of pH on the state of association

The effect of pH on the size of acid protease was followed by gel filtration on HPLC. The native enzyme eluted at 8.5 min, as a single peak, when detected at 280 nm at pH 6.0, where the enzyme is stable (Figure 36). At pH 3.0, two peaks were seen, which elute at 6.2 and 17.2 min. Increase in pH above 6.0 resulted in altered elution time. At pH 7.0 and 8.0, the protein eluted at 6.0 and 5.2 min respectively. The change in the elution time was not reversible with pH reversal, indicating clearly a change in the state of association of the molecule.

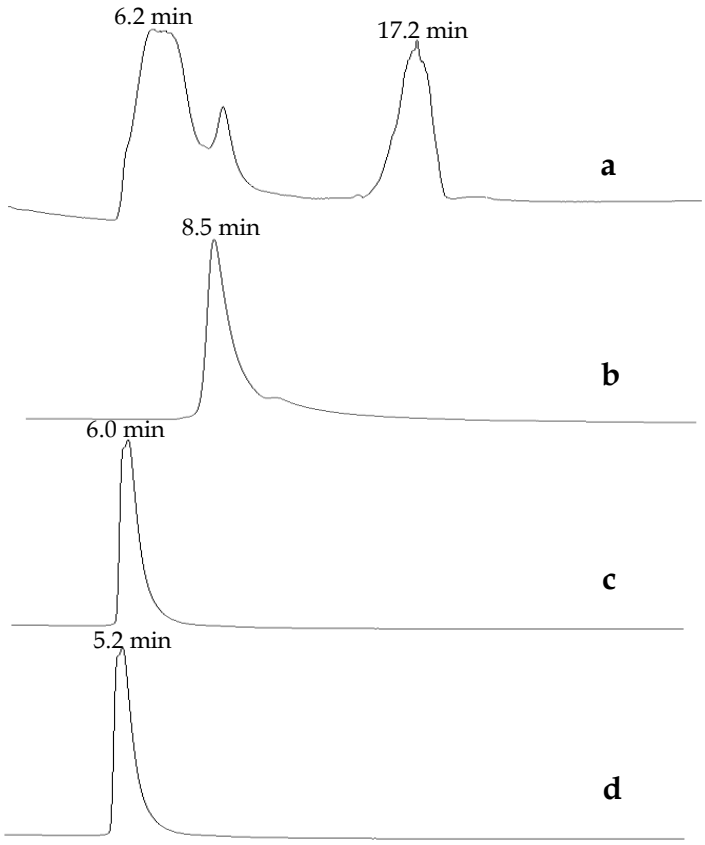


Figure 36. The effect of pH on the elution profile of acid protease by size exclusion chromatography on HPLC: Acid protease at (a) pH 3.0, (b) pH 6.0, (c) pH 7.0 and (d) pH 8.0

Temperature induced unfolding of acid protease

Thermal denaturation of acid protease was studied by various spectroscopic techniques, including UV-absorbance, fluorescence, DSC and CD measurements. The thermal unfolding of acid protease is shown in Figure 37. The transition curves reflect cooperative unfolding process with strong differences in the transition temperature, T_m. Acid protease exhibited an apparent T_m of 57±1°C. Tertiary structure was lost at 57°C, while the enzyme retained some amount of secondary structure up to 71.4°C. The temperature induced structural transition was characterized by an endothermic peak in DSC (Figure 37 inset), a slight decrease in 280 nm absorbance, an obvious rise in the molar ellipticity at 222 nm, combined with a marked decrease in intrinsic fluorescence. Thermal unfolding was found to be irreversible, since no peak was observed on reheating after cooling the sample.

Thermal inactivation kinetics

Thermal inactivation kinetics of the enzyme was determined by incubating the enzyme in 20 mM phosphate buffer (pH 6,), at the test temperature for varying intervals of time. Thermal inactivation was found to follow first order kinetics, indicating the existence of a single inactivation mechanism. The time at which loss of activity reached 50% was taken as the experimental half-life for the enzyme and the rate constant determined (Figure 38A). Thermodynamic parameters were estimated, from the Arrhenius plot constructed, using the rate constants at different temperatures. E_a (activation energy) was determined to be 65.82 ± 0.17 kcal.mol^{-1}, from the slope of the Arrhenius plot

(Figure 38B). The activation enthalpy (ΔH^*), entropy (ΔS^*) and free energy change (ΔG^*) were calculated to be 65.16 ± 0.01 kcal.mol^{-1}, 128.05 ± 0.25 cal.mol^{-1}.$^{\circ}$K^{-1} and 23.35 ± 0.24 kcal.mol^{-1}, respectively.

Thermal aggregation of acid protease

No visible aggregation was observed when the native enzyme was heated at 65ºC. Pepstatin, a known inhibitor of aspartic proteases, was used to monitor its effect on thermal inactivation of acid protease. Addition of 20 μM pepstatin to acid protease resulted in complete inactivation at 27°C. The apparent $T_{1/2}$ of the pepstatin inhibited enzyme was found to be higher than that of native enzyme (63.5ºC as compared to 57ºC) (Figure 39A).

When increase in turbidity was studied as a function of concentration of the pepstatin inhibited enzyme in the range of 0.01-0.1mg/ml, the initial rate of aggregation obeyed a linear relationship with the protein concentration with the kinetic order close to one (Figure 39B). Aggregation and autolysis was also visualized on native and SDS-PAGE gels, respectively (Figure 40A).

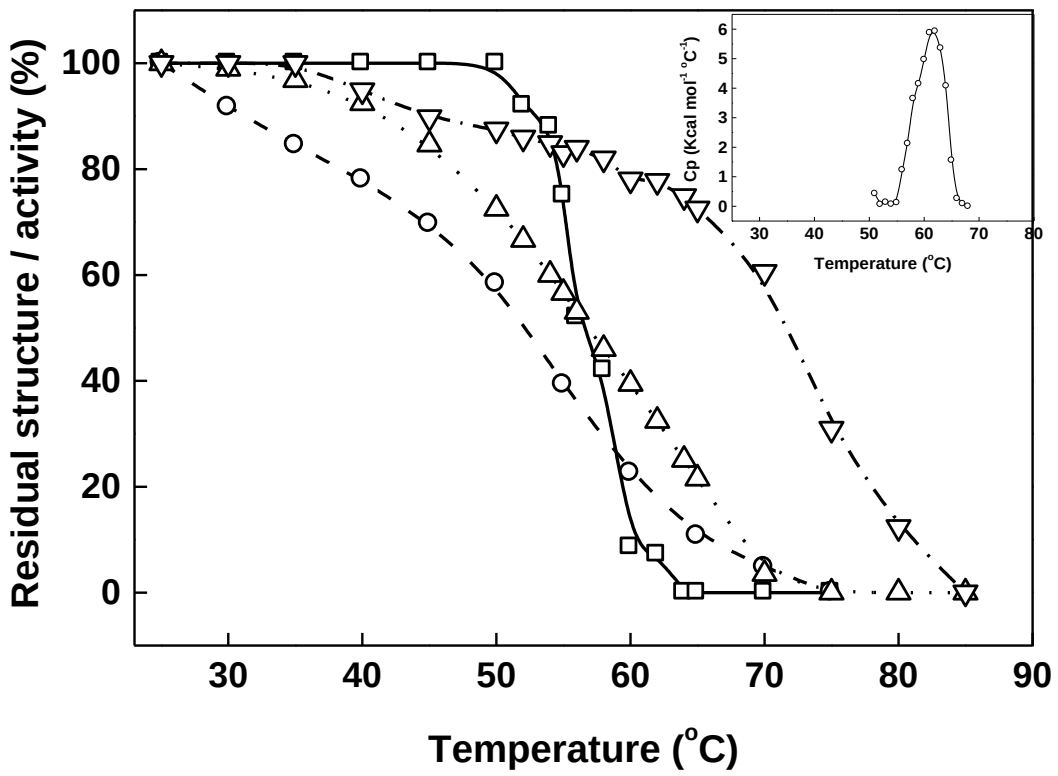


Figure 37. Thermal unfolding of acid protease from *A. oryzae*

Normalized thermally induced unfolding of acid protease was measured by residual activity (solid line,□), recording molar ellipticity at 222 nm (dash line,▽), absorption spectra at 280 nm (dotted line,△) and changes in fluorescence intensity (dash dot line, ○). **Inset** shows the DSC curves for thermal unfolding of acid protease at pH 6.0.

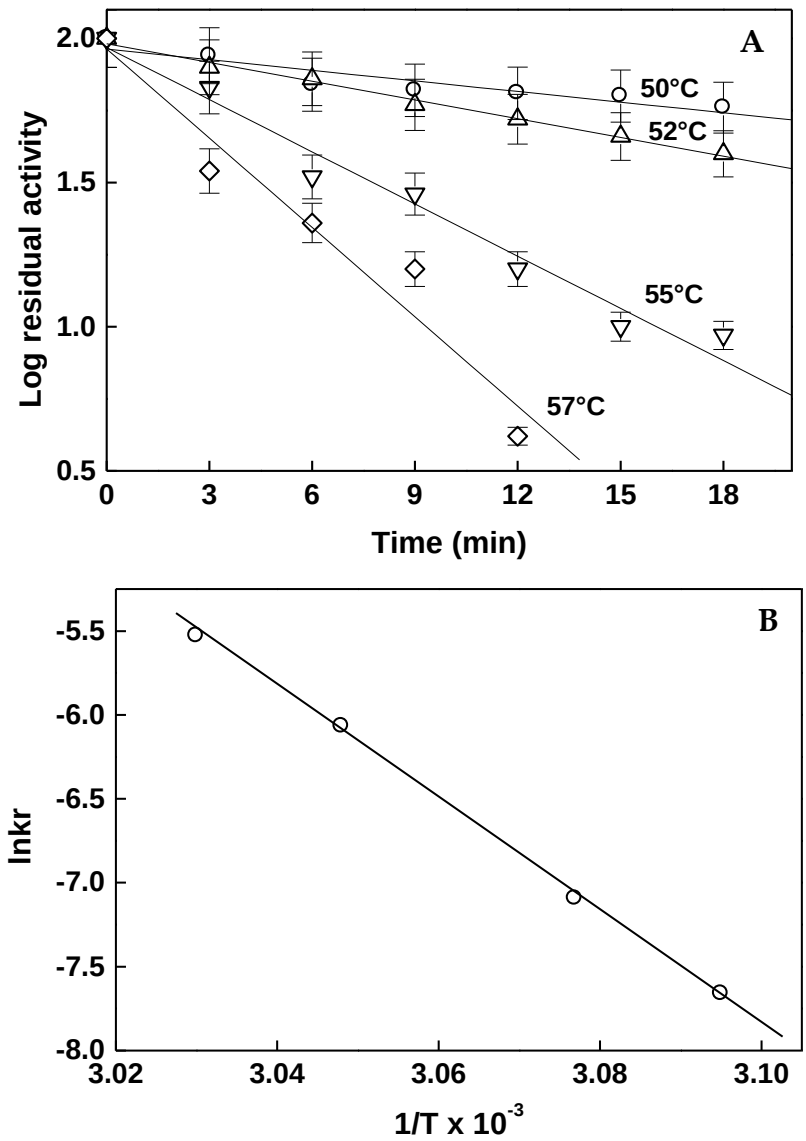


Figure 38. Thermal inactivation kinetics for acid protease from *Aspergillus oryzae*

A. Thermal inactivation profile of acid protease. Enzyme was incubated at 50ºC (○), 52ºC (△), 55ºC (▽) and 57ºC (◇), aliquots were drawn at different intervals of time, cooled to 4ºC and the remaining activity was measured under standard assay conditions.

B. Arrhenius plot for the thermal inactivation of acid protease. The slope of the plot equals $-E_a/R$.

The role of hydrophobic regions in the protein in maintaining stability was studied by intrinsic fluorescence and ANS binding measurements. Figure 41A shows emission spectra of acid protease following excitation at 280 nm. The emission spectra of native protein revealed a peak at 330 nm, indicating that the fluorophores of the protein were in a fairly hydrophobic environment. A diminished intensity, coupled with prominent red shift (+25 nm) observed at higher temperature suggested that the chromophores were more exposed to the solvent. The larger spectral widths of emission spectra also indicated the higher heterogeneity of fluorophore environments in the unfolded state.

ANS fluorescence was followed in the presence of acid protease at 27°C and 65°C. As it can be seen, in Figure 41A inset, the unheated enzyme bound ANS revealed a peak at 496 nm. A red shift of +42 nm with a significant decrease in fluorescence intensity was seen upon heating the same at 65°C.

Fluorescence quenching by acrylamide was studied using pepstatin inhibited enzyme and the Stern-Volmer constant (K_{sv}) calculated at 27°C and 65°C. As given by Figure 41B, K_{sv} increased about 2.5 folds at 65°C, suggesting that the protein fluorophores had become more solvent accessible and/ or mobile at high temperature.

Anti aggregation effect of chaperone-like proteins: caseins

Alpha and beta caseins are reported to have chaperone like character. Acid protease was mixed with α or β- casein separately prior heating at 65°C. Little or no turbidity observed with the addition of chaperone- like proteins in the concentrations used. Both

α and β- casein offered thermal protection in a concentration -dependant manner (Figure 42A and 42B).

Effect of kosmotropic and chaotropic anions on thermal aggregation of acid protease

Thermal aggregation of the enzyme was studied in the presence of anions like, SO_4^{-2}, Cl^{-1} and SCN^{-1}, which differ with respect to their impact on water structure around the protein. At 65°C, both Cl^{-1} and SO_4^{-2} enhanced heat induced aggregation (Figure 43A) but the chaotropic anion SCN^{-1} suppressed the effect (Figure 43B). The anti- aggregation effect of SCN^{-1} showed a clear dependence on its concentration. Addition of SO_4^{-2} gradually induced aggregation, which was otherwise inhibited by 600 mM SCN^{-1} (Figure 43C). These findings strengthen the importance of hydrophobic interactions in maintaining acid protease structure. Chaotropic agent such as, SCN^{-1} induce breakage of water structure thereby weakening the hydrophobic interactions leading to reduced rate and extent of aggregation.

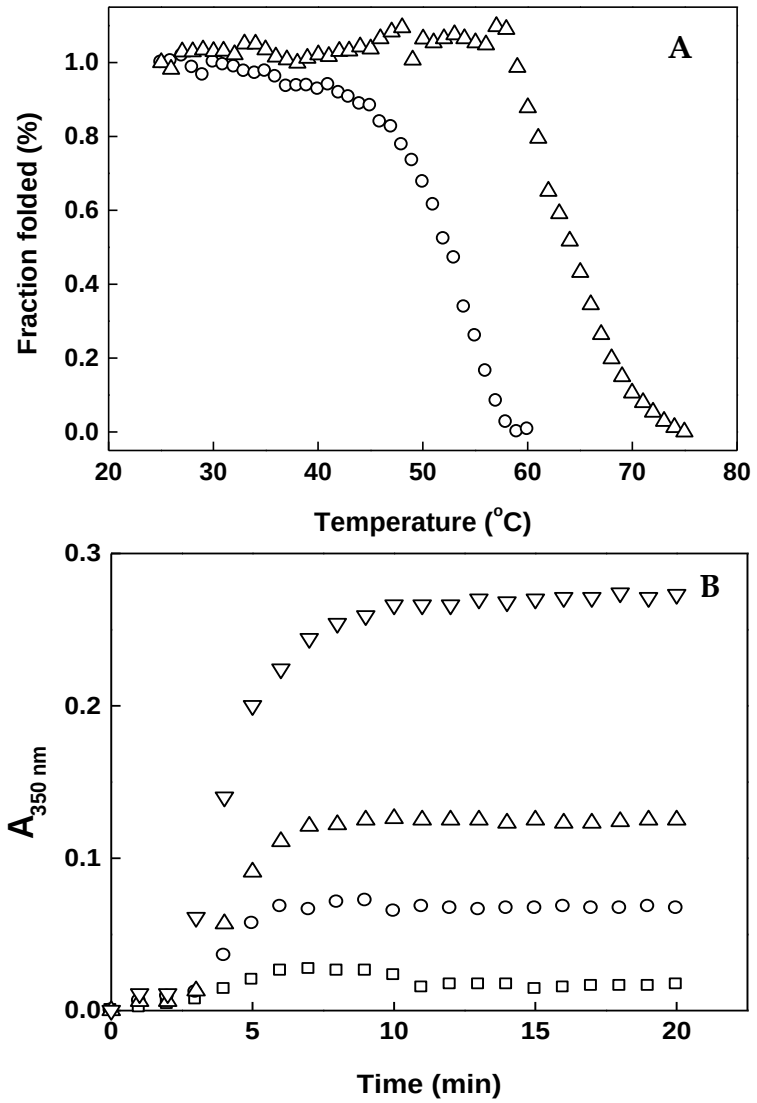


Figure 39. Effect of pepstatin and concentration of protein on thermal unfolding of acid protease.

A. Thermal unfolding of native (○) and pepstatin inhibited (△) acid protease as followed by changes occurring during absorption spectra at 280 nm.

B. Effect of protein concentration on the kinetics of pepstatin inhibited acid protease aggregation at 65 °C, as followed by measuring the relative OD at 350 nm. Protein concentration was 10 (□), 25 (○), 50 (△) and 100 (▽) µg/ml

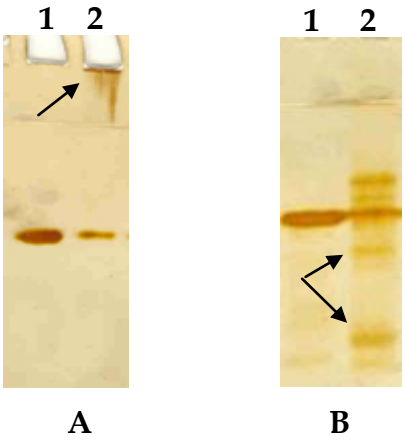


Figure 40. Thermal aggregation and autolysis of acid protease in Native and SDS-PAGE

A. Native PAGE showing aggregation as a result of heating pepstatin inhibited acid protease at 65°C. Lane 1-Native enzyme at 27°C, Lane 2-pepstatin inhibited enzyme at 65°C. Arrow indicates the aggregated enzyme.

B. SDS-PAGE showing autolysis as a result of heating native acid protease at 65°C. Lane 1-native enzyme at 27°C, Lane –native enzyme at 65°C. Arrows indicate hydrolyzed fragments enzyme as a result of autolysis.

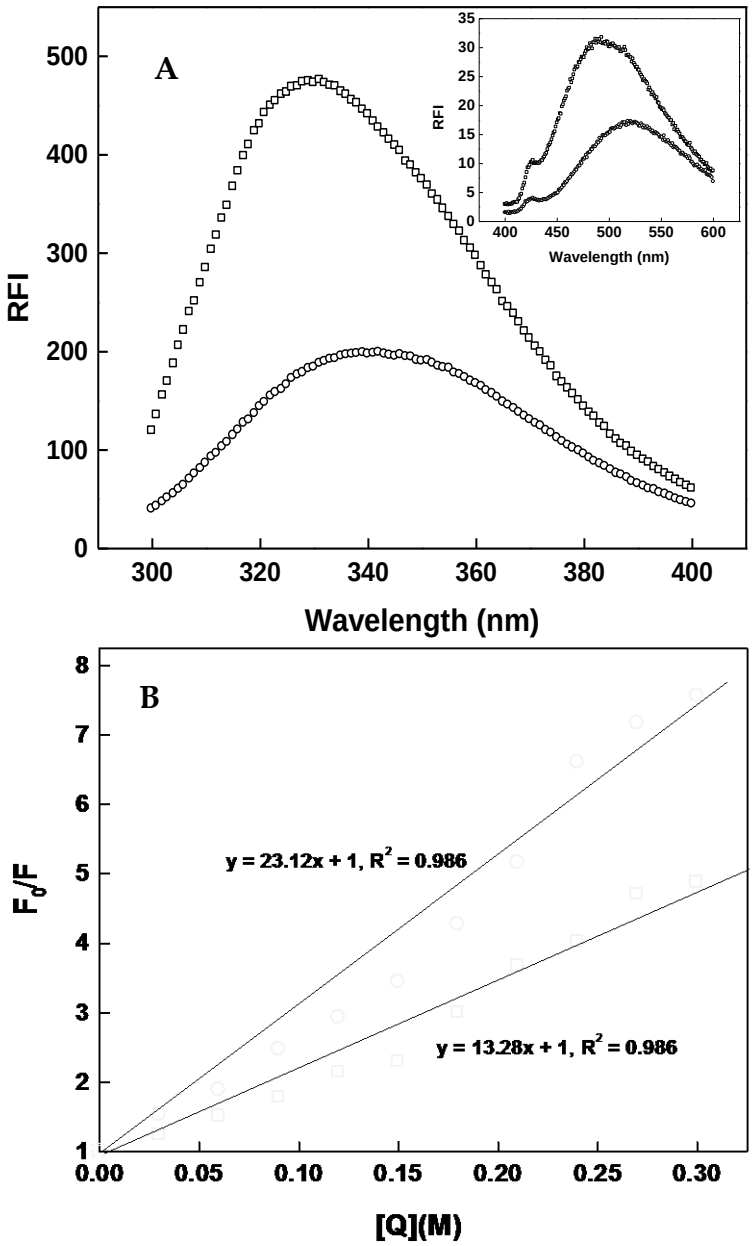


Figure 41 A. Intrinsic fluorescence emission spectrum of acid protease at 25 °C (□) and 65 °C (○) when excited at 280 nm. **Inset:** Fluorescence emission spectra of ANS at 25 °C (□) and 65 °C (○). **B.** The quenching effect of acrylamide on the intrinsic fluorescence of acid protease at 25 °C (□) and 65 °C (○) as a function of quencher concentration. The excitation wavelength was 280 nm.

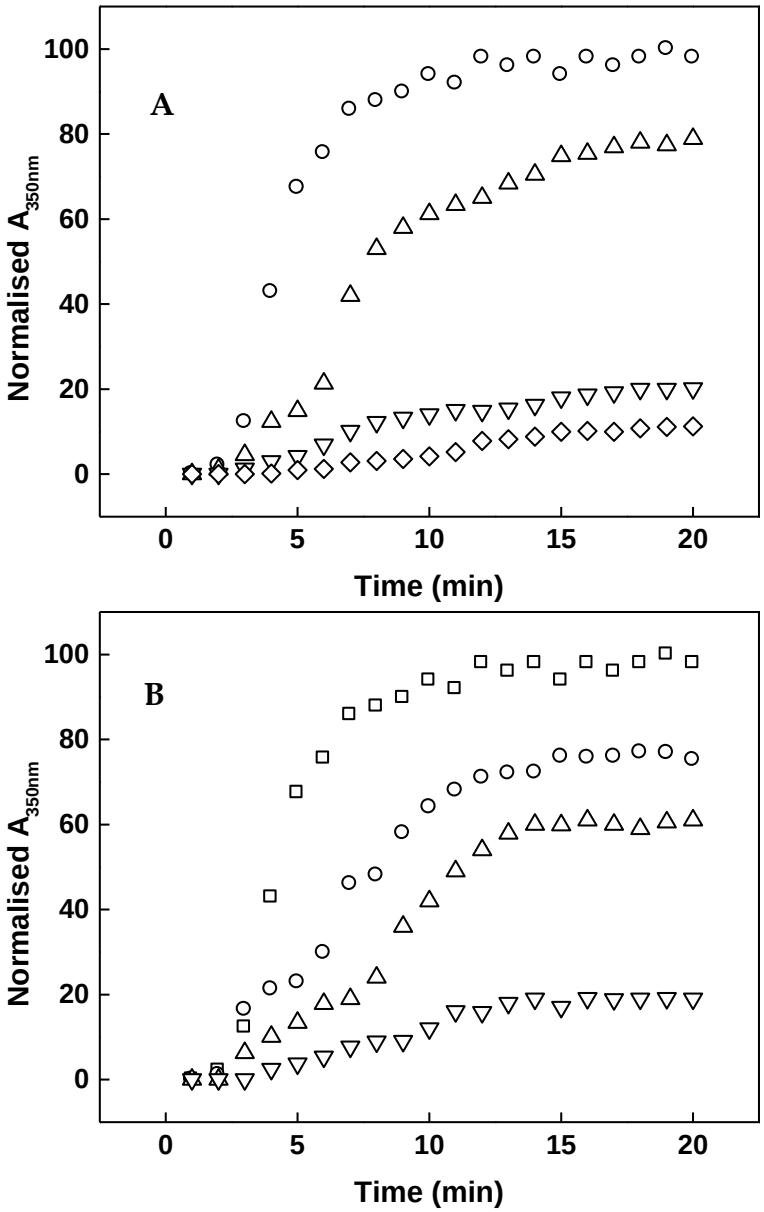


Figure 42 The anti-aggregation effect of α- and β-caseins on acid protease aggregation at 65 °C. Protein concentration was 0.05 mg/ml. **A.** with α-casein concentrations of 0 (○), 0.5 (△), 1 (▽) and 2 (◇) mg/ml. **B.** with β-casein concentrations of 0 (□), 0. 5 (△), 1 (▽) and 2 (◇) mg/ml.

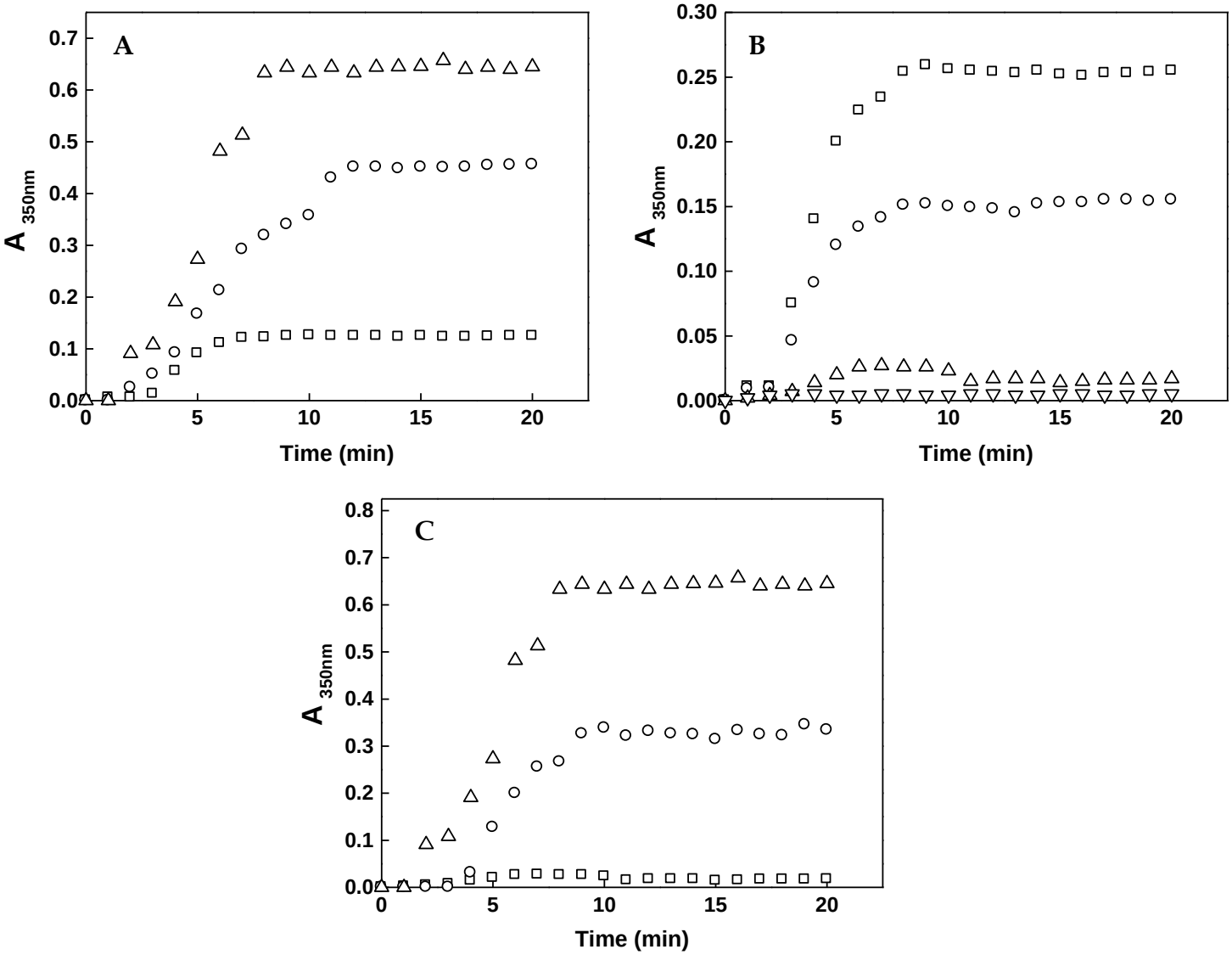


Figure 43. Effect of kosmotropic and chaotropic anions on thermal aggregation of acid protease. **A.** Effect of kosmotropic anions on thermal aggregation of acid protease at 65°C. Figure shows aggregation pattern of native enzyme (□) and enzyme incubated with 250 mM NaCl (○) and 120 mM SO_4^{-2}. **B.** Effect of 0 (□), 100 (○), 250 (△) and 600 mM (▽) SCN^{-1} on thermal aggregation of 0.05 mg/ml acid protease at 65 °C. **C.** Partial removal of the anti-aggregation effect of 250 mM SCN^{-1} by 0 (□), 60 (○) and 120 (△) mM SO_4^{-2}. Acid protease concentration was 0.05 mg/ml and the temperature was 65 °C.

Effect of chemical denaturants

GuHCl induced unfolding

GuHCl induced changes in the conformation of acid protease were followed by CD (ellipticity at 222nm), fluorescence (wavelength emission maximum) and activity measurements. Acid protease was active below a GuHCl concentration of 0.5 M. All structural changes take place between 0.5 -1.2 M GuHCl with a transition midpoint at 0.8 ± 0.1 M GuHCl. Upon unfolding, significant change in fluorescence intensity was observed along with a red shift of +20 nm (Fig 44A). The far UV CD spectrum also showed significant changes. At 4 M concentration of the denaturant, all secondary structure was lost (Fig 44B). Under the said conditions of experiments, GuHCl induced transition curves were sigmoidal (Figure 45). The loss in proteolytic activity coincided with loss in tertiary and secondary structure, thereby reflecting a good correlation between activity and structure of the protein.

Urea induced unfolding

Enzymatic activity of acid protease was sensitive to unfolding by urea. Unfolding followed by fluorescence indicated that, there was significant decrease in fluorescence intensity with increase in emission maximum from 330 nm to 348 nm. This indicated complete exposure of tryptophan residues to solvent (Fig 46A). The change in molar ellipticity values of acid protease also indicated the change in the conformation of the molecule (Fig 46 B). The loss in activity and structure due to urea followed a single transition with a transition midpoint of 1.8 ± 0.15 M urea (Figure 47).

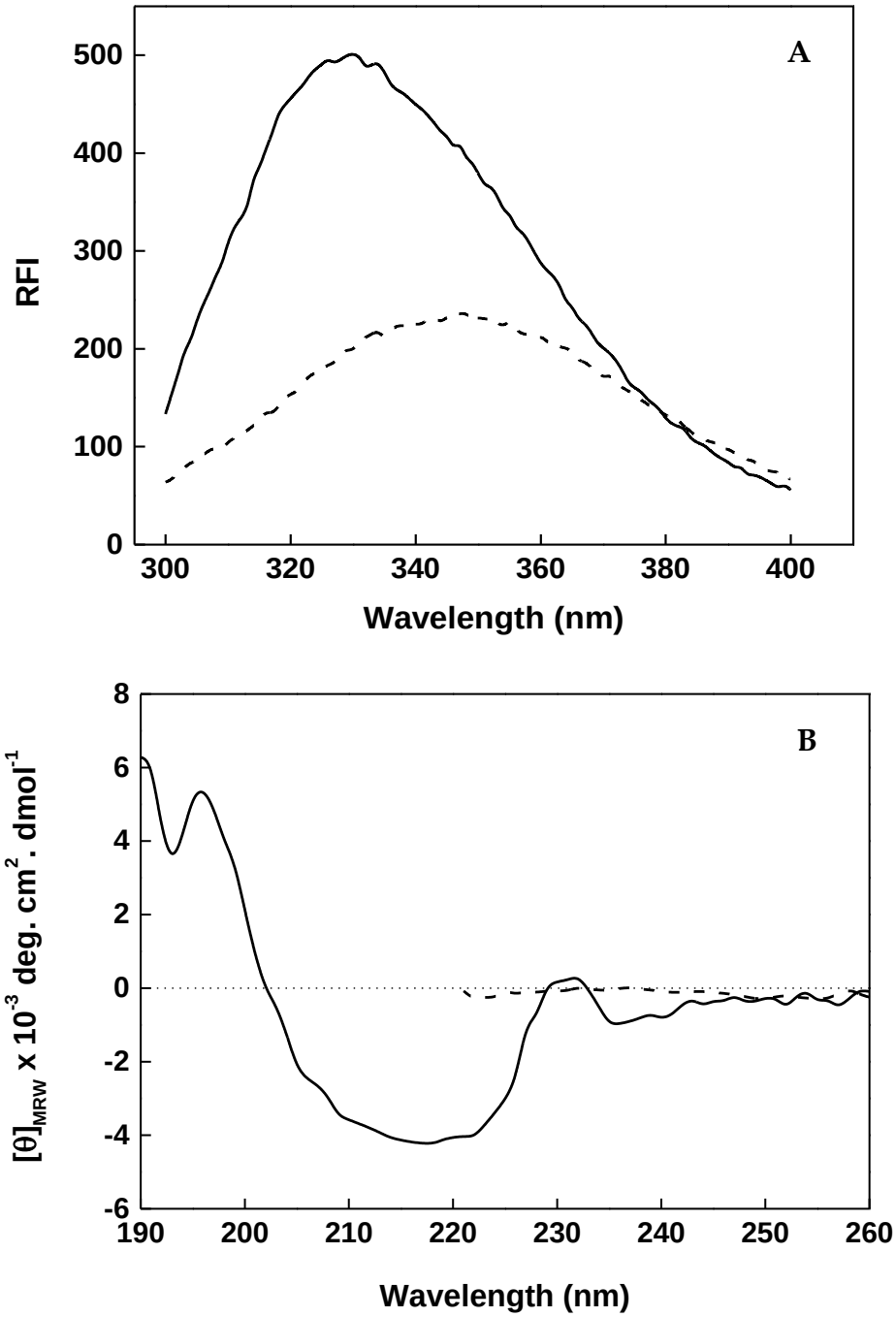


Figure 44. Structural perturbations in acid protease caused by 4M GuHCl followed by **(A)** Fluorescence and **(B)** Far UV CD measurements. The protein was incubated in the denaturant for 24 h before the spectra were recorded. Control is represented by solid line and the denatured sample by broken line.

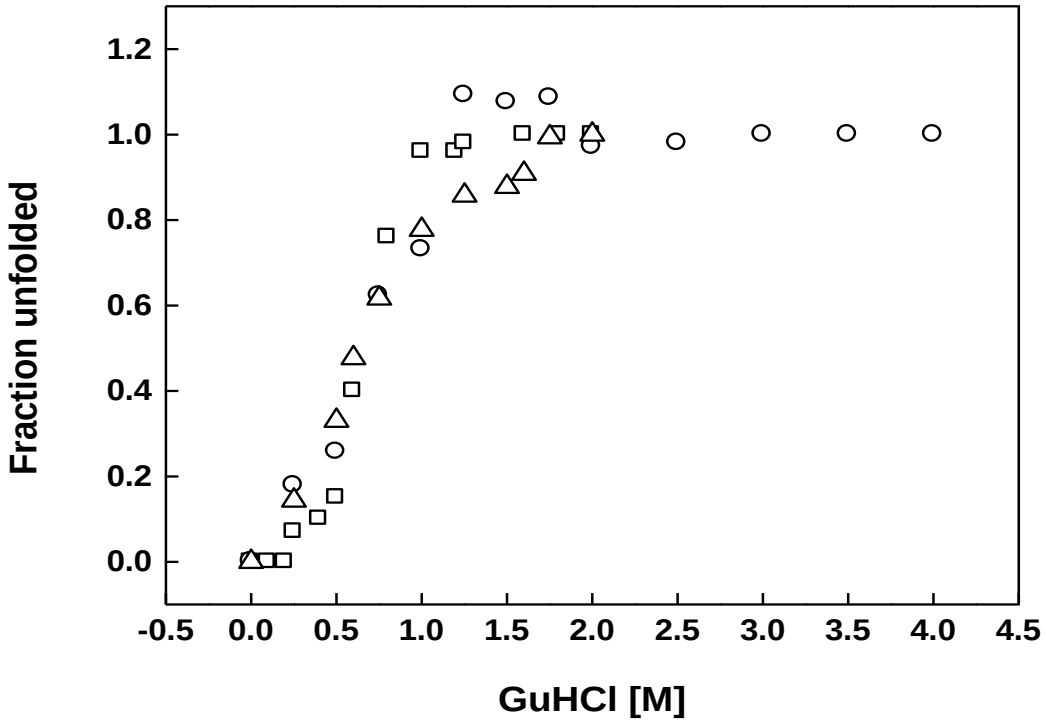


Figure 45. GuHCl induced denaturation of acid protease at pH 6.0. Unfolding was monitored by circular dichroism ellipticity at 222 nm (○), proteolytic activity (□) and fluorescence (△). Protein was incubated at particular protein concentration for 24 h. The data obtained were normalized and presented.

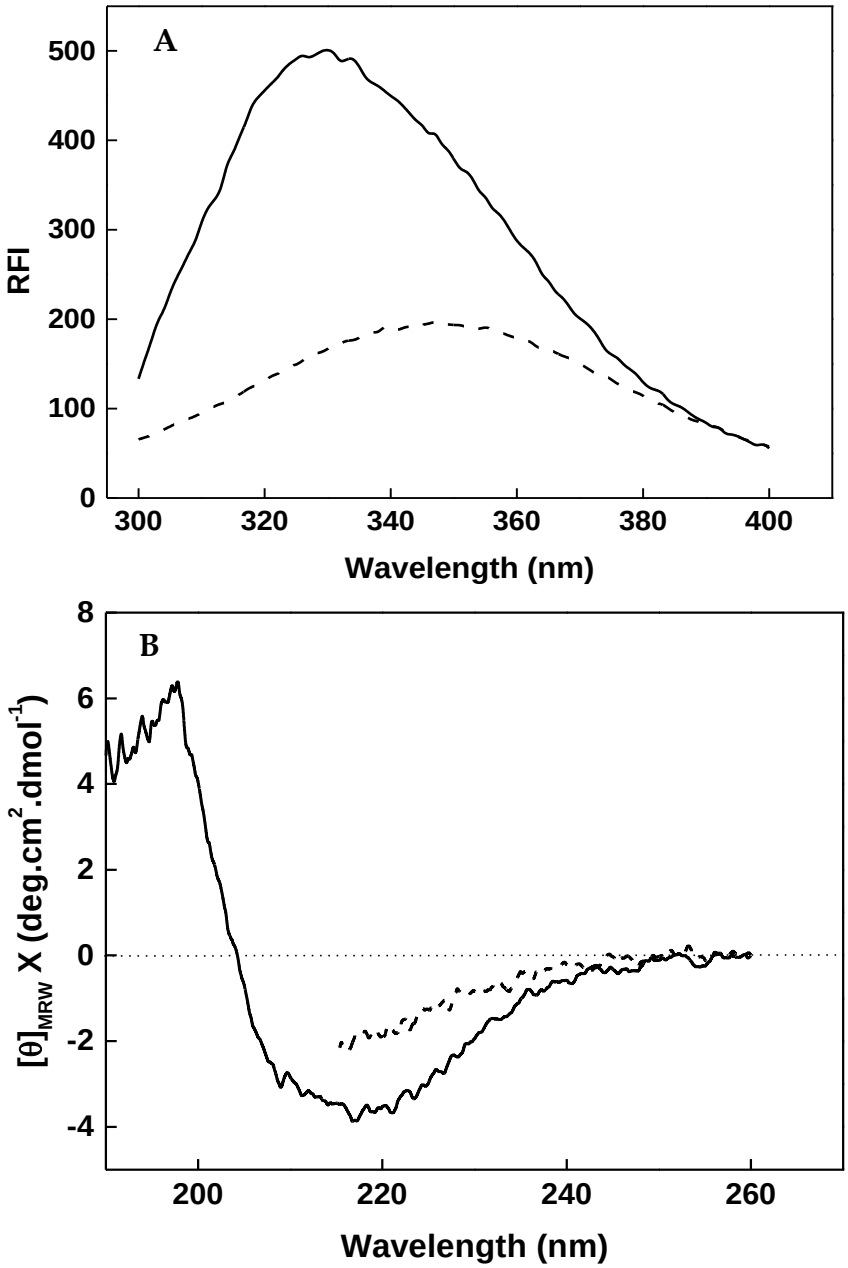


Figure 46. Effect of 5 M urea on structure of acid protease was followed by **(A)** Fluorescence and **(B)** Far UV CD measurements. The protein was incubated in the denaturant for 24 h before the spectra were recorded. Control is represented by solid line and the denatured sample by broken line.

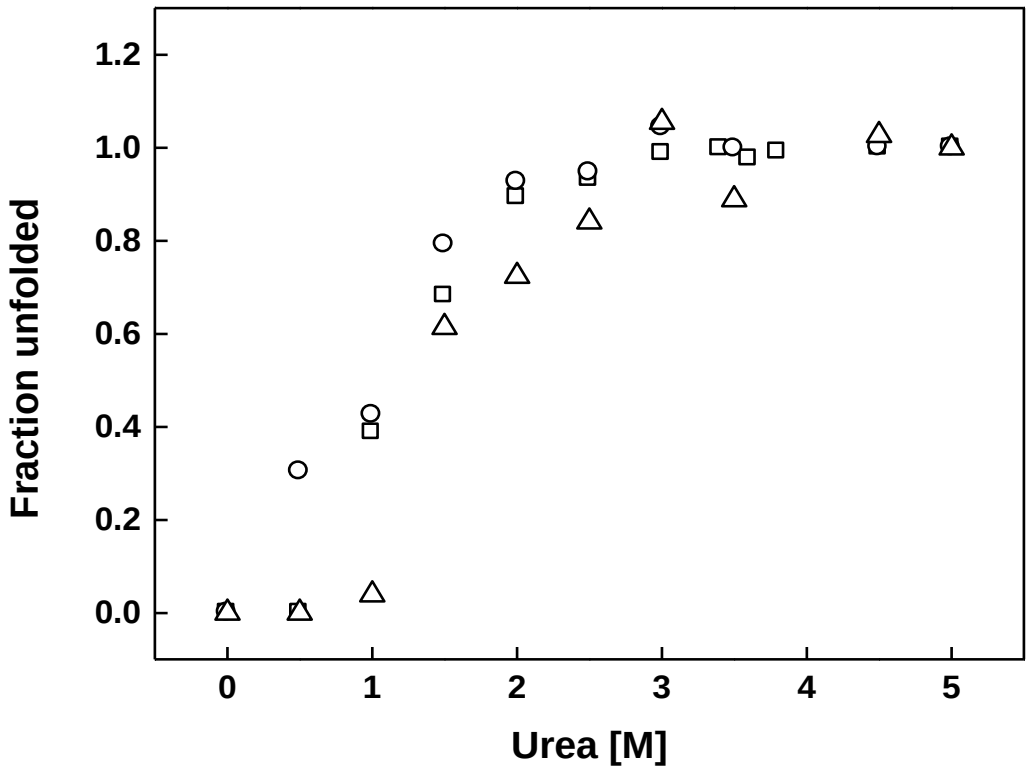


Figure 47. Urea induced denaturation of acid protease at pH 6.0. The unfolding was monitored by circular dichroism ellipticity at 222 nm (△), proteolytic activity (□) and fluorescence (○). Protein was incubated at particular denaturant concentration for 24 h and data obtained were normalized and presented.

Discussion

In the present study, the unfolding and conformational stability of acid protease from *A. oryzae* as a function of pH and denaturants (chemical and thermal) was studied.

Numerous side reactions, such as aggregation and sulfhydryl oxidation, contribute to the irreversible unfolding of proteins (Sudharshan and Rao, 1999). There are a wide variety of approaches that have been utilized in an attempt to solve the protein folding problem and characterization of intermediate state. Analysis of the changes in structural and functional parameters as function of external variables is a requisite for the description of the forces responsible for the folded conformation of the protein as well as stability. Intra-molecular interactions such as van der Waals, hydrophobic, electrostatic and hydrogen-bond interactions stabilize three-dimensional structure of a protein (Dill, 1990; Pace, 1990). Therefore, an accurate determination of the conformational stability under various conditions is vital for the comprehension of the physical interactions that stabilize the functional structure of a protein.

Effect of pH on conformational stability of acid protease

Structural perturbations are generally observed in proteins at extreme pH conditions, due to the disruption of the salt bridges or ion pairs which contribute significantly to the protein stability (Pace, 1999). In order to investigate the effect of pH on the structure of acid protease, the tryptophan fluorescence was recorded for the enzyme in the pH range of 2.0 to 10.0. Significant changes were observed in λ_{max} indicating denaturation

of the enzyme under both acidic and basic pH conditions. This observation shows that acid protease is both acid and base labile. The enzyme is in the active native conformation in the pH range 3.0–6.0. Beyond this range, the enzyme unfolds and is completely denatured at pH below 3.0 and above pH 8.0. Transition in both acidic and basic pH ranges is cooperative with no observable intermediate conformational state(s), indicating that the denaturation process follows an all-or-none process of the simple two-state mechanism of protein unfolding.

At pH 4.5, where acid protease is in its native state, the far UV CD spectrum resembles that typical of a β-sheet-rich protein. At pH range 8.0–10, where related protein like pepsin is in alkaline denatured state and has mostly unstructured N-terminal domain, the spectrum indicates a partial loss of secondary structure and the emergence of α-helical character indicated by the minima at 222 nm (Fig). Similar results are reported earlier on pepsin (Pande et al., 2009; Kamatari et al., 2003). An analysis of the far UV CD spectra indicates that the alkaline denatured state has a larger α-helical and a smaller β -sheet content than that observed in the native state of pepsin resulting in α -helix-like far UV CD spectra. It has been reported that in alkaline denatured state in aspartyl protease N-terminal domain is mostly unstructured but the C-terminal domain retains the secondary structure (Pande et al., 2009).

The possible explanation of the denaturation of N-terminal domain in alkaline denatured state is the ionization of some carboxyl groups with *pKa* values in the neutral and alkaline range. By and large, carboxyl groups of aspartic and glutamic side chains have *pKa* values near pH 4. But the *pKa* of these carboxyl groups can be increased

greatly when they interact with other charged groups or by their burial in the tertiary structure of protein. Structural analysis of aspartic protease indicates that most of these residues are located in N-terminal lobe of the protein and are involved in its alkaline denaturation.

The intrinsic fluorescence of acid protease remains the same between pH 2.5 and 6.5. The pH-rate profiles of the enzyme reactions conform to bell-shaped curves, with p*Ka* values of ~3 and 4.8–6.2 with different substrates (Polgar et al., 1994; Hyland et al., 1991; Ido et al., 1991). These p*Ka* values are attributed to the ionization of the catalytically competent aspartic acids. It is clear, however, from Figure 31 that at pH ~3, there is a considerable conformational change, which may arise from the ionization of one of the catalytic aspartic acids or some other acidic group involved in the stabilization of the protein structure, for example by ion pair formation (Grant et al., 1992). From the pH dependence of fluorescence intensity, it appears that the tryptophan residues are buried to the greatest extent between pH 2.5 and 6.5, where the enzyme is most compact. The compact form of a protein is expected to be more stable than a wobbly form. HIV-1 protease (aspartic protease) also shows similar effects upon extreme pH treatments, where the enzyme loses its structure and stability on either sides of 4.5-7.5 (Szeltner and Polgar, 1996).

Upon decreasing the pH from 4.0 to 2.0, acid protease is irreversibly inactivated. The ANS binding and CD measurements indicated that acid protease at pH 2.5 existed as molten globule-like structure as shown by large exposure of hydrophobic surface to the solvent accompanying the disruption of the tertiary structure. It has been suggested that

a balance exists between the hydrophobic interactions governing the conformational states induced by acid unfolding of proteins (Fink et al., 1998; Goto and Fink, 1989). It has also been reported that under the conditions of very low pH (< 2.0), the main forces to unfold the protein were the repulsion between charged groups on the protein molecule. The high net charge and the increased size of the charged residues were due to the presence of the associated counter ions on the protein molecule (Fink, 1995). This might promote the intrinsic hydrophobic forces to cause a collapse to a molten globule-like structure, which in turn might prevent the formation of native structure and thus stabilize the intermediate conformational states (Privalov, 1996; Jaenicke, 2000).

Thermal unfolding

Thermal unfolding of acid protease is monitored by different measures (CD, fluorescence and activity) is cooperative. The transition curves are also coincidental (Figure 34). This indicates that the molecule unfolds as a single entity. It is apparent from DSC studies (Figure 34 inset) that, thermal unfolding of acid protease is irreversible.

Proteases are believed to be secreted by the source cells as inactive precursor forms (Zymogens) to avoid autolysis. When the protease is required for the function, pro-peptide sequences are excised to make the protein active. Acid protease, when heated, does not visibly aggregate even at 65 °C. Pepstatin was used for irreversibly inhibiting the enzymatic activity of acid protease. Turbidity of pepstatin inhibited acid protease was followed at 65 °C. The activity of protease was completely lost at 25°C using 20 μM

pepstatin. Though the activity is completely lost, the secondary and tertiary structure of the protein remains unchanged upon inhibition. Pepstatin inhibited enzyme showed turbidity at 65 °C (Figure 35B). T_m of pepstatin bound acid protease was found to be higher than that of the non-inhibited enzyme (63.5°C as compared to 57°C). In the protein concentration range tried (0.01-0.1 mg/ml), the kinetic order was close to one. The enzyme showed a well defined lag phase followed by a sharp increase in turbidity during the aggregation period.

When, α-casein and β-casein were incubated separately with pepstatin inhibited enzyme and aggregation was followed at 65 °C, turbidity due to aggregation reduced. Such observations are also made by earlier workers (Rezaei-Ghaleh et al., 2008; Raman and Rao 1997). These proteins, which are widely believed to interfere with inter-molecular hydrophobic interactions (Abgar et al., 2001; Reddy et al., 2006), when added to enzyme solution, afford significant protection against thermal aggregation in a concentration-dependent manner. The stabilizing effect of these chaperone-like proteins may indicate that they preferentially interact with the thermally-unfolded species of acid protease which exposes larger hydrophobic patches over its surface and prevents aggregation. The anti-aggregation effect of these natural chaperones may be due to their subsequent incorporation into the "start aggregates" formed during the apparent lag phase and their retardation effects on the rate of cluster–cluster sticking (Khanova et al., 2007).

Thermal aggregation of acid protease was also studied in the presence of some anions (SO_4^{-2}, Cl^{-1} and SCN^{-1}) which differ with respect to their impact on water structure (Curtis et al., 2002). At the test temperature of 65 °C, the unfolding process was complete. It was found that, at identical ionic strengths, Cl^{-1} and especially SO_4^{-2} enhanced the heat-induced aggregation of acid protease but the chaotropic anion SCN^{-1} considerably diminished it. The anti aggregation effect of SCN^{-1} revealed a clear dependence on its concentration. The possible explanation for this effect could be water structure breakage by a chaotropic anion such as SCN^{-1}. Water, which otherwise leads to the weakening of hydrophobic interactions, is broken by $SCN^{-1}$and thus diminish the rate and extent of aggregation (Rezaei-Ghaleh et al., 2008).

It was also noted that, addition of SO_4^{-2}, a kosmotropic anion, could gradually release inhibition of aggregation provided by 600 mM SCN^{-1}. In total, these observations provide further support for the critical involvement of hydrophobic interactions in thermal aggregation of acid protease.

Effect of chemical denaturants on structure-stability of acid protease

Guanidine hydrochloride and urea are the most common chemical denaturants that are used for protein denaturation. GuHCl is an electrolyte with a p*K*a of about 11, which means that at pH values below this the GuHCl molecule is present in a fully dissociated form, i.e., as GuH^+ and Cl^-. The presence of these ions would influence the stabilizing properties of proteins/enzymes.

The unfolding of acid protease by denaturants exhibited a super-imposable profile for activity, secondary (far UV CD) and tertiary (fluorescence) structural changes, suggesting that the unfolding is a two-state process (F ↔ U). GuHCl is ~2 times more effective as denaturant than urea in unfolding proteins (Vecchio et al., 2002). In case of acid protease, GuHCl also proved to be ~ 2 times more effective than urea in unfolding the enzyme.

The spectral parameters of tryptophan fluorescence emission such as position, shape, and intensity are dependant on the electronic and dynamic properties of the chromophore environment (Akhtar et al., 2002). Steady state tryptophan fluorescence has been used widely to obtain information on structure-stability of many proteins (Prajapati et al., 1998). The changes in the microenvironment around chromophores of acid protease due to denaturants were followed by studying changes in the intensity and emission maxima (λ_{max}) as a function of increasing concentration of GuHCl. Results indicate that, native acid protease has a λ_{max} of 330 nm with significant fluorescence intensity. In general, the buried tryptophan residues in the folded protein show the fluorescence emission maximum at 330-340 nm (Bastians et al., 1992). Between 0-1.5 M GuHCl, acid protease undergoes major changes both in fluorescence intensity and $\lambda_{max.}$ Similar quenching of tryptophan fluorescence are reported with many proteins, which occur due to compaction of native conformation (Akhtar et al., 2002; Ahmad et al., 2001). As the concentration of the denaturant increased, the λ_{max} shifted towards 350 nm, which is a characteristic observation of tryptophan residues moving to more hydrophilic environments leading to unfolding of the molecule (Lakowicz, 1983).

Far UV studies on GuHCl induced unfolding of acid protease also indicated similar results. Native molecule has a characteristic β-sheet formation which shows a large decrease in ellipticity at 222 nm from 100% to almost complete loss of signal was observed.

Urea, being uncharged, does not affect inter and intra-molecular electrostatic interactions in the protein. Hence, $[urea]_{1/2}$ or free energy of unfolding by urea, reveals the net stability of the protein contributed by hydrophobic and electrostatic interactions (Monera et al., 1994). Under the experimental conditions, urea unfolded the acid protease completely. The denaturation curves that were obtained by various measures were sigmoidal and coincidental showing absence of intermediates in the unfolding pathway. The ratio of $[urea]_{1/2}$ to $[GuHCl]_{1/2} > 2$ for thermophilic proteins (Vecchio et al., 2002). For acid protease, the calculated ratio for $[urea]_{1/2}$ to ($[GuHCl]_{1/2}$ is ~2.3, suggesting stability is determined primarily by electrostatic interactions.

In essence, acid protease is susceptible to the unfolding action of pH, temperature and denaturants. The scheme of unfolding by the action of denaturing agents is summarized in Figure 48. It is unfolded at relatively low GuHCl concentrations. The unfolding process is also pH dependent. Hydrophobic interactions play an important role in aggregation at high temperatures. These studies on structure function and stability of acid protease may throw light on the kind of interactions involved in the stabilizing acid protease.

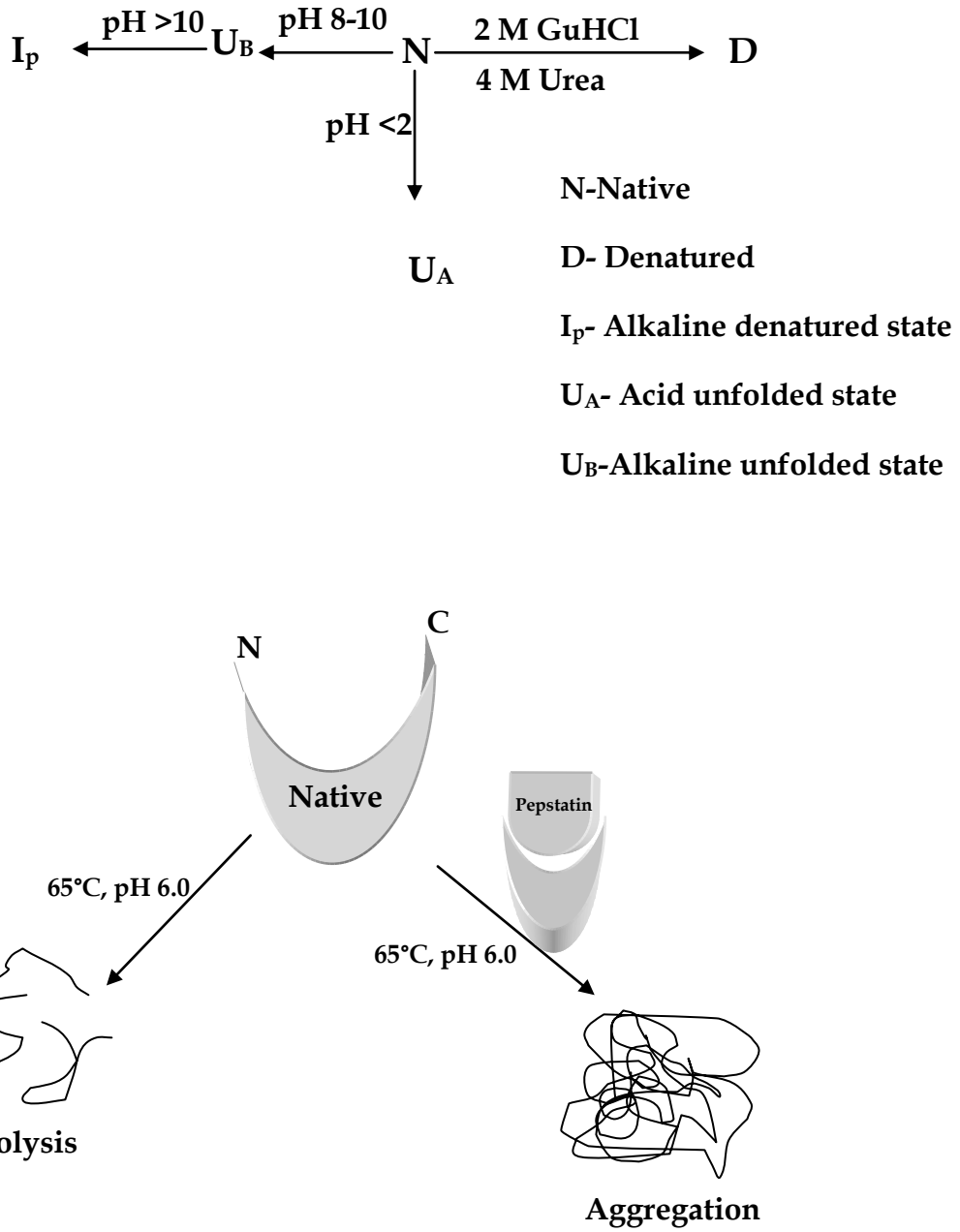


Figure 48. Unfolding scheme of acid protease from *A. oryzae* by the action of denaturing agents

Section 3: Biotechnological applications of acid proteases and milk clotting enzyme from *Aspergillus oryzae* MTCC 5341: with special reference to food applications

I Gluten modification with acid protease from *A. oryzae* MTCC 5341

Acid protease was extracted from moldy bran with 0.1 M NaCl in water was used. The effect of acid protease preparation (crude enzyme) on wheat gluten was studied by changes in the dough rheology as well as the effect on product quality of bread and crackers. The protease had a positive effect on the product quality including sensory quality. The enzyme preparation had acid protease, amylase and lipase activities (Table 24).

Table 24. Enzyme activities in the acid protease preparation

S. No.	Enzyme activity	(U/ mL)
1	Acid protease (HUT)	8.2×10^4
2	Milk clotting (SU)	3800
3	Amylase (FAA)	1000
4	Lipase	184
5	Pectinase	Nil
6	Cellulase	Nil

The crude preparation obtained from moldy bran after extraction was subjected to freeze drying. Sodium alginate (1%) was added as filler to the solution before freeze drying (Figure 49). Acid protease enzyme was obtained in powder form after 18 h of freeze drying. The enzyme had 76% of initial activity (1.56 x 10^4 U/mg). This enzyme powder is used in bread and cracker preparation.

The effect of enzyme preparation from moldy bran on wheat gluten was studied by changes in the dough rheology as well as the effect on product quality of bread and crackers. The protease had a positive effect on the product quality including sensory quality. The results of these studies are given hereunder.

Effect of acid protease from *A. oryzae* on quality of bread

Dough Rheology

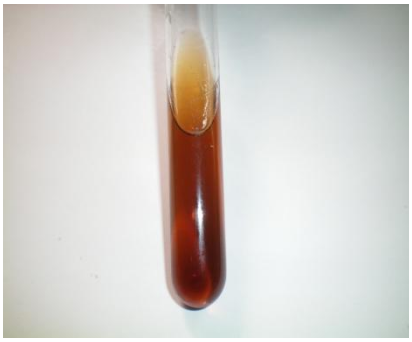
The effect of acid protease (0.01, 0.02, and 0.03%) and commercial protease (*Aspergillus oryzae* protease, BIOCON, Bangalore) (0.02%) on the farinograph characteristics of wheat flour was studied. The results showed that as the level of acid protease increased from 0 to 0.03% and commercial enzyme at 0.02% the water absorption of the flour decreased from 58.9% to 57.7% and 57.5% respectively (Table 25). A decrease in dough development time and stability were observed with the increase in the level of acid protease. The control had a dough stability of 10.5 min as against the stability value of 7.0 min in case of dough with 0.01% acid protease, 0.02% acid protease (5.5 min), 0.03% acid protease (4.5 min) and 0.02% of commercial enzyme

(5.0 min). The mixing tolerance index values increased with the increase in the level of acid protease and addition of commercial enzyme. The control had a mixing tolerance index value of 40 FU whereas it increased to 80 with addition of acid protease; above results indicate that addition of acid protease decreased water absorption and strength of the dough.

Table 25. Effect of acid protease and commercial protease on the farinograph characteristics of wheat flour for bread making

Parameters	Control	Acid protease (%)			Commercial protease (%)
		0.01	0.02	0.03	0.02
Water absorption (%)	58.9	58.5	58.1	57.7	57.5
Dough development time (min)	5.0	4.5	4.5	4.0	4.0
Dough Stability (min)	10.5	7.0	5.5	5.0	5.0
Mixing Tolerance Index (FU)	40	80	80	80	80

Moldy bran



Acid protease

+ 1% Sodium alginate

Freeze dried powder

Figure 49. Freeze drying of acid protease from *A. oryzae* in to powder form

Extensograph characteristics

The effect of acid protease (0.01, 0.02, and 0.03%) and commercial protease (0.02%) on the extensograph characteristics of wheat flour was studied. The resistance to extension for the control was 500 BU and for the dough containing 0.01 - 0.03% acid protease, it ranged from 455-370 BU and commercial protease (394 BU) (Table 26). Thus a decrease in resistance to extension was observed with the use acid protease. The extensibility values showed an increase with the addition of acid protease. The control had an extensibility value of 140 mm, whereas it increased to 142 mm with 0.01% acid protease, 0.02% acid protease (145 mm), 0.03% acid protease (152 mm) while commercial protease showed extensibility value of 150 mm. The Ratio Figure (R/E) of control was 3.5, for the dough. In presence of 0.01 to 0.03% acid protease, it varied from 3.2 to 2.4 and dough with 0.02% commercial protease showed the ratio figure of 2.6. The area of the curve representing strength of the dough decreased from 135 cm^2 of that of the control to 102 – 125 cm^2 for dough treated with acid protease. The above results indicate that addition of acid protease decreased elasticity and increased extensibility of the dough.

Table 26. Effect of acid protease and commercial protease on the extensograph characteristics of wheat flour for bread making

Parameters	**Control**	**Acid protease (%)**			**Commercial protease (%)**
		0.01	**0.02**	**0.03**	**0.02**
Resistance to extension, R (BU)	500	455	405	370	394
Extensibility, E (mm)	140	142	145	152	150
Ratio figure, R / E	3.5	3.2	2.8	2.4	2.6
Area, (cm^2)	135	125	120	102	111

Amylograph characteristics

The effect of acid protease (0.01, 0.02, and 0.03%) and commercial protease (0.02%) on the amylograph characteristics of wheat flour was studied. The results did not show much variation in gelatinization temperature with addition of acid protease. The peak viscosity of control was 878 AU while in the case of wheat flour with 0.01% acid protease, it was 740 AU, 0.02% acid protease (680 AU), 0.03% acid protease (650 AU) and 0.02% commercial protease (674 AU). The above data shows that use of acid protease decreased the peak viscosity.

Bread making characteristics

Effect of different levels of acid protease on bread making characteristics of wheat flour is presented in Table 27 and Figures 50 and 51. Addition of increasing level of acid protease from 0.01 to 0.03% increased the loaf volume of bread from 505 to 630 ml. The commercial protease resulted in increased volume of 635 ml. The crumb firmness, which is a force at 25 %, decreased from 580 g to 420 g with increase in addition of acid protease from 0 to 0.03% and commercial protease showed a crumb firmness value of 395 g indicating improvement in the texture of breads with acid protease. However addition of acid protease at the level of 0.02 % and commercial protease at the level of 0.02% brought about highest improvement in the loaf volume and texture. The improvement brought about in crust color, shape, crumb color and grain was also highest for the bread with 0.02% acid protease and 0.02% commercial protease.

Addition of 0.03% acid protease resulted in stickiness of dough. From the above results it can be concluded that the improvement brought about by addition of 0.02% acid protease is comparable to that of 0.02% commercial protease.

Table 27. Effect of acid protease and commercial protease on the amylograph characteristics of wheat flour for bread making

Parameters	**Control**	**Acid protease (%)**			**Commercial protease (%)**
		0.01	**0.02**	**0.03**	**0.02**
Gelatinization temp (°C)	64.5	64.9	64.7	64.8	65.3
Peak viscosity (BU)	878	740	680	650	674

Table 28. Effect of acid protease and commercial protease on bread making characteristics of wheat flour

Bread		Control	Acid protease (%)			Commercial protease 0.02 (%)	SEM ±
			0.01	0.02	0.03		
Weight (g)		139.8a	140.0a	140.1a	140.2a	140.1a	0.3
Volume (ml)		505a	585b	630c	635c	620c	5.0
Specific volume (ml/g)		3.61a	4.17a	4.49c	4.52c	4.42c	0.05
Crumb firmness value (Force, g)**		580d	500c	420b	395a	430b	5.0
CRUST	**Color (10)*****	8a	8a	9b	9b	9b	0.5
	Shape (15)	11.5a	12.5b	14c	14c	14c	0.48
CRUMB***	**Color (15)**	11a	12b	14d	13c	14d	0.4
	Grain (20)	14a	15a	18d	16c	18d	0.3
Mouthfeel (20)		15a	16b	17b	15a	17b	0.45
Taste (20)		17a	17a	17a	17a	17a	0.35
Overall quality (100)		76.5a	80.5b	89d	84c	89d	1.5

SEM: Standard error of the mean at 15 degrees of freedom

*Means in the same row followed by different letter differ significantly ($p \leq 0.05$)

** Force at 25% compression measured using texture analyzer

*** The figures in the parenthesis indicate maximum score for crust and crumb parameters

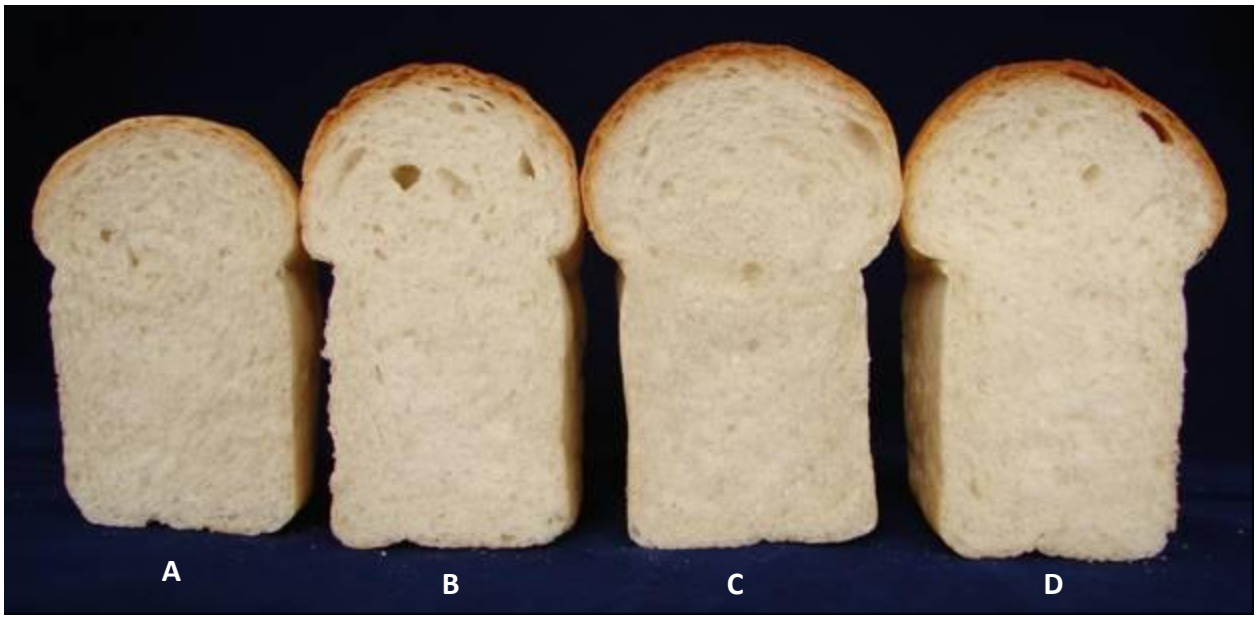


Figure 50. Effect of acid protease on bread making quality

A: Control, B: Acid protease **0.01%, C:** Acid protease **0.02%, D:** Acid protease **0.03%**

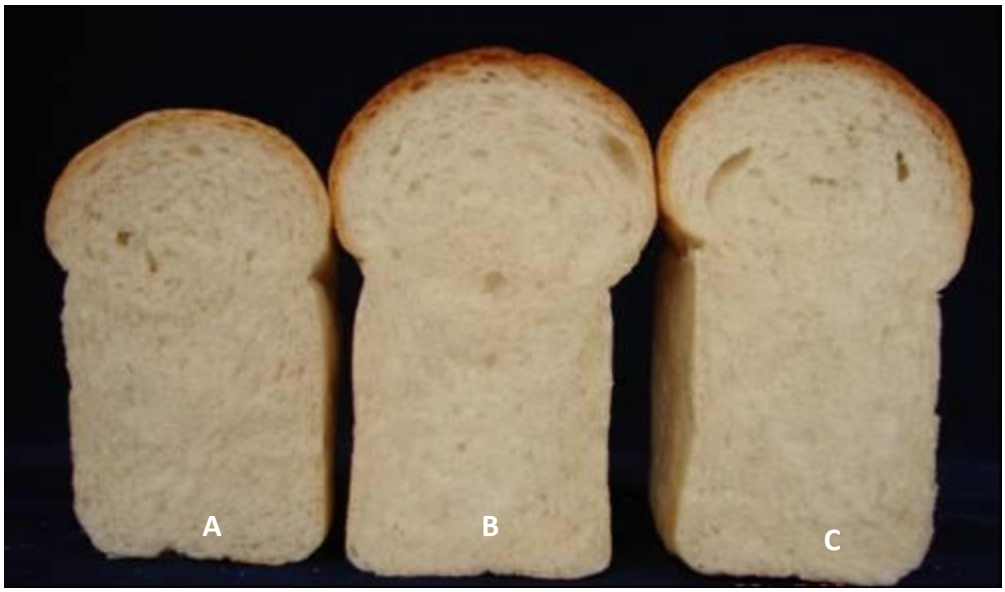


Figure 51. Effect of acid protease from *A. oryzae* MTCC 5341 and commercial protease on bread making quality

A: Control, B: Acid protease **0.02%, C:** Commercial protease **0.02%**

Effect of acid protease from *A. oryzae* on quality of crackers

The flour used for the studies had the following chemical characteristics are 0.46% ash, 9.80% dry gluten, 58 ml SDS value, 10.60% protein and 9% damaged starch. The flour was of medium hard type suitable for the preparation of crackers.

Effect of acid protease on the dough rheological properties

Effect of acid protease on the farinograph characteristics of wheat flour

The effect of incorporation of fungal protease at 1, 2 and 3% on the farinograph characteristics of wheat flour was studied. The data showed that the water absorption of the flour decreased from 59.8% of that of the control to 57.7 for dough with 3% protease (Table 29). Thus the water absorption decreased as the level of fungal protease increased from 1 - 3%. A decrease in dough development time and stability were observed with the increase in the level of protease. The control had a dough stability of 5.2 min whereas the dough stability was 3.5, 3.0 and 2.5 min for dough containing 1, 2, and 3% enzyme respectively. The control had a mixing tolerance index value of 40 FU whereas it increased to 76, 86 and 104 FU with 1, 2, and 3% fungal protease. The farinograph quality number decreased to 47 for dough with 3% protease as against the control value of 83

showing that the dough strength decreased with increasing level of fungal protease.

Table 29. Effect of fungal protease on the farinograph characteristics of wheat flour for cracker making

Fungal protease (%)	Water absorption (%)	Dough development time (min)	Stability (min)	Mixing Tolerance Index, MTI (BU)	Farinograph Quality Number
---	59.8	4.3	5.2	40	83
1	58.4	3.5	3.1	76	55
2	58.0	3.0	3.0	86	54
3	57.7	2.5	3.0	104	47

Effect of fungal acid protease on the extensograph characteristics of wheat flour

The effect of 1, 2 and 3% fungal protease on the extensograph characteristics of wheat flour was studied. The resistance to extension for the control was 410 BU and for the dough containing 1, 2, and 3% fungal protease, it was 283, 244 and 230 BU, respectively (Table 30). Thus a decrease in resistance to extension was observed with increase in enzyme quantity. The control had an extensibility value of 158 mm whereas it ranged between 205-219 mm for dough with 1-3% enzyme. The extensibility values showed an increase as the level of fungal protease increased. The Ratio Figure varied from 1.40 – 1.05 for the dough with 1 - 3% protease as against control value of 2.50. The area value decreased from 135 cm^2 of that of the control to 79 - 62 cm^2 for dough with 1 - 3% protease.

Table 30. Effect of fungal protease on the extensograph characteristics of the wheat flour for cracker making

Fungal protease (%)	**Resistance to extension (R, BU)**	**Extensibility (E, mm)**	**Ratio Number (R/E)**	**Area (cm^2)**
---	410	158	2.50	135
1	283	205	1.40	79
2	244	211	1.20	74
3	230	219	1.05	62

Effect of acid protease on the quality of crackers

The data on the effect of fungal protease on the physical characteristics of crackers is presented in Table 31. The data showed that the diameter and spread ratio of the crackers increased with the increase in the level of protease. The spread ratio of the control was 7.90 whereas it ranged between 8.10 – 8.50 for crackers with 1 – 3% of protease. The breaking strength of the control crackers was 1525 g whereas it was 1460, 1410 and 1315 g for crackers with 1, 2 & 3% protease. The texture of the crackers with protease was lighter and crisp. The data on the sensory characteristics (Table 32) showed that the crackers with 2% protease were rated the best evident from the highest total score of 93.5 out of 100. The crackers possessed golden brown color, smooth surface, creamish white crumb color, distinct and noticeable layers with a crisp flaky texture. The crackers with 3% protease showed slightly dark brown crust color (Fig. 52). The layers were not very noticeable and crackers possessed fragile texture. The crackers had the lowest total score of 84 as against the total score of 88.5 and 90 for control and crackers with 1% protease respectively. Thus use of fungal protease at 2% level was optimum as it improved the overall quality characteristics of the crackers.

Table 31. Effect of fungal protease on the physical characteristics* of crackers

Fungal Protease %	Weight (g)	Diameter (D, mm)	Thickness (T, mm)	Spread ratio (D/T)	Texture** (g)
---	37.20	49.5	6.3	7.90	1525
1	36.80	50.0	6.2	8.10	1460
2	36.50	50.8	6.0	8.40	1410
3	37.30	51.2	6.0	8.50	1315

* Average of 6 crackers

** Measured using texture analyzer

Table 32. Effect of fungal protease on the sensory characteristics of crackers

Fungal Protease %	Crust color (5)	Surface character (15)	Crumb color (10)	Crumb layers (20)	Crumb texture (20)	Flavor (10)	Taste (10)	Mouthfeel (10)	Total score (100)
---	4.0b	13.0a	8.5a	17.5a	18b	9.5a	9.0a	9.0a	88.5b
1	4.0b	13.5a	8.5a	18.0b	18.5b	9.75a	9.0a	9.0a	90.0c
2	4.5b	14.0ab	9.0b	19.0c	19.5c	9.5a	9.0a	9.0a	93.5d
3	3.5a	13.0a	9.0b	16.0a	16.0a	9.0a	8.5a	9.0a	84.0a
SEM±	0.3	0.45	0.35	0.4	0.35	0.25	0.25	0.3	2.0

Means in the same column followed by different tests differ significantly ($P \leq 0.05$)

SEM: standard mean error of the mean at 12 degrees of freedom

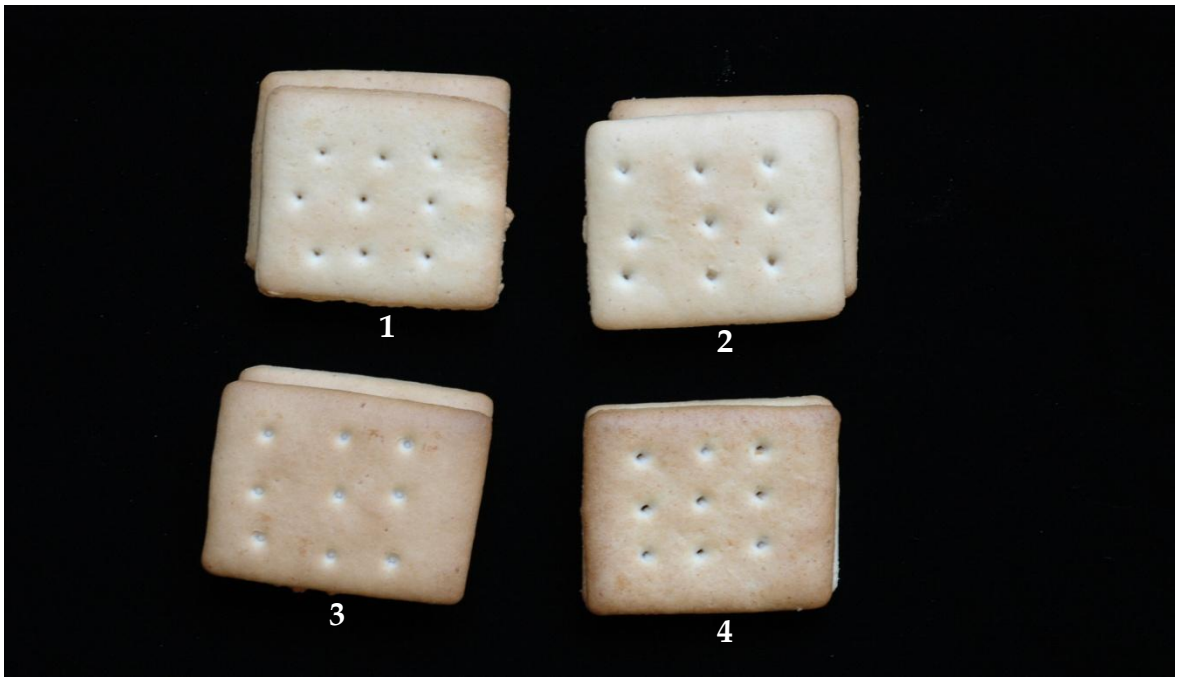


Figure 52. Effect of acid protease from *A. oryzae* MTCC 5341 on cracker quality

1. Control **2.** 1% protease **3.** 2% protease **4.** 3% protease

Cheese making trials

Cheddar cheese was made using milk clotting enzyme from *Aspergillus oryzae*. Cheese was prepared at National Dairy Research Institute (NDRI), Bangalore using the *Aspergillus* rennet. Performance of the *Aspergillus* rennet was compared with standard rennet (Meso™ rennet from *Mucor sp.*) according to standard procedures established in the institute. Cheese was made with 50 l batch milk for both the enzymes. After pasteurizing at 62°C for 30 min, the milk was cooled to 32-35°C and starter culture was added to acidify the milk. After 30 min of starter culture addition, rennets were added to each of the vat to achieve coagulation. Milk clotting was observed after 40 min of rennet addition in both the enzymes. The temperature of curdled milk was raised to 40-42°C to assist separation of whey. After draining the whey, standard procedures like cutting, cheddaring, milling, salting, hooping, pressing and parafinning were performed to obtain cheese. The parafinned cheese was kept at 4°C for ripening.

The enzyme characters like primary yield of the cheese, surface characteristics and clotting time were compared with that of standard rennet. 50 l of bovine milk was used for each trial. The milk coagulated after 40 min of addition of enzymes in both the cases. Thus the clotting time was the same for both the enzymes. After process of the cheddaring and removing the whey by pressing under high pressure, the primary weight of the cheese is taken. When Aspergillus rennet was used for coagulation of milk, the primary yield was 5.1 kg in comparison to 5.35 kg in standard rennet (Table 31). Surface characters like off flavor, texture and firmness were evaluated visually by

expert panel revealed that both the enzymes work in similar way. The cheese obtained by action of these enzymes is parafinned and kept for maturation at 4°C for a period of 8-10 months. Final evaluation of cheese quality is thus incomplete. The whole process of the cheese making is depicted in the Figure 53.

Table 33. Primary characteristics cheese made out of fungal rennet

Source of rennet	**Total milk used (L)**	**Primary yield (kg)**	**Recovery (%)**	**Surface characteristics**
Meso™ (*Mucor sp.*) (1g in 50 ml)	50	5.35	10.7	Smooth, no off-flavors, less whey draining
Aspergillus oryzae (1g in 200ml)	50	5.10	10.2	Smooth, no off-flavor and low whey generation

1. **Filtering milk**

2. **Pasteurization**

3. **Starter culture**

4. **Rennets used**

5. **Rennet addition**

6. **Cutting**

7. **Separating curd**

8. **Cheddaring**

9. **Salting**

10. **Hooping**

11. **Pressing**

12. **Parafinning**

Figure 53. Cheese making using milk clotting enzymes from *Aspergillus oryzae* MTCC 5341 and *Mucor sp.*

Chapter 4

Summary and Conclusions

The salient features of the present investigation are as follows:

Acid protease from *Aspergillus oryzae* MTCC 5341

- Out of 24 fungal cultures screened for acid protease activity, *Aspergillus oryzae* MTCC 5341 produced highest activity levels by SSF. Optimized process parameters for acid protease production by *A. oryzae* MTCC 5341 by SSF are, wheat bran as solid media supplemented with defatted soy flour (4%), time of incubation-120 h, pH- 4.0-5.0 and incubation temperature of 28-30°C. At the end of 120 h of fermentation, *A. oryzae* produced an acid protease activity of 8.6×10^5 U/ g dry bran, which is the highest reported activity from any fungal species
- Down stream processing: Parameters for recovery of acid protease from moldy bran were optimized. Water having 0.1M NaCl was the ideal solvent for extracting the enzyme from the moldy bran. The enzyme could be extracted by soaking the bran in solvent, bran:solvent ratio of 1:10 for 60 min without agitation at 4°C. Decolorization with 2% charcoal removed extraction color. Addition of alginate and freeze drying helped to obtain a stable enzyme powder
- Acid protease was purified to homogeneity by conventional chromatographic techniques. The enzyme characterstics were:

- Acid protease had a specific activity of 43,658 U/mg compared to 2630 U/mg in crude, thus enzyme was ~ 17 folds purified with a yield of 29%
- Molecular weight of the purified protein, deduced by SDS-PAGE and gel filtration on HPLC, was 47 kDa
- K_m and V_{max} for acid protease were determined with acid denatured hemoglobin as substrate to be 0.12 M and 89,445 μmol/min/mg protein, respectively
- The enzyme was active over a wide pH range of 3-4. pH stability of the enzyme showed that it is more stable in the range of 2.5-6.5. The optimum temperature for activity was determined to be 55°C
- Pepstatin inhibited acid protease completely- aspartic protease and other class specific protease inhibitors had no effect on proteolytic activity. Pepstatin is a competitive inhibitor with a K_i of 3.7×10^{-7} M.
- Acid protease activated trypsinogen to active trypsin, Val-$(Asp)^4$-Lys, a hexapeptide towards N-terminal of precursor trypsinogen is cleaved to release trypsin.
- Enzyme had absorption maxima of 278 nm. The emission maximum was 330 nm, when excited at their excitation wavelength of 278 nm indicating that the tryptophan residues are not exposed to solvent

- Secondary structure analysis (CDSSTR, 1986) revealed 57% β structure, 4% α helix and 38% aperiodic structure
- From the rate constants at different temperatures, Arrhenius plot was constructed and inactivation kinetic parameters estimated. E_a (activation energy), was determined to be 65.82 kcal.mol^{-1} from the slope of the Arrhenius plot. The activation enthalpy (ΔH^*), entropy (ΔS^*) and free energy change (ΔG^*) were calculated to be 65.16 kcal.mol^{-1}, 128.05 cal.mol^{-1}°K^{-1} and 23.35 kcal.mol^{-1} respectively
- T_m was determined to be 57°C at pH 6 in 0.05 M phosphate buffer by activity and spectroscopic methods

➢ Acid protease was stable in the pH range 3-6. The midpoints of pH transition for the acidic and alkaline transitions were 3.0 and 6.4, respectively

- Number of ANS molecules bound increased as a result of unfolding triggered by pH. Blue shift in the λmax indicated transfer of non polar groups to hydrophobic environment
- Gel filtration experiments were carried out to study the effect of pH on structure. At pH 3.0, autolysis occurred; at pH 6.0, molecule is more compact and stable. At pH above 6.0, area under the peak increased with change in elution time, indicating a change in the state of association of the molecule. At extreme pH values, enzyme lost activity and structure

- Thermal inactivation of acid protease was due to proteolysis and aggregation. Hydrolysis was prevented by pepstatin, a competitive inhibitor of the enzyme
    - Protein concentration had positive effect on aggregation. Chaotropic salts like NaCl and SO_4^{-2} enhanced aggregation. SCN^{-1}, β- casein and α- casein reversed aggregation showing importance of hydrophobic and ionic interactions in maintaining the structure of acid protease
- GuHCl and Urea unfolded the molecule and resulted in loss of activity. GuHCl is more potent than urea in inducing unfolding. GuHCl obeyed "two fold" rule in unfolding of the molecule. Chemical denaturants induced unfolding curves were cooperative and biphasic- N to U
    - Denaturant concentration at half completion of [denaturant]1/2 were Urea- 1.74 M and GuHCl- 0.85 M. Urea and GuHCl shifted emission λ_{max} towards red indicating unfolding of the molecule, and exposure of aromatic chromophores
- Loss of proteolytic activity upon unfolding coincides with loss in tertiary and secondary structure reflecting good correlation between activity and structural integrity of the molecule
- Primers for acid protease were designed based on the reported sequences from *A. oryzae*. The enzymes have nearly 75-80% homology. The sequence of the amplified fragment of aspartic protease from *Aspergillus oryzae*

MTCC 5341 had 92% homology with reported aspartic proteases of *Aspergillus sp.* The fragment amplified corresponds to a part of the gene coding for an aspartic protease. Further work needs to be carried out to obtain an active aspartic protease in a suitable host organism.

- Acid protease, added to wheat flour, before preparation of bread or crackers helped improve the dough rheology as evidenced from farinograph and extensograph results. Better loaf volumes were obtained for bread. The texture of crackers was considerably improved as shown by sensory studies
- The acid protease from *A. oryzae* could be used to improve the dough rheology, machinability and product quality of bread, bun and crackers. The acid protease preparation improved the dough and product quality of both bread and crackers at 0.2 – 0.3% level (for bread) and 1 – 2% for crackers. The enzyme can be used to substitute chemical additives used in bakery products

Milk clotting enzyme from Aspergillus oryzae MTCC 5341

- Out of 16 fungal cultures screened for milk clotting activity, *Aspergillus oryzae* MTCC 5341 produced highest milk clotting activity by SSF. Media components, optimum solvent, bran: solvent ratio, time of extraction were standardized to obtain 40, 000 U/g dry bran milk clotting activity.

Rhizopus microsporus var. *oligosporus*, *Mucor rouxii* and *Aspergillus oryzae* were the best producers of milk clotting activity. SSF at 6.3 pH was best for enzyme production

- Immobilizing rennet in alginate beads and subsequent elution purified the enzyme with a recovery ~72% with a specific activity of 3,500 U/mg protein
    - Molecular weight of the enzyme–34 kDa
    - Optimum pH of 6.2-6.5 and temperature of 55-60°C
    - Midpoint of heat inactivation- 49°C
    - Rennet hydrolyzed all the casein fraction showing little specificity for hydrolysis
    - Milk clotting enzyme showed more hydrolysis of κ – casein. Hydrolysis of other two fractions, α and β caseins was also seen, which add bitter peptides essential for flavor developing during ripening
- Cheese preparation compared with commercial *Mucor* rennet indicated that, clotting time, yield and surface characteristics were similar to the commercial enzyme

- Both acid protease and milk clotting enzymes were obtained in powder form by freeze drying with a yield of 70-75%. Enzymes retained activity for more than 8 weeks with minimal loss. Enzyme in powder form was utilized to modify wheat proteins.

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